SEROPREVALENCE AND MOLECULAR EPIDEMIOLOGY OF *TOXOPLASMA GONDII* IN THE WESTERN CAPE OF SOUTH AFRICA

By

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DECLARATION

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SUMMARY

Toxoplasmosis is a disease caused by the obligate intracellular parasite *Toxoplasma gondii* and is one of the most widely occurring infections known to man, causing disease in almost all mammals and warm-blooded birds. *Toxoplasma gondii* is an opportunistic pathogen, taking advantage of weakened immune systems to cause disease. The disease toxoplasmosis is known to occur in both active and latent or chronic forms. The latent or chronic form of toxoplasmosis is known to be the most common latent infection in man. Currently, the effects of the latent or chronic form of toxoplasmosis is neither properly studied nor understood.

In Africa, there is currently no systematic monitoring or reporting of toxoplasmosis incidence or *T. gondii* prevalence and this leaves health systems at a disadvantage. The United States Centre for disease Control and Prevention (CDC) currently classify toxoplasmosis as a neglected tropical infection.

In this series of research projects, the seroprevalence of *T. gondii* antibodies and the risk factors to infection were investigated. Following that, the molecular epidemiology and population structure of the pathogen was also investigated. The investigations were conducted in opportunistically selected human and animal populations within the Western Cape Province of South Africa. The data obtained was analyzed from a "One World One Health" perspective and conclusions were then documented.

The selected human populations were, a cohort of human immunodeficiency virus (HIV) positive and human immunodeficiency virus (HIV) negative post parturient women and their HIV exposed and uninfected (HEU) and HIV unexposed and uninfected (HUU)

infants, and a cohort of HIV positive and HIV negative male and female patients presenting with a clinical diagnosis of uveitis at the Tygerberg academic hospital opthalmology clinic. The selected animal populations were a population of feral cats in the greater Cape Town metropole, a population of free ranging sheep on a single farm in Bredasdorp and a population of free ranging caracals in the Cape peninsula region.

Toxoplasma gondii seroprevalence was 23.3% in the cohort of post parturient women, whilst the seroprevalence in the infant cohort was 1.02%. The seroprevalence was 24.7% in the cohort of patients presenting with ocular infections. The seroprevalence was not associated with HIV status but was associated with the consumption of unwashed fruits and vegetables. The similarity between the seroprevalence in the post parturient cohort and the ocular cohort points to the lack of regional differences in seroprevalence rates, whilst the low seroprevalence in the infant cohort implies a low rate of congenital toxoplasmosis in the study area and this comforms to what is observed in other parts of Africa, Europe and North America.

Seroprevalence was extremely high in the felid populations investigated (Feral cats - 37.1% and Caracals- 69.6%). These justifies considerable attention since these felids were free ranging and therefore have access to domestic human and animal populations. The seroprevalence in the sheep flock was also higher at 8.0% than the five point six percent (5.6%) reported in earlier studies in Cape Town.

Current research has established that *T. gondii* genotypes vary in their geographic distribution, with certain genotypes being more predominant in certain geographic areas than others. To establish the genotypes of *T. gondii* present in our selected geographic

area, we investigated the molecular epidemiology of the pathogen. The molecular epidemiology investigations revealed a predominance of Type II genotypes in both human and animal samples. A Type III genotype was detected in human but not in animal samples. Atypical genotypes were also identified in both human and animal samples. The observed population structure is similar to that of South America and other parts of Africa but differs from that observed in Europe and North America.

We have demonstrated the presence of *T. gondii* as well as its active transmission within selected human and animal populations in the Cape Town metropole of South Africa. The presence of atypical *T. gondii* genotypes was also demonstrated. The observed seroprevalence rates investigated in this study implies that more attention needs to be paid to the disease as it could have implications for female reproductive health, infant to adolescent ocular health as well as overall economic losses due to an increase in disease burden in humans (reported as disability-adjusted life years) and for agriculture.

OPSOMMING

Toksoplasmose is 'n siektetoestand wat veroorsaak word deur die intrasellulêre-gebonde parasiet *Toxoplasma gondii* is 'n opportunistiese patogeen, wat voordeel trek uit verswakte immuunstelsels om die siekte te veroorsaak Toksoplasmose is een van die mees wyd verspreide infeksies wat bekend is aan die mens, en affekteer baie, indien nie die meeste, soogdierspesies en voëls. Toksoplasmose kom voor in beide aktiewe en latente vorms, en dit is bekend dat die latente vorm van toksoplasmose die mees algemene latente infeksie in die mens is, en die gevolge daarvan is nog nie behoorlik bestudeer of word verstaan nie.

In Afrika, is daar tans geen sistematiese monitering of verslagdoening oor die voorkoms van toksoplasmose ó f *Toxoplasma gondii* nie en dit benadeel tans gesondheidbestuur. Die “Centre for Disease Control (CDC)” in die Verenigde State van Amerika klassifiseer toksoplasmose tans as 'n afgeskeepte tropiese infeksiesiekte.

In hierdie reeks navorsingsprojekte word die serologiese voorkoms van *T. gondii* teenliggame en die risikofaktore wat tot infeksie lei, ondersoek. Hierop volg dan ‘n verdere ondersoek na die molekulêre epidemiologie en populasiestrukture van die patogeen. Die navorsing is uitgevoer op opportunisties geselekteerde mens- en dierbevolkings binne Suid-Afrika. Die data wat verkry is, is verwerk vanuit ‘n “One World One Health” perspektief en gevolgtrekkings is hiervolgens gemaak.

Die geselekteerde menslike bevolking het bestaan uit 'n groep MIV-positiewe en MIV-negatiewe na-swangerskap vroue-pasiënte met hul MIV-blootgestelde en onbesmette (HEU), en MIV-onblootgestelde en onbesmette (HUU) kinders, asook 'n groep van

manlike en vroulike pasiënte met okulêre infeksies gediagnoseer by die Tygerberg Hospitaal oogkliniek. Die geselekteerde dierebevolkings het bestaan uit 'n bevolking van wilde katte in die groter Kaapse metropool, 'n bevolking van vry weiende skape op n enkele plaas, en 'n bevolking van los-lopende rooikatte in die Kaapse skiereiland, almal in die bestek van die Wes-Kaap Provinsie van Suid-Afrika.

Seroprevalensie van *T. gondii* was 23.3% in die groep van na-geboorte vroue terwyl prevalensie 1.02% in die kindergroep was. Die seroprevalensie was ook hoog, teen 24.7%, in die groep van pasiënte met okulêre infeksies. Die seroprevalensie was nie verbind met MIV-status nie, maar wel met die verbruik van ongewaste vrugte en groente. Die ooreenstemming in die seroprevalensie van die na-geboorte groep en die ooginfeksie groep dui op ‘n area-verskil in seroprevalensie tempo, terwyl die lae seroprevalensie in die kinder-groep dui op ‘n lae tempo van aangebore toksoplasmose in die area van studie en dit stem ooreen met waarnemings in ander dele van Afrika, Europa en Noord-Amerika.

Seroprevalensie was baie hoog in die wilde kat bevolkings (wildekatte –37.1% en in Rooikatte- 69,6%). Hierdie bevinding vereis verdere aandag omdat die katte vrylopend was en daarom toegang gehad het tot die plaaslike mens- en huisdierbevolkings. Die seroprevalensie in die skaapbevolking was ook hoër teen 8.0% teenoor die 5.6% wat in vorige studies gerapporteer was in Kaapstad.

Huidige navorsing het bepaal dat *T.gondii* genotipies varieër in hul geografiese verspreiding, met sekere genotipes meer dominant in sekere geografiese area’s as ander. Om die genotipes van *T.gondii* in ons geselekteerde geografiese gebied te bestudeer , het

ons die molekulêre epidemiologie van die patogeen bepaal. Die molekulêre epidemiologiese ondersoeke het aangedui op 'n oorheersing van Tipe II genotipes in beide mens- en dier monsters. Tipe III genotipes was gevind in die menslike siektevorm, maar nie in die dier-infeksie monsters nie. Atipiese genotipes is ook waargeneem in beide mens- en dieremonsters. Die waargeneemde bevolkingstruktuur van *Toxoplasma gondii* is soortgelyk aan dié van Suid-Amerika en ander dele van Afrika, maar verskil van dié wat waargeneem word in Europa en Noord-Amerika.

Ons het die teenwoordigheid van *T. gondii* asook sy aktiewe oordrag binne die geselekteerde mens en dier bevolkings in die Wes-Kaap van Suid-Afrika bewys. Die teenwoordigheid van atipiese *T.gondii* genotipes word ook beskryf. Die waargeneemde seroprevalensies wat in hierdie studie ondersoek is, dui daarop dat meer aandag gegee moet word aan die siekte, omdat dit ernstige implikasies inhou vir vroulike reproduktiewe gesondheid, baba tot adolessent okulêre gesondheid, asook ‘n hoë lading op die gesondheidstelsel en in die landbou plaas as gevolg van 'n toename in siektelas in die mens.

DEDICATION

I dedicate this thesis to my wife Coretta Maame Panyin Jonah and my son Zaffery Nii Doddoo Hammond-Aryee, with whose support this piece of work has come to fruition.

To these two I say, “The race is never for the swift but for those that are able to persevere”

I am eternally grateful for all your support and unconditional belief in me.

To my father I say even though we lost you before the journey ended, I believe you will always be with us and I also am eternally grateful for all you taught me. Rest in perfect peace.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AF	Allele frequency
APICO	Apical complex
ARV	Antiretroviral
BLAST™	Basic local alignment search tool
Bp	Base pair
BTUB	Beeta tubulin
C	Cytosine nucleotide base
CI	Confidence interval
CD4	Cluster of differentiation 4
CFT	Complement fixation test
Cm	Centimeter
CSF	Cerebrospinal fluid
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanidine triphosphate
dTTP	Deoxythyimine triphoshpate
dNTP	Deoxyribonucleotide triphosphate

EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	gram
G	Guanine nucleotide base
gDNA	Genomic DNA
h	Hour
HAART	Highly active antiretroviral therapy
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HFF	Human foreskin fibroblast cells
IFA	Indirect fluorescent antibody assay
IFL	Indirect immunofluorescence assay
GRA	Dense granule protein
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kDa	Kilo Dalton
Kg	Kilogram
LD	Linkage disequilibruim

M	Molar
MgCl2	Magnesium chloride
MGE	Mobile genetic element
MIHS	Mother infant health study
Min	Minute
Ml	Milliliter
MLST	Multi locus sequence typing
mM	Millimolar
mRNA	Messenger RNA
NCBI	National centre for biotechnology information
NHLS	National health laboratory services
Nm	Nanometer
NPV	Negative predictive value
OD	Odds ratio
^{o}C	Degree celcius
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PK	Proteinase K
PK1	Protein kinase 1
PPV	Positive predictive value
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROP	Rhoptry protein

RPM	Revolutions per minute
s	Second
SAG	Surface antigen protein
SNP	Single nucleotide polymorphism
TAE	Tris/acetate/EDTA
Taq	*Thermos aquaticus*
TBE	Tris/borate/EDTA
TE	Toxoplasmic encephalitis
T. gondii	*Toxoplasma gondii*
Tm	Melting Temperature
TMP-SMX	Trimethoprim plus sulfamethoxazole
ToxoDB	*Toxoplasma gondii* genome database
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µm	Micrometer
V	Volts
WHO	World health organization
%	Percentage
/	Per

CHAPTER 1 INTRODUCTION AND REVIEW OF LITERATURE

1.0 INTRODUCTION

Toxoplasma gondii is a facultative heteroxenous parasite belonging to the Apicomplexan phylum, the class Sporozoasida and the Coccidiasina subclass (Hutchison *et al*., 1970). *Toxoplasma gondii*'s definitive hosts in which sexual reproduction occurs, are strictly members of the felidae family only, but it is able to infect almost all homoeothermic vertebrates, including humans (Tenter *et al*., 2000). The pathogen can cause severe disease in both its definitive and intermediate hosts.

Toxoplasma gondii has a wide range of hosts, and an apparently global distribution. It also has the ability to maintain an apparently benign co-existence with whichever host it infects, thereby maintaining a latent infection. The above listed characteristics have led *T. gondii* to be regarded by some researchers as one of the most common parasites in the world (Carruthers, 2002). Even though *T. gondii* has a global distribution, its prevalence varies widely across different continents and countries and also amongst the different regions of individual countries. Recently *T. gondii* infection has been discovered in some marine wildlife species, specifically sea otters, indicating the presence of the parasite in seawaters and confirming its extremely wide distribution as well as host variability (Conrad *et al*., 2005).

Toxoplasmosis, the disease caused by *T. gondii*, is one of the most commonly occurring and widespread parasitic infections known to man. The pathogen is thought to infect over half a billion of the global population are thought to be currently infected, based on

antibody detection tests for *T. gondii* (Dubey, 2008). Over the years, *T. gondii* has become one of the most well studied parasites in the scientific world, mainly because of its medical and veterinary significance arising from its ability to cause congenital infections as well as abortions in its hosts, and also its appropriateness as an organism for research at the microbiological level (Weiss and Kim, 2000). Interest in toxoplasmosis and its pathogenesis has re-emerged within the current human immunodeficiency virus (HIV) era, particularly since it was discovered that with the onset of the acquired immunodeficiency syndrome (AIDS) epidemic, opportunistic infections such as acute toxoplasmosmic encephalitis and the ensuing cranial calcification can be fatal if not treated in some AIDS patients (Bhopale, 2003).

1.1 DISCOVERY

In 1908, Nicole and Manceaux discovered an organism in the rodent, *Ctenodactylus gundi,* whilst studying leishmaniasis at the Pasteur Institute in Tunis, North Africa. The organism was firstly considered to be a piroplasm, and then was suspected to be a different form of *Leishmaniasis*, but it was later proved that it was distinctly different and therefore an unknown organism. Subsequently, it was named *Toxoplasma gondii* due to its structural appearance, ”toxo" meaning crescent or bow and "plasma" meaning life (Ajioka and Morrissette, 2009). Nicolle and Manceaux had actually mistakenly identified the host of this new organism as *Ctenodactylus gondi* thus naming their discovery *T. gondii* as opposed to *T. gundi* (Weiss and Kim, 2000).

Splendore in 1908 also reported the discovery of the same organism in a rabbit in Brazil, South America. The *gundi* normally inhabit the foothills and mountains in the south of

Tunisia, and since *T. gondii* was found in their bloodstream the researchers hypothesized that the parasites were being transmitted via arthropods which occurred commonly in this habitat. In investigating the mode of transmission of the pathogen, Woke et al. (1953), in Tunisia and other researchers in the USA investigated the role played by various arthropod species in the spread of *T. gondii* with fundamentally no repeatable results. Researchers around that time therefore reported that the *gundi* did not acquire the infection in their natural habitat but instead hypothesized that they had acquired the *T. gondii* infection in captivity (Frenkel, 1970). During the course of the following thirty years, organisms similar to *T. gondii* were discovered in numerous different host species, including in avian species and humans (Dubey, 2002). Viable *T. gondii* in animal species were described by Sabin and Olitsky in 1937 and demonstrated to be the same organism as that identified in humans via the use of cross protection studies (Sabin and Olitsky, 1937).

Janků (1923) described the first human case of *T. gondii* infection in 1923 when he observed tissue cysts in an 11-month-old child's retina in Prague, Czech Republic. Subsequently, Wolf et al. (1937) successfully isolated the parasite from tissues of a neonate with encephalitis, through animal inoculation (Frenkel, 1970; Cox, 2002). This served to be the first example of an organism causing disease *in utero*. Fatal toxoplasmosis, in adults a rare disease especially at the time with its associated rash, pneumonia, encephalitis, myositis, myocarditis and hepatitis was then described by Kass et al (1952). Sabin and Feldman in 1948 had then described the human immune reaction to *T. gondii* infection as comprising inborn as well as adaptive immune mechanisms (Sabin and Feldman, 1948). The human immune protective response to a *T. gondii*

infection discovered later is mainly directed by cells of the lymphatic system (Frenkel, 1967; Suzuki *et al*., 1988). Until 1969, only asexual stages of *T. gondii* were known, the sexual cycle in the definitive host and the environmentally resistant forms of the parasite were discovered in 1970 (Dubey *et al*., 1970).

1.2 ULTRA-STRUCTURE

There are three infectious stages of *T. gondii,* which are linked together in an intriguing lifecycle (Frenkel, 1973). The tachyzoite (derived from "tachos" meaning speed in Greek) is the rapidly dividing form of the parasite and was initially referred to as the trophozoite (derived from "trophicus" meaning feeding in Greek). The tachyzoites have also been referred to as endodyzoites and in some instances endozoites. The bradyzoites (derived from "brady" meaning slowly in Greek) a term initially used by Frenkel (1973) are the slowly multiplying form of *T. gondii* found in the cysts in tissues and also referred to as cystozoites and a third form, the sporozoites that occur in oocysts (Jewell *et al*., 1972). The encysted forms of the parasites, which contains the bradyzoites and are found in the host tissues were described as tissue cysts in order to differentiate them from the oocysts, which contain the sporozoites and are shed by the definitive host (Frenkel *et al*., 1970). There is no certainty as to who first discovered or identified the encysted forms of *T. gondii*, but the ability of *T. gondii* to exist in an encysted form for many months in tissues was documented by Laison (1958).

Tachyzoites

Gustafson et al. (1954) were the first to study the ultra-structure of the tachyzoite. The tachyzoite (Figure1.1) is normally crescent shaped, about 2 by 6 µm, with a narrowed anterior (conoidal) end and a rounded posterior end, the nucleus is situated centrally to the cell and mostly contains chromatin and a centrally positioned nucleolus. Tachyzoites can be observed to aggregate into groups or clones. This stage of the parasite is directly associated with the acute phase of the disease (de Melo and de Souza, 1997).

The tachyzoites can multiply and kill virtually all types of host cells of warm blooded animals and can also cross the placenta in certain species of intermediate hosts. Tachyzoites have no visible means of locomotion but have been observed to glide and flex and in some instances rotate and undulate within the host tissues. There are no differences in mechanisms of locomotion for tachyzoites, bradyzoites and sporozoites. Tachyzoites penetrate the host cell by actively boring through the plasmalemma or by a phagocytic process. Their growth rates as well as ability to invade the host vary from one strain of the parasite to the other and also from one host cell type to the other (Kaufman and Maloney, 1962). The tachyzoites are known to divide and grow asexually in the host cell via endodyogeny, a type of division in which two daughter organisms develop from the parent organism whilst consuming the parent cell in the process. Tachyzoites are similar in ultra-structure to the bradyzoites of *T. gondii* (Sheffield and Melton, 1968).

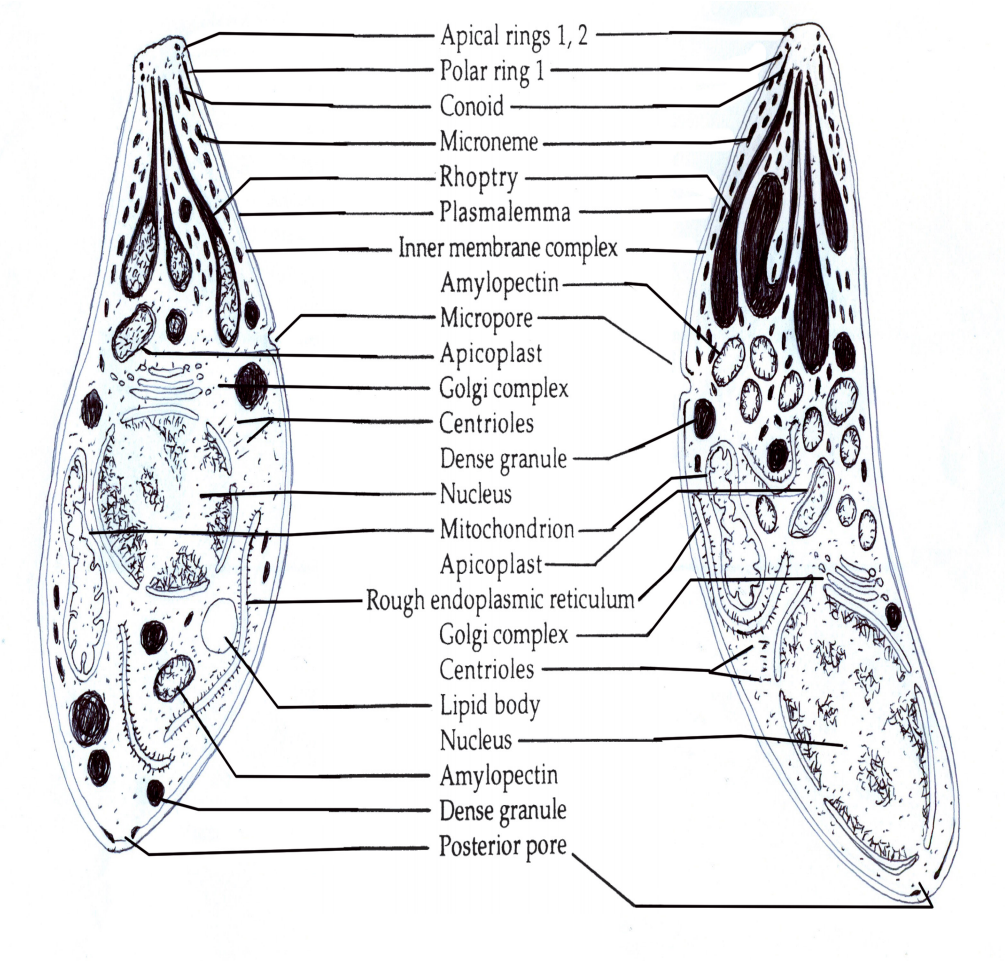

Figure 1.1 Schematic drawings of *Toxoplasma gondii* tachyzoite (left) and bradyzoite (right).

Notes: Drawings are composites of electron micrographs redrawn from Dubey et al. (1998).

Bradyzoites and tissue cysts

Bradyzoites (also known as cystozoites), (Figure 1.1) are the slowly multiplying forms of the organism within the tissue cysts. Dubey and Frenkel carried out the initial detailed studies on the tissue cysts and bradyzoites in 1976 and they described in detail the ontogeny and morphology of the tissue cysts and the bradyzoites (Dubey and Frenkel, 1976).

The bradyzoite differs very slightly from tachyzoites in terms of structure; the nucleus is slightly more posterior and the contents of their rhoptries are usually electron dense and are known to vary with the age of the tissue cyst. Bradyzoites are slighter in shape than the tachyzoites and are also more resistant to digestion by host proteolytic enzymes compared to tachyzoites. Bradyzoites have been documented to be slow dividing or dormant in nature within the tissue cysts, but more recently research has shown that they actually have the ability to replicate actively within the tissue cysts in both sporadic as well as synchronous manner (Watts *et al*., 2015). The tissue cysts (Figure 1.2) remain within the tissue cell cytoplasm as the bradyzoites housed within them multiply via endodyogeny.

The cysts within the tissues are known to differ in size, with immature ones having diameters as small as 5 µm and containing only one bradyzoite, whilst more mature ones may contain numerous organisms with markedly larger diameters (Dubey and Frenkel, 1976). Tissue cysts (Figure 1.2) occur more commonly in the tissues of the muscles and the neurons, particularly the brain, heart and the eyes but can also occur in tissues of the lungs, kidneys and liver. Tissue cysts in the brain can reach sizes of up to 70 µm in diameter and are spheroid in shape whilst those found in the muscles are elongated in shape and can attain sizes of up to 100 µm in diameter. Intact cysts are probably harmless to the host and can remain in the host throughout its lifetime without causing any inflammatory reaction (Dubey *et al*., 1998). The tissue cyst has an elastic and thin wall (<0.5 µm in diameter) and it encloses numerous bradyzoites. The tissue cysts are normally found in the host cell cytoplasm and their cell walls are constituted of both host

and parasite material, which is granular and also occurs in the gaps between the bradyzoites thereby acting as a spacing material (Ferguson and Hutchison, 1987).

Sporozoites and oocysts

Toxoplasma gondii oocysts were firstly discovered in the feces of the cat in 1970, and oocyst shedding by the definitive hosts was included in the description of the lifecycle of the parasite (Dubey and Frenkel, 1976). When unsporulated, oocysts are spherical in shape and are 10 µm to 12 µm in diameter. Under the light microscope, the oocysts are observed to be filled with the developing sporozoites. The oocyst has a cell wall consisting of two colourless layers and has no polar granules, a micropyle can also be clearly seen in the ultra-structure. Sporulation occurs within about 1 to 5 days of the oocyst being excreted into the environment, depending on humidity, temperature and aeration conditions in the environment. Each sporocyst contains four sporozoites (Speer and Dubey, 1998).

Sporozoites appear similar in ultra-structure to the tachyzoite but have fewer rhoptries, micronemes and amylopectin granules. Sporozoites are normally 6 to 8 µm in diameter with no crystalloid or refractile bodies and a subterminal nucleus (Figure 1.3). *Toxoplasma gondii* sporozoites are infectious to both the definitive and intermediate hosts. In either host type they are responsible for initiating the intermediate host cycle. The sporozoites invade the host cells and occupy a temporary parasitophorous vacuole within which they differentiate into tachyzoites (Jerome *et al*., 1998; Dubey *et al*., 1998).

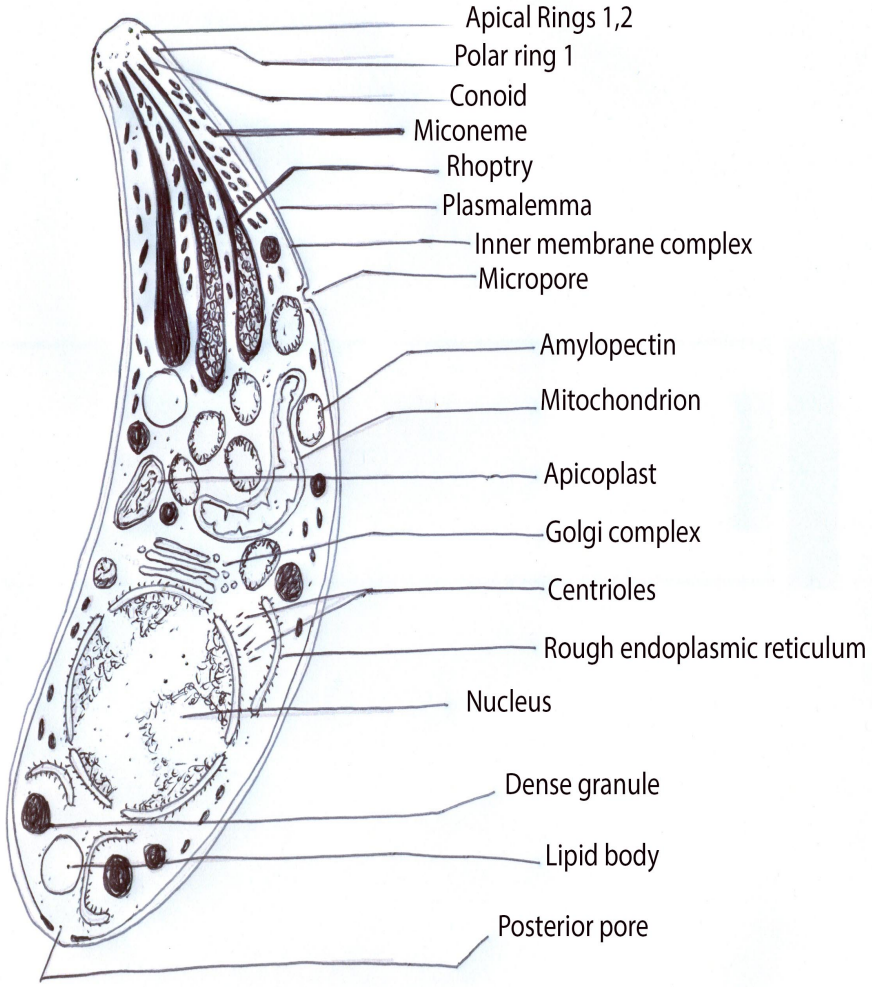

Figure 1.2 Schematic drawing of *Toxoplasma gondii* sporozoite.

Notes: Composites of electron micrographs redrawn from Dubey et al. (1998).

1.3 LIFE CYCLE

The complete life cycle of *T. gondii* was understood in its entirety in 1970 with the detection of the sexual stages in the small intestine of the felid (Dubey *et al*., 1970) (Frenkel, 1970). The *T. gondii* life cycle consists of two independent cycles, intersecting at the formation of the intracellular tachyzoites (Figure 1.4). Asexual replication can

occur in any nucleated animal cell, and consists of the following four steps (Roos *et al*., 1994).

1. Attachment to the host cell via ligands including laminins.

2. Invasion into a specialized parisitophorous vacuole. The mechanism of invasion and precise origin of the vacuole is not known but the process is usually associated with clearing of one or more of the rhoptries that are specialized organelles found at the apical section of the parasite.

3. Multiple rounds of replication within the parasitophorous vacuole via endodygyny leading to the production of two daughter parasites from each division, and the destruction of the parent organisms. All parasites within a parasitophorous vacuole divide synchronously leading to an exponential multiplication in the number of parasites.

4. Host cell lysis and parasite exit facilitated by twisting motility of the parasite. Motility of the parasite during exit contrasts with the quiescence during replication and is probably induced by changes that occur as the host cell membrane starts to degenerate under pressure from the expanding parasitophorous vacuole caused by the rapid multiplication of the parasites (Endo *et al*., 1987; Endo and Yagita, 1990)

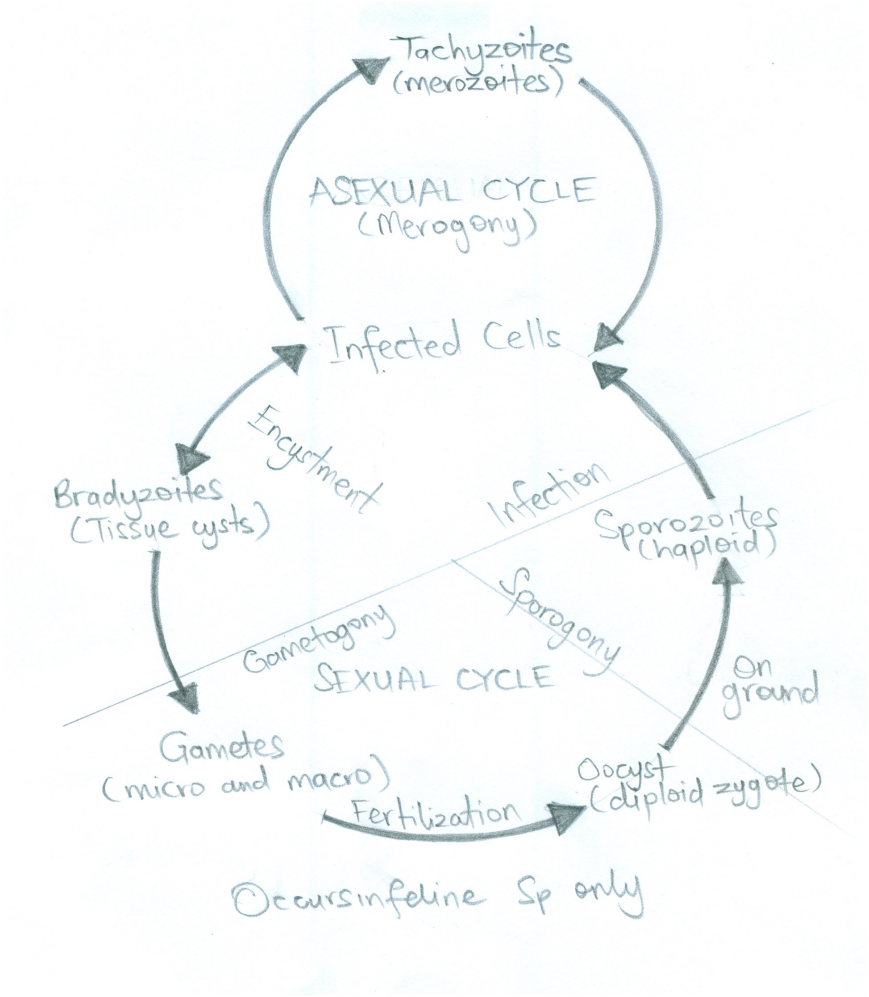

Figure 1.3 A schematic representation of the sexual and sexual stages of *Toxoplasma gondii* lifecycle.

Notes: Figure redrawn from Roos et al. (1994), showing both asexual stages (infected hosts) and sexual stages (only in felid host) of *T. gondii.*

Within tissues of the host, tachyzoites differentiate into a more slowly replicating form termed bradyzoites, the bradyzoites then develop a periodic acid schiff-positive wall when mature. In their matured state, bradyzoites are able to persist within the host tissues hidden away in tissue cysts for months or years. Bradyzoites re-emerge periodically to provide a natural boost to host immunity or cause re-infection and this phenomenon is thought to account for the high frequency of latent infection in human populations

worldwide. Bradyzoites represent a serious danger to immunocompromised individuals (Luft and Remington, 1992; Roos *et al*., 1994).

In contrast to the broad host range of asexual parasite forms, sexual differentiation is known to occur in feline species only (Frenkel, 1973). When bradyzoites invade intestinal epithelial cells (following ingestion of infected prey), the parasites can differentiate into macro- or microgametes. The microgametes rupture out of infected cells and fuse with macrogametes to produce diploid oocysts, which then develop a thick impermeable wall. When exposed to oxygen and ambient temperature, the oocyst undergoes sporogony consisting of two meiotic divisions and a single mitotic division, which produce eight haploid sporozoites localized in two sporocysts within the oocyst wall. Sexual crosses can be carried out in cats under laboratory conditions, but although bradyzoite cysts have been observed in culture it has not yet been possible to reproduce the complete *T. gondii* sexual cycle *in vitro* (Lindsay et al. 1991; Roos *et al*., 1994)

1.4 *TOXOPLASMA GONDII* GENOTYPE DESIGNATION, GLOBALIZATION AND POPULATION STRUCTURE

The determination of *T. gondii* genotypes can provide much useful information for epidemiology studies and help understand pathogenesis. These genotyping methods include a microsatellite analysis based method, a multi-locus sequence typing, a PCR-restriction fragment length polymorphism based method and a random amplification polymorphic DNA-PCR based methodology, all of which will be discussed below.

Toxoplasma gondii genotype designation via microsatellite (MS) analysis is based on the analysis of short tandem repeat motifs of DNA called microsatellites which occur at

random in the eukaryote genome. The repeats may occur as many as 2 to 20 times and the short repeat units are normally up to two base pairs in length. These short DNA repeat units are highly variable due to ongoing insertions and deletions of repeat units. The numbers of repeat units therefore differ in a population and this gives rise to multiple alleles at any microsatellite locus (Ajzenberg *et al* 2002a; Liu *et al*., 2015). A system for determination of *T. gondii* genotypes using 15 microsatellite loci in a single reaction has been developed (Ajzenberg *et al*., 2010). This method has gained in popularity due to its ease of application and high specificity. Multilocus locus sequence typing (MLST) works best when there is enough starting DNA material for genotype designation. Under such circumstances, it is one of the best techniques for resolving differences in *T. gondii* strains. This method focuses on sequence polymorphisms in the DNA sequences including single nucleotide polymorphisms (SNPs), as well as deletions and insertions of nucleotides (Khan *et al*., 2006). However, this method is not suitable for genotype determination directly from clinical samples due to the fact that it requires comparatively large quantities of starting *T. gondii* DNA.

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) is a genotyping technique based on the ability of restriction endonucleases to digest specific sections of the DNA. The digested DNA when amplified by PCR and electrophoresed shows clear distinct bands on the gel and these bands are unique for each genotype of *T. gondii* (Howe and Sibley, 1995). This technique also relies on single copy polymorphic sequences and therefore needs a large amount of starting DNA material in order to give accurate results. An improvement on the method is a multiplex multilocus nested PCR-RFLP technique based on 10 genetic markers including SAG1, SAG2, SAG3, BTUB,

GRA6, c22-8, c29-2, L358, PK1 and Apico. This method is highly sensitive and has currently been used to generate large amounts of data on *T. gondii* genotypes and their distribution globally (Su *et al*., 2012). However, a major drawback is the ease with which the system can be contaminated leading to incorrect genotype assignments, due to its high level of sensitivity.

Another PCR based technique is that of random amplified polymorphic DNA-PCR (RAPD-PCR) (Williams *et al*., 1990). This DNA polymorphism assay is based on restriction fragment analysis of randomly amplified DNA using arbitrary primers. It is highly sensitive and requires only small amounts of highly pure DNA as starting material, which therefore limits its use in *T. gondii* genotyping directly from biological material (Guo and Johnson, 1995).

Despite its apparent global distribution and ability to infect nearly all-mammalian and avian species, the genetic variability of *T. gondii* was originally thought to be low and geographic variations almost absent. Based on initial genetic variability studies, the structure of *T. gondii* populations was thought to comprise three predominant clonal lineages, denoted Types I, II and III on the basis of the strain virulence in mice. This population structure was also thought to be highly conserved and recombination amongst the three clonal lineages was thought to be rare (Howe and Sibley, 1995; Lehmann *et al*., 2006).

Recently, it has been shown that sexual recombination in *T. gondii* strains, especially in South America, does play a role in the development of the genetic structure of *T. gondii* although it is rare. There are minor genetic variations in *T. gondii* population from Africa,

Eurasia and North America, but large variations in *T. gondii* populations from South America (Khan *et al*., 2011). There have been different genotypes of *T. gondii* reported from various geographical locations according to the literature and there are dominant genotypes in the various regions from which typing studies have been reported (Table 1.1). The data available, suggests a South American genesis of *T. gondii* species, followed by two possible migrations: the first from South America to Eurasia via bird migration and the second via long distance migration to other continents via rats, possibly in slave ships (Lehmann *et al*., 2006).

Human toxoplasmosis is predominantly caused by the Type II strains, but strains of Types I and III have also been isolated in human clinical cases of toxoplasmosis and also these human infections have been shown to have varying degrees of severity. Research findings suggest that, as with mice, at least part of this variability in severity of the human infections can be ascribed to the strain type that causes the infection, (Weiss and Kim, 2007). Recently, two new genotypes, types A and X of *T. gondii*, have been discovered in sea otters in the USA (Miller *et al*., 2004; Conrad *et al*., 2005; Sundar *et al*., 2008). Two other haplogroups, *Africa* 1 and *Africa* 3 have also been reported in *T. gondii* isolates from animals in Gabon (Mercier *et al*., 2010).

Currently a number of studies are ongoing to properly understand the genetic diversity of *T. gondii* as well as its association with disease presentation and geographic distribution. These studies will enable the development of a proper classification of the parasite strains and hence eliminate the uncertainty that exists in the present reported population structure and genetic variations in the *T. gondii* genome (Robert-Gangneux and Darde, 2012).

Table 1.1 *Toxoplasma gondii* genotype distribution in different geographic location from Mercier et al (2010).

Geographic Location	**Reported genotypes/Haplogroups**
Asia	Types I, II, III (haplogroup 3), Chinese 1
America (North)	Type III (haplogroup 3); haplogroup12; Type II (haplogroup 2)
America (South and Central)	Type II; Type III; African (haplogroup 6), other atypical genotypes
Europe	Type III; Type II (haplogroup 2); other genotypes reported
Africa	Type II; Type III (haplogroup 3); African 1,2,3 (haplogroup 6), atypical

Notes: There is a dominance of Type III (haplogroup 3) genoypes in Asia, whislt North America shows a dominance of Type III (haplogroup 3), haplogroup 12 and Type II (haplogroup 2), South and Central America on the other hand show a predominance of Type 1, Type II, African (haplogroup 6) and other stypical species. Europe has a dominance of Type III, Type II (haplogroup 2) and Africa shows a dominance of Type II, Type III (haplogroup 3), and African 1,2,3 (haplogroup 6).

The strains generally fall into one of two extremes, highly virulent type 1 strains, with an LD100 (the dose of infection at which 100% of murine hosts are killed) of one (single) parasite and mildly virulent to avirulent types 2 and 3 strains, with an LD100 of several thousands. Although the degree of difference between type 1 and types 2 or 3 in murine infection (mouse phenotype) is dramatic, there is no guarantee that the differences observed in mice will also be similar to those in other hosts. Virulence is strictly contextual and a strain that causes severe disease in one host may be benign in another one (Howe and Sibley, 1995).

The reasons for the differences in disease presentation or parasite virulence in different subjects are currently unknown, but research suggests these differences may be as a result of parasite strain characteristics, host variability, host genetics or other factors which are yet unknown. The majority of exposed human adults and animals do not develop clinical symptoms. The association between clonal ancestry and human *T. gondii* infections may have implications for drugs development, (treatment or prophylaxis) vaccines for prevention of infection as well as effective protocols for diagnosis of the toxoplasmosis (Sibley and Boothroyd, 1992; Dubey, *et al*., 1998).

1.5 MODES OF TRANSMISSION

Toxoplasma gondii has over its years of existence acquired an impressive and broad spectrum of transmission mechanisms. However even with the existing knowledge of these known mechanisms of transmission, it still has not been possible to establish the epidemiological importance or significance of the different mechanisms. Even though there has been much research on the vertical transmission of the parasite (congenital or trans-placental transmission) there is not much known about the horizontal transmission of the parasite, as well as the main stores of the organism in nature and the epidemiological significance of the various infection sources in human as well as animal populations (Tenter *et al*., 2000; Dubey *et al*., 2014). Knowledge of the most probable routes of horizontal transmission of the parasite within the human population and also on the most likely source of infections in a given populace are required for the design, development and implementation of appropriate schemes and programs for the prevention of infection in groups that are at risk. These are, for example, immunocompromised individuals such as patients undergoing immunosuppressive

treatments or chemotherapy, patients suffering from AIDS and tuberculosis and pregnant women, whether immune compromised or not.

The mechanism or mechanisms by which *T. gondii* infects man and animals was not understood for a long time. This situation begun to change when in 1956, Weinman and Chandler suggested that *T. gondii* could be spread via a meat-to-man route and demonstrated the presence of infectious *T. gondii* cysts in diaphragms of sheep and swine. They further explained how carnivorous animals become infected but they believed that there must be other routes of infection besides the congenital and meat-eating routes since herbivores and human populations that eat little or no meat may also show a high incidence of seropositivity to the parasite (Weinman and Chandler, 1956; Smith, 1993). With the discovery of the infectious oocyst form of *T. gondii* in the feces of cats, it was then possible to show that herbivorous animals and vegetarians could be infected by *T. gondii* through inhalation of oocysts from dried cat feces or by ingesting water or food contaminated with oocysts from the feces of infected felids (Frenkel, 1973).

Congenital infection from tachyzoites crossing the placental barrier when a female acquires a primary infection during pregnancy, carnivorous ingestion of bradyzoites in tissue cysts and environmental exposure to oocysts are the three major routes of transmission of *T. gondii* infection in humans. However, the relative contribution of the various sources of *T. gondii* infection to the overall prevalence of the disease in specific human populations is unknown and currently there are no laboratory means of identifying the particular infectious stages of the parasite that are responsible for a particular infection; that is whether it is from ingestion of an oocyst or from infected meat or food

or water. In the USA, the current increase of infection in teenagers and the low levels of sero-prevalence in children suggest that parasite transmission by consumption of meat is of some significance (Dubey, 2008).

Despite lack of absolutely clear quantitative evidence, the most important source of infection or route of transmission of the parasite in humans and animals is probably the bradyzoite-containing tissue cysts of *T. gondii*. These cysts develop as early as 6 to 7 days post-infection via ingested tissue cysts or oocysts and they are believed to remain throughout the life of the host. It is worth noting that the parasitized locations or areas within any particular intermediate host as well as the proliferation of tissue cysts within the intermediate hosts differ from one intermediate host to the other. *Toxoplasma gondii* tissue cysts occur frequently in the tissues of animals such as sheep, goats, pigs, rabbits when they become infected, but not frequently in tissues of infected poultry, horses and dogs (Dubey, 2008). Although cattle and buffalo may demonstrate high titers of antibodies to *T. gondii,* demonstrating the existence of past infections or exposures to infection, their tissues rarely contain tissue cysts of *T. gondii* (Tenter *et al*., 2000, Dubey *et al*., 2008). Game animals have been observed to demonstrate high sero-prevalence rates for *T. gondii* antibodies, with hares, cervids and black bears, kangaroos, wild boars and also white tailed deer demonstrating high prevalence levels. In the same study done in the USA, *T. gondii* antibodies were present in 80% and 60% of black bears and raccoons, respectively (Dubey *et al*., 2011).

Infected felids are known to shed millions of oocysts into the environment through their feces, which then develop into sporulated cysts and these cysts in the environment become potential sources of parasite transmission amongst humans and other

intermediate host populations. Toxoplasmosis is virtually absent in attolls and small islands which are uninhabited by felids (Wallace, 1969). These observations highlight the epidemiological significance of the felids and the oocysts they shed, as major vectors of toxoplasmosis.

In addition to the bradyzoite and oocyst infective stages discussed above are the tachyzoites, *T. gondii* tachyzoites are vital to the vertical transmission route of *T. gondii*, although they are killed very quickly outside the host due to their extreme sensitivity to external environmental conditions. Even though they may occur in limited numbers outside the host, because of their low viability, horizontal transmission of the parasite via tachyzoites is unlikely to be epidemiologically significant (Tenter *et al*., 2000). Tachyzoites and tissue cysts have been implicated in infections through transplantation of kidney, heart, bone marrow and liver tissues. Tachyzoites are also known to be transmitted via the leukocyte fraction of whole blood products and can also be transmitted horizontally in the laboratory through accidental injection (Siegel *et al*., 1971).

In addition to blood, milk fractions from goats, sheep, cows and camels have also been observed to contain *T. gondii* tachyzoites and there have been reports of infection via consumption of unpasteurized goats milk and also in a breastfed infant with a mother known to have acquired a primary infection with *T. gondii* (Sacks *et al*., 1983; Bonametti *et al*., 1997). *Toxoplasma gondii* tachyzoites have been observed in other body fluids of humans and animals, such as saliva, urine, semen, sputum and tears, although currently there have been no reports of the parasite being transmitted through these fluids in humans or animals. The predominant thinking is that parasite transmission horizontally

amongst humans and animals is via active or passive ingestion of the persisting stages of the organism (namely the oocysts from feline feces) in contaminated food or water and tissue cysts in infected (contaminated) meat or offal (Tenter *et al*.,2000). Transmission has also been demonstrated to occur during the handling and evisceration of infected animals (Weiss and Kim, 2007).

Sources of *T. gondii* infections in human populations vary, as can be seen from acute outbreaks of toxoplasmosis from various parts of the world. These variations are sometimes due to eating and cultural habits of the different human populations and most of the reports documented indicate that such acute toxoplasmosis outbreaks have originated from a single point or source of infection (Choi *et al*., 1997) Recently the likelihood of transmission of the disease via contaminated water sources has come to the fore (Smith, 1993), and this has serious implications for underdeveloped countries, especially in Africa, where the lack of clean potable water sources is a huge problem. The implications of toxoplasmosis as a waterborne disease are far reaching and hence environmental and epidemiological studies need to be intensified to properly understand how the parasite circulates within the ecosystem in order to design accurate interventions to curb an otherwise likely surge in disease prevalence.

1.6. PATHOGENESIS

No more than a small proportion of exposed adult humans or animals actually develop clinical toxoplasmosis. Experimentally, irrespective of the dosage orally administered, infections induced by oocysts (in animals) tend to be more severe than those induced by bradyzoites and tissue cysts (Dubey and Frenkel, 1976; Bhopale 2003). The outer wall of

oocysts or tissue cysts is digested by enzymatic activity within the host and the sporozoites and bradyzoites are released. These then invade the intestinal lumen of the host, rapidly occupy and proliferate within the cells and mature into tachyzoites, which migrate to the other organs of the intermediate host through the lymphoid system or bloodstream. Tachyzoites can infect all cells that have nuclei, and rapidly multiply thereby causing total destruction. With the onset of the host immune reaction, the parasites are then transformed into tissue cysts within the host tissues resulting in a lifelong infection (Bhopale, 2003).

1.6.1 CLINICAL CHARACTERISTICS

Typical clinical charactristics of a *T. gondii* infection is known to depend on the interplay of a number of factors. These include but are not limited to the type of host being infected, the virulence of the parasite causing the infection, the developmental stage of the organism ingested (that is tachyzoite, tissue cyst or bradyzoite), the number of parasites ingested, as well as the immune state of the host being infected (Dubey, 2008).

1.6.1.1 TOXOPLASMOSIS IN THE IMMUNOCOMPETENT HUMAN HOST

In the majority of people with competent immune systems, infection after birth is asymptomatic. However, a minority of apparently healthy persons who acquire primary *T. gondii* infection after birth may exhibit mild symptoms such as fever and lymphadenopathy. These symptoms last for only a few weeks and subsequently disappear. The infection progresses from an acute stage to a chronic or latent stage in the host. Once a primary infection is acquired, the infection is regarded to last throughout the individual's lifetime. Infected individuals generally remain asymptomatic unless they

become immunosuppressed, which might then lead to a reactivation of the apparently latent infection (Dubey and Jones., 2008). However in certain unusual cases, previously healthy persons have been known to acquire and develop severe and sometimes fatal toxoplasmosis, with pulmonary and multivisceral clinical presentations possibly from more potent form of the parasite (Demar *et al*. 2007). Up to 2% of healthy subjects who become infected with *T. gondii* in the USA develop ocular complications, leading to disease and severe visual impairment or blindness, most often retinochoroiditis. This proportion is even higher in some countries such as Brazil, where 17.7% ocular toxoplasmosis prevalence has been reported in a study of 1,042 individuals in Southern Brazil (Glasner *et al*., 1992; Dubey and Jones, 2008).

1.6.1.2 CONGENITAL TOXOPLASMOSIS

Congenital toxoplasmosis is said to occur when a mother is able to transmit a previously acquired *T. gondii* infection to the fetus *in utero*. This occurs normally when the mother has an acute *T. gondii* infection just before or during a preganacy, even though in some rare instances a mother carrying a latent *T. gondii* infection is able to transmit the infection to the fetus due to the reactivation of the latent infection brought about by severe immune suppression in the mother (Mitchell *et al.,* 1990; Remington, 2006). The risk of transplacental transmission of the infection to the developing fetus is relatively low when maternal primary infection happens during the first three months of the pregnancy (10-15%) and is highest when the primary infection occurs during the final three months of the gestation period (60-90%)(Remington, 2006). However congenital infection is generally more severe if acquired during the first trimester of pregnancy than if acquired during the second or third trimesters of pregnancy.

Congenital toxoplasmosis affects the development of the fetus very severely and it leads to spontaneous abortions, stillbirths, or live infants with hydrocephalus or microcephalus, retinochoroiditis and cerebral calcifications. Some newborns have been known to demonstrate central nervous system involvement and complications. In some healthy appearing children, a congenital infection leads to retinochoroiditis, chorioretinitis and central nervous system complications later in life. Most children are born asymptomatic but may develop health complications as they grow (Dubey and Jones, 2008). Despite various screening programs for congenital toxoplasmosis in a number of European countries, the effectiveness of prenatal treatment for congenital toxoplasmosis on the health outcomes of the offspring is currently not known (SYROCOT (Systematic Review on Congenital Toxoplasmosis) study group *et al*. 2007).

Research has shown that ocular toxoplasmosis infection is an important cause of chorioretinitis worldwide. In the USA it accounts for approximately 30-50% of all cases of posterior uveitis and hence it is an important cause of blindness and visual impairment. Patients begin to show signs of disease from the second decade of life, which include lesions in the eyes due to cyst rupture, which leads to visual complications. Chorioretinitis is characteristically bilateral in patients with congenitally acquired infection, but more often unilateral in individuals with recently acquired toxoplasma infection (Roberts and McLeod, 1999).

1.6.3.1 TOXOPLASMOSIS IN THE IMMUNOSUPPRESSED HUMAN HOST

Toxoplasmosis as a result of immunosuppression is more often than not due to a reactivation of a (latent) chronic infection stemming from the persisting immune depression and manifests primarily as toxoplasmic encephalitis (TE) (Luft *et al*., 1983; Luft *et al*., 1984), also known as central nervous system toxoplasmosis. Toxoplasmosis is the most common opportunistic infection causing encephalitis or intracerebral lesions in patients with immune deficiencies or complications (Luft and Remington, 1992). Patients at risk include those on immunosuppression treatment due to organ transplants, patients with AIDS and patients with depressed immunity due to chemotherapy or auto immune conditions (Seigel *et al*., 1971). TE has a sub-acute onset and the focal neurologic abnormalities are frequently accompanied by mild bilateral headache, altered mental states and sometimes fever (Renold et al. 1992). Disease progression leads to severe manifestations such as confusion, lethargy, mental state changes, seizures, ataxia and coma, and the outcome is often fatal (Hill and Dubey, 2002).

In the pre HAART (highly active anti- retroviral therapy) era, TE was one of the common causes of death in patients with AIDS worldwide and was known to occur in 3-40% of all patients with acquired AIDS (Jones *et al*., 2003; Lindström *et al*., 2006; Jones and Dubey, 2012). However with the onset of HAART especially in the developed countries, morbidity, mortality and healthcare utilization related to TE has reduced noticeably (Saadatnia and Golkar, 2012).

In developed and developing countries, the occurrence of neurological infections as well as other opportunistic disease in human immunodeficiency virus infected individuals has

significantly decreased with increasing access to HAART. In developing countries with access to HAART, the impact is notably lower compared to the developed countries. This could be due to a myriad of reasons, some being the lack of opportunities for timely treatment of infected individuals as well as the delayed diagnosis of patients with HIV. Neurological complications as a result of TE lead to increased rates of death and disease burden in less developed countries with limited access to HAART (Pereira-Chioccola *et al*., 2009).

1.6.3.2 *TOXOPLASMA GONDII* INFECTION AND ITS INFLUENCE ON HOST BEHAVIOR

Toxoplasma gondii infection and its effect on host behavior has become the focus of research in recent times as outlined below. Specifically in human infections, past research has covered areas such as *T. gondii* infection and its effect on motor abilities, human behavior in general and mental illness (Flegr and Hrdý, 1994).

Rodents, such as rats associated with human dwellings, are important intermediate hosts of *T. gondii*. In brown rats, where toxoplasmosis is a sexually transmitted infection, uninfected and healthy female rats have been demonstrated to become attracted to infected male rats as opposed to uninfected male rats, instead of showing aversion towards such males. This behavior was attributed to the parasite presence in the male, which causes it to become sexually attractive to the healthy females. Hence, the parasite enhances the attractiveness of the male rats thereby influencing mate choice and continuing the chain of infection in the population (Dass *et al*., 2011).

Chronically infected rodents have been observed to demonstrate other changes in their behavior. These rodents are known to lose their inhibition or aversion towards their felid

predators and become attracted to the odor of their felid predators, which results in an increase in predation on infected rodents. In turn, this leads to uptake of the parasite by the definitive host thereby ensuring the sexual phase of the lifecycle and eventual propagation of the parasite. This behavior is highly specific towards feline odors and was not induced by other predators investigated. It also had no observed effects on anxiety and conditioned fear in the infected rodents (Prandovszky *et al*., 2011).

Studies done in the Czech Republic have revealed that latent *T. gondii* infection may have an effect on psychomotor performance as well as personality profiles of infected human individuals, which is consistent with the effects observed in the rodent models (Flegr, 2013). The studies concluded that the presence of the parasite in the central nervous system and the brain is not accidental, but could actually be a deliberate progression of the infection in order to give the parasite access to the behavioral control mechanisms of the host and thereby affect or modify the host behavior to suit the continued propagation of the parasite (Flegr, 2007; Flegr, 2013). The mechanism is not understood. Stibbs. (1985) reported an increase in the global dopamine content in the brain of mice chronically infected with *T. gondii*, whereas concentrations of other neurotransmitters remained unchanged. Similarly Prandovszky et al. (2011) reported the up-regulation of dopamine metabolism in mammalian cells responsible for dopamine regulation when infected with *T. gondii* tachyzoites.

Researchers have reported increased levels of anti *T. gondii* IgG and IgM antibody levels in patients suffering from schizophrenia at the onset of the disease (Yolken *et al*., 2001). It has also been reported that latently infected human females have increased suicidal tendencies, whilst latently infected males tend to become erratic or reckless in behavior

(Carruthers and Suzuki, 2007; Fuks *et al*., 2012). Mothers infected with *T. gondii* have been found to be more likely to indulge in self-directed violence (Pedersen *et al*., 2012). *Toxoplasma gondii* infection has also been associated with bipolar disorder type 1 disease in studies in the USA and France (Hamdani *et al*., 2012; Pearce *et al*., 2012). Fuks et al. (2012) have shown that the mechanism for this is the invasion of dendritic cells and subsequent modification of gene expression in these cells to produce and secrete GABA, a well-known neurotransmitter. In the brain, up-regulation of GABA reduces fear and anxiety and has the potential to lead to self-destructive behavior.

1.6.4 HOST IMMUNE RESPONSE

In the normally immune human host, both cellular and humoral immune systems are known to control *T. gondii* infection, parasite virulence as well as the destruction of infected tissue, the extent of which has been suggested to be dependent on the strain causing the infection. Tachyzoites are known to stimulate macrophages, which then start producing interleukin (IL-12), which in turn activates the natural killer (NK) cells and T cells which then begin to produce interferon-gamma (INF) which is required for initial defence against parasitic infection. Interferon gamma and tumor necrosis factor (TNFα) organize and direct the attempted destruction of the tachyzoites by the macrophages. The combination of these two cytokines leads to the production of free radicals and nitric oxide (NO), which have been observed to be important in carrying out the destruction of the parasites and hence afford protection against infection (Gazzinelli *et al*. 1993; Sher and Sousa, 1998; Bhopale, 2003)

The T cells responsible for the *T. gondii* immune response are largely the CD8+ cells and the CD4+ T cells support these. Immune CD8+ cells from both mice and humans infected with *T. gondii* have been observed to secrete IFN which exhibits in vitro cytotoxicity towards the *T. gondii* infected cells (Gazzinelli *et al.* 1993). Infection with *T. gondii* also leads to the production of anti *T. gondii* specific antibodies of the classes IgG, IgM, IgA and IgE (extracellular) in response to the parasite membrane and excretory antigens. These specific antibodies can destroy the tachyzoites within the extracellular milieu (Yong *et al*., 1991).

1.6.5 DIAGNOSIS

Diagnosis or identification of *T. gondii* infection in humans can be carried out by means of a number of methods, which may be biological (parasite isolation), histological (parasite identification), serological (detection of antibodies against *T. gondii* in blood of host organism) or molecular (detection of *T. gondii* DNA), or by a combination of any of these methods. These will be briefly discussed below. The toxoplasma infection is known to mimic a number of commonly occurring infections hence clinical signs are considered non-specific and therefore are not sufficient for a conclusive or definitive diagnosis (Hill and Dubey, 2002).

1.6.5.1 DIRECT DETECTION OF PARASITE BY HISTOLOGICAL METHODS

Directly observing or identifying parasites in specimens from patients with infection can diagnose clinical toxoplasmosis. Secretion, excretions, body fluids, and tissues are potential specimens for direct observation of parasites. Tachyzoites may be observed as free organisms or within host cells such as leukocytes smeared on microscopic slides

stained with Giemsa. Well- preserved tachyzoites appear to be crescent shaped when stained with Giemsa and are actually known to stain very well, but degenerating organisms may be oval and stain poorly. Tissue imprints stained with Giemsa may reveal *T. gondii* cysts (Hill and Dubey, 2002; Garcia, 2004).

1.6.5.2 PARASITE ISOLATION

Parasites can be isolated with limited success by inoculating patient tissue or body fluids into live mice or tissue culture cells (Dubey *et al*., 2011). Cytopathic effects may be detected on direct examination of cells after 24 to 96 hours in culture. The use of tissue culture for isolation permits more rapid diagnosis than mouse inoculation but may be less sensitive; both methods can be useful for diagnosis of congenital toxoplasmosis (Garcia, 2004).

1.6.5.3 SEROLOGICAL METHODS

Since *T. gondii* organisms are rarely directly detected in humans with toxoplasmosis, serologic examination is used to indicate infection by detecting toxoplasma specific antibodies (Jones *et al*., 2003). Serological tests are widely used, yet they have limitations as they often provide ambiguous results (Kompalic-Cristo *et al*., 2004). There are different serology tests in use currently (Table 1.2), which have their advantages and disadvantages These include indirect fluorescent antibody assay (IFA), latex agglutination test (LAT), complement fixation test (CFT) and the enzyme-linked immunosorbent assay (ELISA) (Hill and Dubey, 2002). The various serology methods are listed in table 1.2 below. The modified agglutination test (MAT) is based on the use of formalin fixed tachyzoites to detect IgG antibodies and has a limitation of giving false

positive results during acute early phase of an infection (Desmonts and Remington, 1980).

The indirect fluorescent agglutination test (IFAT) uses killed whole tachyzoites to capture IgG and IgM antibodies and it is highly sensitive. Commercial versions of the test are available and it is used for *T. gondii* diagnoses in both humans and animals with relative ease (Arthur and Blewett, 1988), however, a major point to note is that it requires a fluorescence microscope to generate results. The indirect hemagglutination test (IHA) works on the principle that *T. gondii* soluble antigen can be used to sensitize red blood cells and these sensitized red blood cells when in contact with positive serum are able to agglutinate. Its major limitation is that it will miss early acute phase as well as congenital infections (Esch, 2010). The enzyme linked immunosorbent assay (ELISA) is also extensively used to test for *T. gondii* serologically and it is based on the principle of enzymatic reactions. The system usually consists of a solid phase antigen or antibody, an enzyme labeled antibody or antigen and a substrate of the enzymatic reaction, which has the flexibility of testing for both antigen and antibody. A major drawback is the absence of a unified inter-laboratory standardization whilst a major advantage is that the system can be automated and therefore large numbers of tests can be carried out at the same time (Turunen *et al*., 1983).

The immunosorbent agglutianation assay (Youssef *et al*., 1992) uses anti-human IgM antibodies to test for the presence of IgM antibodies in infected serum. The test can identify both early phase and congenital infection but it requires an overnight incubation step, which makes it time consuming. Another commonly used test is the latex agglutination test (LAT). The LAT (Trees *et al*., 1989), is based on the use of soluble

antigen coated latex particles and it tests for the presence of both IgG and IgM antibodies. This method is very simple and easy to use it is therefore very useful as an initial screening tool for large-scale epidemiological testing projects, where short turnaround times and large numbers of samples are the expected to be processed. The piezoelectric immunoagglutination assay (PIA), uses a piezoelectric device to detect the agglutination reaction between antigen coated gold nanoparticles and specific IgM antibodies, however, the test is not very common as it requires the use of a piezoelectric device (Wang *et al*., 2004). Although there are commercially available versions of most of these tests it is worth noting that there are always problems relating to inter-laboratory standardization of test results.

Table 1.2 Summary of common methods used in laboratory serologic diagnosis of *Toxoplasma gondii.*

Serological method	Antigens or antibodies used	Antibody/Antigen type tested	Reference
Modified agglutination test (MAT)	Formalin fixed tachyzoites	IgG	Desmonts and Remington. (1980)
Indirect fluorescent antibody test (IFAT)	Killed whole tachyzoites	IgG, IgM	Arthur and Blewett. (1988)
Indirect hemagglutination test (IHA)	Tanned red blood cells sensitized with soluble antigen	IgG	Esch. (2010)
Enzyme-linked immunosbsorbent assay (ELISA)	Tachyzoite lysate antigen, recombinant antigen, specific antibodies	IgG, IgM, IgA, antigens	Turunen et al. (1983)
Immunosorbent agglutination assay (ISAGA)	Anti-human IgM antibodies	IgM	Youssef et al. (1992)
Latex agglutination test (LAT)	Soluble antigen coated with latex particles	IgG, IgM	Trees et al. (1989)
Piezoelectric immunoagglutination assay (PIA)	Antigen coated gold nano particles	IgM	Wang et al. (2004)
Western blotting (WB)	Tachyzoite lysate antigen, recombinant antigen	IgG, IgM	Rilling et al. (2003)
Immunochromatographic test (ICT)	Antigen or antibody labeled with colloidal gold	IgG, ESA	Wang et al. (2011)
Avidity test	Tachyzoite lysate antigen, recombinant antigen	IgG, IgA, IgE	Ashburn et al. (1998) Hedman et al. (1989)

Notes: data presented focuses on the serology methods used for diagnosis of *T. gondii* infection, antigens or antibodies used as well as the antibody and/or antigen type targeted.

In adult humans, antibodies of the classes IgG, IgM, IgA and IgE become detectable within 7-21days post-infection with *T. gondii.* The presence of IgG antibodies indicates exposure to parasite at some unknown time and may imply a chronic infection. The

presence of IgM, IgA and IgE antibodies may indicate an acute or a primary infection (Jones *et al.,* 2003; Garcia, 2004). In a primary *T. gondii* infection, IgM antibodies appear within 7 days of infection, followed by IgA and IgE. Concentrations of these acute phase antibodies peak after about two months and are usually undetectable by serological tests after six to nine months, but can persist for longer periods of time (Montoya and Rosso, 2005; Sukthana, 2006). IgG on the other hand appears within three weeks of an infection and peaks or plateaus at about two to three months post infection in the absence of treatment (Gras *et al*., 2004).

A problem with serological tests is that the detection of antibodies in immune-compromised individuals may be difficult due to the deterioration of the immune system (Schneider *et al*., 1992). A further problem in immunocompromised individuals is that IgM may persist for longer than expected periods and discrimination between recent and older infections may therefore be a problem (Montoya *et al*., 2004). This is an important factor when diagnosing toxoplasmosis in immunocompromised individuals, since the presence of IgG indicates a risk for the reactivation of a latent infection, and IgM indicates the possibility of an acute infection. In pregnant women, positive IgM results indicate the likely acquisition of infection during gestation and a positive IgG and negative IgM result indicates a previous infection (Montoya, 2002).

Antibody avidity tests help to differentiate between recently and distantly acquired infections and hence assist in dating *T. gondii* infections within clinical and research settings. Antibody avidity tests are based on the principle that during acute or recently acquired infections*, T. gondii* specific IgG antibodies bind antigen relatively weakly and therefore are classified as having low avidity. However during chronic or latent

infections, *T. gondii* specific antibodies mature and hence possess stronger antigen binding abilities and therefore they are classified as high avidity anti-*T. gondii* antibodies (Lappalainen and Hedman, 2004).

1.6.5.4 MOLECULAR METHODS

Application of polymerase chain reaction (PCR) technology to detect toxoplasma DNA has proved to be useful in situations for which knowledge of recent infection is essential. PCR detection of toxoplasma in blood, urine, cerebrospinal fluid (CSF), amniotic fluid, ocular fluid and bronchial alveolar lavage is a strong indicator of an acute infection, however the absence of toxoplasma DNA does not exclude toxoplasmosis (Garcia, 2004). Both multiplex and monoplex PCR have proved useful for the identification of *T. gondii* (using specific segments within the *B1* repeat element, and/or *SAG2, ITS, P30, 529* bp repeat element genes as the target sequence) in various kinds of host tissues (Burg *et al*., 1989; Dabil *et al*., 2001). The *B1* gene covers a 2.2 kb region and it is repeated approximately 35-fold in the *T. gondii* genome (Burg *et al*., 1989), whlist the 529 bp target is repeated about 200 to 300 times within the *T. gondii* genome (Homan *et al*., 2000). The *P30* PCR target on the otherhand is a single copy target gene spanning a 957 base pair region of the 32 Kda surface antigen of *T. gondii* (SAG1) and it is therefore less sensitive as a PCR target than the multiple copy gene targets (Burg *et al*., 1988). The 18S rRNA (ITS-1) PCR diagnostic target is similar in sensitivity to the B1 gene target PCR (Calderaro *et al*., 2006) and is repeated approximately within the genome. The *B1* gene target is used more often than the 529 bp repeat element and this is because even though both occur in repeats within the genome, the *B1* gene occurs in all *T. gondii* strains and hence is thought to be reliable, whilst the 529 bp repeat element has been

shown not to occur in some strains of *T. gondii* and also to differ in number of repeats from one strain to another (Costa and Bretagne, 2012).

1.6.6 EPIDEMIOLOGY

Toxoplasmosis is known to be one of the most widespread parasitic infections globally. The discovery of the methylene blue dye test also referred to as the gold standard test in 1948 by Sabin and Feldman actually allowed for accurate detection of the parasite in both the definitive and intermediate hosts (Sabin and Feldman, 1948). This enabled researchers to investigate the prevalence of *T. gondii* infection in various human and animal populations worldwide and led to the generation of credible evidence in support of the diverse distribution as well as varied prevalence rates of *T. gondii* in many populations. Documented *T. gondii* seroprevalence differs widely from population to population, from one geographic region to the other, from one ethnic group to the other within the same area and amongst different regions within the same country (Pappas *et al.*, 2009). Reports on *T. gondii* antibody sero-prevalence rates over the past three decades range from 0 to 100% in various adult human populations assessed globally from inspection of the literature.

Seroprevalence is an indication of the build up or cumulative exposure to a particular infectious agent over the course of an individual's lifetime within a specific or unique social setting, thus seropositivity rates can be taken as representing a quantitative measure of exposure. Sero-prevalence rates are therefore surrogate to assess the relative risk of acquiring a parasitic infection for an individual found within such a population. Therefore in populations or settlements where the general seroprevalence is low, the risk

for an individual to be infected is regarded as low, on the other hand in populations or settlements where the population seroprevalence is high, it can be suggested that the probability of an individual acquiring a primary *T. gondii* infection is high in the instance where the individual is not already carrying an infection (Pappas *et al*., 2009). Globally, *T. gondii* seroprevalence rates have been observed to be dynamic through time and when analyzed carefully there seems to be a trend towards a decrease in seroprevalence rates in various populations. This is particularly the case in the developed world over time, although this cannot be generalized. The increasingly mobile global population can negate this trend and hence migration from *T. gondii* endemic areas to areas of low prevalence may lead to the resurgence or increase in seroprevalence in these non-endemic areas (Jones, 2003).

Seroprevalence rates may vary widely, even doubling, according to the chosen method of assessment and its associated specificity, sensitivity or predictive value (Tenter *et al.,* 2000). Therefore, reported or measured seroprevalence data usually differ from actual or true prevalence rates within the various populations assessed. However such data are useful for comparison if they are applied as approximations of the actual values and hence an indication of rates of seroprevalence in populations, corrected for compounding factors such as age, environmental factors and cultural habits which might adversely affect epidemiological investigations of *T. gondii* infections (Hill *et al*., 2005).

The epidemiology of a parasite as versatile as *T. gondii* is impacted by a variety of factors, which include, but are not limited to, the different habits of the human or animal consumer, density of cats or felids (definitive hosts) in a particular population, the type of management and production systems of livestock for human consumption, food

processing technologies, environmental conditions which will affect the sporulation of the oocysts (humidity, wind and temperature), hygienic standards of abattoirs and even geographical location of the population in question. Seroprevalence has also been shown to be directly related to the age of the individual as well as exhibit an association with exposure to different risk factors over time (Tenter *et al.,* 2000; Dubey *et al*., 2008). Only a few countries in the world are known to have policies in place to regularly monitor toxoplasmosis in humans and even fewer countries monitor the disease in animal populations, despite credible public health organizations (such as the World Health Organization, WHO) recommending that such monitoring systems are implemented and accurate epidemiological data recorded on a regular basis and documented in order to facilitate management of disease outbreaks as well as spread of disease. Differences in sero-prevalence rates may help in investigations into the different risk factors involved in the disease and also aid in the determination and implementation of appropriate health policies by different countries affected (Hill *et al*., 2005).

1.7 PUBLICATIONS

In reviewing the literature for the thesis, two manuscripts were prepared and submitted for publication, these papers were accepted for publication and then eventually published. The first paper reviewed the existing online literature for all published articles on Toxoplasma seroprevalence studies in humans and animals on the African continent in order to bring to the attention of the scientific community the lack of any planned or regular *T. gondii* seroprevalence tracking schemes on the continent even though the published work available indicate that seroprevalence rates are high in numerous populations on the continent.

The second publication attempted to revive the conversations around toxoplasmosis within the South Africa context and therefore was a review of the salient points regarding the disease and the research done specifically withing the South African context.

The publications have been included in the thesis as they were accepted for publication and can also be viewed online.

1.7.1 *TOXOPLASMA GONDII* SEROPREVALENCE STUDIES IN HUMANS AND ANIMALS IN AFRICA

Hammond-Aryee, K., Esser, M., and van Helden, P. D. 2014. "*Toxoplasma gondii* seroprevalence studies on humans and animals in Africa." *South African Family Practice* 56 (2): 119-124.

Affiliations 1: MSc student division of Molecular Biology and Human Genetics at Stellenbosch University. His research interests is toxoplasmosis particularly its genetics, molecular epidemiology immunology and vaccine development. Toxoplasmosis is a co-infection of immunosuppressive diseases particularly HIV and has implications for impoverished cultures, he also holds a BSc and an MBA.

Affiliation 2: MMed Paed (Rheum) head of NHLS Immunology Unit Tygerberg, Division Medical Microbiology, Department of Pathology, Stellenbosch University and also coordinator of Pediatrics Clinical Immunology and Rheumatology Clinics, Department of Pediatrics and Child Health, Tygerberg Hospital. Principal Investigator for the Primary Immunodeficiency Register of South Africa

Affiliation 3: Professor and head of the division of Molecular Biology and Human Genetics Stellenbosch University. His research interest is tuberculosis ranging from diagnostics, through immunology and genetics, to clinical trials and veterinary TB. TB is intimately linked to culture, society, poverty and other infectious and chronic conditions.

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Keywords: *Toxoplasma gondii*, Toxoplasmosis, Sero-prevalence, Disease burden, Africa.

Abstract

Background: Toxoplasmosis is a disease caused by *Toxoplasma gondii*, which can infect nearly all mammalian and avian species. Approximately 25% of the global human population is thought to be infected. Interest in Toxoplasmosis has surged since it was discovered that with the onset of AIDS, acute toxoplasmosis can induce cranial calcification and the ensuing encephalitis can be fatal. The African HIV epidemic, increasing levels of other immunosuppressive infections such as tuberculosis, poor sanitation practices, and lack of any monitoring of at risk populations points to a problem that maybe under-rated.

Objective: to review the body of research available on the sero-epidemiology of Toxoplasma gondii in Africa, in order to establish the existing prevalence trends and to draw attention to the information available on the pathogen in Africa.

Materials and Methods: The NCBI, Google Scholar and ToxoDB databases were searched for all peer reviewed articles focusing specifically on the seroprevalence studies of Toxoplasma gondii in Africa up until year end 2012.

Results: Seroprevalence rates on the continent are high in humans and animals. There is a geographical trend, with decreasing sero-prevalence from the North to the South and from the West to the Eastern regions of the continent. Most sero-prevalence studies in humans were reported between 1981 and 2000.

Conclusions: There is a need for further and more consolidated information on the prevalence of Toxoplasma gondii in Africa, in order to address morbidity and mortality from opportunistic but treatable diseases such as toxoplasmosis in the ongoing HIV pandemic and to improve lives of the African population.

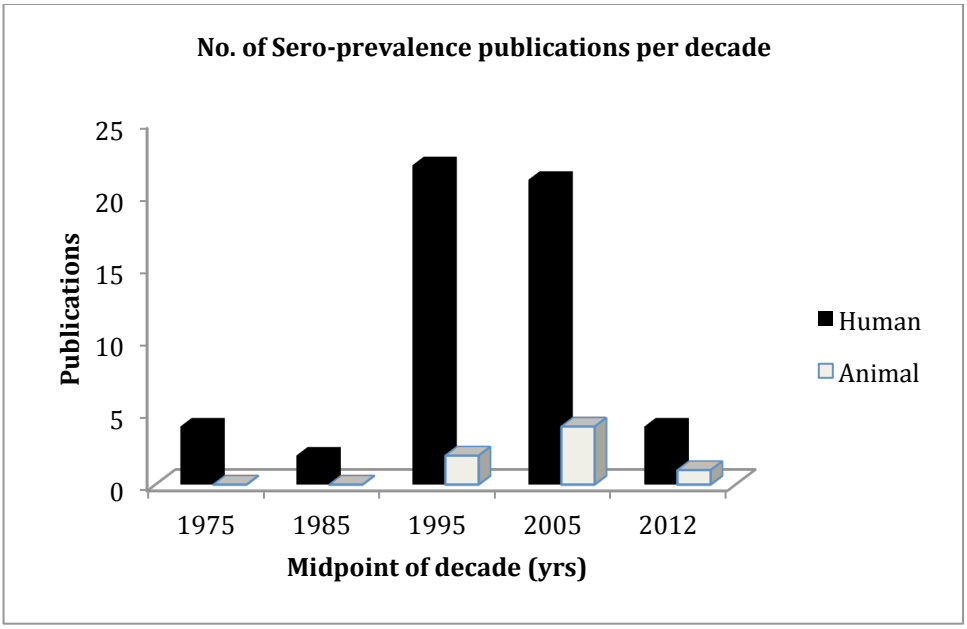
Introduction

Over the years there have been scattered, sporadic (and usually small) *Toxoplasma gondii* seroprevalence studies on the African continent and this state of affairs leaves much to be desired. The current HIV epidemic on the continent, increasing levels of other immunosuppressive infections such as tuberculosis, poor sanitation and hygiene practices, inadequate veterinary services, and high levels of poverty, coupled with the lack of any proactive monitoring of at risk populations and reporting of these investigations is part of the problem. The lack of public health schemes targeted at managing the spread of this specific pathogen, places African populations at risk for ongoing and possibly increasing incidence and prevalence as well as a corresponding increase in mortality and morbidity from toxoplasmosis. This may be particularly the case for toxoplasma encephalitis which is currently one of the leading causes of mortality and morbidity in HIV patients who have progressed to full blown AIDS and are not on HAART therapy, in Africa [1].

There have been few studies on the seroprevalence rates of *Toxoplasma gondii* in animal species on the African continent. Given the large numbers of domestic and wild animals on the continent as well as the lack of sophisticated animal husbandry, there is a high likelihood of transmission and hence mobility of the pathogen, from animal hosts to human hosts together with increased risk of morbidity and mortality.

Sero-prevalence surveys

Figure I. Number of seroprevalence studies per decade (1971-1980, 1981-1990, 1991-2000, 2001-2010, 2011 - 2012 (incomplete decade))



Surveys have also not focused on comparable cohorts over time and therefore one must be cautious in interpreting the data. The data presented in Table 1.1a-c (and 1.2 for animals) below, classify the investigations according to the locations at which they were conducted, the sample size of the study, the year in which the study was conducted in chronological order, the level of seroprevalence as a percentage as well as the type of population investigated.

Table 1.1-*Toxoplasma gondii* seroprevalence studies in Africa (Human).

Immunocompetent individuals (1.1a)

Country/Region	Sample size (absolute numbers)	Seroprevalence% (95% confidence intervals)	Year	reference
South Africa/Transvaal	806	37%	1974	(2)
South Africa/Nationwide	3379	20%	1978	(3)
Somalia	356	43%	1980	(4)
Kenya	322	42%	1983	(5)
Zambia	189	11%	1991	(6)
Uganda	93	27%	1991	(6)
Sudan/Gazira	368	41.7%	1991	(7)
Ethiopia/Nationwide	1016	74.4%	1993	(8)
Niger/Niamey	371	18%	1996	(9)
Tunisia	564	58.4%	2001	(10)
Mali/Bamako		21%	2001	(11)
Egypt/Qualyobia	152	57.9%	2001	(12)
Nigeria	144	20.8%	2005	(13)
Sao Tome and Principe/Nationwide	161	74.5%	2007	(14)
Egypt	260	59.6%	2009	(15)
Tanzania/Tanga	199	45.7%	2009	(16)
Tunisia/Sfax	1691	13.7%	2010	(17)
South Africa/Gauteng	497	6.4%	2011	(18)
Mali/Kolle	760	27%	2012	(19)

Women of reproductive age (1.1b)

South Africa/Bloemfontein	**600**	**3%**	**1975**	**(20)**
Ivory Coast	>2000	37.2-70%	1989	(21)
Cameroun/Yaounde	192	77.1%	1992	(22)
South Africa/Kwazulu Natal		31.3	1992	(23)
Senegal/Dakar	353	40.2%	1993	(24)
Senegal/Dakar	720	40.3%	1993	(25)
Benin/Coutonou	211	53.6%	1995	(26)
Madagascar/Antanavario	599	83.5%	1995	(27)
Tanzania/Dar es Salaam	849	35%	1995	(28)
Nigeria		75.4%	1996	(29)
Gabon/Franceville	767	71.2%	1998	(30)
Egypt/Qualyobia	31	58.1%	2001	(12)
Egypt/Qualyobia	38	44.7%	2001	(12)
Sudan/Khartoum, Omdurman	487	34.1%	2003	(31)
Ivory Coast/Yopougon	1025	60%	2004	(32)
Burkina Faso/Ougadougou	336	25.3%	2006	(33)
Morroco/Rabat	2456	50.6%	2007	(34)
Sao Tome and Principe/Nationwide	499	75.2%	2007	(35)
Egypt/El Fayoum	68	20.45%	2009	(36)
Egypt/El Fayoum	20	17.9%	2009	(36)
Tunisia/Sfax	40,566	39.3%	2012	(17)
South Africa/Gauteng	376	12.8%	2011	(18)

HIV and AIDS individuals (1.1c)

Zambia	**187**	**4%**	**1991**	**(6)**
Uganda	186	34%	1991	(6)
Kenya/Nairobi	94	54%	1991	(37)
Ivory Coast	294	21%	1993	(38)
Burkina Faso	45	55.5%	1995	(39)
Burkina Faso	50	52.0%	1995	(39)
Burkina Faso	40	62.5%	1995	(39)
South Africa/western cape	327	43%	1995	(40)
South Africa/western cape	91	26%	1995	(40)
South Africa/western cape	61	15%	1995	(40)
Ethiopia/Addis Ababa	170	80%	1998	(41)
South Africa/Kwazulu natal		24.6%	1998	(42)
Burkina Faso/Bobo-Dioulasso	1828	25.4%	2000	(43)
Mali/Bamako		60%	2001	(11)
Nigeria	219	38.8%	2005	(13)
Uganda/Malago	130	54%	2006	(1)
South Africa /Gauteng	307	8%	2007	(44)
Mozambique	150	18.7%	2010	(45)
Tunisia/Sfax	78	11.7%	2010	(17)
Nigeria/Lagos	380	54%	2010	(46)
South Africa/Northeast	160	18.1%	2010	(47)
South Africa/Gauteng	376	9.8%	2011	(18)
Morroco/Marrakesh	95	62.1%	2012	(48)
Nigeria/ Northern	219	38.7%	2013	(49)

1.2-*Toxoplasma gondii* seroprevalence studies in Africa (Animal species).

South Africa/various	**68**	**74%**	**1999**	**(50)**
Ghana/Nationwide	732	33.2%	2000	(51)
Ghana/Nationwide	526	26.8%	2000	(51)
Botwana/Chobe	53	92%	2002	(52)
Namibia/Windhoek	21	100%	2002	(52)
South Africa/Kruger park	12	100%	2002	(52)
South Africa/Hluhluwe-Imfolozi park	30	100%	2002	(52)
Botwana/Chobe	1	100%	2002	(52)
South Africa/Kruger park	7	86%	2002	(52)
Zimbabwe/Nationwide	335	67.9%	2005	(53)
South Africa/Nationwide	600	4.3%	2007	(54)
Ghana/ Kumasi	64	64.1%	2008	(55)
Egypt/El Fayoum	62	98.4%	2009	(36)
Egypt/El Fayoum	24	41.7%	2009	(36)
Tunisia/nationwide	158	17.7%	2011	(56)

Toxoplasmosis in immunocompetent individuals

Studies of *Toxoplasma gondii* in asymptomatic individuals in Africa are limited in number and none of the countries follow a systematic planned pattern of reporting (Table 1.1a). Seroprevalence rates range from 6.4% to 74.5%, with a median of 37%. The first study reported was from South Africa by Mason *et al* in 1974 where a seroprevalence of 20% was found in a cohort made up of Blacks, Whites, Indians and Colored immunocompetent individuals. Since then, there have been other studies, the most recent being that of Kistiah *et al* (2011), who reported 6.4% seroprevalence in a generalized cohort, but biased towards pregnant women. Within the SADC region, Swai and Schoonman (2009) reported a seroprevalence of 45.7% in a Tanzanian cohort predominantly made up of immunocompetent individuals who were exposed to livestock via their occupations (abattoir workers, livestock keepers, animal health workers). In the eastern part of the continent, Zumla *et al* (1991) reported a seroprevalence of 27% in Uganda whilst (Griffin and William., 1983) reported a seroprevalence of 42% in Kenya. Further north, Abdel-Hameed (1991) reported a seroprevalence rate of 41.7% in Sudan, whilst in the north of Africa Elsheikha *et al* (2009) and Bouratbine *et al* (2001) reported seroprevalence of 59.6% and 58.4% in Egypt and Tunisia respectively. In the west of Africa, the highest seroprevalence reported was 74.5% by C-K Fan *et al* in 2009 in Sao Tome and Principe and the lowest seroprevalence of 20.8% by Uneke *et al* in 2005 in Nigeria. Prevalence rates in Northern Africa followed decreasing trend overtime from 58.4% in 2001 to 13.7% in 2010. In Southern Africa, there was also a decreasing trend from 37% in 1974 to 6.4% in 2011, whilst in Western Africa there was an increasing

trend from 21% in 2001 to 27% in 2012 and in Eastern Africa there was also an increasing trend from 43% in 1980 to 45.7% in 2009.

Women of reproductive age and pregnant women

There have been a number of studies done on this risk group, with most of the studies done in the west and north of Africa, whilst the south and east are under- represented in studies (Table 1.1b). Seroprevalence rates range from 12.8% to 83.5%, with a median of 42.5%. High rates of seroprevalence have been reported in the west of Africa, where Ndumbe *et al* (1992) reported a prevalence of 77.1% in Cameroun and Hung *et al* (2007) reported 75.2% prevalence in Sao Tome and Principe, The highest rate on the continent was recorded in the southern part of Madagascar by Lelong *et al* (1995), where a seroprevalence of 83.5% was reported. The lowest seroprevalence was reported in South Africa, where Kistiah *et al* (2011), reported a prevalence of 12.8%. Seroprevalence rates in North Africa have generally been high, with the highest of 58.1% in Egypt, reported by Hussein *et al* in 2001. Seroprevalence rates in Northern Africa showed decreasing trend overtime from 58.1% in 2001 to 39.3% in 2012. In Southern Africa there was an increasing trend from 3% in 1975 to 12.8% in 2011, whilst in Western Africa there was also an increasing trend in seroprevalence from 70% in 1989 to 75% in 2007. Seroprevalence rates are generally high in women of reproductive age. It is interesting to note that such high levels are comparable to those reported in similar South American cohorts[58].

HIV and AIDs-related toxoplasmosis

With the advent of the HIV-AIDS era, the significance of toxoplasmosis was realized, resulting in an increase in the number of seroprevalence investigations, particularly in HIV-positive compared to HIV-negative populations (Table 1.1c). Seroprevalence rates range from 4% to 80%, with a median of 36.35%. The highest seroprevalence of *Toxoplasma gondii* amongst HIV patients was reported in Ethiopia by Woldemichael *et al* in 1998, with an 80% seroprevalence in 170 patients tested. In West Africa, the average seroprevalence figure was about 52%, ranging from 62.5% in Burkina Faso to 21% in the Ivory Coast, by Ledru *et al* (1995) and Lucas *et al* (1993) respectively. The lowest rate on the continent, 4%, was recorded in Zambia by Zumla *et al* (1991). Lindstrom *et al* (2006) and Brindle *et al* (1991) reported seroprevalence rates of 54% in both Uganda and Kenya respectively. There has been only one study reported from the North of Africa. The reported study was in Morocco where a seroprevalence rate of 62.5% was reported by Addebbous *et al* (2012). In the southern part of Africa reported seroprevalence studies have been mostly from South Africa. Sonnenberg *et al* (1998) reported the highest seroprevalence rate of 24.6% in Kwazulu-Natal province and the lowest rate of 8% were reported in Gauteng province by Hari *et al* (2007). Seroprevalence rates follow an increasing trend in Northern Africa, from 11.7% in 2010 to 62.1% in 2012. In Southern Africa there was also an increasing trend from 4% in 1991 to 9.8% in 2011, whilst in Western Africa there was increasing trend overtime from 21% in 1993 to 38.2% in 2013. In Eastern Africa there was also increasing trend overtime from 34% in 1991 to 54% in 2006.

Toxoplasmosis in animal species

Most of the studies reported to date have been done in the southern part of Africa (Table 1.2) where Samra *et al* (2007), demonstrated a seroprevalence of 4.5% in a 600 sheep cohort in South Africa and in Zimbabwe, Hove *et al* (2005) reported a seroprevalence of 67.9% in 335 sheep and goats investigated. Cheadle *et al* (1999), reported a seroprevalence of 74% in wild felids in South Africa and Penzhorn *et al* (2002) reported a seroprevalence of 100% in lions from the Kruger National Park and Hluhluwe-Imfolozi park in South Africa and 92% in lions from Botwana (Chobe national park). *Toxoplasma gondii* antibodies have also been detected in felids in the wild, and in chinchillas, ferrets, cheetah, a dog, as well as leopards in Southern Africa. In Ghana, Dubey *et al* (2008) reported a seroprevalence of 64.1% in free range chickens, whilst a seroprevalence of 38.2% in sheep and 26.8% in goats respectively was reported by Van der Puije *et al* (2002). There have been no documented reports on seroprevalence in animals from the eastern part of Africa but in Egypt, Ghoneim *et al* (1998) reported a seroprevalence of 98.4% in sheep. Reported seroprevalence rates range from 4.3% to 100%, with a median of 74%. Overtime seroprevalence rates show a decreasing trend from 98.4% in 2009 to 17.7% in 2011 in Northern Africa. In Southern Africa there was also a decreasing trend from 74% in 1999 to 43% in 2007, whilst in Western Africa there was an increasing trend from 33.2% in 2000 to 64.1% in 2008.

General conclusions

Overall, seroprevalence rates on the continent are very high in both human and animal populations. The data presented does suggest a geographic trend, with decreasing sero-prevalence from the North to the South and from the West to the Eastern regions of the continent. Most sero-prevalence studies in humans were reported between 1981 and 2000. The majority of the sero-surveys were carried out on immunocompetent individuals followed by women of reproductive age and then HIV/AID patients. Only a limited number of these studies take into consideration the risk factors most important for infection with the pathogen. Thus, very few surveys have been done in animals, to understand the zoonotic risk. Considering the risk factors from studies in other parts of the world, it is possible to infer that large numbers of people on the African continent are at risk of a primary infection or reactivation of a chronic infection, such as pregnant women and HIV positive individuals. In all groups studied, seroprevalence rates generally demonstrated decreasing trends overtime for three regions namely Northern, Southern, and Eastern Africa. The exception of note was in Western Africa where seroprevalence rates showed increasing trend overtime for all the groups and in HIV-AIDS patient cohorts where prevalence rates showed an increasing trend overtime for all the regions. . This data suggests that overall seroprevalence rates follow an increasing gradient from a relatively low prevalence in the southern part of the continent, moderate to high in the west and east and highest in the northern part of the continent. These findings must be taken seriously and further structured investigations need to be carried out in order to ascertain the causative factors and action taken to mitigate their effects.

This information is important because if even only 5% of individuals infected, present with clinical manifestations, the implications or the burden of disease for Africa with a population approaching 1 billion is staggering. Given that Toxoplasmosis is associated with clinical manifestations in HIV positive individuals, given that reactivation of a latent infection can be life threatening [58], due to a number of factors including the increased immunosuppression and encephalitis [49], it is possible that this disease may be a neglected driver for clinical diseases such as Tuberculosis or other chronic or infectious illnesses that might otherwise not manifest themselves. In this case, the Burden of disease and DALYs cost for Africa from Toxoplasmosis is considerable and deserving attention.

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1.7.2 TOXOPLASMOSIS IN SOUTH AFRICA- OLD DISEASE IN A NEW CONTEXT

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Abstract

Toxoplasmosis is one of the most widespread parasitic infections known. Clinical manifestation of toxoplasmosis because of immunosuppression is typically due to a reactivation of a chronic infection. According to the UNAIDS 2008 report on the global AIDS epidemic, about 5.7 million South Africans were infected and living with HIV in 2007, with obvious risk and health resource implications for toxoplasmosis. *T. gondii* has been largely neglected as a health risk to the general population in the HIV era. Currently South Africa is burdened with ongoing HIV and TB pandemics. South Africa accounts for 17% of the global HIV burden and has a TB incidence of 950 per 100,000 as at 2012. Such high incidence of immunosuppressive infections puts the population at a high risk

of opportunistic infections such as toxoplasmosis. Seroprevalence rates in Africa are high in both human and animal populations, but there are no reports on the significance of the pathogen within the food or water chain of African cultures. Future work should focus on a more systematic approach towards *Toxoplasma gondii* seroprevalence data gathering and analysis in order to inform on effective approaches to its prevention and disease reduction, and on the molecular epidemiology of the pathogen within the South African context.

Keywords: *Toxoplasma gondii*, behavior, toxoplasmosis, mental health, sero-prevalence, South Africa, disease burden, HEU, HUU.

1. Introduction

Toxoplasmosis, a disease caused by *Toxoplasma gondii*, is one of the most widespread parasitic infections known. It has a global distribution, is able to infect nearly all mammalian and avian species and approximately 25% of the global human population is thought to be infected (Fuks *et al*., 2012).

T. gondii has both medical and veterinary significance owing to manifestations such as congenital infections as well as abortions in its human and animal hosts (Kim and Weiss, 2004). Interest in Toxoplasmosis and its pathogenesis has resurfaced since it was discovered that with the onset of the Acquired Immunodeficiency Syndrome epidemic, opportunistic infections such as acute toxoplasmosis induced cranial calcification and the ensuing encephalitis could be fatal if not treated (Bhopale, 2003; Kistiah *et al*., 2011). The predominant thinking is that parasite transmission in humans and animals is via

active or passive ingestion of the persisting stages of the organism (namely the oocysts from feline feces) in contaminated food or water and tissue cysts in infected (contaminated) meat or offal (Frenkel, 1970) (Jacobs and Mason, 1978) (Smith, 1993) (Tenter *et al*., 2000). Infected felids are known to shed millions of oocysts into the environment through their feces, which then develop into sporulated cysts, which become sources of infection. Not unexpectedly, toxoplasmosis is virtually absent in islands which are uninhabited by felids (Munday, 1972) (Wallace, 1969).

2. Pathogenesis

The majority of exposed human adults do not develop apparent clinical symptoms, however, a minority of apparently healthy persons who acquire primary *T. gondii* infection after birth may exhibit mild symptoms such as fever and lymphadenopathy. Recent research suggest that this variability in human infection may in part be ascribed to the causative *T. gondii* strain type (Kim and Weiss, 2004). The infection progresses from an acute stage to a chronic or supposedly latent (cyst) stage in the host (Bhopale, 2003). Infected individuals remain asymptomatic unless they become immunosuppressed. However in certain unusual cases, previously healthy individuals have been known to acquire and develop severe and fatal toxoplasmosis, with pulmonary and multivisceral clinical presentations which are possibly related to virulence of the parasite (Demar et al., 2007). Healthy subjects may also develop ocular complications with severe visual impairment (Glasner *et al*., 1992)(Dubey and Jones, 2008).

Congenital Toxoplasmosis, as the second most common intrauterine infection, remains a public health problem throughout the world. It can result in some of the most serious consequences with a wide range of clinical manifestations, including, but not limited to,

spontaneous abortions and, stillbirths, or in live infants hydrocephalus, microcephaly, and retinochoroiditis and cerebral calcifications. 70-90% of congenitally infected infants are asymptomatic at birth but affected neonates may already demonstrates central nervous system involvement and related complications or a healthy appearing child may present with retinochoroiditis, chorioretinitis and central nervous system complications later in life (Dubey and Jones, 2008).

The risk of transplacental transmission of *T. gondii* to the developing fetus is low when maternal primary infection occurs during the first three months of the pregnancy (10-15%) and is highest when the primary infection occurs during the last trimester of gestation (60-90%). The frequency of transmission is inversely related to the severity of the disease (Remington, 2006). The clinical course in the infant or child is however not related to the presence or absence of symptoms of the disease in the mother.

Clinical manifestation of toxoplasmosis because of immunosuppression, typically due to reactivation of a chronic infection or acquisition of a new infection, manifests primarily as toxoplasmic encephalitis (TE) also known as central nervous system toxoplasmosis (Luft et al., 1983)(Luft *et al*., 1984). Disease progression leads to severe manifestations such as confusion, lethargy, mental state changes, seizures, and coma, and the outcome is fatal (Hill and Dubey, 2002). Approximately two thirds of all people currently living with HIV are found in the sub-Saharan region of Africa. According to UNAIDS 2008 report on the global AIDS epidemic, about 5.7 million South Africans were infected and living with HIV in 2007, with obvious risk and health resource implications for reactivation or acquisition of infections including toxoplasmosis (Kistiah et al., 2011).

In the pre-HAART era, toxoplasmic encephalitis was the leading cause of death in

patients with AIDS worldwide and was known to occur in 3 to 40% of all patients with acquired immunodeficiency syndrome (Jones et al., 2003)(Jones and Dubey, 2012). However fortunately with the onset and wider availability of HAART, morbidity, mortality and healthcare utilization related to Toxoplasma encephalitis has declined noticeably (Saadatnia and Golkar, 2012).

Focus has shifted recently to the growing population of infants born to HIV- positive mothers, but who themselves are HIV negative (HEU). These infants compared to their HIV unexposed, uninfected counterparts appear to be at increased risk of hospitalization due to serious infections (Slogrove *et al*., 2012). Especially those born to mothers who are infected simultaneously with HIV and *T. gondii* should be evaluated for congenital toxoplasmosis, as there is an increased risk of reactivation and disease particularly in the more severely immune-compromised mother.

There is limited literature on toxoplasmosis and tuberculosis co-infection. However, tuberculosis is a common co-infection of HIV and hence compounds the potential for cerebral toxoplasmosis. Toxoplasmosis together with toxocariasis and tuberculosis, are the most common causes of lymphadenitis in children (Guneratne *et al*., 2011). Hwang et al., recently reported the co-infection of tuberculosis and cerebral toxoplasmosis in an otherwise immunocompetent individual and they attribute the opportunistic toxoplasmosis infection to immunosuppression caused by the coexistent tuberculosis (Hwang *et al*., 2012).

2.1 Modification of Host Behavior

The dormant, latent or chronic form of human toxoplasmosis was thought to be asymptomatic during the latent period, however; recently the adverse effect of the supposedly latent infection on the infected individual's reaction time, tendency for accidents, behavior and mental illness has become a focus of research.

In animal studies, rodents that are chronically infected with toxoplasmosis, lose their aversion towards their felid predators and are attracted to their odor which results in more successful predation (Dass *et al.*, 2011). Latent *T. gondii* infection may even have an effect on psychomotor performance as well as on personality profiles of infected humans, consistent with effects observed in the rodent models (Flegr and Hrdý, 1994)(Flegr, 2007)(Flegr, 2013).

Increased levels of anti *T. gondii* IgG antibody levels have been reported in schizophrenia patients at the onset of the disease and increased suicidal tendencies in latently infected human females, whilst latently infected males have been observed to become erratic or reckless in behavior (Fuks *et al.*, 2012)(Carruthers and Suzuki, 2007).

Mothers infected with *T. gondii* were found to be more likely to indulge in self-directed violence (Pedersen et al., 2012). *T. gondii* infection has also been associated with bipolar disorder type 1 disease (Hamdani *et al*., 2012)(Pearce *et al*., 2012).

Fuks *et al*. 2012, proposed that the mechanism for this as being the invasion of dendritic cells and subsequent modification of gene expression in these cells to produce and secrete GABA, a well-known neurotransmitter (Fuks *et al*., 2012).

These observations give rise to speculations on the etiology of violence and the potential

for intervention particularly for mental health disorders, the global and national burden of which is on the rise (Sorsdahl *et al*., 2011).

3. Prevalence of Human Toxoplasmosis in South Africa

Global *T. gondii* antibody sero-prevalence rates ranges from 0 to 100% (Dubey and Beattie, 1988). The first prevalence study from South Africa in 1974 reported a 37% positivity in the former Transvaal region, the highest sero-prevalence in this study was amongst Indians (58%), followed by coloreds (43%), whites (33%) and blacks 29% (Mason *et al*., 1974).

In 1978, national sero- prevalence of 20% was reported, on samples representing all provinces and ethnic groups (Jacobs and Mason, 1978). In 1992, Schneider, Schutte and Bommer, reported a sero- prevalence of 12.5% in whites, in coloreds 28.3%, 36.9% in Indians and 46.2% in blacks (Schneider *et al*., 1992).

Fielder et al. in 1995, reported sero-prevalence rates of 43% in blacks, 26% in colored and 15% in whites in 480 consecutive HIV infected individuals in the Western Cape (Fielder *et al*., 1995). Sonnenberg, Silber and Jentsch (1998), documented a sero-prevalence rate of 24.6 in black HIV (+) patients from the Eastern Cape and KwaZulu-Natal Provinces, Lesotho and Mozambique (Sonnenberg *et al*., 1998).

However, a study from the Gauteng Province by Hari *et al*., in 2007 was of 307 black HIV infected in-patients who were not on antiretrovirals or receiving cotrimoxazole reported a sero-prevalence rate of only 8% (Hari *et al*., 2007).

Bessong and Mathomu (2010) reported a sero-prevalence of 18.1% in a retrospective study of *T. gondii* infection in HIV positive individuals in Venda, Northeastern South

Africa, but they noted that females between the ages of 21 to 35 had a sero-prevalence rate of 82% (Bessong and Mathomu, 2010). However, Kistiah *et al*., 2011 reported a sero-prevalence of 9.8% amongst a cohort of HIV positive and negative patients and only 12.8% prevalence in a cohort of HIV positive pregnant women in Gauteng Province (Kistiah *et al*., 2011).

The above studies were not systematic; they focused on different populations and different diagnostic tests, making comparison difficult. Risk factors were not detailed and at-risk groups not monitored over time for clinical relevance. Extremely high seroprevalence rates 82% (northeastern South Africa) were reported as well as low prevalence (8%, 9.8%) in the coastal surveys.

4. Diagnostic And Treatment Challenges

The most commonly used initial laboratory test for diagnosis is the serological detection of specific IgG, IgM and IgA antibodies to *T. gondii* by commercially available test kits. Although IgM antibodies decline faster than IgG antibodies, they may persist at high levels for up to 18 months making the diagnosis of congenital infection difficult. In this setting and where only a single sample is available the more recently available IgG avidity test may be helpful and assist in differentiating recent infection with low avidity (weak antigen binding) from chronic infection with high avidity IgG antibodies (strong antigen binding) to help rule out infections in the last 4 to 5 months. PCR diagnosis on amniotic fluid samples of gestations identified at risk serological or suggestive ultrasonographic features of the foetus have a reported specificity of close to 100% for *T. gondii*. Serologic methods for diagnosis in AIDS patients and the immune compromised are unreliable due to deficient specific antibody production. Prevention strategies for

infection with *T. gondii*, especially in pregnant females revolve around health education for personal hygiene and food handling (Bope and Kellerman. 2013).

Specific treatment of the immune competent, non-pregnant patient is generally not indicated, as infection is usually subclinical and self-limited. For pregnant women (if infection is diagnosed) and the immune compromised however, treatment with anti-toxoplasmic agents such as a combination of pyrimethamine and sulphonamides and spiramycin directly after diagnosis, is indicated (Bope and Kellerman. 2013).

5. Conclusions

Currently South Africa is burdened with ongoing HIV and TB pandemics. South Africa accounts for 17% of the global HIV burden and has a TB incidence of 950 per 100,000, as at 2012 (Mayosi and Benatar, 2014). Overall, reported seroprevalence rates in Africa are high in both human and animal populations. The likelihood of disease transmission via contaminated food and water sources in the HIV era has serious implications for underdeveloped countries. Hence the increased risk of toxoplasmosis as a waterborne or food borne disease (Conrad *et al.*, 2005). Toxoplasmosis presents a challenge in its diagnosis and treatment of recent infection and reactivation, as it requires a combination of tests, which have to be interpreted in the context of clinical examination and history for appropriate intervention especially in pregnancy and for the immune suppressed. A more systematic approach towards *Toxoplasma gondii* seroprevalence data gathering and analysis is called for in Africa, for effective approaches to prevention and disease reduction.

T. gondii has been neglected as a health risk to the general population in the HIV era and there is lack of accurate information on prevalence in human and animal populations and

its importance in the food chain and in water sources. The emerging evidence concerning its potential effects on the mental health of infected but apparently physically healthy individuals should prompt further research.

Future work should also focus on the monitoring of sensitive populations such as pregnant women, cats (both feral and domestic), HIV and TB patients and small ruminants such as sheep. The molecular epidemiology of the pathogen also needs to be investigated in order to have an understanding of the strains that are causing infection in South Africa and where they fit in within the African as well as global phylogeny and also their virulence phenotypes.

Finally, there is the need to institute policies on advocacy and active surveillance of at risk populations in order to create adequate awareness and hence aid in prevention of any unexpected outbreaks.

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1.8 STUDY RATIONALE, HYPOTHESES AND AIMS

Within a given ecosystem, the health of humans, animals and the environment are linked inextricably and investigations into pathogens that exist in such an ecosystem should not be studied in isolation. In order to implement effective interventions to manage the effects of disease-causing pathogens in such ecosystems, there is a need to gather as much information as possible on the pathogen, its host or hosts and the environmental factors, and then by applying aholistic “One Health” (Thompson, 2013) approach, we may be able to systematically analyze data in context, draw conclusions and make recommendations (van Helden *et al*., 2013). Such an approach also allows the investigators to ascertain which interventions may have significant impact and also where possible what the end results of such interventions will be for alternative hosts and parasite vectors within the ecosystem. The study applies an adaptation of the One-Health

(Thompson, 2013) framework in order to gain an understanding of the current status quo regarding the pathogen *T. gondii* within the selected setting. In applying such an approach to the study of *T. gondii* in the Western Cape Province of South Africa, we reasoned that,

- Knowledge of *T. gondii* prevalence and strain diversity within the local population is relevant information, due to its largely under-recognized importance and the potential for associated long-term health complications.

- Prevalence studies within at-risk groups such as HIV endemic populations and women of child bearing ages, will provide information which will enable health authorities to anticipate *T. gondii* disease reactivation rates and monitor individuals who are at risk of morbidity or mortality. It will also enable us make predictions concerning the level of risk for primary infections within the population and hence devise schemes to educate the population and manage primary infections during pregnancy.

- The sampling of animal reservoirs of *T. gondii* such as feral cats and caracals near the urban edge will provide us with invaluable data on the prevalence patterns of the pathogen as well also inform us on transmission dynamics. The sampling of vulnerable livestock animals such as sheep provides us information on the infection burden in such livestock at a selected point in time and this will then inform on the need or not thereof for interventions.

- There is no data for South Africa, concerning the effect of HIV exposure to the fetus *in utero* and the subsequent susceptibility of the infant to toxoplasmosis or other infectious diseases. One can therefore investigate whether the HIV exposed but uninfected infant is therefore at a higher risk of acquiring congenital toxoplasmosis or a future infection when compared to the HIV unexposed and uninfected infant (A pilot study of innate immune abnormalities in HIV exposed but uninfected infants in comparison to their unexposed and uninfected counterparts, conducted in South Africa, has shown statistically significant increase of serious infections in the exposed but uninfected population (Slogrove *et al*., 2012).

- There is a lack of recent data on ocular and congenital Toxoplasmosis in South Africa in general and the Western Cape specifically.

Rather than follow the traditional hypothesis driven approach, this thesis follows a different approach, mainly due to the uniqueness of the pathogen being studied and the fact that the different populations investigated were extremely difficult to obtain at the onset of the project. A data driven scientific approach (van Helden, 2013) was followed, which allowed the investigator to generate data as and when the populations or sample specimens became available and then analyze and interprete the data and present it in a structured thesis.

In opting to organize the thesis from a one-health point of view certain limitations have to be stated. The populations studied did not cover the whole of the Western Cape province, but were selected as and when they became available to the investigator. Secondly,

certain aspects of the investigations were built on as the project progressed and data and sample collection were not necessarily optimal, for example they were opportunistic and had to rely on how others collected samples in earlier times. . The thesis therefore presented attempted to mimic the one health approach as best as possible and therefore conclusions drawn were aimed at being conservative.

The aims of the study were therefore:

- To determine the seroprevalence of *T. gondii* antibodies in a population of HIV positive and HIV negative post parturient mother, and to compare the seroprevalence of the HIV positive subset to that of the HIV negative subset
- To investigate risk factors for *T. gondii* infection in the cohort of post parturient mothers
- To determine the seroprevalence of *T. gondii* antibodies at predetermined time intervals in two subsets of a cohort of infants, one subset being made up of infants who have been exposed to HIV *in utero* but are not infected with HIV at birth (HEU) and the other subset being made up of infants who have not been exposed to HIV *in utero* and are also uninfected (HUU), and then to compare the seroprevalence rates of the two sub populations and also to investigate the time to acquisition of a *T. gondii* infection in the infant cohorts from birth to 56 weeks of age.
- To determine and compare the prevalence of *T. gondii* infection in a cohort of patients presenting with ocular complications at the Tygerberg Academic Hospital ophthalmology unit via both serology and molecular investigations

- To determine the seroprevalence of *T. gondii* antibodies in selected animal populations (feral cats, caracals and sheep)
- To investigate the molecular epidemiology and population structure of *T. gondii* in infected human and animal samples opportunistically obtained in the Western Cape of South Africa
- To attempt the revovery of viable *T. gondii* tachyzoites from collected runoff water effluent and infected animal tissues through mouse bioassays.

1.9 STUDY DESIGN

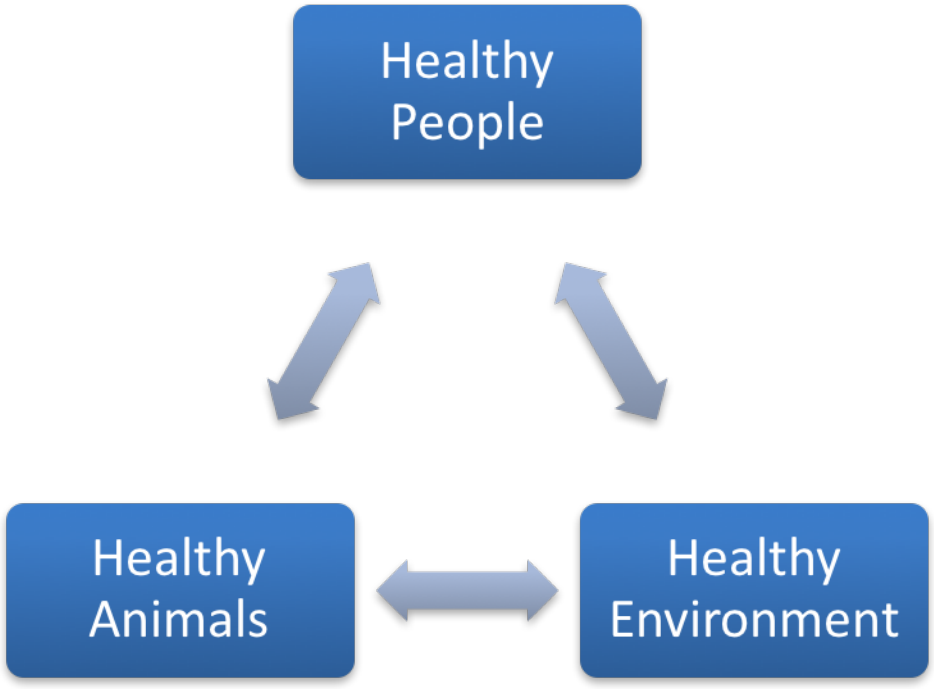

Figure 1.4 The One Health framework adapted from (Thompson, 2013).

Notes: Figure depicts the interplay between human, animals and the environment within a connected ecosystem.

The One-Health triad approach divides the ecosystem into three main parts (Figure 1.4) and enables us to look at total health as opposed to individual components. The well-being of the different components is interrelated and hence one cannot obtain a complete picture of the epidemiology of a disease without looking at all the components.

It is important to look at health from a holistic perspective because it is only such an approach that will ensure that the interrelations between animals, human and the environment are taken cognizant of in the drive to attain optimum well being within an ecosystem (Helden *et al.,* 2013). The components of the One-Health triad system are, the people, the environment and the animals. The current study therefore follows an adapted version of the framework above (Figure 1.4) the adaptations are shown in figures 1.5, 1.6 and 1.7 below.

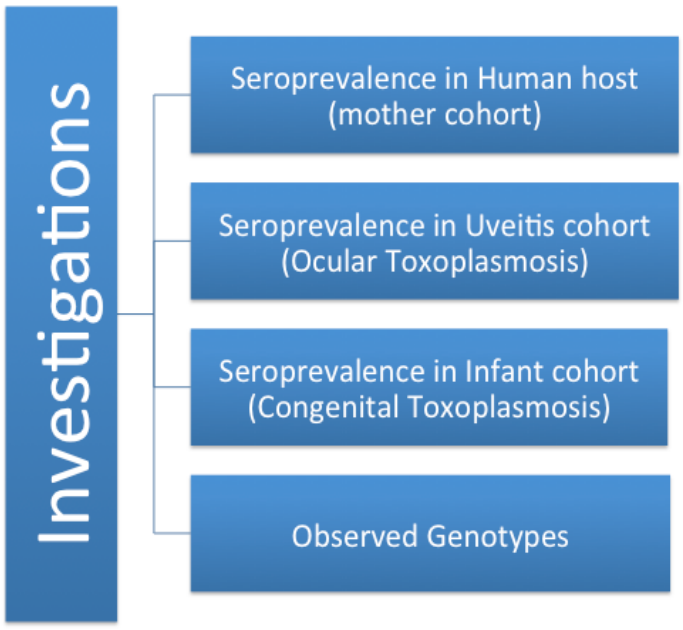

Figure 1.5 Part one, human host Investigation.

Notes: Investigations will cover systemic toxoplasmosis, ocular toxoplasmosis, congenital toxoplasmosis and genotypes of *Toxoplasma gondii* causing infection in opportunistic human samples.

The first part of the investigations (Figure 1.5) focuses on *T. gondii* infections in the human host (systemic, ocular and congenital) as well as investigations into the genotypes causing infection in selected human samples.

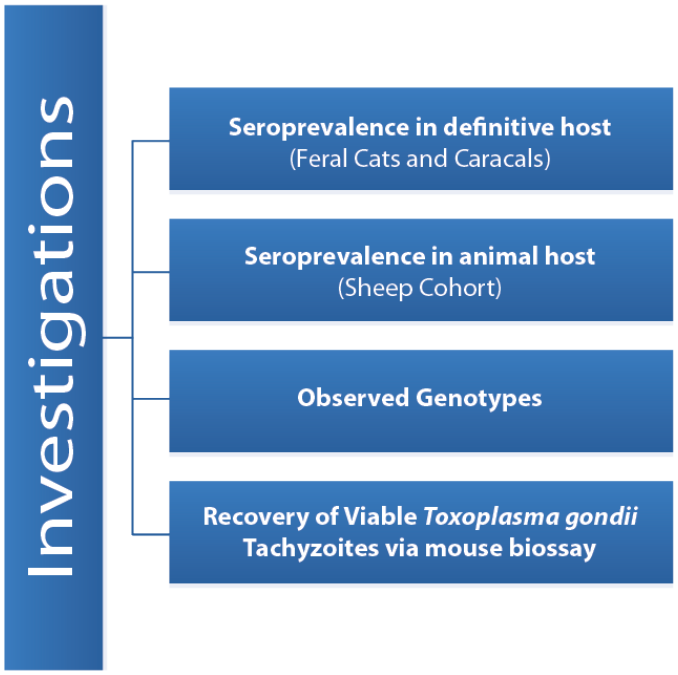

Figure 1.6 Part two, animal host Investigation.

Notes: Investigations will cover seroprevalence in animal hosts (feral cats, caracals and sheep) as well as genotypes of *Toxoplasma gondii* causing infection in opportunistic animal samples and recovery of viable *Toxoplasma gondii* tachyzoites from infected and animal tissues through mouse bioassays.

The second part of the investigations (Figure 1.6) will focus on investigating *T. gondii* antibody seroprevalence in selected animal populations (feral cats, caracals and sheep), the genotypes of *T. gondii* causing infection in infected animal samples and the recovery of viable *T. gondii* tachyzoites from infected animal tissues through bioassay in mice.

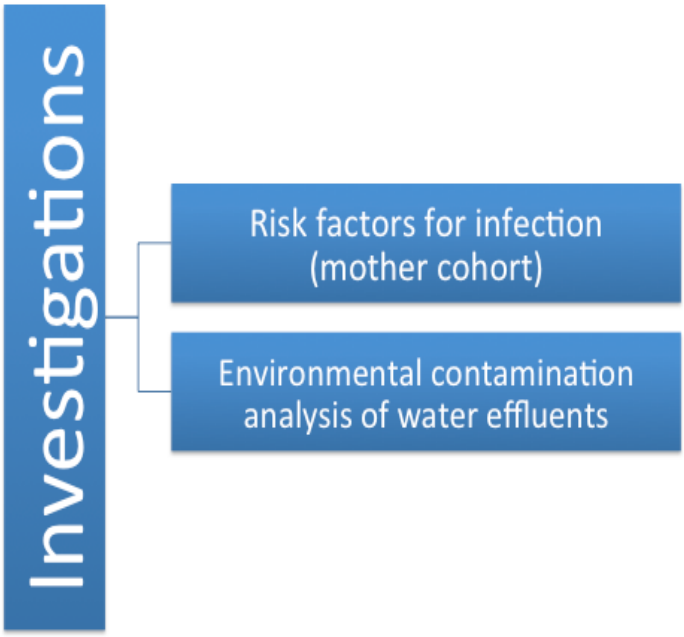

Figure 1.7 Part three, the environmental investigations.

Notes: Investigations will cover risk factors for *Toxoplasma gondii* infection in the population of post parturient mothers as and the recovery of viable *T. gondii* tachyzoites from collected runoff water effluent.

The final part of the investigations (Figure 1.7) will focus on investigations into the risk factors for *T. gondii* infection in the cohort of post parturient mothers and the recovery of viable *T. gondii* tachyzoites from runoff water effluent and infected animal and human tissues through mice bioassays. In so doing the thesis will attempt to provide a holistic picture of *T. gondii* seroprevalence and molecular epidemiology within the studied populations in the Western Cape of South Africa, thereby adding significantly to the existing body of knowledge.

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Note: References listed here are specific to contents of chapter one only and do not include those from the two publications in the chapter.

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CHAPTER 2 PREVALENCE AND RISK FACTORS ASSOCIATED WITH *TOXOPLASMA GONDII* INFECTION IN SELECTED HUMAN POPULATIONS IN THE CAPE TOWN METROPOLITAN AREA.

2.1 INTRODUCTION

Toxoplasma gondii infects humans globally as well as a broad range of warm blooded animals. It is not clear how the parasite has evolved to become so widespread but it is suggested that the existence of different biological as well as infective stages of the parasite may have a role to play. In many settings where seroprevalence studies have been reported, the rates reported have often times exceeded 50% of adults at any given point in time (Pappas *et al*., 2009; Hammond-Aryee *et al*., 2014).

In humans, primary infection in 90% of immune-competent children and adults are normally asymptomatic, although rarely some patients may develop lymphadenopathy of the occipital and cervical lymph nodes. This usually lasts for a limited time and the immune response is adequate enough to control the infection and there is therefore often no need for treatment. Unfortunately this phenomenon has resulted in toxoplasmosis often being regarded as generally unimportant and ignored. This primary infection is thought to lead to an immune response that leaves an immune memory, able to protect the host from secondary exposure to a *T. gondii* infection (Dupont *et al*., 2012). In about 10% of cases the primary infection may be severe and lead to hospitalization and patients may require treatment. In certain outbreaks (Ekman *et al*., 2012), symptoms such as lymphadenitis, chorioretinitis, pneumonia, hepatitis and rarely myocarditis and myositis are observed. The immune response after a primary infection results in the formation of dormant tissue cysts, which are not affected by antibiotic treatment, and which

characterizes the latent stage of the infection. These cysts form a reservoir of infective forms of the pathogen which is able to establish chronic infections or become reactivated in immune-compromised hosts such as patients with AIDS, organ transplantation patients, cancer patients or patients taking immunosuppressants. Specifically in AIDS patients the reactivated infection may cause toxoplasmic encephalitis (TE), which can be fatal (Ruskin and Remington, 1976).

In women who become primarily infected during pregnancy or just before conception, the infective stages are able to cross the placenta and may establish a congenital infection in the developing fetus. This may result in severe malformations and death to the developing fetus or a seemingly healthy infant who may suffer ocular complications later on in life (Dubey *et al*., 2012). It was previously thought that a primary infection in a host leads to an immunological memory that gives rise to protection of the host from further re-exposure, this thinking was based on research with strains from Europe and North America. Currently research with strains from other settings such as South America have shown that these strains are able to re-establish a secondary infection in the host after a primary exposure and some of these secondary infections have been shown to be lethal to the host: this implies that even if a pregnant woman has prior exposure to *T. gondii* there is still the possibility of a secondary infection occurring with certain strains which can be detrimental to the fetus, which is in contrast to the earlier dogma (Jensen *et al*., 2015).

With humans being the limiting hosts when it comes to *T. gondii* infections, domestic animals and wildlife form an integral component of the infective lifecycle of *T. gondii* and also transmission from the environment to the human host. One may speculate that

human toxoplasmosis may actually be non-existent in the absence of the animal hosts (Schlüter *et al*., 2014).

In Africa, poverty as well as the provision of clean and safe drinking water and hygiene are major health problems, according to the World Health Organization. It is reported that 42% of people in sub-Saharan Africa, lack access to clean water and only 31% of rural dwellers in developing countries have access to proper sanitation ("WHO | Water Sanitation and Health", 2012). In many rural communities in Africa, the informal slaughtering of livestock is common practice and numerous people come into contact with raw meat during these activities. In these communities, consumption of undercooked meat, eating with hands as well as communal eating are cultural norms. There are also inadequate veterinary and food quality control services such as meat inspection and produce certification, and a general lack of access to clean running water (Samra *et al*., 2007), these factors together with predominantly poor sanitary conditions suggest a high risk for *T. gondii* infection within these communities.

There are various factors reported to be associated with *T. gondii* infection in humans, these include but are not limited to the, prevalence of oocysts in the environment, specifically sandboxes, gardening soil, school playing grounds and cat litter boxes (Boyer *et al*., 2005; Akanmu *et al*., 2010; Dabritz and Conrad, 2010; Ferreira *et al*., 2013; Carellos *et al*., 2014), consumption of raw or undercooked meat which harbors tissue cysts (Jones and Dubey 2012; Djurković-Djaković *et al*., 2013), consumption of oocyst contaminated water or fruits and vegetables (Demar *et al*., 2007; Ekman *et al*., 2012; Risebro *et al*., 2012) as well as humid and tropical climates as opposed to dry climates (Afonso *et al*., 2013).

2.1.1 TOXOPLASMOSIS IN IMMUNOSUPPRESSED INDIVIDUALS

Extensive immune suppression in solid organ or stem cell transplant or advanced HIV disease is known to predispose patients to toxoplasmosis. It is therefore of concern that in high prevalence HIV settings data on toxoplasmosis regarding prevalence in livestock, wildlife, meat for consumption as well as prevalence in sentinel populations remains scattered and scarce (Lewis *et al*., 2015). Toxoplasmosis as a result of immunosuppression is typically due to a reactivation of a chronic infection and manifests primarily as TE (Luft *et al*., 1983; Luft *et al*., 1984), also known as central nervous system toxoplasmosis. Disease progression leads to severe manifestations such as confusion, lethargy, mental state changes, seizures, ataxia and coma, and the outcome may be fatal (Hill and Dubey, 2002). In the pre-highly active antiretroviral therapy (HAART) era, TE was one of the causes of death in patients with AIDS and was known to occur in 3 to 40% of patients with acquired immunodeficiency syndrome (Jones *et al*., 2003; Jones and Dubey, 2012). However with the onset of HAART, morbidity, mortality and healthcare utilization related to TE has declined noticeably (Saadatnia and Golkar, 2012). However toxoplasmosis is still considered to be a life-threatening opportunistic infection in patients living with HIV despite the availability of various anti retroviral regimes. The relationship or the absence of a relationship between latent *T. gondii* infection and HIV if any is currently poorly understood. The immune response to a *Toxoplasma gondii* infection involves different immune cells of the host. T cells and natural killer (NK) cells act to control the initial parasite infection through the production of interleukin 12 (IL12) and interferon gamma (IFN –gamma), whilst T and B cells mediate longer-term protection (Beran *et al*., 2015). Therefore one may speculate that

low CD4 count individuals with a previous exposure to *T. gondii* may be at an increased risk for reactivation of a latent *T. gondii* infection (Lazenby, 2012).

Currently approximately two-thirds of all people living with HIV are found in the sub-Saharan region of Africa. According to the UNAIDS 2008 report on the global AIDS epidemic, about 5.7 million South Africans were infected and living with HIV in 2007. In areas such as these, toxoplasmosis could occur more commonly than in the more developed world (Kistiah *et al*., 2011). There is also a growing population of infants who have been born to HIV-positive mothers, who may be either HIV- positive themselves, or due to current effective treatment regimens, be HIV- negative. A recent pilot study has shown that these HIV exposed but negative infants (HEU) are more likely to be hospitalized due to serious infections, compared to their HIV unexposed and uninfected counterparts (Slogrove *et al*., 2012). Unlike in adults infected with HIV, central nervous system toxoplasmosis is not a common opportunistic infection in children infected with HIV. It is also important to note that the risk of concurrent infections of congenital toxoplasmosis and HIV as well as the clinical sequelae of such occurrence are not well studied nor understood (Machala *et al*., 2013; Campos *et al*., 2014; Beran *et al*., 2015).

In a multi-country survey on the disease pattern and causes of death in hospitalized HIV-positive adults in West Africa, Lewden et al (2014) concluded that out of a total of 823 HIV-positive adults hospitalized, the most frequent underlying cause of hospitalization was an AIDS defining condition (54%), followed by non-AIDS defining infections (32%), other diseases (8%) and non specific illnesses (6%). The most common diseases observed were tuberculosis (29%), pneumonia (15%), malaria (10%) and toxoplasmosis

(10%). Of the patients who died from AIDS during the study, cerebral toxoplasmosis accounted for 31 (9.8%) of the deaths (Lewden *et al*., 2014).

2.1.2 MOTHER TO CHILD TRANSMISSION OF *TOXOPLASMA GONDII*

The major concern of an acute *T. gondii* infection during the early stages of a pregnancy remains the probability of the vertical transmission of the infection to the developing fetus and the severe outcomes associated with the infection in the fetus and the newborn. Even though it is treatable, congenital toxoplasmosis remains a threatening condition in certain parts of the world, either due to unavailability of screening programs or lack of diagnoses, missed diagnoses and also ineffective treatment regimes. Most pregnant women do not experience any noticeable symptoms when they become infected during pregnancy. In a study in North America, Boyer et al (2005) reported that out of 131 mothers with infants congenitally infected with toxoplasmosis, 52% did not recall experiencing any infection related symptoms or any other identifiable risk factor during their pregnancies. The frequency of mother to child transmission of *T. gondii* increases with gestational age even though the symptoms are more severe in cases where the infection is transmitted during the earlier stages of gestation. It is important to establish whether a pregnant woman has been infected with *T. gondii* or not anytime before and during pregnancy especially in countries with a high incidence of congenital toxoplasmosis. In the case where a positive diagnosis is established, the time of infection is important for fetal risk assessment. If the mother tests negative then it is ideal to screen repeatedly throughout the pregnancy in order to identify if sero-conversion occurs during the pregnancy so that treatment can be initiated immediately (Montoya and Remington, 2008; Paquet and Yudin, 2013). However, it should be noted that several

reports have argued against the effectiveness of treatment of pregnant women diagnosed with acute toxoplasmosis and its success in improving the disease manifestation in the newborn (Gras *et al*., 2005; SYROCOT (Systematic Review on Congenital Toxoplasmosis) study group *et al*., 2007).

In countries with a low incidence of congenital toxoplasmosis, routine screening is not recommended due to the high associated costs. It is therefore important to make information on *T. gondii* infection sources and transmission risk available to all pregnant women or all women planning pregnancies (Singh, 2003). *Toxoplasma gondii* mother to child transmission rates have been reported to be between 400 to 4000 cases each year in the USA (Jones *et al*., 2001). There have also been reported rates of congenital toxoplasmosis ranging from as low as 0.07/1000 births in Sweden (Evengård *et al*., 2001), to as high as 0.1-0.2/1000 births in Italy (Tomasoni *et al*., 2014), 1.9-3.2/1000 births in Paris (Varella *et al*., 2009), and 5.0/1000 births in Brazil (Segundo *et al*., 2004). In South Africa there is increasing evidence that infants whose mothers are HIV-infected, even when the infants themselves remain HIV-uninfected, are more likely to be hospitalized or die from infectious diseases during early childhood, than are infants whose mothers are HIV-uninfected (Newell *et al*., 2004; Slogrove *et al*., 2012).

To investigate the seroprevalence of *T. gondii* within the selected experimental setting two studies were accessed to provide comprehensive samples as well as to provide an indication of the seroprevalence of *T. gondii* within these study settings and baseline information from which further research could be conducted. The two studies accessed were, “The Mother Infant Health Study” and a study on “The causes of intraocular inflammation in HIV-positive and HIV-negative patients in the Western Cape Province,

South Africa". The Mother Infant Health study provided samples of post parturient mothers and their infants, which enabled us to investigate *T. gondii* seroprevalence in women of childbearing ages as well as congenital toxoplasmosis. The polymerase chain reaction amplification methodology was used for *T. gondii* diagnosis of infants at weeks two, eight and sixteen due to the low amount of blood available from the infants as well as the method of collection (blood spots on filter paper, owing to ethical requirements). Whilst at weeks twenty-six and fifty- two there was enough blood available for serum to be isolated and hence the *T. gondii* specific enzyme linked immunoassay methodology was used for *T. gondii* IgG and IgM sero-diagnosis.

The uveitis study allowed access to a pool of samples, which enabled us to investigate the incidence of ocular toxoplasmosis. Both studies provided us the opportunity to investigate the role played by HIV-status in *T. gondii* infections as well as the opportunity to investigate diverse risk factors associated with *T. gondii* infections within the local context, since this information is not available within the study settings. Even though the two studies were not directly linked to each other, they were both based in Cape Town and therefore study participants were from the same area. The studies provided us with the opportunity to investigate the occurrence of *T. gondii* in important groups, such as women of child bearing age, infants, HIV- positive individuals and individuals with ocular complications. The insights gained we believe will be very useful as baseline findings on which to build further research when funding is available.

2.2 STUDY AIMS AND OBJECTIVES

The aims of this study were to,

- Determine and compare the seroprevalence of *T. gondii* infection in a cohort of HIV- positive and HIV- negative mothers two weeks after childbirth
- Determine infant time to acquisition of a *T. gondii* infection in a cohort of HIV exposed uninfected (HEU) and HIV unexposed and uninfected (HUU) infants and to compare *T. gondii* infection seroprevalence in the two groups of infants at the pre-determined time points
- Employ the use of structured interviews of individuals in the recruited post parturient mother cohort to investigate the factors associated with *T. gondii* infection within the study setting
- Determine the prevalence of antibodies to *T. gondii* in serum, in a cohort of HIV-positive and HIV- negative individuals presenting with clinically diagnosed uveitis at the Tygerberg academic hospital ophthalmology clinic
- Test for ocular toxoplasmosis in HIV- positive and HIV- negative patients presenting with clinically diagnosed uveitis via molecular (PCR) testing of aqueous fluid

The objectives of the study were,

i. To determine the *T. gondii* IgG and IgM antibody seroprevalence in a cohort of HIV- positive and HIV- negative mothers two weeks after childbirth

ii. To compare *T. gondii* IgG, IgM and IgM+IgG seroprevalence between the HIV-positive and HIV- negative mothers two weeks after childbirth

iii. To determine the *T. gondii* IgG antibody avidity profile in *T. gondii* IgG positive mothers two weeks after childbirth

iv. To determine infant *T. gondii* infection status at weeks 2, 8 and 16 via *T. gondii* DNA specific PCR methodology

v. To determine infant *T. gondii* IgG and IgM seroprevalence at 26 and 52 weeks via enzyme linked immunosorbent assay methodology

vi. To compare *T. gondii* IgG and IgM antibody seroprevalence in the two groups of infants at weeks 26 and 52

vii. To administer a baseline questionnaire to the mothers to obtain fundamental demographic information after completion of recruitment into the study

viii. To investigate the associations between the *T. gondii* antibody sero-status (IgG and IgM) of the mothers recruited unto the study and the selected demographic and socioeconomic factors.

ix. To determine the *T. gondii* IgG and IgM antibody seroprevalence in a cohort of HIV- positive and HIV- negative individuals presenting with clinically diagnosed uveitis

x. To compare *T. gondii* IgG and IgM seroprevalence between the HIV- positive and HIV- negative individuals presenting with clinically diagnosed uveitis

xi. To analyze patient DNA samples extracted from ocular (aqueous) fluid of individuals presenting with clinically diagnosed uveitis for the presence of *T. gondii* DNA via PCR

2.3 MATERIALS AND METHODS

2.3.1 Determination and comparison of *Toxoplasma gondii* antibody sero-prevalence in a cohort of HIV (+) and HIV (-) mothers two weeks after childbirth

2.3.1.1 Study setting

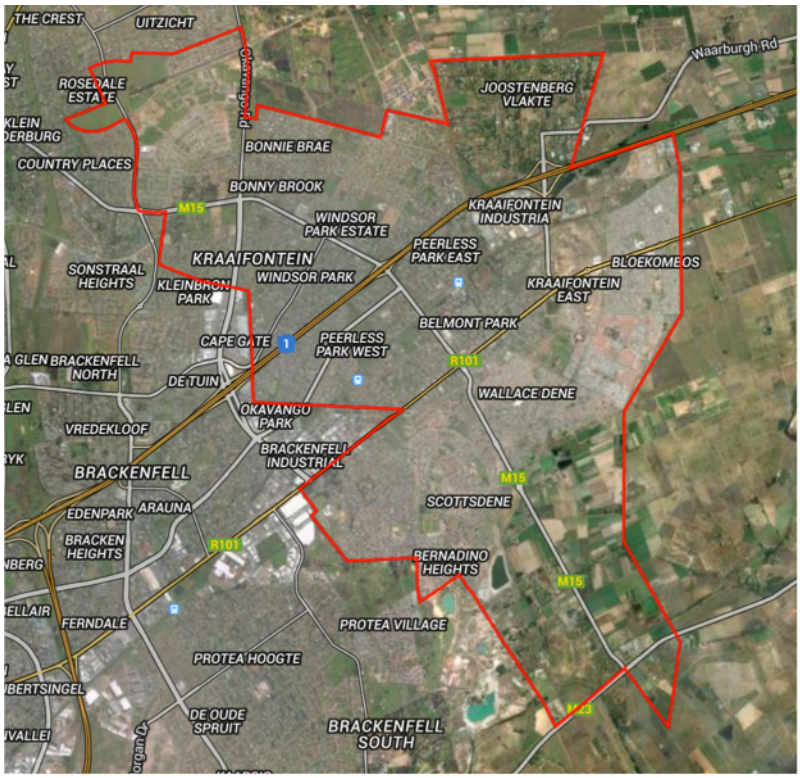


Figure 2.1: Map showing the recruitment area for the study.

Notes: Mother infant pairs were recruited from Kraaifontein municipal area (outlined in red), a suburban area located in the north of Cape Town in the Western Cape province of South Africa

2.3.1.2 Sample size determination

In consultation with the statistics department at the faculty of health sciences, a target sample size of 300 mothers with a 150 HIV- positive and 150 HIV- negative split was selected to be adequate, since one required a minimum of 130 HIV- positive and 130

HIV- negative mothers to be able to have 83.14% power to detect a difference of 17 percentage points between the two groups using a t-test for independent proportions (Dupont, 1988).

2.3.1.3 Ethics clearance

Ethical clearance was obtained from the Stellenbosch University ethical review committee, for this study as a sub-study of a large Mother-Infant Health Study Ethics Reference #: S12/01/009 (Appendix 1 and 4).

2.3.1.4 Statistical analysis

Stata version 13.1 (Statacorp, Texas, USA) was used for all data analysis. Associations between categorical outcome data and other factors or covariates were assessed using chi-square test, Fisher's exact test or logistic regression. A *p*-value equal to or less than 0.05 was considered for statistical significance, all statistical analysis was done at the biostatics department of the University of Stellenbosch.

2.3.1.5 Recruitment and follow up period

The study was a prospective cohort study of 258 mother-infant pairs recruited at delivery from the Karl Bremer Hospital labour ward and the Kraaifontein midwife obstetric unit. The mother infant health study started in July 2012, recruitment was completed in June 2013 and final 1-year study follow-up was completed in December 2014. Mother-infant pairs were scheduled to participate in the study for 12 months from birth of the infants. There was no follow up testing of post parturient mothers due to budget constraints. The study was set in Kraaifontein (Figure 2.1), which is a located in the north of Cape Town

and it is recognized that use of one site only is a limitation of the study. The selection of the catchment area was because the research team was based in Tygerberg Academic Hospital and owing to sample collection and processing time requirements, Kraaifontein presented the best option for the funding available owing to its close proximity to the lab. Secondly for ease of managing recruited participants who had to be transported to Tygerberg academic hospital for every visit, any location, which was more than a thirty minute drive from the data collection center, would have been problematic.

2.3.1.6 Study procedures

Visit 0: Informed consent was obtained from all participants and the study team conducted baseline interviews for demographic and contact details, review of appropriate health records to determine eligibility for the study. Inclusion, exclusion and withdrawal criteria are included in appendix 6.

2.3.1.7 Data management

For all mothers and infants selected for inclusion into the study a unique anonymous study number was assigned and data was collected on coded de-identified case report forms (CRF's) at enrolment at birth and at each study visit.

Methodologies

Maternal investigations

Whole blood samples were collected from enrolled mothers via venipuncture. Whole blood (5 ml) was collected in BD Vacutainer SST^{TM} tubes (BD) and immediately put on ice before being transported to the National Health Laboratory Services in Tygerberg Hospital in Cape Town. The collected blood was then centrifuged at 3500g for 5 minutes in a Beckman GS-6 centrifuge (Beckman Ltd) to isolate serum. Serum was stored at -80 ^{o}C for further analysis. CD4 lymphocyte counts were performed using standard Tygerberg NHLS Immunology laboratory procedures using the Beckman Pan-Leukogating (PLG) method (Glencross *et al*., 2002).

ELISA procedure for *T. gondii* IgM and IgG antibody determinations

Human serum samples were analyzed for the presence or absence of IgM and IgG antibodies to *T. gondii* via Enzyme Linked Immunosorbent Assay (ELISA) methodology, one of the standard methods for detection of *T. gondii* antibodies (Montoya, 2002), using the *T. gondii* IgG and IgM antibody commercial ELISA kits (Euroimmun AG) according to the manufacturers instructions. A pre-incubation step was included for *T. gondii* IgM antibody determination.

Procedure

Thawed serum sample (10 μl) was incubated with 1ml of sample incubation buffer and the resulting sample was mixed briefly by vortexing, the mixture was then allowed to

incubate at room temperature for 15 minutes. However, in the case of *T. gondii* IgG antibody determinations, thawed serum sample (10 µl) was pipetted into a test tube containing 1 ml of sample diluent and mixed briefly by vortexing, with no incubation.

All reagents were allowed to come to room temperature before being used for the procedure. Diluted patient samples (100 µl), calibrator (100 µl), positive control (100 µl) and negative control (100 µl) were independently transferred into individual wells of a 96 well microplate pre-coated with *T. gondii* antigen and incubated at room temperature for 30 minutes. The microplate wells, were emptied by tapping gently but firmly unto an absorbent paper towel with the openings facing downwards, in order to remove all unbound sample. The microplate was then washed three times with 300 µl of wash buffer by repeated pipetting and emptied of all residual wash buffer by tapping gently but firmly on absorbent paper towel with the openings facing downwards. In the next step, 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgM for IgM determinations and peroxidase labeled anti-human IgG in the case of IgG determinations) was then added to each well of the microplate, and the microplate was incubated at room temperature for 30 minutes. The microplate wells were then emptied and washed as described above. Enzyme substrate (TMB H_2O_2 (3,3', 5,5'-tetramethylbenzidine, hydrogen peroxide) (100 µl), was then pipetted into each well and the microplate was incubated for 15 minutes at room temperature in the dark. After incubation, stop solution (0.5 M sulphuric acid) (100 µl) was then pipetted into each well in the same order and at the same speed as the substrate was added to stop the enzyme-antibody conjugate- substrate reaction. Within 30 minutes of stopping the reaction, the microplate was shaken gently to mix the contents and the optical densities of the reactants in the sample wells was measured at a

wavelength of 450nm on the Bio-Rad PR 3100 TSC (BIO-RAD) spectrophotometric microplate reader. For IgM antibody determination, optical densities were translated into semi-quantitative results according to the formula,

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of the calibrator}} = \text{Ratio}$$

The ratios were interpreted as thus

Ratio less than 0.8 = Negative

Ratio between 0.8 to 1.1 = Borderline (equivocal)

Ratio greater than or equal to 1.1 = Positive

For IgG antibody determination, optical densities were translated into quantitative results by reading of an internally generated standard curve of optical densities against calibrator concentrations in international units per ml

The results were interpreted as thus

Reading less than 8 IU/ml = Negative

Reading between 8 IU/ml to 11.1 IU/ml = Borderline (equivocal)

Reading greater than or equal to 11.1 IU/ml = Positive

Toxoplasma gondii IgG antibody avidity serology assays

Toxoplasma gondii IgG avidity assays were performed on all patient samples that had a positive *T. gondii* IgG result with an optical density of 0.140 and above. This is because research shows that measurement of *T. gondii* IgG antibody avidity in samples with

optical densities lower than 0.140 after incubation without urea treatment does not yield reliable results, as the samples do not contain diagnostically significant concentrations of specific antibodies. *Toxoplsma gondii* IgG antibody avidity assay was performed using an enzyme linked immunosorbent assay methodology using the Euroimmun *T. gondii* IgG antibody avidity kit (Euroimmun AG) according to manufacturer's instructions.

Thawed patient serum samples (10 µl) were pipetted into 1 ml of sample buffer in a test tube and thoroughly mixed by vortexing gently. High avidity anti *T. gondii* (IgG, Human) control (100 µl), low avidity anti *T. gondii* (IgG, Human) control (100 µl) and diluted serum samples (100 µl) were then pipetted into the wells two 96 well microplates pre-coated with *T. gondii* antigen in the same order and incubated for 30 minutes at room temperature. The microplate wells were then emptied by tapping gently but firmly unto an absorbent paper towel with the openings facing downwards, in order to remove all unbound sample. The microplates were then washed once with 300 µl of wash buffer by repeated pipetting. The microplates were emptied of all residual wash buffer by tapping gently but firmly on absorbent paper towel with the openings facing downwards. Urea (200 µl) was added to each well of first 96 well microplate and phosphate buffer (200 µl) was added to each well of the duplicate 96 well microplate. Both microplates were then incubated for 10 minutes at room temperature. After incubation, the two microplates were then washed three times with 300 µl of working strength wash buffer and the wells emptied of all residual wash buffers by tapping gently but firmly on absorbent paper towel with the openings facing downwards. Enzyme conjugate (peroxidase-labelled anti-human IgG) (100 µl) was then pipetted into each well of the two microplates and the microplates were incubated for 30 minutes at room temperature. The microplate wells

were emptied and washed three times as described above. Enzyme substrate, TMB H_2O_2 (3,3', 5,5'-tetramethylbenzidine, hydrogen peroxide) (100 µl), was then pipetted into each well and the microplates were incubated for 15 minutes at room temperature in the dark. After incubation, stop solution (0.5 M sulphuric acid) (100 µl) was then pipetted into each well of the microplates in the same order and at the same speed as the substrate was added to stop the enzyme-antibody conjugate- substrate reaction. Within 15 minutes of stopping the reaction, the microplates were shaken gently to mix the contents and the optical densities of the reactants in the sample wells was measured at a wavelength of 450nm on the Bio-Rad PR 3100 TSC (BIO-RAD) spectrophotometric microplate reader.

Optical densities were translated into relative avidity scores according to the formula,

$$\frac{\text{Extinction of the sample with Urea treatment}}{\text{Extinction of the sample without Urea treatment}} \times 100 = \text{Relative avidity index (RAI)}$$

The relative avidity indices were interpreted as thus,

Relative avidity index (RAI) less than 40% = Indication of low-avidity antibodies

Relative avidity index (RAI) between 40% and 60% = Equivocal range

Relative avidity index (RAI) greater than 60% = Indication of high-avidity antibodies

Significance of a low or high relative avidity index

Avidity tests are only useful if applied in combination with standard IgG and IgM serology tests. A low avidity index result implies an infection was acquired within twelve weeks of testing. It is important to note that since low avidity antibodies have been shown to persist for years after an infection, a low avidity index cannot be used alone as an

indication of a recent infection. A high avidity result on the other hand implies the infection was acquired more than twelve weeks prior to testing and hence a recent infection can be ruled out conclusively. Avidity testing is very important in the case where both the IgG and IgM are positive, since the avidity result can be used to rule out a recent infection. Failing that, follow up samples or PCR testing may be used to confirm a recent infection (Montoya *et al*., 2004).

2.3.2 Investigation of infant time to acquisition of *Toxoplasma gondii* infection from two weeks up until fifty-two weeks post birth

Ethical clearance, statistical analysis, recruitment and data management are same as that for mother cohort and are reported in section 2.3.1 above.

Blood samples

Capillary blood (200 μl) was isolated from infants via heel puncture, blood was collected onto dry Whatman filter paper (Sigma) at weeks 2, 8, and 16. These were then air dried in a sterile room and stored at room temperature for further analysis. Venous blood (600 μl) was isolated from the infants at weeks 26 and 52 via venipuncture into EDTA tubes (BD). The samples collected venous blood samples were centrifuged at 3500g for 5 minutes in a Beckman GS-6 centrifuge (Beckman Ltd) to isolate the plasma. The isolated plasma was then stored in 100 μl aliquots at -80 ^{o}C for further analysis. Infant *T. gondii* infection status at 2 weeks, 8 weeks and 16 weeks was determined via PCR amplification of the 35 repeat *B1* gene, using an adaptation of the Burg *et al*., (1989) protocol.

The *B1* gene was chosen as a target for amplification and detection of *T. gondii* due to its repetitive nature, which allows for sensitive and specific detection via amplification of *B1* specific DNA.

Toxoplasma gondii DNA was extracted from Type 1 RH strain of *T. gondii* ATCC® PRA-310™ tachyzoites obtained from Dr. D Hardie at Groote Schuur Hospital in Cape Town and used as positive control. The tachyzoites had been maintained *in vitro* in a culture of human foreskin fibroblast cells (HFF) and cryopreserved in liquid nitrogen (-160 ^{o}C).

The primers used were T1 and T2 below, which amplify a 194 to 196 base pair region on the *T.gondii B1* gene.

T1 5′GGA ACT GCA TCC GTT CAT GAG 3′(Forward primer)

T2 5′ TCT TTA AAG CGT TCG TGG TC 3′ (Reverse primer)

***Toxoplasma gondii* genomic DNA extraction from tachyzoites**

Manual DNA extraction was done using the ZR genomic DNA™ –tissue miniprep kit (Zymo Research Corporation).

Cryovial (2 ml) of *T. gondii* tachyzoites in culture was allowed to thaw to room temperature and then the culture suspension was centrifuged at 1500x *g* for 5 minutes and the supernatant was discarded and the cell pellet was resuspended in 2 ml of phosphate buffered saline (Sigma), and used as starting material for the DNA extraction procedure.

Thawed tachyzoites in phosphate buffered saline (100 µl) were pipetted in a 2 ml micro centrifuge tube (Eppendorf). A solution of 2X digestion buffer (95 µl) and 5 µl proteinase K (20 mg/ml) was then added to the tachyzoite suspension. The sample was then mixed by vortexing for 30 seconds and incubated on an Accublock™ digital dry bath (Labnet International Inc) for 30 minutes at 55^{0}C with brief mixing after every 10 minutes of

incubation. After incubation, 700 µl of genomic cell lysis buffer was then added to the tube and the contents of the tube mixed thoroughly by vortexing for 30 seconds. The tube was then centrifuged for one minute at 10,000x *g* in a Sigma D-37520 micro-centrifuge (Sigma), to remove all insoluble debris. The supernatant (850 µl) was then gently transferred onto a Zymo-Spin ^{TM} IIC column in a collection tube, without the pipette touching the sides of the tube. The column was placed in a Sigma D-37520 micro-centrifuge (Sigma Aldrich) and centrifuged for one minute at 10,000x *g*. The collection tube was replaced with a new collection tube and DNA pre-wash buffer (200 µl) added to the spin column. The spin column was then centrifuged in a Sigma D-37520 micro-centrifuge for one minute at 10,000x *g*. The collection tube was replaced and g-DNA wash buffer (400 µl) was then added to the spin column. The column was placed in a Sigma D-37520 micro-centrifuge and centrifuged at 10,000x *g* for one minute. The spin column was transferred to a clean micro centrifuge tube (Eppendorf) and DNA elution buffer (100 µl) was added to the spin column and the spin column was incubated at room temperature (25-27 ^{o}C) for 5 minutes. After incubation, the spin column was placed in Sigma D-37520 micro-centrifuge and centrifuged for 30 seconds at 16,000x *g* to elute the DNA from the spin column. The eluted DNA integrity was analyzed and quantified on the Biodrop 80-3006-51 spectrophotometer (Whitehead Scientific). The eluted DNA was then diluted to an effective 1µl in 9 µl of PCR grade water (Qiagen^{R}) and aliquoted into 25 µl aliquots and stored at -80 ^{0}C for further analysis.

Polymerase chain reaction for diagnosis of infant *T. gondii* status

The PCR reaction was performed using the Thermo Scientific™ Phusion™ blood direct kit (Thermo scientific), which is designed to perform PCR directly from whole blood with no prior DNA extraction. The starting material for *T. gondii* DNA detection from the infant blood was infant blood impregnated into Whatman™ filter paper (Sigma). The positive control was 1 µl of extracted *T. gondii* genomic DNA also impregnated into Whatman™ filter paper discs (Sigma). PCR grade water 1µl (Qiagen®) was used as the no template control (NTC).

Each PCR reaction mixture was made up of 10 µl of 2x Phusion blood PCR buffer (Thermo scientific), which contained dNTP mix (10 mM) and $MgCl_2$ (3 mM), 0.4 µl of Phusion blood II DNA polymerase (5 U/µl) (Thermo scientific), 5 mM each of forward and reverse primers (Integrated DNA technologies), 1 µl of positive control DNA (1 mm impregnated Whatman™ filter paper disc), 5% infant blood sample (1 mm impregnated Whatman™ filter paper disc) in a final reaction volume of 20 µl. The amplification conditions were 94˚C for 8 minute, 35 cycles of (94˚C for 15 seconds, 60˚C for 30 seconds and 72˚C for 45 seconds with a final extension at 72˚C for 5 minutes in a Veriti 96 well thermal cycler (Applied Biosystems).

Gel electrophoresis

PCR product (10 µl) was mixed with 2 µl of 6x DNA staining and visualizing dye, novel juice (Genedirex), and electrophoresed on 2% agarose gel (Sigma). The gel was then visualized and photographed in the Uvitec gel documentation system (Uvitec). Images were saved as .jpeg files for further analysis.

Infant *T. gondii* IgG and IgM antibody serology assays (26 and 52 week samples)

Infant plasma samples were analyzed for the presence or absence of IgM and IgG antibodies to *T. gondii* at 26 and 52 weeks respectively via an Enzyme Linked Immunosorbent Assay (ELISA) methodology using the *T. gondii* IgG and IgM antibody commercial ELISA kits (Euroimmun AG) according to the manufacturers instructions. The methodology was same as that described for maternal determinations earlier in this section.

2.3.3 Investigation of possible risk factors associated with *Toxoplasma gondii* infection in a metropolitan area in the Western Cape of South Africa

Interviews with mother

a) Maternal baseline interview for necessary obstetric, maternal demographic and contact details was performed using standardized structured questionnaires.
b) A detailed socioeconomic interview was administered once at 2 weeks after delivery using standardized structured questionnaires.
c) A detailed maternal health questionnaire was administered to all mothers at 2 weeks and 6 months after delivery using standardized structured questionnaires.

Study demographic and socio-economic variables are included (appendix 1).

2.3.4 Investigation into the prevalence of *Toxoplasma gondii* antibodies in serum of HIV-positive and HIV-negative individuals presenting with a clinical diagnosis of uveitis at Tygerberg academic hospital ophthalmology clinic

2.3.4.1 Study area

The study was based at the ophthalmology clinic at the Tygerberg Academic Hospital, in Cape Town in the Western Cape of South Africa.

2.3.4.2 Sample size determination

After consultation with the statisticians at Stellenbosch University biostatistics unit, it was determined that it would be impossible to perform a sample size determination at the outset of this study since no local data is available regarding the prevalence of ocular toxoplasmosis. It was therefore decided to choose an initial sample size of 50 HIV positive and 50 HIV negative participants as a pilot.

2.3.4.3 Study recruitment plan

The study was a cross-sectional analytical study. Fifty HIV positive and 50 HIV negative consecutive patients presenting to the ophthalmology clinic at the Tygerbeg Academic Hospital with a clinical diagnosis of uveitis were recruited unto the study over a period of one year, inclusion and exclusion criteria are listed in appendix 8.

2.3.4.4 Ethical clearance

Ethical clearance for the study was obtained from the health research ethics committee of Stellenbosch University, Cape Town, ethics clearance certificate number N13/10/146 (Appendix 3)

2.3.4.5 Data management

Data from this study was recorded on an online database called REDCap™ which was developed by Vanderbilt University in the USA. When necessary, the data was then exported to a software program for statistical analysis.

2.3.4.6 Statistical analysis

Stata version 13.1 (Statacorp, Texas, USA) was used for data analysis. Associations between categorical outcome data and other factors or covariates were assessed using chi-square test, Fisher's exact test or logistic regression. A *p*-value of less than 0.05 was considered for statistical significance

Sample collection

Whole blood

Whole blood samples were collected from enrolled patients via venipuncture, after informed consent. Whole blood (5 ml) was collected in BD Vacutainer SST^{TM} tubes (BD), and immediately put on ice before being transported to the National Health laboratories in Tygerberg academic hospital in Cape Town. The collected blood was then centrifuged at 3500x *g* for 5 minutes in a Beckman GS-6 centrifuge (Beckman Ltd) to isolate serum. Serum was stored at -80 ^{o}C for further analysis. Socio-economic as well as demographic data was collected through interviews using closed ended questionnaire administration.

Ocular (aqueous) fluid

Aqueous humour (0.1 ml – 0.2 ml) was obtained from patients in the outpatient department or in theatre at the Tygerberg Academic Hospital after informed consent was taken. Aseptic techniques were employed throughout the procedures, patients received topical anesthesia, followed by cleaning with povidone-iodine 5% solution. Thereafter, a sterile lid speculum (Schott) was used and the anterior chamber paracentesis made with a 28-gauge 12.7mm needle (Lasec). Post-procedure another drop of povidone-iodine 5% was instilled cold chain technique was maintained throughout procedure. The collected aqueous fluid was then aliquoted into 200 µl aliquots in sterile 2 ml collection tubes (Eppendorf) and stored at -80^{o}C for further analysis.

DNA extraction from ocular (aqueous) fluid

The stored aqueous fluid was allowed to thaw on ice and DNA was extracted using the nuclisens easyMAG automated extraction system (bioMerieux) (bioMerieux 2016). The starting volume of aqueous fluid was 100 µl. the DNA was extracted into a final volume of 50 µl elution buffer in a 2 ml collection tube (Eppendorf) and stored at -80 ^{o}C for further analysis.

***Toxoplasma gondii* IgG and IgM antibody serology assays**

Human serum samples were analyzed for the presence or absence of IgM and IgG antibodies to *T. gondii* via ELISA methodology using the *T. gondii* IgG and IgM antibody commercial ELISA kits (Euroimmun AG) according to the manufacturer's instructions as described previously in section 2.3.1.9 of this chapter.

2.3.5 Detection of *Toxoplasma gondii* DNA in DNA from ocular (aqueous) fluid of patients presenting with intraocular inflammation via PCR methodology

Toxoplasma gondii diagnostic PCR

Ocular (aqueous) fluid DNA extracts were analyzed for the presence of *T. gondii* DNA via polymerase chain reaction amplification of the 35 repeat *B1* gene, using an adaptation of the (Burg *et al*. 1989) protocol. The primers used were T1 and T2 below, which amplify a 194 base pair region on the *T.gondii B1* gene.

T1 5′ GGA ACT GCA TCC GTT CAT GAG 3′ (Forward primer)

T2 5′TCT TTA AAG CGT TCG TGG TC 3′ (Reverse primer)

Each PCR consisted of 10 µl of crude DNA extract or 1 µl of control DNA, 10x PCR buffer (Mg-) (Roche diagnostics), 10 mM dNTP mix (2.5 mM each of dATP, dCTP, dGTP, dTTP) (Roche diagnostics), 25 mM $MgCl_2$, 25 µM each of reverse and forward primers (Integrated DNA Technologies) and faststart taq DNA polymerase 5 U/µl (Roche diagnostics) in a final volume of 50 µl. The amplification conditions were 94˚C for 2 minute, 35 cycles of (94˚C for 20 seconds, 58˚C for 30 seconds and 72˚C for 45 seconds) with a final extension at 72˚C for 7 minutes in a Veriti 96 well thermal cycler (Applied biosystems).

Gel electrophoresis

PCR product (10 µl) was mixed with 2 µl of 6x DNA staining and visualizing dye, novel juice (Genedirex), and electrophoresed on 2% agarose gel (Sigma). The gel was then

visualized and photographed in the Uvitec gel documentation system (Uvitec). Images were saved as .jpeg files for further analysis.

2.4 RESULTS

2.4.1 Determination and comparison of *Toxoplasma gondii* antibody sero-prevalence in a cohort of HIV (+) and HIV (-) mothers two weeks after childbirth

To determine and compare the *T. gondii* antibody sero-prevalence in mother cohort of HIV positive and HIV negative individuals two weeks after childbirth, we targeted a minimum of 130 HIV positive and 130 HIV negative mother-Infant pairs within the Kraaifontein metropole of Cape Town in South Africa. In a prospective and longitudinal cohort study, two hundred and sixty four (264) mother-infant pairs were recruited from July 2012 to January 2014. Four mother infant pairs were excluded from study voluntarily leaving a total of 260 at the onset of the study. Two mothers were withdrawn from the study later on due to HIV sero-conversion during the course of the study hence in total two hundred and fifty eight (258) mothers were included in the laboratory analysis part of the study (Figure 2.2). The minimum target of 130 HIV-positive and 130 HIV-negative mothers at baseline as well as mother infant pairs at the two-week visit time point was achieved although it was the minimum, but recruitment could not be extended owing to financial, logistics and ethical permission difficulties.

To determine characteristics of the mother cohort that may be associated with a *T. gondii* infection, we collected demographic, biological and socioeconomic data on the mothers through questionnaire administration and baseline laboratory tests, and tested their association with *T. gondii* IgG and/or IgM sero-positivity through Pearson chi squared test and the results reported as (p) values. The recruited mother cohort was made up of

235 (90.4) individuals of black African ethnicity, 22 (8.5%) individuals of South African Coloured ethnic origins and 3 (1.1%) individuals of unknown ethnicity (Figure 2.3). The ages of the women ranged from 18.4 years to a maximum of 42.7 years with a mean age of 26.8 years (Figure 2.4), there were 131 (50.38%) HIV-positive individuals and 129 (49.6%) HIV-negative individuals (Figure 2.5) on the study. Overall, *T. gondii* IgG antibody seropositive rate was 23.3% (60 out of 256) with a 95% confidence interval of 18.5- 28.8%. *T. gondii* IgM antibody seropositive rate was 18.7% (48 out of 256) with a 95% confidence interval of (14.3-23.9%) and 15 (5.8%) were both IgG and IgM positive with a 95% confidence interval of (3.3-9.4%), (Figure 2.7).

The study cohort was made up of 90.4% black and 8.5% coloured individuals and the observed seroprevalence rates for IgG were 21.5%, (95% CI 16.4-27.3%%) in the black ethnic group and 40.9%, (95% CI 20.7-63.6%) in the coloured ethnic group, whilst IgM antibody seroprevalence was 18.5%, (95% CI 13.7-24.0%) in the black ethnic group and 22.5%, (95% CI 7.8-45.4%) in the coloured ethnic group. There were a higher percentage of IgG positive mothers in the group of mothers of coloured ethnicity 40.9% (95% CI 34.6-47.2%), than the group of mothers of black African ethnicity 21.5% (95% CI 16.4-27.3%) (Table 2.6), whilst there was also a higher percentage of IgM positive mothers in the group of mothers of coloured ethnicity 22.7% (95% CI 17.3-28.1%), than the group of mothers of black African ethnicity 18.5% (95% CI 13.7-24.0%) (Table 2.7).

We also determined the *T. gondii* IgG and IgM seroprevalence variation with age within the study cohort, the mean age for IgG negative group was 26.95 (95% CI 26.3-27.7%), whist the mean age for the IgG positive group was 27.5 (95% CI 26.0-29.0%) and there was no significant variation of *T. gondii* IgG seroprevalence with age within the studied

cohort (p = 0.7687) (Table 2.4). The mean age for the IgM negative group was 27.14 (95% CI 26.4-27.9%), whilst the mean age for the IgM positive group was 26.83 (95% CI 25.4-28.2%). There was also no significant variation of IgM antibody seroprevalence with the ages of the mothers in the study cohort (p = 0.3449) (Table 2.5).

To determine whether or not there was an association between CD4 cell count and *T. gondii* status, we also measured the CD4 counts of all the study participants, the lowest absolute CD4 cell count was 5.5 cells/mm^3 whilst the highest was 1741 cells/mm^3 and the average CD4 count was 467.4 cells/mm^3 (Figure 2.6).

In order to investigate the risk of congenital infection in the infant cohort, we determined approximately the time at which the *T. gondii* infection was acquired by determining the IgG antibody avidity index. *Toxoplasma gondii* IgG antibody avidity index was determined for all IgG positive samples with an optical density of greater than 0.140 after incubation without urea (n=56), Fifty-one (91%) of IgG positive mothers had high avidity *T. gondii* IgG antibodies, implying previous exposure to *T. gondii* (greater than 12 weeks prior to being tested for *T. gondii* infection), whilst five (8.9%) of IgG positive mothers had low avidity *T. gondii* IgG antibodies implying a possible recent exposure to *T. gondii* (less than twelve weeks of being tested for *T. gondii* infection) (Table 2.1). We also determined the *T. gondii* IgG and IgM seroprevalence of the HIV positive group of mothers and also for the HIV negative group of mothers and investigated if there was any association between *T. gondii* IgG and IgM serostatus and HIV serostatus for the cohort. There was a higher *T. gondii* IgG antibody seroprevalence in the HIV negative group, 25.2% 95% CI (18.4-33.5%) than there was in the HIV positive group of mothers, 21.4% 95% CI (15.2-29.3%) in the study, but this difference was not significant (p = 0.4670)

(Table 2.2). There was a higher *T. gondii* IgM seroprevalence in the HIV negative group of mothers, 25.2% (95% CI 18.4-33.5%) than there was in the HIV positive group of mothers 12.2% (95% CI 7.6-19.1%) in the study and this difference was statistically significant ($p = 0.008$) (Table 2.3). *Toxoplasma gondii* IgG seroprevalence did not vary significantly with the ages of mothers tested for *T. gondii* infection in the study ($p = 0.7687$) (Table 2.4), and *T. gondii* IgM seroprevalence also did not vary significantly with the ages of mothers tested for *T. gondii* infection in the study ($p = 0.3549$) (Table 2.5).

To determine whether the educational level attained by an individual had any effect on *T. gondii* sero-positivity, we collected data on the educational background of the mothers in the cohort and determined its association with *T. gondii* IgG and IgM sero-status through a Pearson chi-squared test. The highest level of education attained by respondents was incomplete further education post grade 12 whilst the lowest was grade one. 84 (32.5%) of mothers had completed grade 12 level education, 65(25.2%) of mothers had completed grade 11 level education, 51(19.8%) of mothers had completed grade 10 level education, 29 (11.2%) of mothers had completed grade 9 level education, whilst there was 1 (0.4%) with lower than grade 1 level education and also 1 (0.4%) with a higher than grade 12 level education. *Toxoplasma gondii* IgG antibody seroprevalence did not vary significantly with the level of education attained by study participant ($p = 0.0830$). The highest IgG seroprevalence was in the groups with a minimum of grade one and grade three level education respectively, where it was one out of a total of one individual (100%), whilst the lowest IgG seroprevalence was in the group with a minimum of incomplete further education post grade 12, where there was no IgG positive individual

(0%), (Table 2.8). *Toxoplasma gondii* IgM antibody seroprevalence did not vary significantly with the level of education attained by study participant ($p = 0.6060$). The highest IgM seroprevalence was in the groups with a minimum of grade seven level education, where it was two (2) out of a total of seven (7) individuals (28.6%), whilst the lowest IgM seroprevalence was in the groups with a minimum of grade one, grade three, grade three and incomplete further education, where there were no IgM positive individuals (0%), (Table 2.9).

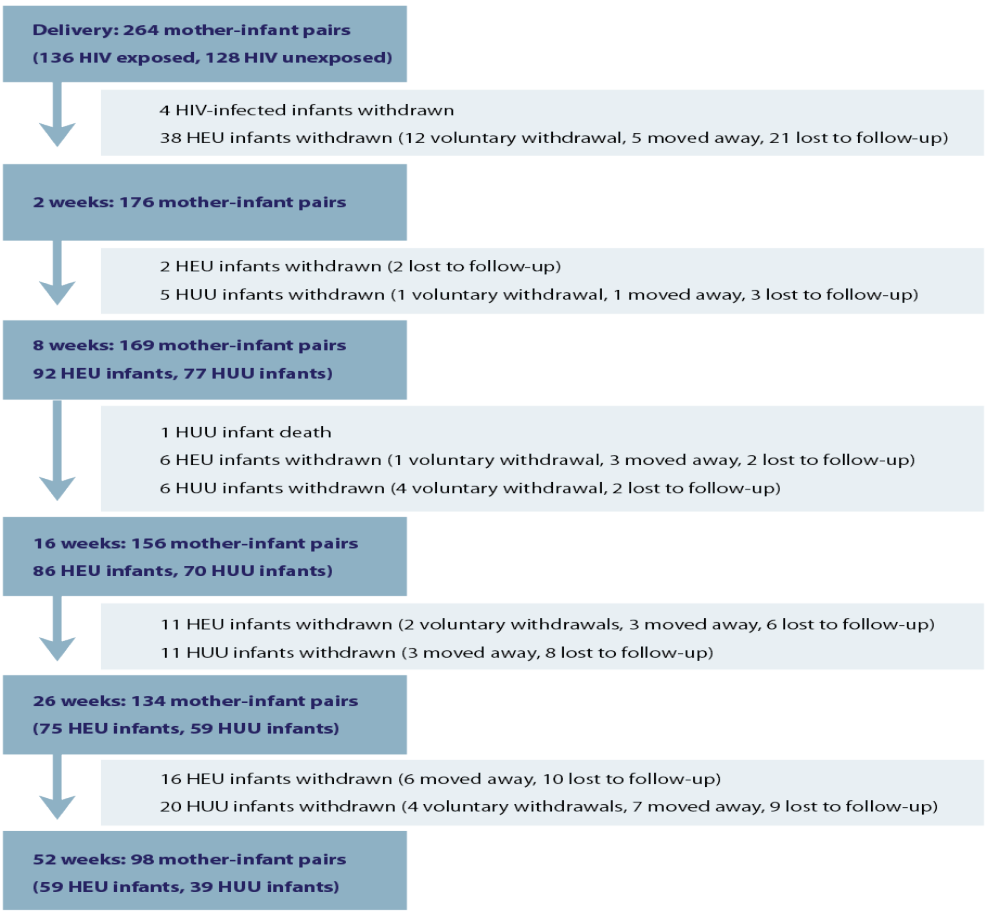


Figure 2.2 A schematic depiction of study recruitment profile for the mother infant health study.

Notes: Two hundred and sixty four mother infant pairs were recruited unto the study, infants were followed up for 52 weeks. The infant cohort was made up of 136 human immunodeficiency virus exposed but uninfected infants and 128 human immunodeficiency virus unexposed and uninfected infants at recruitment but at the onset of the study at week two there were 176 infants on the study, total loss to follow up over the course of the study were 77 human immunodeficiency virus exposed but uninfected infants and 89 human immunodeficiency virus unexposed and uninfected infants, therefore infant cohort at week 52 was made up of 59 human immunodeficiency virus exposed but uninfected infants and 39 human immunodeficiency virus unexposed and uninfected infants.

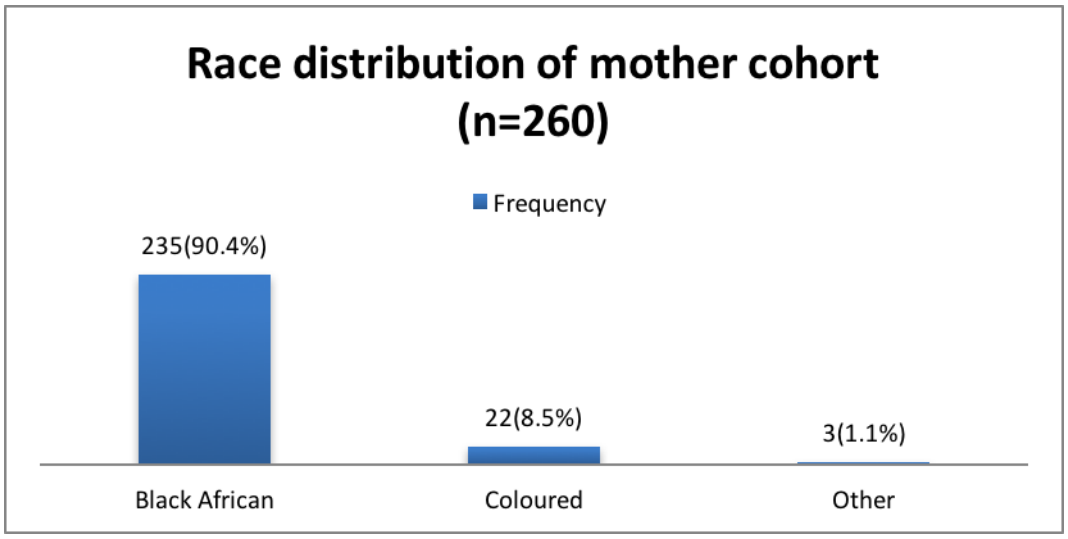


Figure 2.3 Ethnic distribution of study cohort.

Notes: Of the 260 mothers on the study, 235 (90.4%) were of black African ethnic origins, 22 (8.5%) were of coloured African ethnic origins and 3 (1.1%) were of unknown ethnic origins.

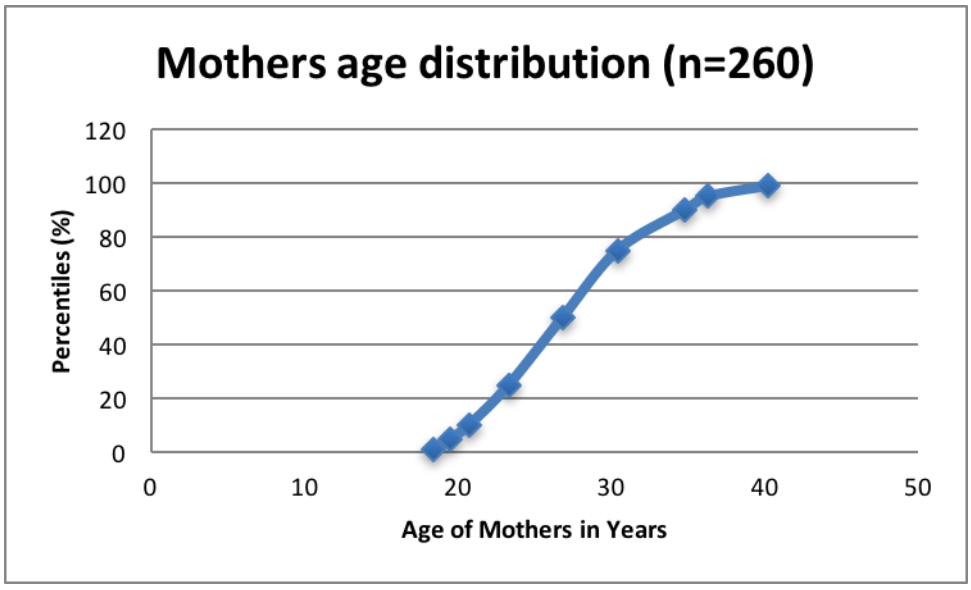


Figure 2.4 Age frequency distribution of study cohort.

Notes: Ages of the mothers on the study ranged from a minimum age of 18.4 years to a maximum age of 42.7 years, with a mean age being 26.8 years.

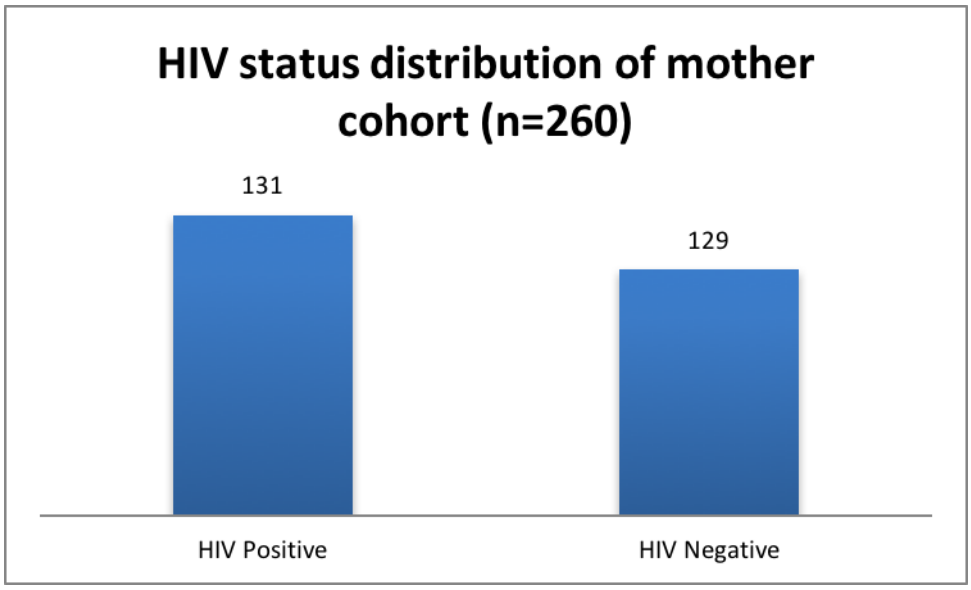


Figure 2.5 Mother cohort human immunodeficiency virus status profile.

Notes: The figure above shows the distribution of human immunodeficiency virus positive and human immunodeficiency virus positive negative mothers in the study cohort, there were 131 human immunodeficiency virus positive positive mothers and 129 human immunodeficiency virus positive negative mothers in the cohort.

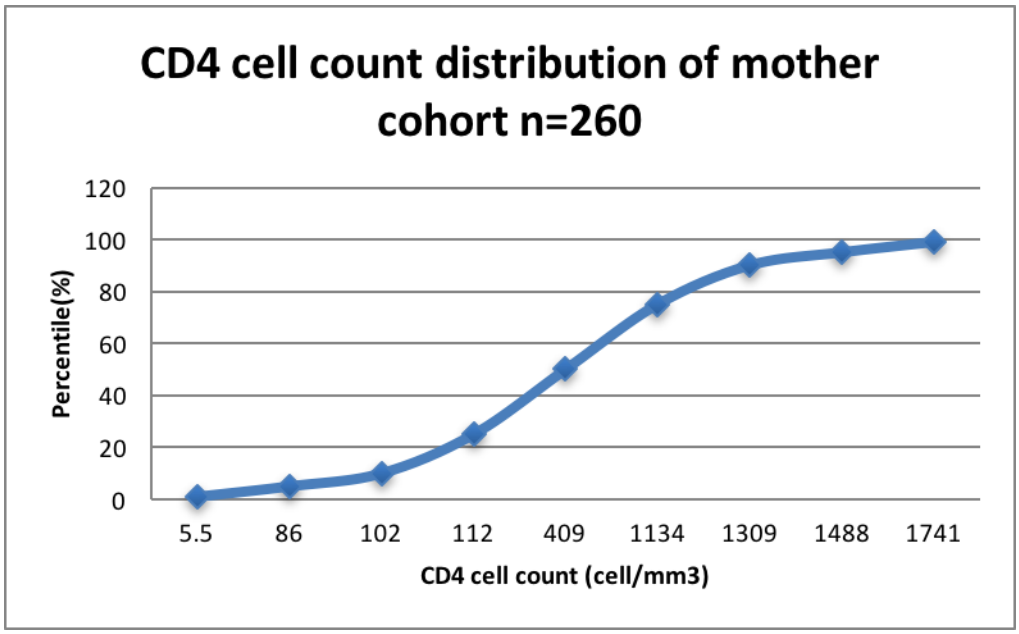


Figure 2.6 CD4 cell count frequency distribution of cohort.

Notes: The CD4 cell count were reported in cells/mm^3 and ranged from a minimum of 5.5 cells/mm^3 to a maximum of 1741 cells/mm^3

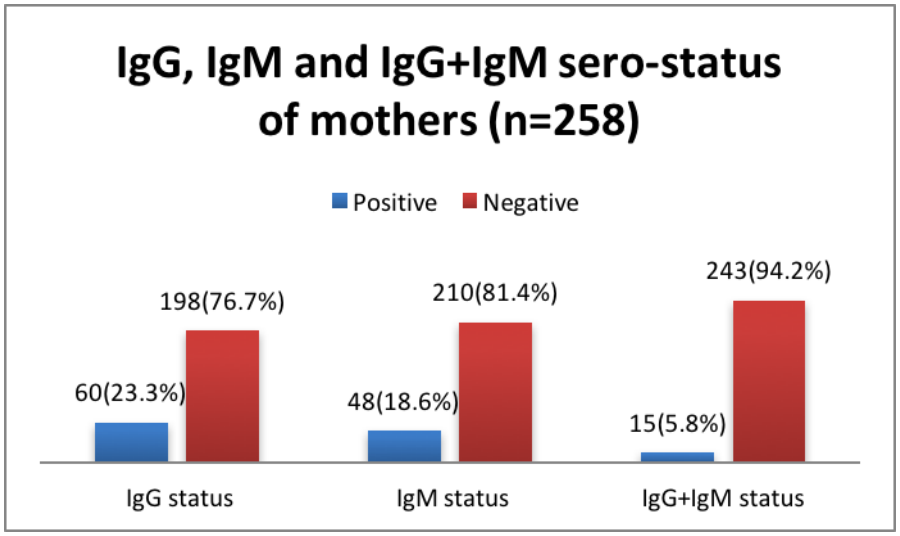


Figure 2.7 *Toxoplasma gondii* IgG only, IgM only and (IgG + IgM) antibody seroprevalence in mother cohort

Note: IgG seroprevalence in mother cohort was 23.3% (95% CI 18.5-28.8%), IgM seroprevalence was 18.7% (95% CI 14.3-23.9%) and IgG+IgM seroprevalence was 5.5% (95% CI 3.3-9.4%)

Table 2.1 *Toxoplasma gondii* IgG antibody avidity status of IgG antibody positive mothers in the study cohort

IgG Avidity Index (%)	Frequency
Less than 60 95% CI (%)	5 (8.9%) (3.7-20.2)
Greater than or equal to 60 95% CI (%)	51 (91.0%) (79.8-96.3)
Total	56 (100%)

Notes: Of the 56 mothers whose IgG antibody avidity indices were determined, 5 of them (8.9%, 95% CI 3.7-20.2%) had low avidity antibodies and 51 of them (91%, 95% CI 79.8-96.3%) had a high avidity index

Table 2.2 *Toxoplasma gondii* IgG antibody seroprevalence versus human immunodeficiency virus positive status of study cohort

	IgG Negative	IgG Positive	Total
HIV Positive 95% CI (%)	103 (78.6%) (70.7-84.8)	28(21.4%) (15.2-29.3)	131 (100%)
HIV Negative 95% CI (%)	95 (74.8%) (66.5-81.6)	32 (25.2%) (18.4-33.5)	127 (100%)
Total 95% CI (%)	198 (76.7%) (71.2-81.5)	60 (23.3%) (18.5-28.8)	258 (100%)

Notes:IgG antibody seroprevalence in the human immunodeficiency virus negative group was 25.2% (95% CI 18.4-33.5%), which was higher than that of 21.4% (95% CI 15.2-29.3%) in the human immunodeficiency virus positive group, but the difference was not significant $p = 0.4670$

Table 2.3 *Toxoplasma gondii* IgM antibody seroprevalence versus human immunodeficiency virus status of study cohort

	IgM Negative	IgM Positive	Total
HIV Positive 95% CI (%)	115 (87.8%) (80.9-92.4)	16 (12.2%) (7.6-19.1)	131 (100%)
HIV Negative 95% CI (%)	95 (74.8%) (66.5-81.6)	32 (25.2%) (18.4-33.5)	127 (100%)
Total 95% CI (%)	210 (81.4%) (76.1-85.7)	48 (18.6%) (14.3-23.9)	258 (100%)

IgM antibody seroprevalence was 25.2% (95% CI 18.4-33.5%) in the human immunodeficiency virus negative group and this was higher than that of 12.2% (95% CI 7.6-19.1%) in the human immunodeficiency virus positive group. This difference was significant ($p = 0.008$)

Table 2.4 *Toxoplasma gondii IgG* seroprevalence variation with age of mothers in the study cohort

	Observations	Mean Age	95% CI (%)
Negative	198	26.95	26.3-27.7
Positive	60	27.51	26.0-29.0

Notes: Mean age for IgG negative group was 26.95 (95% CI 26.3-27.7%), whist the mean age for the IgG positive group was 27.5 (95% CI 26.0-29.0%). There was no significant variation of *T. gondii* IgG seroprevalence with age within the studied cohort (p = 0.7687)

Table 2.5 *Toxoplasma gondii* IgM seroprevalence variation with ages of mothers in the study cohort

	Observations	Mean Age	95% CI (%)
Negative	210	27.14	26.4-27.9
Positive	48	26.83	25.4-28.2

Note: Mean age for the IgM negative group was 27.14 (95% CI 26.4-27.9%), whilst the mean age for the IgM positive group was 26.83 (95% CI 25.4-28.2%). There was no significant variation of IgM antibody seroprevalence with the ages of the mothers in the study cohort (p = 0.3449)

Table 2.6 *Toxoplasma gondii* IgG antibody seroprevalence within ethnic groups in mother cohort

	T. gondii IgG antibody sero-status		
Ethnicity of Mother	IgG Negative	IgG positive	Total
Black African	183 (78.5%) (95% CI 73.2-83.8%)	50 (21.5%) (95% CI 16.4-27.3%)	233 (100%)
Coloured	13 (59.1%) (95% CI 52.8-65.4%)	9 (40.9%) (95% CI 34.6-47.2%)	22 (100%)
Other	2 (66.7%) (95% CI 60.1-72.8%)	1 (33.3%) (95% CI 27.3-39.4%)	3 (100%)
Total	198 (76.7%) (95% CI 71.27-82.0%)	60 (23.3%) (95% CI 17.87-28.73%)	258 (100%)

Note: There were a higher percentage of IgG positive mothers in the group of mothers of coloured ethnicity 40.9% (95% CI 34.6-47.2%), than the group of mothers of black African ethnicity 21.5% (95% CI 16.4-27.3%)

Table 2.7 *Toxoplasma gondii* IgM antibody seroprevalence within ethnic groups of mother cohort

	T. gondii IgM antibody sero-status		
Ethnicity of mother	IgM Negative	IgM Positive	Total
Black African	190 (81.6%) (95% CI 76.8-86.4%)	43 (18.5%) (95% CI 13.7-24.0)%	233 (100%)
Coloured	17 (77.3%) (95% CI 71.9-82.7%)	5 (22.7%) (95% CI 17.3-28.1%)	22 (100%)
Other	3 (100%)	0 (0.00%)	3 (100%)
Total	210 (81.4%)	48 (18.6%)	258 (100%)

Note: There were also a higher percentage of IgM positive mothers in the group of mothers of coloured ethnicity 22.7% (95% CI 17.3-28.1%), than the group of mothers of black African ethnicity 18.5% (95% CI 13.7-24.0%)

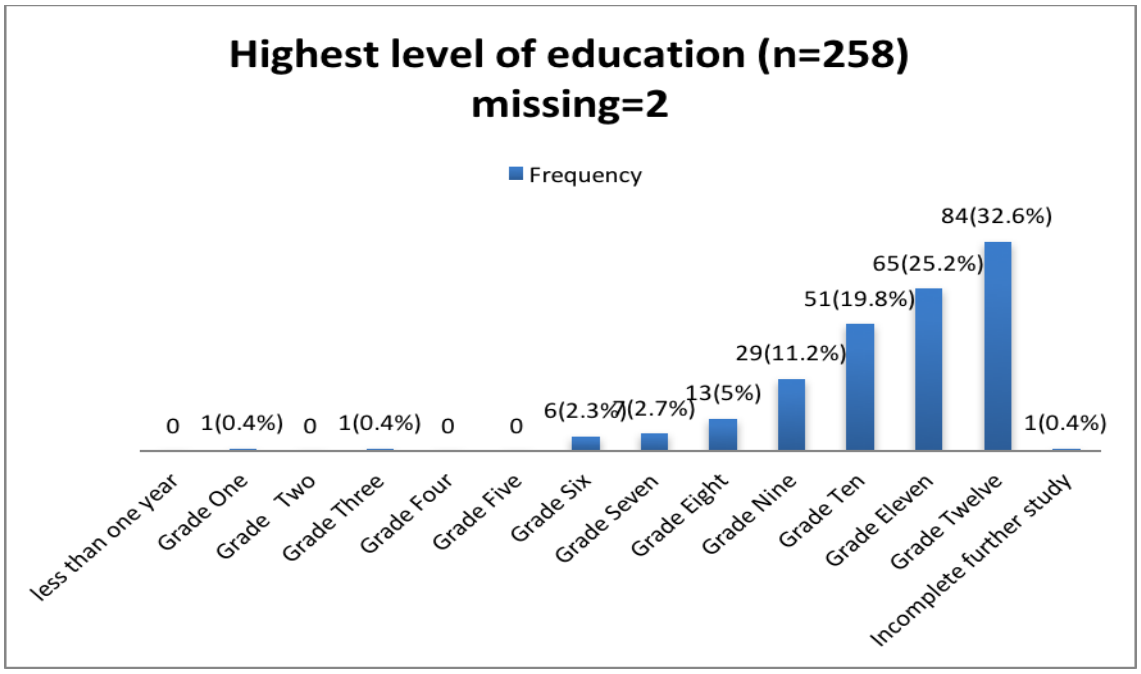


Figure 2.8 Educational background distribution of mother cohort (n=258)

Note: Figure depicts the educational background of the mother cohort. Educational background spanned from grade one to uncompleted further education post grade twelve. The 32.5% of mothers had completed grade twelve level of education, whilst 0.4% had completed grade one level education.

Table 2.8 *Toxoplasma gondii* IgG antibody seroprevalence versus educational level (meducoth) of participants

	T. gondii IgG antibody sero-status		
meducoth	Negative	Positive	Total
1	0	1 (100%)	1 (100%)
3	0	1 (100%)	1 (100%)
6	4 (66.7%)	2 (33.3%)	6 (100%)
7	3 (42.9%)	4 (57.1%)	7 (100%)
8	12 (92.3%)	1 (7.7%)	13 (100%)
9	20 (69%)	9 (31%)	29 (100%)
10	39 (76.5%)	12 (23.5%)	51 (100%)
11	49 (77.8%)	14 (22.2%)	63 (100%)
12	68 (80.9%)	16 (19.1%)	84 (100%)
13	1 (100%)	0	1 (100%)
Total	196 (76.6%)	60 (23.3%)	256 (100%)

Note: IgG antibody seroprevalence was highest in the group of mothers with grade one as the last education level, whilst the lowest IgG seroprevalence was in the group of mothers with grade eight level education. There was no IgG positive mother in the group of mothers who had attained incomplete further education. Seroprevalence did not vary significantly with the educational level of mothers in the study cohort ($p = 0.0830$).

Table 2.9 *Toxoplasma gondii IgM* sero-status versus educational level of participant (meducoth)

	T. gondii IgM antibody sero-status		
meducoth	Negative	Positive	Total
1	1 (100%)	0	1 (100%)
3	1 (100%)	0	1 (100%)
6	6 (100%)	0	6 (100%)
7	5 (71.4%)	2 (28.6%)	7 (100%)
8	10 (76.9%)	3 (23.1%)	13 (100%)
9	23 (79.3%)	6 (20.7%)	29 (100%)
10	41 (80.4%)	10 (19.6%)	51 (100%)
11	56 (88.9%)	7 (11.1)	63 (100%)
12	64 (76.2%)	20 (23.8%)	84 (100%)
13	1 (100%)	0	1 (100%)
Total	208 (81.3%)	48(18.7%)	256 (100%)

Note: IgM antibody seroprevalence was highest in the group of mothers with grade seven as the last education level, whilst the lowest IgM seroprevalence was in the group of mothers with grade eleven level education. There was no IgM positive mother in the group of mothers who had attained grade one, grade three, grade six and grade thirteen levels of education. Seroprevalence did not vary significantly with the educational level of mothers in the study cohort ($p = 0.6060$).

2.3.2 Investigation of infant time to acquisition of *Toxoplasma gondii* infection from two weeks up until fifty-two weeks post birth

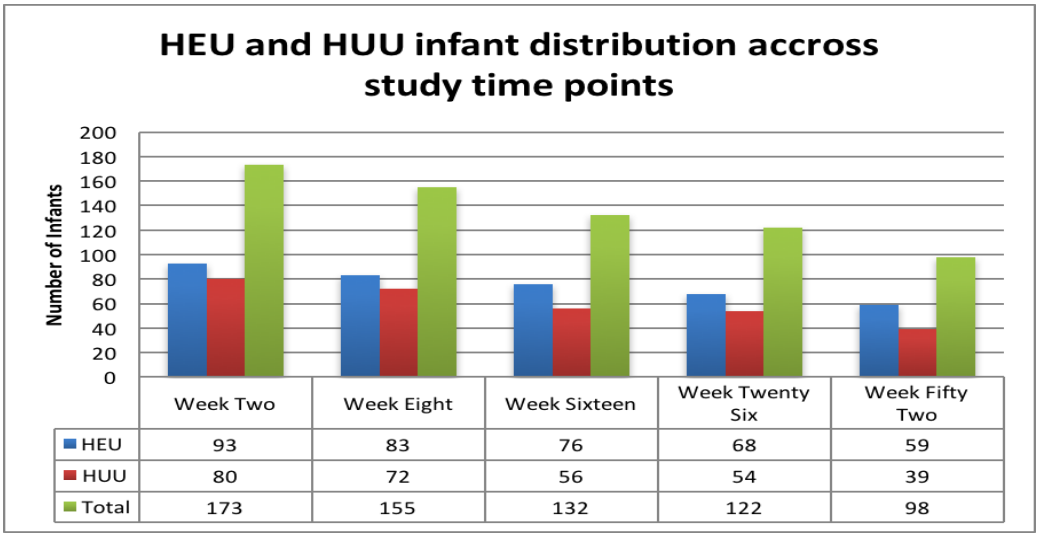
There was a high rate of loss to follow up at all visits, largely due to the fact that many mothers on the study, although they indicated that they resided in the study area, did not actually reside in the area permanently but had come in from other parts of the Cape region to access the health facilities within the study area and hence after child birth returned to their original areas of residence. This was regardless of all the incentives that were allowed according to the ethical protocol and offered, including transportation and home pickup services and regular phone calls to monitor their participation. There were a total of 173 infants at week two, made up of 93 HEU and 80 HUU infants (3 infants out of the 176 recruited at week two were withdrawn because they did not have consecutive samples). At week eight there were a total of 155 infants made up of 83 HEU and 72 HUU, at week sixteen there were 132 infants made up of 76 HEU and 56 infants, at week twenty six there were 122 infants made up of 68 HEU and 54 HUU and at week 52 there were 98 infants made up of 59 HEU and 39 HUU infants (Figure 2.9).

In order to investigate the incidence of congenital toxoplasmosis in our cohort of HEU and HUU infants and also to monitor the time to acquisition of *T. gondii* infection of the infants, we followed up the infants from birth to 12 months of age and investigated *T. gondii* serostatus at predetermined time points. We determined the sero-status of the infants at weeks two, eight and sixteen through PCR diagnosis and determined the *T. gondii* sero-status at weeks twenty-six and fifty two via *T. gondii* IgG and IgM ELISA serology assays. All the HEU and HUU infants tested at weeks two, eight and sixteen were negative for *T. gondii* DNA (Tables 2.10, 2.11 and 2.12). Further to that, at time

point four (twenty-six weeks after birth), there was one (1) 1.47% (1 out of 68) HIV exposed uninfected (HEU) infant with an equivocal *T. gondii* IgG antibody sero-status and one (1) 1.47% (1 out of 68) HIV exposed uninfected (HEU) with a positive *T.gondii* antibody sero-status. There were no HIV unexposed uninfected (HUU) infants with a positive or equivocal *T.gondii* infection sero-status. Hence, scoring conservatively, overall IgG seroprevalence at week twenty-six (26) was 0.82% (1 out of 122 infants) (Table 2.13). At time point four (twenty-six weeks after birth), there were no HIV exposed uninfected (HEU) infants or HIV unexposed uninfected infants (HUU) with an equivocal or positive *T. gondii* IgM antibody sero-status. Hence overall IgM seroprevalence at week twenty-six (26) was 0.00% (Table 2.15).

At time point five (fifty- two weeks after birth), there were no HIV exposed uninfected (HEU) infants with an equivocal *T. gondii* IgG antibody sero-status and there was one infant (1) 1.69% (1out of 59) HIV exposed uninfected (HEU) with a positive *T.gondii* antibody sero-status (Table 2.14). There were no HIV unexposed uninfected (HUU) infants with a positive or equivocal *T.gondii* infection sero-status, hence overall IgG seroprevalence at week fifty-two (52) was 1.02% (1 out of 98 infants) (Table 2.14). At time point five (fifty-two weeks after birth), there were two (2) 3.39% (2 out of 59) HIV exposed uninfected (HEU) infants with an equivocal *T. gondii* IgM antibody sero status and no HIV exposed uninfected (HEU) with a positive *T.gondii* IgM antibody sero status. There was one (1) 2.56% (1 out of 39) HIV unexposed uninfected (HUU) infants with a positive *T.gondii* IgM antibody sero-status and there were no HIV unexposed uninfected infants with an equivocal *T. gondii* IgM antibody sero-status, hence overall *T. gondii* IgM antibody seroprevalence at week fifty-two (52) was 1.02% (1 out of 98 infants) (Table

2.16). The incidence of *T. gondii* infection from birth till fifty two weeks post birth cumulatively in the infant cohort was 1.7% that is 1 IgM positive and 2 IgG positives out of the starting cohort of 173.



	Week Two	Week Eight	Week Sixteen	Week Twenty Six	Week Fifty Two
HEU	93	83	76	68	59
HUU	80	72	56	54	39
Total	173	155	132	122	98

Figure 2.9 Graph showing the distribution of human immunodeficiency virus exposed but uninfected (HEU) infants and human immunodeficiency virus unexposed and uninfected (HUU) infants across study time points

Note: There were a total of 173 infants at week two, made up of 93 human immunodeficiency virus exposed but uninfected and 80 human immunodeficiency virus unexposed and uninfected infants. At week eight there were a total of 155 infants made up of 83 human immunodeficiency virus exposed but uninfected and 72 human immunodeficiency virus unexposed and uninfected infants, at week sixteen there were 132 infants made up of 76 human immunodeficiency virus exposed but uninfected and 56 human immunodeficiency virus unexposed and uninfected infants, at week twenty six there were 122 infants made up of 68 human immunodeficiency virus exposed but uninfected and 54 human immunodeficiency virus unexposed and uninfected infants and at week 52 there were 98 infants made up of 59 human immunodeficiency virus exposed but uninfected and 39 human immunodeficiency virus unexposed and uninfected infants.

Table 2.10 *Toxoplasma gondii* sero-status of human immunodeficiency virus exposed uninfected and human immunodeficiency virus unexposed uninfected infants at week two (2)

Infant category	Number tested	Number of PCR positives
HIV exposed uninfected	93	0
HIV unexposed uninfected	80	0
Total	173	0

Note: Out of 173 infants, made up of 93 human immunodeficiency virus exposed but uninfected and 80 human immunodeficiency virus unexposed and uninfected infants tested for *Toxoplasma gondii* infection at week two via PCR, none tested positive

Table 2.11 *Toxoplasma gondii* sero-status of HIV exposed uninfected and HIV unexposed uninfected infants at week eight (8)

Infant category	Number tested	Number of PCR positives
HIV exposed uninfected	83	0
HIV unexposed uninfected	72	0
Total	155	0

Note: Out of 155 infants made up of 83 human immunodeficiency virus exposed but uninfected and 72 human immunodeficiency virus unexposed and uninfected infants tested for *Toxoplasma gondii* infection at week eight via PCR, none tested positive

Table 2.12 *Toxoplasma gondii* sero-status of human immunodeficiency virus exposed uninfected and human immunodeficiency virus unexposed uninfected infants at week sixteen (16)

Infant category	Number tested	Number of PCR positives
HIV exposed uninfected	76	0
HIV unexposed uninfected	56	0
Total	132	0

Note: Out of 132 infants made up of 76 human immunodeficiency virus exposed but uninfected and 56 human immunodeficiency virus unexposed and uninfected infants tested for *T. gondii* infection at week sixteen via PCR, none tested positive

Table 2.13 Table depicting the *Toxoplasma gondii* IgG antibody sero-status of human immunodeficiency virus exposed uninfected infants and human immunodeficiency virus unexposed and uninfected infants at week twenty-six (26)

Infant category	*Toxoplasma gondii* IgGserostatus		
	Negative	Equivocal	Positive
HIV exposed uninfected	66	1	1
HIV unexposed uninfected	54	0	0
Total	120	1	1

Note: The IgG sero-status of 122 infants determined at week twenty six, yielded 66 negative human immunodeficiency virus exposed and uninfected infants, 1 equivocal human immunodeficiency virus exposed and uninfected infant and 1 positive human immunodeficiency virus exposed and uninfected infant, whilst the human immunodeficiency virus unexposed and uninfected infants all tested negative

Table 2.14 Table depicting the *Toxoplasma gondii* IgG antibody sero-status of human immunodeficiency virus exposed uninfected infants and human immunodeficiency virus unexposed and uninfected infants at week fifty- two (52)

Infant category	*Toxoplasma gondii* serostatus		
	Negative	Equivocal	Positive
HIV exposed uninfected	58	0	1
HIV unexposed uninfected	39	0	0
Total	97	0	1

Note: The IgG sero-status of 98 infants determined at week fifty two, yielded 58 negative human immunodeficiency virus exposed and uninfected infants, and 1 positive human immunodeficiency virus exposed and uninfected infant, whilst the human immunodeficiency virus unexposed and uninfected infants all tested negative

Table 2.15 Table depicting the *Toxoplasma gondii* IgM antibody sero-status of human immunodeficiency virus exposed uninfected infants and human immunodeficiency virus unexposed and uninfected infants at week twenty-six (26)

Infant category	*Toxoplasma gondii* serostatus		
	Negative	Equivocal	Positive
HIV exposed uninfected	68	0	0
HIV unexposed uninfected	54	0	0
Total	122	0	0

Note: The IgM sero-status of 122 infants determined at week twenty six, were all negative

Table 2.16 Table depicting the *Toxoplasma gondii* IgM antibody sero-status of human immunodeficiency virus exposed uninfected infants and human immunodeficiency virus unexposed and uninfected infants at week fifty- two (52)

Infant category	*Toxoplasma gondii* serostatus		
	Negative	Equivocal	Positive
HIV exposed uninfected	57	2	0
HIV unexposed uninfected	38	0	1
Total	95	2	1

Note: The sero-status of 98 infants determined at week fifty two, yielded 57 negative human immunodeficiency virus exposed and uninfected infants, 2 equivocal human immunodeficiency virus exposed and uninfected infants, 1 positive human immunodeficiency virus unexposed and uninfected infant and 38 negative HUU infant

2.3.3 Investigation of possible risk factors associated with *Toxoplasma gondii* infection in a metropolitan area in the Western Cape of South Africa

We investigated selected socioeconomic and behavioural characteristics (Appendix 7), to ascertain whether they were risk factors for *T. gondii* IgG and IgM seropositivity in the mother cohort through the administration of questionnaires, followed by analysis of the collected data via logistic regression. The risk factors (variables) investigated, includedpresence of household pets, source of household water consumed, use of public toilet, absolute CD4 cells count, household toilet usage, how well meat is cooked before consumption, how well fruits and vegetables are washed before consumption, age and HIV serostatus (Table 2.17 and 2.18). The outcomes of the tests of associations were then reported as odds ratios (OR).

Out of one hundred and seventy six respondents (68.2%) of recruited mothers who were interviewed at two weeks after birth, 86.4% had not had a previous test for *T. gondii* and 13.6% did not know whether they had been tested previously or not, only 1 individual

(0.6%) had a household member previously tested for toxoplasmosis, whilst 63.6% had not had any household member previously tested for toxoplasmosis and 35.2% did not know whether or not any household member had been previously tested for toxoplasmosis (Table 2.17). A small proportion of mothers (11.9%) had household pets (hhpets), whilst 88.1% had no household pets. One hundred and one (63.1%) respondents used shared household toilet facilities, whilst 36.9% did not use-shared households toilets. Partially cooked meat was consumed by 5.1% of respondents whilst 94.9% consumed well-cooked meat. Fruits and vegetables were washed completely always before consumption by 55.7% of respondents, and washed often by 5.7% of respondents whilst 38.6% of respondents washed their fruits and vegetables sometimes before consumption (Table 2.17). Respondent household types were made up of 39.8% standalone houses, 4% flat in a block of flats, 4.6% house/flat/room in backyard, 32.0% shack in backyard, 16.5% shack not in backyard, 2.8% room/flat or shared property and 1.1% other. Household water type was piped into dwelling (47.1%) or piped into site or yard (44.5%), with 7.4% using public taps. Household toilet type was predominantly flush toilet with sewage connection (98.3%), then flush with septic tank (1.1%) whilst 0.6% had no toilet facility or used the bush (Table 2.18).

Of the selected variables investigated as risk factors to IgG and IgM sero-positivity, there was no association between the presence of household pets and both IgG seropositivity (OR 1.12, 95% CI 0.33-3.57, p= 0.842) (Table 2.19) and IgM sero-positivity (OR 1.10, 95% CI 0.34-3.44, *p*= 0.895) (Table 2.20), there was also no association between water being piped into the household and both IgG sero-positivity (OR 0.41, 95% CI 0.18-1.00, p= 0.074) (Table 2.19) and IgM sero-positivity (OR 0.82, 95% CI 0.38-1.77, *p*= 0.837)

(Table 2.20). There was no association between the use of a public tap and IgG sero-positivity (OR 0.51, 95% CI 0.1-2.5, *p*= 0.074) (Table 2.19) as well as with IgM sero-positivity (OR 0.71, 95% CI 0.14-3.54, *p*= 0.837) (table 2.20). There was no association between the absolute CD4 cell counts and IgG sero-positivity (OR 1.00, 95% CI 0.99-1.02, *p*= 0.412) (Table 2.19), but there was a significant association between absolute CD4 cells count and IgM sero-positivity (OR 1.00, 95% CI 1.00-1.02, *p*= 0.021) (Table 2.20). IgG and IgM sero-positivity was not associated with household toilet usage (OR 0.85, 95% CI 0.39-1.85, *p*= 0.686) (Table 2.19) and (OR 0.77, 95% CI 0.35-1.69, *p*= 0.507) (Table 2.20) respectively.

There was no association between consumption of well cooked meat and both IgG seropositivity (OR 1.30, 95% CI 0.80-2.00, *p*= 0.590) (Table 2.19) and IgM sero-positivity (OR 1.10, 95% CI 0.70-1.90, *p*= 0.126) (Table 2.20), there was also no association between the consumption of partially cooked meat and both IgG sero-positivity (OR 1.90 95% CI 1.20-3.00, *p*=0.590) (Table 2.19) and IgM sero-positivity (OR 1.00 95% CI 0.70-1.80, *p*=0.126) (Table 2.20). Although there was no significant association between IgG sero-positivity and the washing of vegetables and fruits often (OR 0.45, 95% CI 0.65-3.77, *p*= 0.55) and hardly washing of vegetables and fruits before consumption (OR 1.25, 95% CI 0.59-2.64, *p*= 0.55) (Table 2.19), we observed a statistically significant association between IgM sero-positivity and washing of vegetables and fruits often (OR 0.71, 95% CI 0.08-0.61, *p*= 0.018) and washing of vegetables and fruits sometimes (OR 2.85 95% CI 1.31-6.22, *p*= 007) (Table 2.20). The variables HIV status and age were not associated with IgG sero-positivity (OR 1.24, 95% CI 0.69-1.05, *p*= 0.467) and (OR 0.98, 95% CI 0.93-1.05, *p*= 0.708) (Table 2.19), whilst

there was a significant association between HIV status and IgM seropositivity (OR 2.42, 95% CI 1.25-4.68, *p*= 0.007) (Table 2.20). There was no association between the variable age and IgM sero-positivity (OR 1.02, 95% CI 0.97-1.08, *p*= 0.463) (Table 2.20).

Table 2.17 Variables and response frequencies for interviews with mothers at visit one

Variable	**Response category**	Frequency	Percent
Mtoxtest	No	152	86.4%
	Do not know	24	13.6%
hhtoxtest	Yes	1	0.6%
	No	112	63.6%
	Do not know	62	35.2%
	Missing data	1	0.6%
hhpets	Yes	21	11.9%
	No	155	88.1%
Household toilet facility usage	Shared with other households	111	63.1%
	Not shared with other households	65	36.9%
How do you and your household eat your meat	Well cooked	167	94.9%
	Partially cooked	9	5.1%
Do you or your household wash your fruits and vegetables before consumption	Always	98	55.7%
	Often	10	5.7%
	Sometimes	68	38.6%

Note: Respondents data from questionnaire administration to mothers at visit one (n=176). 86.4% of mothers had not had a previous test (mtoxtest) for toxoplasmosis and 13.6% did not know whether or not they had been tested before. One mother (0.6%) knew of a household member who had been tested for toxoplasmosis (hhtoxtest), 63.6% did not have anyone in their households tested for toxoplasmosis whilst 35.2% did not know whether or not any household member had ever been tested for toxoplasmosis. 11.9% of mothers had household pets (hhpets), whilst 88.1% had no household pets. One hundred and one (63.1%) respondents used shared households toilet facilities, whilst 36.9% did not use-shared households toilets. Partially cooked meat was consumed by 5.1% of respondents and 94.9% consumed well-cooked meat. Fruits and vegetables were washed completely always before consumption by 55.7% of respondents, and washed often by 5.7% of respondents whilst 38.6% of respondents washed their fruits and vegetables sometimes before consumptio

Table 2.18 Variables and response frequencies for interviews with mothers at visit one

Variable	**Response category**	Frequency	Percent
Household type	Stand alone	70	39.8%
	Flat in block of flats	7	4%
	House/Flat/Room in backyard	8	4.6%
	Shack in backyard	56	32.0%
	Shack not in backyard	29	16.5%
	Room/flatlet or shared property	4	2.8%
	Other	2	1.1%
Household water type	Water piped into dwelling	83	47.1%
	Water piped into site/yard	80	44.5%
	Pblic tap	13	7.4%
Household toilet type	Flush toilet (connected to sewage	173	98.3%
	Flush toilet (septic tank)	2	1.1%
	No facility/bush/field	1	0.6%

Note: Respondent household types were made up of 39.8% standalone houses, 4% flat in a block of flats, 4.6% house/flat/room in backyard, 32.0% shack in backyard, 16.5% shack not in backyard, 2.8% room/flat or shared property and 1.1% other. Household water type was piped into dwelling 47.1% or piped into site or yard 44.5%, with 7.4% using public taps. Household toilet type was predominantly flush toilet with sewage connection 98.3%, then flush with septic tank 1.1% and then 0.6% had no toilet facility of used the bush.

Table 2.19 Univariate analysis of selected characteristics of the mother cohort and their association with IgG antibodies to *Toxoplasma gondii* infection

Patient's characteristics	Univariate analysis		*p*-value
	Odds Ratio (OR)	95% CI	
Presence of household pets (cats)	1.12	0.35-3.57	0.842
Water piped into yard	0.41	0.18-1.00	0.074
Use of public tap	0.51	0.1-2.5	0.074
Absolut CD4 count	1.00	0.99-1.02	0.412
Household toilet usage	0.85	0.39-1.85	0.686
Well cooked meat	1.30	0.80-2.00	0.590
Partially cooked meat	1.90	1.20-3.00	0.590
Wash vegetables and fruits often	0.45	0.65-3.77	0.551
Hardly wash vegetables and fruits	1.25	0.59-2.64	0.551
HIV status	1.24	0.69-2.21	0.467
Age	0.98	0.93-1.05	0.708

Note: Univariate analysis was done via logistic regression and *p* values were determined through Pearson Chi Square, associations or non associations between the selected characteristics and *Toxoplama gondii* IgG sero-positivity were reported as odds ratios

Table 2.20 Univariate analysis of selected characteristics of the mother cohort and their association with IgM antibodies to *Toxoplasma gondii* infection

Patient's characteristics	Univariate analysis		*p*-value
	Odds Ratio (OR)	95% CI	
Presence of household pets (cats)	1.10	0.34-3.44	0.895
Water piped into yard	0.82	0.38-1.77	0.837
Use of public tap	0.71	0.14-3.54	0.837
Absolut CD4 count	1.00	1.00-1.02	0.021
Household toilet usage	0.77	0.35-1.69	0.507
Well cooked meat	1.10	0.70-1.90	0.126
Partially cooked meat	1.00	0.70-1.80	0.126
Wash vegetables and fruits often	0.71	0.08-0.61	0.018
Hardly wash vegetables and fruits	2.85	1.31-6.22	0.018
HIV status	2.42	1.25-4.68	0.007
Age	1.02	0.97-1.08	0.463

Note: Univariate analysis was done via logistic regression and *p* values were determined through Pearson Chi Square, associations or non associations between the selected characteristics and *Toxoplama gondii* IgM sero-positivity were reported as odds ratio

2.6.5 Investigation into the prevalence of *Toxoplasma gondii* antibodies in serum of HIV-positive and HIV-negative individuals presenting with a clinical diagnosis of uveitis at Tygerberg academic hospital ophthalmology clinic

In a cross-sectional analytical study, consecutive HIV positive and HIV negative patients presenting with a clinical diagnosis of uveitis were recruited at the ophthalmology clinic of the Tygerberg Academic Hospital in Cape Town. In total, 106 patients were recruited

into the study, although 9 (8.4%) patients withdrew voluntarily, therefore a total of 97 (91.6%) patients took part in the study. The study was made up of 56 (57.7%) females and 41 (42.3%) male participants (Figure 2.10). The ages of the cohort ranged from 18 years to 76 years with the mean of 36.5 years (Figure 2.11). Of the ninety-seven individuals in the study, the ages of the females ranged from 18 years to 70 years with a mean age of 35.9 years whilst the ages of the males ranged from 19 years to 65 years with a mean age of 35 years. The cohort was made up of 40 (41.2%) HIV positive and 57 (58.8%) HIV negative individuals (Figure 2.12) of whom 59% females and 58.5% males were HIV negative individuals and 41% females and 41.5% males were HIV positive individuals (Table 2.21).

Overall, the *T. gondii* IgG antibody seroprevalence rate was 24.7% (24 out of 97) (95% CI 14.93-31.51%), the *T. gondii* IgM seroprevalence rate was 1% (1 out of 97) (95% CI 0.02-5.09%) whilst the *T. gondii* IgG+IgM seroprevalence rate was also 1% (1 out of 97) (95% CI 0.12-2.66%) (Figure 2.13). Ten (10, 25%) individuals were both HIV positive and *T. gondii* IgG positive, whilst fourteen (14, 24.6%) individuals were HIV negative and *T. gondii* IgG positive. There was no difference between *T. gondii* IgG seroprevalence in the HIV positive group (25%) and the HIV negative group (24.6%) (Table 2.22). One (1) individual 2.5% was both HIV positive and *T. gondii* IgM sero-positive, whilst no (0) individual was HIV negative and *T. gondii* IgM positive, there was a higher *T. gondii* IgM seroprevalence in the HIV positive group (2.5%) than in the HIV negative group (0%) but this difference was not significant $p = 0.4120$ (Table 2.23). There was a higher *T. gondii* IgG seroprevalence in males 29.3%, twelve (12) out of forty one (41) individuals, than in females where *T. gondii* IgG seroprevalence was 21.4%

(twelve (12) out of fifty six (56) individuals), the difference was not significant p = 0.3770 (Table 2.24). There was a higher *T. gondii* IgM seroprevalence in males (2.4%, one (1) out of forty one (41)) individuals, than in females where *T. gondii* IgM seroprevalence was 0.00% (zero (0) out of fifty six (56) individuals), and the difference was not significant p = 0.4230 (Table 2.25). There was no association between age and *T. gondii* IgG antibody sero-status (p = 0.2517) and there was also no association between age and *T. gondii* IgM antibody sero-status(p = 0.4858).

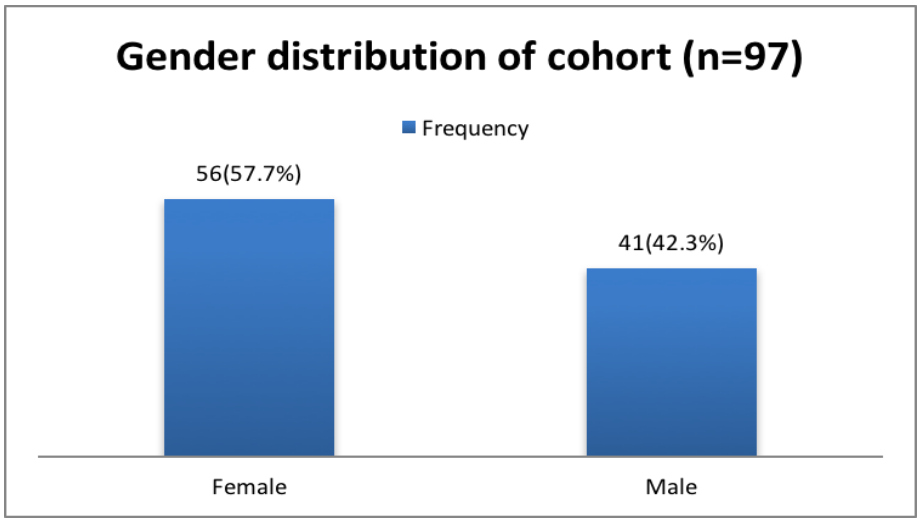


Figure 2.10 Gender distribution of ocular cohort

Note: The ocular cohort investigated was made up of 56 (57.7%) females and 41(42.3%0 males

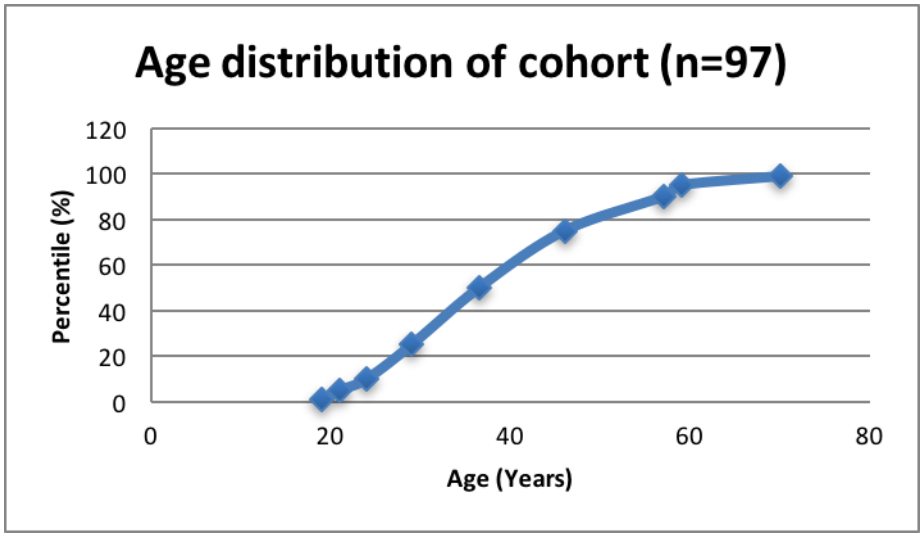


Figure 2.11 Age distribution of ocular cohort

Note: The mean age of the ocular cohort was 36.5years, ages of females ranged from 18 years to 70 years with a mean of 35.9 whilst the ages of the males ranged from years to 65 years with a mean of 35 years

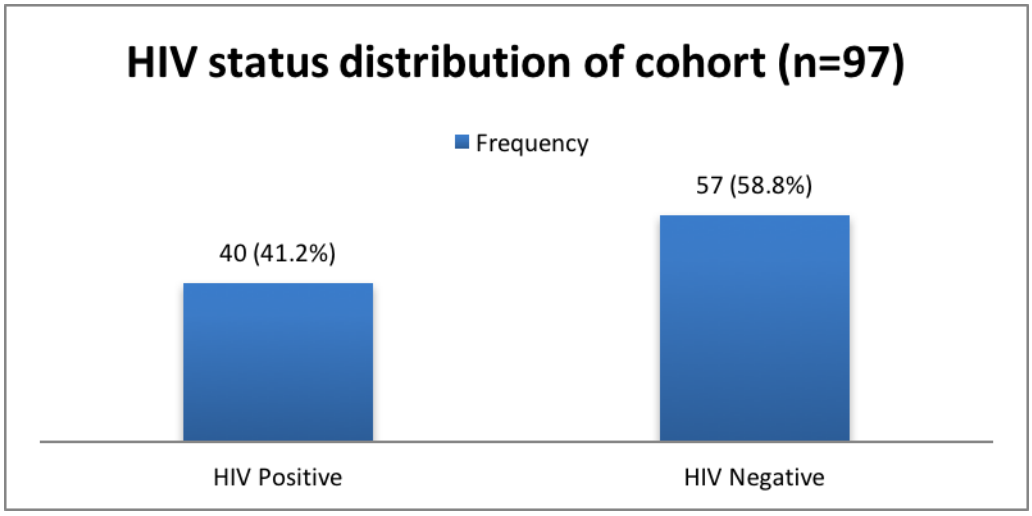


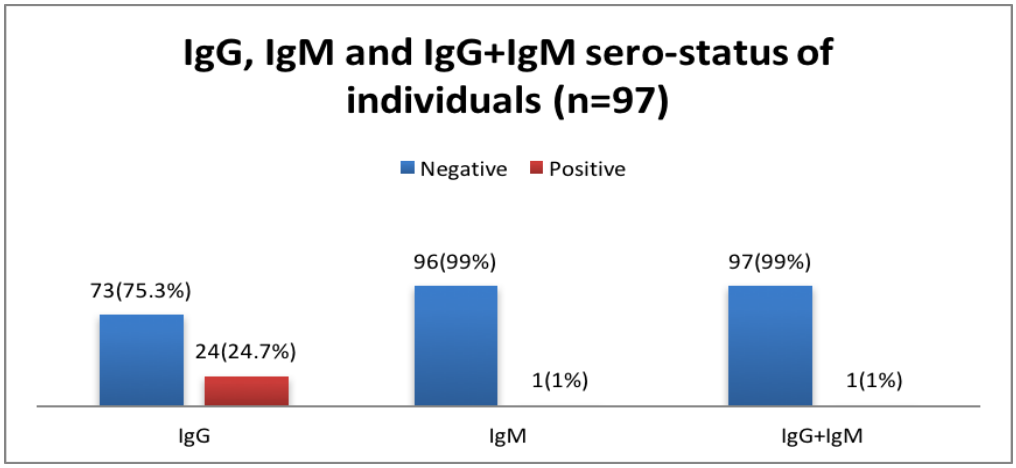
Figure 2.12 Distribution of human immunodeficiency virus status of ocular cohort

Note: The ocular cohort was made up of 40 (41%) human immunodeficiency virus positive individuals and 57 (58.8%) human immunodeficiency virus negative individuals

Table 2.21 Distribution of human immunodeficiency virus status by gender of ocular cohort

	HIV status		Total
Gender	Negative	Positive	
Female	33 (59%)	23 (41%)	56 (100%)
Male	24 (58.5%)	17 (41.5%)	41 (100%)
Total	57	40	97

Note: The ocular cohort was made up of 59% female and 58.5% male human immunodeficiency virus negative individuals together with 41% female and 41.5% male human immunodeficiency virus positive individuals



Note: IgG seroprevalence was 24.7% (95% CI 14.93-31.51%), IgM seroprevalence was 1% (95% CI 0.02-5.1%) and 1% (95% CI 0.12-2.66%) tested positive for both IgG and IgM

Figure 2.13 *Toxoplasma gondii IgG* antibody, IgM antibody and IgG+IgM antibody seroprevalence distribution of cohort

Table 2.22 *Toxoplasma gondii IgG* antibody status versus human immunodeficiency virus status of individuals in the ocular cohort

	T. gondii IgG sero-status		
HIV status	Negative	Positive	Total
Negative	43 (75.4%)	14 (24.6%)	57 (100%)
Positive	30 (75%)	10 (25%)	40 (100%)
Total	73 (75.3%)	24 (24.7%)	97 (100%)

Note: Ten (10) 25% of the human immunodeficiency virus positive individuals tested positive for *Toxoplasma gondii* IgG antibodies, 14 (24.6%) of the human immunodeficiency virus negative individuals also tested IgG positive. IgG seroprevalence was higher in the human immunodeficiency virus positive group (25%) than in the human immunodeficiency virus negative group (24.6%) but the difference was not significant ($p = 0.9610$)

Table 2.23 *Toxoplasma gondii IgM* status versus human immunodeficiency virus status of individuals in the ocular cohort

	T. gondii IgM sero-status		
HIV status	Negative	Positive	Total
Negative	57 (100%)	0 (0%)	57 (100%)
Positive	39 (97.5%)	1 (2.5%)	40 (100%)
Total	96 (99%)	1 (1%)	97 (100%)

Note: There was no human immunodeficiency virus negative individual with a positive IgM result, one (2.5%) human immunodeficiency virus positive individual tested positive for Igm antibodies. The IgM seroprevalence was higher in the human immunodeficiency virus positive group than it was in the negative group but this difference was not significant ($p = 0.4120$)

Table 2.24 Association between gender and IgG sero-status of individuals in cohort

	T. gondii IgG sero-status		
Gender	Negative	Positive	Total
Female	44 (78.6%)	12 (21.4%)	56 (100%)
Male	29 (70.7%)	12 (29.3%)	41 (100%)
Total	73 (75.3%)	24 (24.7%)	97 (100%)

Note: IgG seroprevalence was higher in males 29.3% than in females 21.4%, the difference was not significant ($p = 0.3770$)

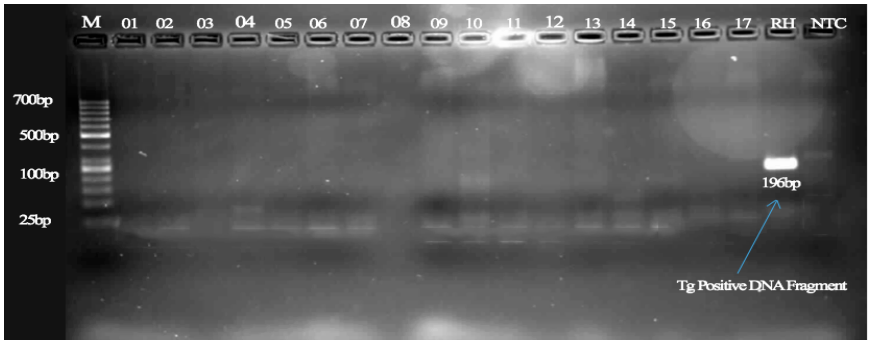
Table 2.2 1 Association between gender and IgM sero-status of individuals in cohort

	T. gondii IgM sero-status		
Gender	Negative	Positive	Total
Female	56 (100%)	0 (0%)	56 (100%)
Male	40 (97.6%)	1 (2.4%)	41 (100%)
Total	96 (99%)	1 (1%)	97 (100%)

IgM seroprevalence was higher in males 2.4% than in females, but the difference was not significant ($p = 0.4230$)

2.6.6 Detection of *Toxoplasma gondii* DNA in DNA from ocular (Aqueous) fluid of patients presenting with a clinical diagnosis of uveitis at the Tygerberg Academic Hospital ophthalmology clinic by means of PCR methodology

The presence or absence of *T. gondii* DNA in ocular fluid of recruited HIV positive and HIV negative individuals was determined by PCR amplification methodology targeting the 35 fold repeat *B1* gene of the organism see section 2.3.2 chapter 2. All the samples tested were negative for the *T. gondii* diagnostic PCR as depicted in figure 2.14 below.



M O1 O2 O3 O4 O5 O6 O7 O8 O9 O10 O11 O12 O13 O14 O15 O16 O17 RH NTC

Figure 2.14 Gel image showing migration pattern of DNA fragments after gel electrophoresis of PCR amplified product from *Toxoplasma gondii* diagnostic PCR of DNA extracted from aqueous fluid of patients presenting with ocular infections.

Note: Gel image showing migration pattern of DNA fragments after gel electrophoresis of PCR amplified product from *Toxoplasma gondii* diagnostic PCR of DNA extracted from eye fluid of patients presenting with ocular infections. RH-Positive control, O1 to O17 human ocular fluid DNA samples, NTC-No template control, M-Marker (low range DNA ladder, Roche diagnostics)

2.4 DISCUSSIONS

Summary

This chapter focuses on investigations into *T. gondii* prevalence and risk factors to infection in selected populations within a metropolitan area in Cape Town in the Western Cape province of South Africa. The populations studied were namely a cohort of HIV positive and HIV negative post parturient women and their infants, and a cohort of HIV positive and HIV negative individuals with a clinical diagnosis of uveitis.

The overall *T. gondii* IgG seroprevalence in the cohort of HIV positive and HIV negative post-parturient women was 23.3% (95% CI 18.5-28.8%), the IgM seroprevalence was 18.7% (95% CI 14.3-23.9%) and the IgG+IgM seroprevalence was 5.8% (95% CI 3.3-

9.4%). The IgG seroprevalence in the HIV positive group was 21.4% (95% CI 15.2-29.3%), whilst IgG seroprevalence in the HIV negative group was 25.2% (95% CI 18.4-33.5%). The IgM seroprevalence in the HIV positive group of post parturient mothers was 12.2% (95% CI 7.6-19.1%) whilst the corresponding HIV negative group had an IgM seroprevalence of 25.2 (95% CI 18.4-33.5%), hence the HIV negative group had both a higher IgG and IgM seroprevalence than the HIV positive group. The incidence of congenital toxoplasmosis in the monitored infant cohort was 1.47% at week 26 and 1.7% at week 52. In the cohort of uveitis patients, *T. gondii* IgG seroprevalence was 24.7% (95% CI 14.9-31.5%), whilst the IgM seroprevalence was 1% (95% CI 0.02-5.1%) and the IgG+IgM seroprevalence was also 1% (95% CI 0.12-2.66%). The IgG seroprevalence in the HIV positive group of uveitis patients was 25%, which was similar to that in the HIV negative group of uveitis patients at 24.6%, IgM seroprevalence in the HIV positive group of uveitis patients was 2.5% and there was no IgM seropositive patient in the HIV negative group of uveitis patients.

In investigating the risk factors for a *T. gondii* infection in the post parturient mother cohort, there was a statistically significant association between IgM sero-positivity and washing of vegetables and fruits often (OR 0.71, 95% CI 0.08-0.61, p= 0.018) and washing of vegetables and fruits sometimes (OR 2.85 95% CI 1.31-6.22, p= 007). Furthermore, IgM sero-positivity was significantly associated with the absolute CD4 cell count (OR 1.00, 95% CI 1.00-1.02, p= 0.021) and HIV status (OR 2.42, 95% CI 1.25-4.68, p= 0.007).

2.4.1 Determination and comparison of *Toxoplasma gondii* antibody sero-prevalence in a cohort of HIV (+) and HIV (-) mothers two weeks after childbirth

Toxoplasma gondii seroprevalence rates have been documented to vary from country to country, amongst different racial and ethnic groups, amongst similar populations in different countries or locations within the same country and amongst different populations within the same country (Pappas *et al*., 2009). Various reasons have been ascribed to the observed differences and these include but are not limited to, differences in cultural practices, socioeconomic factors, geographical and climatic conditions, feeding and dietary practices, sampling size of populations assessed and different forms of assays used for the surveys (Ayi *et al*., 2009; Koffi *et al*. 2015). When compared to other documented studies in South Africa in particular and Africa in the broader context, our studies follow a similar pattern.

The first report of *T. gondii* seroprevalence amongst different ethnic groups in South Africa was by Mason et al in 1974, where they reported seroprevalence rates of 58% in Indians, 43% in coloureds, 33% in whites and 29% in blacks with an overall seroprevalence rate of 37% in a study in the Transvaal region of South Africa. Then in 1978 a follow-up study on *T. gondii* seroprevalence rates in ethnic populations in four regions of South Africa namely, KwaZulu-Natal, Western Cape, Eastern Cape as well as Namibia and Botswana, reported a 34% rate amongst blacks, 33% amongst Indians, 9% amongst the San (Bushmen) and 12% amongst whites, with an overall rate of 20% (Jacobs and Mason., 1978). This was followed by a study in Durban in the KwaZulu-Natal province of South Africa in 1992 by Schneider et al who reported an overall seroprevalence rate of 31.3% with the highest seroprevalence rate within the population

of black ethnicity (46.2%), followed by Indian (36.9%), and then coloured (28.3%), with the white group having the lowest seroprevalence of 12.5% (Schneider *et al*., 1992). Even though our study cohort was split into coloured (8.5%) and black (90.4%) populations and the methods of testing differed from the aforementioned studies, the seroprevalence rate within both the black and the coloured groups was significantly different when compared to those in the earlier studies. In our study, the observed seroprevalence rates for IgG were 21.5%, (95% CI 16.4-27.3%) in the black group and 40.9%, (95% CI 20.7-63.6%) in the coloured group, whilst IgM antibody seroprevalence was 18.5%, (95% CI 13.7-24.0%) in the black group and 22.5%, (95% CI 7.8-45.4%) in the coloured group with an overall seroprevalence rate of 23.3% for IgG and 18.7% for IgM. In a study of 600 pregnant women in Bloemfontein in the Free State of South Africa, Brink et al. reported an overall seroprevalence of 3% and seroprevalence rates of 8% amongst blacks, 10.5% amongst whites and 12.5% amongst the coloured population of women (Brink *et al*., 1975), which was similar to our study and the Mason et al. study where the seropevalence rate was higher in the coloured than in the black population. In our study both *T. gondii* IgG and IgM antibody seroprevalence rates were higher in the coloured group than in the black group. This may possibly be owing to the highly significant difference in the number of individuals of black ethnicity compared to those of coloured ethnicity on the study. There was no association between ethnic background and IgG or IgM antibody seroprevalence in our study but such associations have been reported in other studies (Gilbert *et al*., 1993; Kapperud *et al*., 1996; Flatt and Shetty., 2013).

In South Africa specifically and Africa in the broader context, there have been no reported studies of *T. gondii* seroprevalence in HIV positive and HIV negative women

two weeks post childbirth, hence for the purposes of discussion we compare the rates obtained in our study to those obtained in studies of *T. gondii* seroprevalence in women of reproductive age, pregnant women and HIV positive or HIV negative women. Toxoplasmosis is a very important infectious disease in the case of women of reproductive age not only because of the possibility of vertical transfer of the infection to the fetus during a pregnancy and the complications that arise in the developing fetus and the prepuberscent adolescent (Lebech *et al*., 1996; Montoya and Remington., 2008) but also because of the fact that an uninfected pregnant women will always be at risk of a primary infection which is detrimental to the fetus. Generally on the African continent, reported seroprevalence rates for pregnant women or women in their reproductive ages have mostly been higher than those reported in South Africa, from 75.2% in a cohort of pregnant women in Sao Tome and Principe (Hung *et al*., 2007), 60% in a cohort of childbearing women in Abidjan (Adou-Bryn *et al*., 2004), 73.6% in a cohort of 159 pregnant women in Accra Ghana, (Ayi *et al*., 2009), 52.9% in a cohort of pregnant women in Rabat, Morocco (El Mansouri *et al*., 2007), 34.1% in a cohort of pregnant women in Omdurman in Sudan (Elnahas *et al*., 2003) and 25.3% in a cohort of pregnant women in Ougadougou (Simpore *et al*., 2006). In South Africa, the first reported study of *T. gondii* seroprevalence in women of child bearing age was in 1975 by Brink et al who reported a seroprevalence of 3% in a cohort of 600 pregnant women in Bloemfontein in the Free state province (Brink *et al*., 1975), the next reported study was Schneider et al in 1992 who reported a seroprevalence rate of 31% (Schneider *et al*., 1992) in Durban, this was then followed by Kistiah et al in 2011 who reported a seroprevalence rate of 12.8% in a cohort of HIV negative pregnant women in Gauteng province (Kistiah *et al*., 2011).

In our study we report an IgG seroprevalence rate of 25.2% in the HIV negative cohort and 23.3% overall IgG seroprevalence rate, even though the populations studied are not exactly identical and the laboratory methods used for the surveys differ, we can still make some inferences from the data presented. The rates from Durban (31%) and Cape Town (23.3% and 25.2%) (One metropolitan area) are high when compared to those from Bloemfontein (3%) and Johannesburg (12.8%), but the same rates are low when compared to those from other African locations as discussed above. This could be due to climatic conditions, cultural dispensations and habits, laboratory diagnosis methods and even the times at which the surveys were conducted (Samra *et al*., 2007; Afonso et al. 2013). South Africa is made up of different ethnic groupings with their own distinct lifestyle preferences, therefore one could expect such variability from one province to the other. The seroprevalence rate of 23.3% reported reflects an increase when analyzed within the South African context where the last reported seroprevalence rate in a similar cohort was 12.8% in Gauteng (Kistiah *et al*., 2011). There is therefore a need to undertake follow up surveys to actually confirm whether seroprevalence rates in these areas are going up or not in order to be able to put measures in place to limit transmission. The current study must be taken as exploratory in nature as it gives the seroprevalence in a defined metropolis and a defined population at just one time point. The seroprevalence needs to be monitored consistently so that the health system will be able to identify an increasing prevalence and then be prepared and able to manage the situation.

In order to provide further information regarding the *T. gondii* infection statuses of the mothers in the cohort, the IgG relative avidity index was determined for all IgG positive

mothers. Of the fifty six (56) samples analyzed, fifty one (51) 91%, (95%, CI 78.91-96.34%) had a relative avidity index value higher than 60%, which implied that the infection had been acquired greater than twelve weeks before the diagnosis, whilst five (5) 8.9% (95%, CI 3.66-20.19%) had a relative avidity index lower than 40% implying the infection had been acquired less than twelve weeks before the diagnosis or that the low avidity antibodies were persisting from an old infection (Liesenfeld *et al*., 2001). IgG avidity testing can only rule out a recently acquired infection as signified by the presence of high avidity antibodies (Liesenfeld et al. 2001). Through this study, I established the use of the *T. gondii* IgG antibody relative avidity index assay as an additional test in our laboratories at Tygerberg Academic Hospital in order to improve the efficiency of diagnosis of *T. gondii,* especially in cases of pregnant women and HIV-positive individuals. This has enabled us improve significantly the quality of care afforded these at risk groups within our setting.

The observed *T. gondii* IgM seroprevalence was18.7% (14.3-23.9%), which gives an indication of the number of mothers in the cohort that possibly had an acute *T. gondii* infection at the time sampling was done, that is two weeks post delivery. In other countries such as the USA, UK or France these mothers would be tested again with three to four week repeat samples sent to a reference center for confirmatory testing (Montoya., 2002), mainly due to the high rates of false positive testing associated with *T. gondii* IgM testing with commercially available kits in general (Liesenfeld *et al*., 1997; Garry *et al*., 2005). In the case of pregnant women, this issue is made more complicated with the associated difficulties with the interpretation of results (Sensini 2006; Tekkesin 2012).

We did not test follow up samples on our study due to funding constraints, although this would have been informative.

Toxoplasma gondii seroprevalence has been reported to increase with age, mainly due to the fact that with age comes an increased probability of repeat exposures and therefore an increased level of *T. gondii* antibodies (Frenkel., 1970). That said, there have been numerous studies on *T. gondii* seroprevalence in which this phenomenon was not observed. In our study there was no relationship between the IgG seroprevalence and the age of the mothers in the cohort and there was also no observed relationship between the IgM seroprevalence rate and the age of the mothers in the cohort. Even though some studies have reported a relationship between the *T. gondii* IgG seroprevalence and the age of various cohorts investigated, Bobic et al. reported an increasing IgG seroprevalence rate with increasing age in a cohort of 1157 females of reproductive age in Belgrade with ages ranging from 15 to 45 over a period of four years (Bobić *et al*., 1998). Nash et al. also reported a similar observation in a cohort of 1897 pregnant women in Kent UK, with ages ranging from 15 to 44 years over a period of two years (Nash *et al*., 2005) and in a study of a cohort of 963 pregnant women over a period of four months in northeastern Brazil, Sroka et al. also reported an increasing seroprevalence rate with age with the ages of the participants ranging from 12 to 44 years (Sroka *et al*., 2010). Even though the size of our cohort does not compare to those in the aforementioned studies, the age ranges are comparable. We did not observe this phenomenon (increasing seroprevalence with age) in our cohort and this could be due to the fact that at the observed ages primary infections are likely to have already occurred and also re-exposure if it occurs should be occurring at a consistent rate as those form the active years of life (Flatt and Shetty., 2013).

Not many studies in Africa have reported the *T. gondii* IgM antibody seroprevalence within the cohorts investigated. Most studies have been focused on determination of exposure to *T. gondii* within the cohorts using IgG antibody seroprevalence rates. This study reports an overall IgM antibody prevalence rate of 18.7%, with a rate of 12.2% in the HIV positive group and 25.2% in the HIV negative cohort. The IgM seroprevalence rate of 25.2% in the HIV negative group of post parturient mothers is higher than that reported in a survey of HIV negative pregnant women in Ivory Coast by Koffi et al in which there were no IgM positive individuals (Koffi *et al*., 2015), whilst the overall rate of 18.7% is also higher than the 11.7% reported in a cohort of HIV-positive and 2.0% in a cohort of HIV-negative females in Ethiopia (Walle *et al*., 2013). There was also a significantly higher IgM antibody seroprevalence in the HIV negative group of mothers (25.2%, 95% CI 18.4-33.5%) than in the HIV positive group of mothers 12.5%, (95% CI 7.6-19.1%) $p = 0.008$. There was no association between the IgG sero-status of the mothers in the cohort and the HIV status ($p = 0.467$, OR 1.2390, 95% CI 6.95-22.1). There was an observed association between the *T. gondii* IgM sero-status and the HIV status of the mothers in the cohort ($p = 0.007$, OR 2.4210, 95% CI 1.3-4.7). I have no obvious explanation for the fact that both IgG and IgM seroprevalence was higher in the HIV negative cohort than in the HIV positive cohort. The initial hypothesis was that HIV infection would result in immune suppression and therefore we should expect to see HIV positive individuals being more susceptible to infections such as *T. gondii* than HIV negative controls. The results for both IgG and IgM have proven to be otherwise. We can speculate that the exposure to *T. gondii* in the majority of the individuals in the cohort whether HIV positive or HIV negative must have occurred in the distant past and

therefore what we are actually seeing as IgM positive could be rather a possible reactivated chronic infection instead of an acute one, and therefore HIV infection did not have to play a role in acquisition of a *T. gondii* infection, also since all HIV positive mothers in the cohort were on first line cART treatment this could have boosted their immunity and improved their CD4 cell counts which may afford them increased protection against opportunistic infections such as *T. gondii* . There have been reports of wide variations of *T. gondii* seroprevalence rates in HIV- positive individuals from as low as 3 to 22% (Grant *et al*., 1990; Wallace *et al.*, 1993), whilst some reports indicate a higher seroprevalence of *T. gondii* IgG antibodies in HIV- positive compared to HIV-negative individuals. But some studies report no significant differences and others also report higher IgG seroprevalence rates in HIV- negative individuals when compared to HIV- positive individuals (Sýkora, *et al*., 1992; Morvan *et al*., 1999; Hari *et al*., 2007; Akanmu *et al*., 2010; Walle *et al*., 2013). The seroprevalence rates observed in this study are inconsistent with results obtained in other studies namely in Bahir Dar, Ethiopia (Walle *et al*., 2013) who reported an IgG and IgM seroprevalence of 87.4% and 10% respectively in a HIV- positive pre-ART cohort and an IgG and IgM seroprevalence of 70.3% and 2.97% respectively in HIV- negative asymptomatic healthy blood donor cohort, and also in a study in Mali where an IgG seroprevalence rate of 60% in HIV-positive individuals compared to 21% in HIV- negative individuals (Maïga *et al*., 2001), and in Addis Ababa where IgG seroprevalence rates of 93.3% and 86.7% were reported in HIV-infected and HIV-uninfected individuals respectively (Shimelis *et al*., 2009).

Toxoplasma gondii IgG and IgM antibodies were observed in 15 (5.8%) (95% CI 3.3-9.4%), of the mothers in the study cohort. This suggests that these mothers may have had

a reactivation of an already existing infection or that they are at a point during a primary infection where the IgM antibody levels in the serum are declining and the IgG antibody levels are now increasing in concentration. Both scenarios are ideal for onward transmission of the infection to the unborn infant and hence these individuals and their infants should ideally be monitored and if they develop any clinical symptoms should be treated (Kaul *et al*., 2004). Active toxoplasmosis is suggested by the presence of IgM antibodies, but it must be noted that the presence of elevated anti-IgM titers is often absent in immunocompromised patients. In addition, elevated IgM antibody levels can persist from an acute infection that may have occurred as long as 12 months in the past. There are also problems associated with diagnosis of primary infection in immunocompromised individuals, such as false positive IgM results, and the presence of rising IgG and IgM titres in the absence of clinically apparent infection must be taken into consideration (Takahashi and Rossi., 1997; Montoya., 2002). Hence if an active infection is suspected, then there is a need for confirmatory testing either by PCR or three to four week serial sample testing preferably in a recognized reference center (Montoya *et al*., 2004).

Our study was limited mainly because it catered for testing of the post parturient mothers at only one time point that is two weeks after childbirth, hence there was no opportunity to follow up these mothers to make sure or confirm the suspected infections through the testing of serial samples or PCR testing. This was made more difficult by the high rate of loss to follow up in the cohort, which made monitoring the mothers highly problematic. That said we did not have any hospitalization of any individual that stayed on the study till the end of the study.

2.4.2 Investigation of infant time to acquisition of *Toxoplasma gondii* infection from two weeks up until fifty-two weeks post birth

In the study, a cohort of infants were investigated for the presence of congenital infection two weeks after birth, and then followed up at predetermined time points up until fifty - two weeks post birth to determine the time to acquisition of a *T. gondii* infection if any. The rate of vertical transmission of a *T. gondii* infection as well as the severity of infection in the infant has been correlated to the gestational age at which the infection was transmitted from the mother to the fetus (Sterkers *et al*., 2012) and also to the presence of high levels of IgG and or IgM antibodies as well as low avidity IgG antibodies in the mother during the early stages of the pregnancy (Reis et al., 2006). There is no information on the rate of vertical transmission of *T. gondii* infection specifically in the Kraaifontein metropolitan area of Cape Town, and secondly any information that exists on congenital toxoplasmosis in the Western Cape or South Africa as a whole is not recent. This study therefore acts as a preliminary study from which further investigations should be developed, despite any limitations. If no other information is forthcoming, it at least provides some data to calculate sample size for future studies.

From two weeks after birth until sixteen weeks after birth no *T. gondii* infections were observed amongst the infants tested. Since no infections were observed in either infants born to HIV positive mothers or HIV negative mothers in this case referred to as HEU and HUU, from testing from two weeks after birth till sixteen weeks after birth, we conclude that there were no congenitally infected infants in our cohort of infants from the Kraaifontein metropolitan area in Cape Town. Although there have been reports of weak

association between HIV positive, *T. gondii* chronically infected women and vertical transmission of *T. gondii* to their infants (Fernandes *et al*., 2009; Campos *et al*., 2014), we did not observe this in our cohort.

The prevalence of congenital toxoplasmosis or rates of mother to child transmission of *T. gondii* has been reported to be low in most settings, even within the background of a high seroprevalence amongst pregnant mothers (Campos *et al*., 2014). In the USA it is reported to be approximately 0.01%, and in Europe it has been reported to be between 0.01% and 0.1%, whilst in certain countries in South America it has been reported to be relatively high, such as in Brazil where it has been reported to range from 0.05% to 0.15% (Fernandes *et al*., 2009). In Africa congenital toxoplasmosis rates of 0.3% have been reported in Tunisia in North Africa, (Ben Abdallah *et al*., 2009), and 0.8% in Tanzania in East Africa (Doehring et al. 1995). In a unique case in Egypt, a congenital toxoplasmosis rate of 26.7% was reported when 60 out of 80 neonates, recruited from neonatal intensive care units (NICUs) of Cairo University hospitals with a congenital toxoplasmosis risk of greater than 60% were tested for *T. gondii* through PCR targeting the *T. gondii B1* gene (Said *et al*., 2011). Such a high rate of congenital toxoplasmosis is rare and unique and could be explained by the fact that the cohort tested was a high -risk cohort.

The *T. gondii* IgG seroprevalence in the infants tested at twenty -six weeks post birth was 0.82% (1 out of 122), with 0.82% (1 out of 122) giving an equivocal result. It must be noted that both the infant that tested positive and the infant that tested equivocally for *T. gondii* IgG antibodies were infants born to HIV-positive mothers and hence were HEU infants and the mothers were both *T. gondii* IgG and IgM negative. The fact that the

mother was sero-negative for both IgG and IgM suggests that this was a false positive result, the infant did not show any signs of *T. gondii* infection at the fifty two week visit to the test center and since we did not follow the infants beyond fifty two weeks we must conclude that at the end of the study the infant had not seroconverted. All the infants born to HIV uninfected mothers tested negative for *T. gondii* infection at week twenty six and the IgM seroprevalence at twenty six weeks after birth was also 0.00%. Therefore *T. gondii* infection incidence as indicated by the IgG and IgM sero-diagnosis, within the cohort over a twenty six week period was 0.82% (1 out of 122 infants).

The *T. gondii* IgG and IgM seroprevalence at fifty two weeks after birth was 1.02% (1 each out of 98) respectively, In this cohort the infant that tested positive was born to an HIV-positive mother hence was an HEU infant, and the mother in this case was also IgG positive for *T. gondii* infection. Two HIV exposed but uninfected infants tested equivocal for *T. gondii* IgM antibodies at week fifty- two whilst one HIV unexposed and uninfected infant tested positive for *T. gondii* IgM antibodies at week fifty- two post birth. Hence without taking the equivocal results into consideration, it can be suggested that *T. gondii* infection incidence as indicated by the *T. gondii* IgG and IgM antibody sero-diagnosis was 1.7% cumulative (2 IgG positives and 1 IgM positive out of 173 infants in the initial cohort) in the infant cohort over a fifty-two week time period. Whether or not their exposure to HIV in-utero was associated with the acquisition of a *T. gondii* infection cannot be concluded due to the small number of infants that tested positive, even though studies have suggested that such infants have a higher rate of infectious morbidity when compared to their HIV unexposed and uninfected counterparts (Newell *et al*., 2004; Koyanagi *et al*., 2011; Slogrove *et al*., 2012). We also know that cases of vertical

transmission of *T. gondii* infection from mothers co-infected with *T. gondii* and HIV to their infants are rare (Campos *et al*., 2014).

The *T. gondii* infections in the infants tested can be said to be acquired post birth as opposed to being congenital infections due to the fact that all the infants were negative for *T. gondii* infections from birth up until twenty -six weeks post birth when the first infections were observed. The rate of infection did not differ between weeks twenty- six and fifty-two week time points and the numbers of positive testing infants were not significantly high enough to draw any conclusions on whether or not the *T. gondii* incidence increased with the age of the infants. Although much research attention has been focused on mother to child transmission of *T. gondii* infections, there is not much information on post- natal acquisition of *T. gondii* infection and its long-term effects on the individual. The effect of a primary infection with a pathogen, which has a preference for the central nervous system, on the continuous development of a child, will be very interesting to investigate. The impact of congenital versus childhood-acquired toxoplasmosis in an individual during adulthood will also be of interest.

In the USA, a study on 6 to 11 year old children in the Midwest reported a 3.6% seroprevalence (Muñoz-Zanzi *et al*., 2013) with 7.8% of the children being seropositive by 10 years of age. In another study in Panama, 48% of 2 to 13 year old children were found to be *T. gondii* seropositive, with a mean incidence of 3.6% per year (Sousa *et al*., 1988). Such data suggests the existence of significant exposure of children to *T. gondii* in these and similar settings and therefore the need to further investigate the incidence of post -natal childhood acquired *T. gondii* as well as the risk factors that are associated with

the infection in order to device initiatives to aid prevention and control when the need arises.

2.4.3 Investigation of possible risk factors associated with *Toxoplasma gondii* infection in a metropolitan area in the Western Cape of South Africa

Several factors have been reported as risk factors for acquisition of *T. gondii* infections in different populations globally (Paul, 1998; Kolbekova *et al*., 2007). In our study we looked at selected variables and investigated their role as risk factors for *T. gondii* infection in a cohort of post parturient mothers in a metropolitan area of Cape Town in the Western Cape of South Africa.

In South Africa, there have been sporadic studies on *T. gondii* seroprevalences in different provinces although none that studied an entire province or at the nationwide level. Furthermore, none of the reported studies investigated risk factors for *T. gondii* infection within the settings in which they were carried out. Our study therefore is the first (as far as we can ascertain) within South Africa to investigate risk factors to *T. gondii* infection within a captive cohort in a defined locality. Several studies have been reported on risk factors for *T. gondii* infection, at the global level as well as within different populations on the African continent. These risk factors have not been limited to the types of populations studied, although it can be noted that certain risk factors are more associated with certain cultures than others. It is confusing that manyrisk factors have not emerged worldwide and this may be due to differing cultural practices and levels of development in the varied settings investigated (Flatt and Shetty., 2013).

Specifically, within our population of interest which is women of child bearing age, reported risk factors include: contact with soil and lack of knowledge of the disease, cat contact and hygiene practices (Kapperud *et al*., 1996; Bobić *et al*., 1998; Paul., 1998; Baril *et al*., 1999; Weigel *et al*., 1999; Kolbekova *et al*., 2007; El Mansouri *et al*., 2007; Elsheikha *et al*., 2009). In our study we did not find any association between knowledge of the disease as investigated through the question of prior testing at the individual level and the household level and sero-positivity and we also elected to leave out questions on soil contact and gardening due to the fact that most of our recruitment area dwellings do not have gardens. We did not find any association between cat or pet ownership and sero-positivity and hygiene practices, as a unique risk factor was not investigated. There was also no association between both IgG and IgM sero-positivity and household types, educational levels as well as household toilet facility type and facility usage and household source of water, even though there have been other studies that have reported these factors as risk factors for sero-positivity elsewhere (Bobić *et al*., 1998; Osunkalu *et al*., 2011). Our results are also consistent with those of (Baril *et al*., 1999: Flatt and Shetty, 2013) who did not find these as risk factors for toxoplasmosis, which illustrates the variability in risk factors for toxoplasmosis in research conducted in different cultural and environmental settings.

Consumption of undercooked meat and/or raw and unwashed vegetables as well as drinking unpasteurized milk have been repeatedly reported as risk factors over the years in studies of different population types (Elnahas *et al*., 2003; Fallah *et al*., 2008; Han *et al*., 2008; Flatt and Shetty, 2013; Walle *et al*., 2013). In our study, the consumption of undercooked meat was not a risk factor but we observed an association between IgM

sero-positivity and washing of vegetables and fruits often (OR 0.7094 95% CI 0.0828-0.6072) and washing of vegetables and fruits sometimes (OR 2.8527 95% CI 1.3093-6.2152). There was also no association between the absolute CD4 cell count and the *T. gondii* IgG sero-status of the mothers in the cohort $p = 0.4117$ with an Odds ratio of (1.000, 95% CI 0.9993-1.0015%). The absolute CD4 cell counts were significantly higher in the IgM positive group of mothers than in the IgM negative group of mothers on the study $p = 0.0025$, and there was also an association between absolute CD4 cell count and IgM sero-status of the mothers in the cohort ($p = 0.021$, OR 1.0013, 95% CI 1.0002-1.0025). It is interesting to note the association between the CD4 cell counts and the IgM seroprevalence; this is because $CD4^+$ cells are integral to the response to a *T. gondii* infection together with $CD8^+$ cells (Johnson and Sayles 2002, Lang *et al*., 2007), and since in immunosuppression there is an associated decline in CD4 cells it should be expected that in such a host there should be reduced ability to fight a *T. gondii* infection. Of note is the fact that this phenomenon was not observed in the case of IgG seroprevalence, and this was also the case in Osunkalu et al. (2011) who studied the association of IgG seroprevalence with CD4 cell count in a cohort of HIV-positive patients in Nigeria, and also did not find any significant association. Hence we can infer that CD4 cell count itself is not a risk factor for acquisition of a *T. gondii* infection within our investigated setting, even though it could play a role in the case of reactivation or progression of an already existing infection as observed by its association with *T. gondii* IgM antibody sero-status.

The association of *T. gondii* IgM sero-positivity with cleaning of fruits and vegetables does in our study suggest the environmental contamination with oocysts of *T. gondii* as

the main means of spread of *T. gondii* infections within the investigated setting as opposed to infection through consumption of meat contaminated with tachyzoites or bradyzoites in tissue cysts of *T. gondii*.

2.4.4 Investigation into the prevalence of *Toxoplasma gondii* antibodies in serum of HIV-positive and HIV-negative individuals presenting with a clinical diagnosis of uveitis at Tygerberg academic hospital ophthalmology clinic

Overall the *T. gondii* IgG antibody seroprevalence was 24.7% (95% CI 14.9-31.5%), which is comparable to the 23.3% obtained for the cohort of post parturient mothers recruited from Kraaifontein, and the *T. gondii* IgM antibody seroprevalence was 1% (95% CI 2.0-9.0%), which was significantly lower than the 18.7% obtained for the cohort of post parturient mothers. The *T. gondii* IgG+IgM antibody seroprevalence was also 1% (95% CI 1.2-26.6%) which was also significantly lower than that obtained for the cohort of post parturient mothers. The lower *T. gondii* IgG and IgG+IgM seroprevalence rates in this cohort when compared to the cohort of post parturient mothers could be ascribed to the fact that the two cohorts were from different geographical locations within the Western Cape of South Africa and it is well known that *T. gondii* seroprevalence rates vary within different regions in same countries, whilst the comparable IgG seroprevalence suggests lack of regional differences in rates of *T. gondii* exposure as both cohorts were from Cape Town.

In this cohort, there was no difference in the *T. gondii* IgG antibody seroprevalence in the HIV positive group (25%) and that of the HIV negative group (24.6%). There was a higher *T. gondii* IgM antibody seroprevalence in the HIV positive group (2.4%) than in the HIV negative group (0.00%), however, this difference was not significant $p = 0.4120$.

The results observed in this cohort are not consistent to those observed in the cohort of post parturient mothers where the *T. gondii* IgG and IgM antibody seroprevalence rates were higher in the HIV-negative cohort than in the HIV-positive cohort but are consistent with other studies where similar patterns were observed in Africa (Maïga *et al.,* 2001; Shimelis *et al*., 2009; Akanmu *et al*., 2010 Walle *et al*., 2013). This is typical of *T. gondii* seroprevalence studies in HIV- positive and HIV- negative cohorts globally where there has been no established trend regarding the *T. gondii* IgG or IgM antibody seroprevalence within the HIV- positive group when compared to the HIV- negative control groups. The inference from this may be that HIV status, be it positive or negative, does not impact the *T. gondii* IgG or IgM antibody seroprevalence rate or that the existence of such a phenomenon has not been established beyond reasonable doubt. Studies in the USA and Czech Republic have also reported similar outcomes (Falusi *et al*., 2002; Machala *et al*., 2013). The time of *T. gondii* infections can also not be determined in these cohorts, since there is the probability that the *T. gondii* infections were acquired before the HIV infections were acquired or vice versa.

There was a higher *T. gondii* IgG seroprevalence in males (29.3%) than in females (21.4%) but the difference was not significant $p = 0.3770$, whilst there was also a higher *T. gondii* IgM seroprevalence in males (2.4%) than in females (0.00%), this difference was also not significant ($p = 0.4230$). *Toxoplasma gondii* IgG and IgM seroprevalence were not associated with gender or age in this cohort: the non-association with age was also observed in the cohort of post parturient mothers.

2.4.5 Detection of *Toxoplasma gondii* DNA in DNA from ocular (aqueous) fluid of patients presenting with a clinical diagnosis of uveitis at the Tygerberg academic hospital ophthalmology clinic via PCR methodology

The PCR analysis targeting the *T. gondii B1* gene, in all ocular (aqueous) fluid samples from all patients yielded negative results, hence the *T. gondii* infections in the cohort can be suggested to be systemic and not ocular in nature. Since there was only one acutely infected individual, the likelihood of ocular infection in the cohort was regarded to be very low. Ocular toxoplasmosis diagnosis from DNA amplification techniques has been shown to be very challenging due to the normally low levels of *T. gondii* DNA present in the ocular fluid and in most cases a combination of biological techniques (PCR, calculation of Goldmann-Witmer coefficient and Western blot analysis) is recommended as being useful in establishing a correct diagnosis (Garweg *et al*., 2004; Bourdin *et al*., 2014). Historically, ocular infections with *T. gondii* have been attributed to congenital infections but recently due to the observed low incidence of congenital toxoplasmosis globally and high ocular toxoplasmosis incidence rates in some regions especially in South America, it is being suggested that post natal *T. gondii* infections may play a more important role in ocular disease than previously thought (Vasconcelos-Santos., 2012). The relationship between systemic and ocular *T. gondii* infections has not been very well investigated and hence this study provides the basis for further research to be done in this regard to analyze whether there is any relationship at all between active or prior exposure to *T. gondii* systemically and how it impacts on ocular disease occurrence or progression.

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CHAPTER 3 *TOXOPLASMA GONDII* ANTIBODY SEROPREVALENCE IN SELECTED ANIMAL POPULATIONS IN DEFINED AREAS IN CAPE TOWN IN THE WESTERN CAPE OF SOUTH AFRICA

This chapter will present two published papers and a manuscript prepared for submission.

Hammond-Aryee, Kenneth, Monika Esser, Lesley van Helden and Paul van Helden. "A high Seroprevalence of *Toxoplasma gondii* antibodies in a population of feral cats in the Western Cape province of South Africa." *Southern African Journal of Infectious Diseases* 2015; 30(4):141–144 http://dx.doi.org/10.1080/23120053.2015.1107295

Hammond-Aryee, K., Van Helden, L.S. & Van Helden, P.D., 2015, "The prevalence of antibodies to *Toxoplasma gondii* in sheep in the Western Cape, South Africa", *Onderstepoort Journal of Veterinary Research* 82(1), #993, 5 pages. http:// dx.doi.org/10.4102/ojvr. v82i1.993

3.1 GENERAL INTRODUCTION

Toxoplasmosis is a globally important zoonosis with potentially devastating health impacts for both humans and a range of domestic and wildlife species. The World Health Organization has repeatedly recommended the collection of accurate epidemiological data on toxoplasmosis, yet despite recognized links between infection of wildlife, domestic animals and humans, seroprevalence in animals, both domestic and wild, is rarely monitored if at all (Chadwick *et al.*, 2013).

Members of the family *Felidae* are the only known definitive host for *T. gondii*. Cats can become infected by feeding on prey already infected with *T. gondii* dormant cysts or tachyzoites and also by drinking oocyst-contaminated water. When infected, a cat is known to shed infective oocysts in its feces 5 to 12 days post ingestion of oocysts (Sheffield and Melton, 1969), thereby contaminating the environment and posing a transmission risk to other individuals of the same or other species.

Toxoplasmosis is responsible for massive economic losses in sheep farming globally and was first described in the ovine host in 1954 (Buxton *et al*., 2007). Primary *T. gondii* infections in livestock, in particular sheep and goats, poses a health risk to these animals, since infection is known to cause abortions, stillbirths and neonatal mortalities. In the United Kingdom for example, ovine toxoplasmosis causes 1-2% of fetal losses per annum (Buxton *et al*., 2007; Innes *et al*., 2009).

Toxoplasma gondii infection in immunocompetent cats is usually asymptomatic, although immunosuppression may lead to a reactivation of a latent infection, which can have serious health implications (Davidson *et al*., 1993). Feral cats are free ranging and generally fend for themselves via hunting and eating in an unmanaged manner, although many humans provide supplementary feed for such animals. They are therefore expected to give an indication of the presence or absence of infectious disease agents such as *T. gondii* within the territories where they are found. *Toxoplasma gondii* infection specifically in sheep is mainly acquired postnatally, as congenital infections usually lead to abortions. Rarely, congenitally affected lambs are born and can also be a source of infection for humans (Dubey and Welcome, 1988; Williams *et al*., 2005).

There are various risk factors reported to be associated with *T. gondii* infection in the animal hosts. Seroprevalence in sheep and cats is known to increase with age and is therefore higher in old animals than in younger animals (Dubey, 2009). Risk factors for *T. gondii* infection in sheep include the presence of cats on farms, commercial versus non-commercial nature of farms, intensive or semi –intensive as opposed to free range or open farm management practices, climatic conditions under which the farming is taking place with higher average minimum temperature regions having higher seroprevalence than lower minimum temperature regions, presence of surface drinking water sources, coastal as opposed to inland lying farm locations, and size of the farm (Mainar *et al*., 1996; Samra *et al*., 2007; Andrade *et al*., 2013).

Although infected adult sheep do not always show symptoms of clinical toxoplasmosis, *T. gondii* infection has been noted as an important cause of ovine abortions in the USA and Europe (Dubey, 2009). In the United States, *T. gondii* seroprevalence rates from 20.8% (Huffman *et al*., 1981) to 73.8% (Dubey and Welcome, 1988) have been reported in sheep. It has also been suggested that some breeds of sheep may be more susceptible to *T. gondii* infection than others (Dubey and Welcome, 1988; Williams *et al*., 2005).

The ingestion of partially cooked, undercooked or raw meat has been documented as a significant mode of *T. gondii* infection worldwide. Studies in Europe have shown that ingestion of undercooked sheep or lamb was a risk factor in the acquisition of *T. gondii* infection in a cohort of pregnant women (Cook *et al*., 2000). In other studies in the USA, 50% of a cohort of 131 women who had vertically transmitted *T. gondii* to their infants recalled having eaten raw or uncooked mutton sometime during their pregnancies (Boyer *et al*., 2005). Owing to the ability of sheep to transmit *T. gondii* via consumption of their

meat, as well as the ability of ewes to vertically infect their young, sheep can play a very important role in the epidemiology of *T. gondii*.

Investigations into the seroprevalence of *T. gondii* antibodies in animal populations in Africa, have been far and few between. Most of the studies reported to date have been done in the southern part of Africa where Samra et al. (2007), demonstrated a seroprevalence of 4.5% in a 600 sheep cohort in South Africa and in Zimbabwe, (Hove et al. (2005) reported a seroprevalence of 67.9% in 335 sheep and goats investigated. Cheadle et al. (1999) reported a seroprevalence of 74% in wild felids in South Africa and Penzhorn et al. (2002) reported a seroprevalence of 100% in lions from the Kruger National Park and Hluhluwe-Imfolozi Park in South Africa and 92% in lions from Botswana (Chobe national park), although the numbers tested were very low. *Toxoplasma gondii* antibodies have also been detected in felids in the wild, and in chinchillas, ferrets, cheetah, a dog, as well as leopards in Southern Africa. In Ghana, Dubey et al. (2008) reported a seroprevalence of 64.1% in free-range chickens and a seroprevalence rate of 38.2% in sheep and 26.8% in goats has also been reported by van der Puije et al. (2000). There have been no documented reports on seroprevalence in animals from the eastern part of Africa but in Egypt, Ghoneim et al. (2010) reported a seroprevalence of 98.4% in sheep and 41.7% in goats.

Given the interconnectedness of multiple species in an ecosystem, we can suggest that investigations into the presence or absence of *T. gondii* antibodies in animal host populations such as felids and sheep will provide us with significant insight into the risk of toxoplasmosis transmission within a particular ecosystem (van Helden *et al*., 2013). Without such information there is no way of assessing the public health impact of these

all-important animal hosts within the health ecosystem as well as the role they may play in transmission of the parasite within a South African context.

3.2 A HIGH SEROPREVALENCE OF *TOXOPLASMA GONDII* ANTIBODIES IN A POPULATION OF FERAL CATS IN THE WESTERN CAPE PROVINCE OF SOUTH AFRICA

Hammond-Aryee, Kenneth, Monika Esser, Lesley van Helden and Paul van Helden. "A high Seroprevalence of *Toxoplasma gondii* antibodies in a population of feral cats in the Western Cape province of South Africa." *Southern African Journal of Infectious Diseases* 2015; 30(4): 141–144 http://dx.doi.org/10.1080/23120053.2015.1107295

Abstract

Toxoplasma gondii is an obligate intracellular protozoan pathogen of global importance, which causes toxoplasmosis. *Toxoplasma gondii* is very successful as a pathogen of both humans and animals, due to its ability to infect almost all mammals and birds. Felids are the only known definitive host of *T. gondii*. The aim of this study was to determine the seroprevalence of *T.gondii* antibodies in the serum of a convenience sample of feral cats (*Felis catus*), trapped in population control programs in the Western Cape Province of South Africa. Overall, 159 feral cats were included in this study. There were 95 (59.8%) females and 64 (40.3%) males. 121 (76.1%) of the cats were adults (>12 months old) and 38 (23.9%) were juvenile (≤ 12 months old). The sera were tested by an Indirect Immunofluorescence test. Anti-IgG and IgM antibodies were detected in 59 (37.1%, 95% CI: 29.6-44.6) and 14 (8.8%, 95% CI: 4.4-13.2) cats respectively. Both IgG and IgM antibodies were detected in 10 cats (6.3%). Correlation between serum IgG, IgM, sex,

and age of cats were investigated. This is the first report on surveillance of feral cats for *T. gondii* in South Africa

Introduction

Toxoplasma gondii is an obligate intracellular protozoan pathogen of global importance. It is responsible for causing the disease toxoplasmosis that affects about a third of the global human population. *T. gondii* is very successful as a pathogen due to its ability to infect almost all mammals and birds (1) (2). *Toxoplasma gondii* is therefore of both clinical and public health importance.

Infections with *T.gondii* can be mild or severe depending on the host that is being infected and/or the strain that causes the infection. *T.gondii* infections are known to cause massive abortion storms in sheep and other small ruminants, resulting in high economic losses in commercial operations. Infections with *T.gondii* are also known to lead to death in certain vulnerable species such as new world marsupials (2) (3).

In humans, *T. gondii* infections are acquired via the ingestion of tissue cysts in partially cooked meat or oocysts in water contaminated with infected felid feces. Other infection pathways include handling of tissues of animals infected with tissue cysts or consumption of food contaminated with oocysts. Vertical transmission of tachyzoites from mother to fetus can also occur (4) (5). Primary *T. gondii* infections are generally asymptomatic in immunocompetent individuals, but certain individuals may exhibit symptoms that are normally mild and flu-like in nature and disappear within a few weeks (6) (7).

In immunosuppressed individuals such as those undergoing chemotherapy or organ transplantation or in AIDS patients a reactivation of a latent *T. gondii* infection is often

fatal. T. gondii has been identified as an important opportunistic infection in HIV/AIDS patients and a major contributor to death of AIDS patients in the developing world. In HIV/AIDS patients, it is known to lead to brain calcifications and central nervous system complications which lead to death (8).

Members of the family Felidae are the only known definitive hosts for *T. gondii* (9). Felids can be infected by feeding on prey already infected with *T. gondii* tissue cysts or tachyzoites and by drinking oocyst-contaminated water. Infected felids most often shed infective oocysts 5 to 12 days post infection (10). Felids therefore play an important role in the epidemiology of the disease. *T. gondii* infection in cats is usually asymptomatic in immunocompetent cats, although immunosuppression may lead to a reactivation of a latent infection which can have serious health implications (11). For this reason, surveillance and epidemiological studies on *T. gondii* is important. Feral cats are free ranging and generally fend for themselves via hunting and eating in an unmanaged manner, although humans sometimes provide supplementary food to such animals. They are therefore expected to give an indication of the presence or absence of *T. gondii* within the territories where they are found.

In South Africa an average human *T. gondii* seroprevalence of 20% was reported in 1978 (12), however, since then there have been a limited number no other comprehensive of sero-surveys in human or animal populations.

Notably Kistiah et al, (13), Schneider et al, (14) and Sonnenberg et al, (15). With such limited information there is no way of assessing the public health impact of this all-

important definitive host within the health ecosystem as well as the role it plays in transmission of the parasite within a South African context.

The aim of this study is to investigate the seroprevalence of *T.gondii* antibodies in a population of feral cats in the Western Cape province of South Africa.

Materials and methods

Feral cats

In total, 159 feral cats (*Felis catus*) were live trapped from October 2013 to July 2014, within the Drakenstein (Paarl) and City of Cape Town (Cape Town) municipalities of the Western Cape. The cats were captured to be neutered in a feral cat population control program. Blood samples were collected from the cats via venipuncture by provincial registered veterinarians. Blood was collected in serum separator tubes and immediately put on ice before being transported on ice to the National Health laboratory Services in Tygerberg Academic Hospital in Cape Town, and centrifuged at 3500g for 5 minutes to isolate serum and/or plasma. Serum was stored at -80 degrees for further analysis. In our study, there were 95 (59.8%) females and 64 (40.3%) males, 121 (76.1%) adults (> 12 months old) and 38 (23.9%) juvenile (≤12 months old) feral cats. Ethical clearance was obtained from the Animal Health Research Ethics Committee of the Western Cape Department of Agriculture for this study.

Serology investigations

Cat serum samples were analyzed qualitatively for the presence or absence of antibodies to *T. gondii* by Indirect Immunofluorescence test (IFAT) using the IFAT *T. gondii* IgG

and IgM antibody commercial kits (Euroimmun AG, Germany) according to the manufacturer's instructions. Briefly: 1 in 100 dilutions of thawed serum samples were prepared in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) to prevent non-specific fluorescent reactions. The diluted sera were then incubated with *T. gondii* trophozoite smears immobilized on test slides at 37°C for 30 minutes. Bound antigen – antibody complexes were then stained with fluorescein labeled goat derived anti cat *T. gondii* IgM or IgG antibody after washing for 5 minutes in PBS-T and 5 minutes in distilled water. Stained slides were then washed again as described above and then dried. Dried slides were covered with mounting fluid and fluorescence was visualized under a fluorescence microscope. Samples that showed only apical fluorescence were recorded as negative. Positive and negative controls were included in each test slide.

Statistical analysis

Statistical analysis was done using the Pearson Chi square test with the SAS/STAT (statistical analysis software version 9.3). Correlations between IgG and IgM seroprevalence and gender and age of cats were investigated. Differences were deemed statistically significant when $P \leq 0.05$.

Results

Toxoplasma gondii IgG and IgM antibodies were detected in 59 (37.1%, 95% CI: 29.6-44.6) and 14 (8.8%, 95% CI: 4.4-13.2) of 159 cats respectively, both IgG and IgM antibodies were detected in 10 feral cats (6.3%) (Table 1).

Table 1.*T. gondii* IgM and IgG antibody seroprevalence for feral cats (n= 159)

	IgM +	IgM -	Total
IgG +	10 (6.3%)	49 (30.8%)	59 (37.1%) Adult 52 (42.98%) Juvenile 7 (18.42%)
IgG –	4 (2.5%)	96 (60.4%)	100 (62.9%) Adult 69 (57.02%) Juvenile 31 (81.58%)
Total	14 (8.8%) Adult 11 (9.09%) Juvenile 3 (7.89%)	145 (91.2%) Adult 110 (90.91%) Juvenile 35 (92.11%)	159

Overall, IgG seroprevalence was statistically significantly higher in adult cats than in juvenile cats (43% and 18.4%, p= 0.0069). Even though overall IgM seroprevalence was also higher in adult cats (9.1%) than in juvenile cats (7.9%), the difference was not significant (p= 1.0000).

Table 2. *T. gondii* IgG and IgM seroprevalence by Gender of feral cats (n= 159)

	Female	Male	Total
IgG +	35 (36.8%)	24 (37.5%)	59
IgG –	60 (63.2%)	40 (62.5%)	100
Total	95	64	159
IgM +	6 (6.3%)	8 (12.5%)	14
IgM –	89(93.7%)	56 (87.5%)	145
Total	95	64	159

IgG seroprevalence in females was 36.8% and 37.50% in male feral cats. IgM seroprevalence was 6.32% in female and 12.50% in male feral cats.

Discussion

Overall, IgG seroprevalence was statistically significantly higher in adult cats than in juvenile cats (43% and 18.4%, p= 0.0069). Even though overall IgM seroprevalence was also higher in adult cats (9.1%) than in juvenile cats (7.9%), the difference was not significant (p= 1.0000).

The low percentage of feral cats in the sampled population with an IgM positive test result (8.8%, 14 out of 159), implies that there is a low percentage of newly or acutely infected feral cats in the sampled population and it can therefore be concluded that there is a minimal number of feral cats in the sampled population actively shedding *T. gondii* oocysts in their feces at the time of sampling.

The IgG seroprevalence of 37.1% in feral cats from the current study is comparable to studies in stray cat populations from some parts of North Africa, the Middle East and Asia, (14) (15) (17)(16) (18) (19)) as well as in North and South America (20).

However, it was shown in the United States that the infection prevalence can vary from 0% to 100% depending on the regions from which the surveys were conducted (14)(13) (21)(20). Prevalence is also known to be higher in feral cats than in domestic cats in any given region (22)(21) (23).

There have been two previous reports of extremely high *T. gondii* seroprevalence rates in small populations of free-ranging big cats in South Africa.

In 1999, Cheadle et al, ((26) reported a 74% (50/68) *T. gondii* seroprevalence rate in captive and free ranging populations of lions (*Panthera leo*), leopards (*Panthera pardus*)

and cheetahs (*Acinonyx jubatus*), in the Kruger and Hluhluwe-Umfolozi National Parks in South Africa.

The second study was done in 2002 by Penzhorn, B.L, (27) who reported T. gondii antibodies in 100% of 12 lions (*Panthera leo*) from the Kruger National Park and in 100% of 30 lions from the Hluhluwe-Umfolozi Park and 86% of 7 leopards (*Panthera pardus*) sera also from the Kruger National Park all in South Africa.

The significance of cats, especially free-roaming, to the epidemiology of toxoplasmosis cannot be underestimated. Feral cats have access to the same places as humans and other probable animal hosts (domestic or wild). The cats are able to contaminate the environment with *T. gondii* oocysts, shed in their feces (28). Previous reports suggest that about 1% of cats are expected to be shedding infectious oocysts in their feces at any given point in time (9).

Understanding the interplay between the human hosts, the animal host and the environment is a vital step in establishing and documenting the epidemiology of *T. gondii* in any region.

As traveling and migration within and between countries becomes more prevalent, pathogens will also gain movement advantages and new strains of parasites could be introduced into new areas progressively. The active monitoring and surveillance is thus very important to prevent or manage outbreaks of disease.

In order to achieve this, there is a need to actively monitor or survey pathogens within the human, animal as well as environmental systems as one holistic unit. Failure to do this implies that one misses the opportunity to gather information regarding the development

of pathogenic reservoirs as well as gain an understanding of how the pathogen is able to transition from one system to the other and how the evolution of the pathogen within these systems develops (29).

Conclusion

In South Africa, there is limited information about toxoplasmosis in humans and animals, however there is currently no active surveillance of human and/or animal populations for toxoplasmosis. The objective of this study is to provide preliminary data on the prevalence of *T. gondii* in feral cats, and the information obtained does provide motivation for further studies in the effort to understand the epidemiology of *T. gondii* in South Africa.

Since passive anthropogenic factors such as poverty, poor hygiene, consumption of undercooked meat, dirty water and poor housing are commonplace in South Africa, the risk of infection with *T. gondii* is likely to be very high.

Our study is the first report on active surveillance of feral cats for *T. gondii* in South Africa, and the observed high rate of antibody prevalence indicates that a more planned approach and hence increased attention to *T. gondii* is justified.

A main limitation of this study is that it was done in two municipalities of the Western Cape Province of South Africa only. Knowing that *T. gondii* seroprevalence could vary significantly depending on the regions where the surveys are conducted (4) (13), there is a need to investigate the prevalence elsewhere in the country.

Future studies should focus on sensitive or sentinel populations such as HIV (+) patients, pregnant women, cats (both feral and domestic) and selected groups of clinically healthy individuals.

This will provide accurate data for active monitoring and surveillance purposes as well as aid in policy and health care planning.

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3.3 THE PREVALENCE OF ANTIBODIES TO *TOXOPLASMA GONDII* IN SHEEP IN THE WESTERN CAPE PROVINCE OF SOUTH AFRICA

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Abstract

Toxoplasma gondii is found worldwide and is a protozoan pathogen of global importance. Infections are known to be common at lower altitudes and in warm and humid climates. *T. gondii* has been found in many animal species in varying environments and locations. Primary *T. gondii* infections in food animals, especially sheep and goats, pose a risk for abortions, stillbirths and neonatal mortalities to these animals and have health and economic implications.

The seroprevalence of *T. gondii* antibodies in a sample of 292 sheep (Merino breed) farmed in a semi- intensive manner in the Cape Agulhas municipality of the Western Cape, South Africa was investigated. *T. gondii* antibody seroprevalence was determined by ELISA methodology.

Overall, 23 (8%, 95% CI: 4.7688-10.9846) of the sheep tested positive for *T. gondii* antibodies. There was no statistically significant relationship between seroprevalence and age of the sheep. The highest number of seropositive sheep (17 out of 33) was in the 28 to 40 months old group; a total number of 19 sheep were seropositive by 40 months and

23 were seropositive by 76 months, there were no seropositive sheep in the 16 to 28 months old group.

The reported seroprevalence rate of 8% in this study is higher than the previously reported figures of 6.0% in the Western Cape Province and 4.7% overall in South Africa in 2007. Given that the farming of sheep is economically significant in South Africa this may pose a threat to the production of the small stock industry as well as to public health and food security. We therefore recommend that further and ongoing surveillance should be done in order to identify high-risk animal populations so that local control measures can be put in place to prevent inter-species disease spread.

Introduction

Toxoplasma gondii is an Apicomplexan obligate intracellular protozoan parasite of global importance. Infections by *Toxoplasma gondii* in humans and animals result in the disease toxoplasmosis. *T. gondii* antibodies are known to be present in about a third of the global human population, although local and regional prevalence varies widely. *T. gondii* is very successful as a pathogen due to its ability to infect almost all mammals and birds (Dubey 2002, Dubey and Beattie 1988). Toxoplasmosis is found worldwide, but infections are known to be common at lower altitudes and in warm and humid climates.

Members of the family *Felidae* are the only known definitive host for *T. gondii*. Cats can become infected by feeding on prey already infected with *T. gondii* dormant cysts or tachyzoites and also by drinking oocyst-contaminated water. When infected, a cat is known to shed infective oocysts in its feces 5 to 12 days post ingestion of oocysts (Dubey

1995, Elmore et al. 2010, Al-Kappany et al. 2010), thereby contaminating the environment and posing a transmission risk to other species.

Toxoplasmosis is responsible for massive economic losses in sheep farming globally and was first described in the ovine host in 1954 (Buxton et al. 2007, Jones and Dubey 2012, Dubey and Beattie 1988). Primary *T. gondii* infections in livestock, in particular sheep and goats, poses a health risk to these animals, since infection is known to cause abortions, stillbirths and neonatal mortalities. In the United Kingdom for example, ovine toxoplasmosis causes 1-2% of fetal losses per annum (Buxton et al. 2007, Innes et al. 2009).

In humans and animals, *T. gondii* infections are acquired post-natally via the ingestion of tissue cysts in partially cooked meat, or ingestion of infective oocysts in food or in water contaminated with infected felid feces or handling of tissues of animals infected with tissue cysts. Infection can also occur by vertical transmission from mother to fetus in humans, sheep, goats and small rodents (Jones and Dubey 2012, Smith 1993,Hill and Dubey 2002). In humans, infection can occur via blood transfusions and organ transplantation, although this is rare. Infection has been known to occur via inhalation of aerosols containing infective oocysts or from contact with contaminated soils in both humans and animals.

T. gondii infection specifically in sheep is mainly acquired post-natally, as congenital infections usually lead to abortions. Rarely, congenitally affected lambs are born and can be a source of infection for humans (Williams et al. 2005,Dubey and Welcome 1988).

There are various risk factors reported to be associated with *T. gondii* infection in sheep. Seroprevalence in sheep is known to increase with age and is therefore higher in ewes or rams than in lambs (Dubey 2009). Other risk factors for *T. gondii* infection in sheep include the presence of cats on farms, commercial versus non-commercial nature of farms, intensive or semi –intensive as opposed to free range or open farm management practices, climatic conditions under which the farming is taking place with higher average minimum temperature regions having higher seroprevalence than lower minimum temperature regions, presence of surface drinking water sources, coastal as opposed to inland lying farm locations, and size of the farm (Mainar et al. 1996,Samra et al. 2007,Andrade et al. 2013). Although infected ewes do not always show symptoms of clinical toxoplasmosis, *T. gondii* infection has been noted as an important cause of ovine abortions in the USA and Europe (Dubey 2009). In the United States *T. gondii* seroprevalence rates from 20.8% (Huffman et al. 1981) to 73.8% (Dubey and Welcome 1988) have been reported in sheep. It has also been suggested that some breeds of sheep may be more susceptible to *T. gondii* infection than others (Williams et al. 2005,Dubey and Welcome 1988).

In South Africa, some seroprevalence studies have been reported in human populations (both asymptomatic and HIV-AIDS cohorts) (Hammond-Aryee, Esser, and van Helden 2014). However, these studies are very limited in number and very few of them have been in the post-HIV era. There have been equally few studies in animal populations and only one reported study in sheep, which reported rates of 4.3% via ELISA and 5.6% via IFA (Samra et al. 2007). The study also reported the Western Cape as the province with the highest consumption of mutton in South Africa.

The ingestion of partially cooked, undercooked or raw meat has been documented as a significant mode of *T. gondii* infection worldwide. Studies in Europe have shown that ingestion of undercooked lamb was a risk factor in the acquisition of *T. gondii* infection in a cohort of pregnant women (Cook et al. 2000). In other studies in the USA, 50% of a cohort of 131 women who had vertically transmitted *T. gondii* to their infants recalled having eaten raw or uncooked mutton sometime during their pregnancies (Boyer et al. 2005). Owing to the ability of sheep to transmit *T. gondii* via consumption of their meat, as well as the ability of ewes to vertically infect their young, sheep can play a very important role in the epidemiology of *T. gondii*. Investigation into the presence or absence of *T. gondii* antibodies in a population of sheep will provide us with significant insight into the risk of toxoplasmosis transmission in a particular ecosystem.

The current study is focused on the seroprevalence of *T. gondii* antibodies in a flock of sheep in South Africa and therefore seeks to add to the knowledge of this important pathogen and the role played by animals in its epidemiology.

Materials and methods

Study area and Climate

Sheep were sampled from the farming area in Bredasdorp, in the Cape Agulhas local municipality in the Overberg region located in the Western Cape province of South Africa. The study area receives about 344mm of rain per year, most of which occurs in the winter. It is has a Mediterranean climate. It receives the lowest rainfall (13mm) in December and the highest (44mm) in August. The average midday temperatures range from 17.5°C in July to 26.2°C in January. Livestock farmed in the region consist of

mostly sheep and cattle. The sample consisted of 292 Merino sheep (4 rams and 288 ewes) randomly selected from a flock of approximately 1000-1500 sheep. The sheep were farmed in a semi-intensive manner, grazing planted pastures and crop stubble.

Blood Collection

In May 2014, blood samples were collected from the 292 sheep via venipuncture. Blood was collected in serum separator and EDTA tubes and immediately put on ice, after which it was transported to our labs and centrifuged at 3500g for 5 minutes to isolate serum/plasma. The serum/plasma was then stored at -80°C for further analysis. In this study only the plasma was used for further analysis.

Serology investigations

An enzyme linked immunosorbent assay (ELISA) was used for the detection of IgG antibodies to *T. gondii* in sheep plasma samples. The ELISA test was performed via a commercially available enzyme immuno-assay (EIA) kit (IDEXX Toxotest Ab, IDEXX Laboratories, Switzerland AG) according to the manufacturer's instructions.

Briefly, thawed sheep plasma samples and controls were diluted 1 in 400 in wash buffer to prevent non-specific reactions. The diluted plasma samples were then dispensed into microtitre plates precoated with inactivated *T. gondii* antigen and mixed by shaking the plate gently.

The microplate was then covered with an adhesive plate cover and incubated at 37 degrees Celsius for 60 minutes. Each well was then washed with approximately 300ul of wash buffer three times; all residual wash solution was removed by gently tapping the

microplate onto an absorbent material. Bound antigen-antibody complexes were then conjugated with 100ul of peroxidase labeled anti-ruminant immunoglobulin G conjugate. The plate was covered and incubated at 37 degrees Celsius for 60 minutes. The wash step was repeated as described above, so as to eliminate any residual unbound complexes. Enzyme bound complexes were visualized via the addition of 100 ul of enzyme substrate to the wells of the microplate and the plate incubated at 26 degrees Celsius for 15 minutes. The enzyme –substrate reaction was stopped by the addition of 100ul of stop solution to each well of the microplate. The absorbance was read on a photometer at a wavelength of 450 nm.

Positive and negative controls provided in the kit were included with each batch of test samples on each microplate.

The optical densities of the positive control (PCx) and the optical density of the samples (SampleA_{450}) are corrected by subtracting the optical density of the negative control (NCx). The samples are analyzed in relation to the positive and negative controls according to the formula

$$S/P\ \% = 100 \times \frac{\text{Sample } A_{450} - NCx}{PCx - NCx}$$

Statistical Analysis

Statistical analysis was done using the Pearson Chi square test with the SAS/STAT (statistical analysis software version 9.3). Correlations between *T. gondii* sero-status and

age of sheep were investigated. Differences were deemed statistically significant when $P \leq 0.05$.

Ethical clearance

Ethical clearance was obtained from the Animal Health Research Ethics Committee of the Western Cape Department of Agriculture for this study.

RESULTS

A total of 292 sheep were included in this study. The sheep sample was made up of 288 (99%) ewes and 4 (1%) rams. The rams were between 16 to 64 months old, whilst the ewes were between 4 to 76 months old, with the modal age group being between 4 to 16 months (Figure 1).

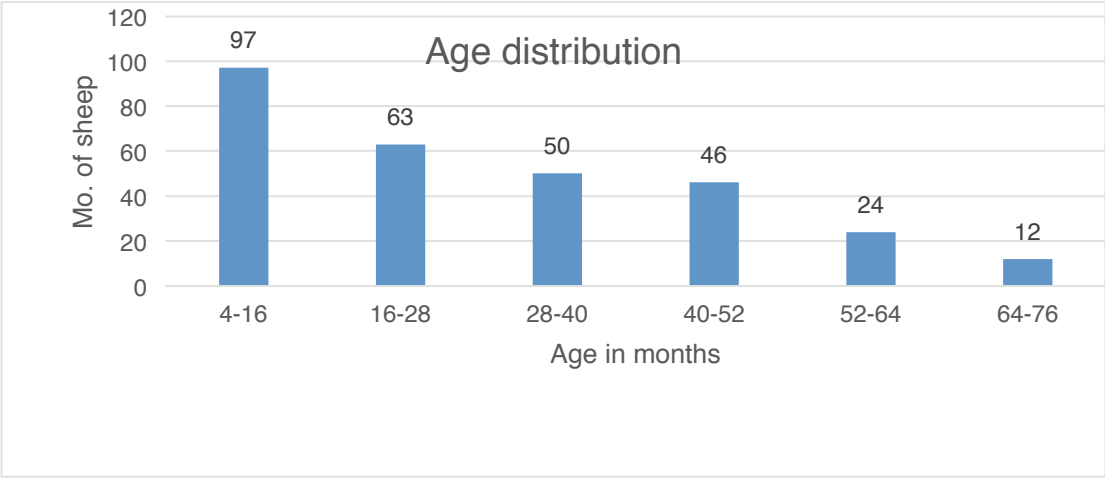


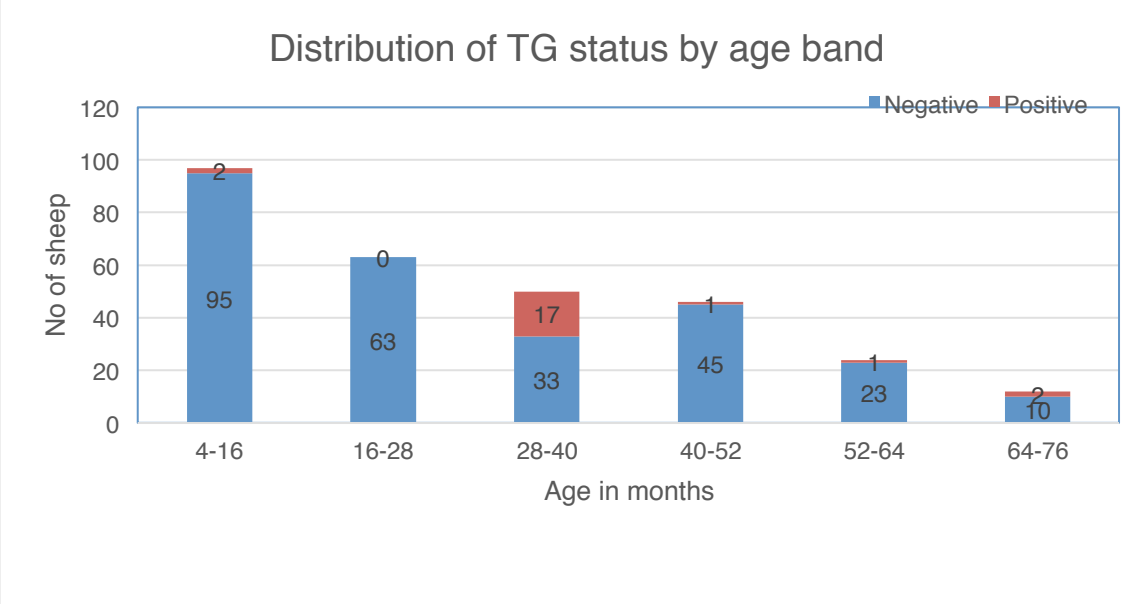
Figure 1. Age distribution of study sample

Overall, 23 (8%, 95% CI: 4.7688 to 10.9846) of the sheep tested positive for *T. gondii* antibodies

Within the 6 different age categories, there was distinct *T. gondii* antibody seroprevalence patterns as depicted in Figure 2 below.

The highest number of seropositive sheep (17 out of 33) was found in the group 28 to 40 months old whilst the lowest number of seropositive sheep (0 out of 63), was found in the 16 to 28 months old group. The 4 to 16 months old group had the highest number of seronegative sheep, 95 out of 97 sheep, whilst 10 out of 12 sheep were seronegative in the 64 to 76 months old group.

Figure 2. Age vrs *T. gondii* antibody seroprevalence in sheep sampled



Discussion

Infectious diseases such as toxoplasmosis have a huge impact on herd productivity in sheep-farming regions of the world. Some of these diseases may remain undetected in flocks for prolonged periods of time and may lead to unforeseen and unexplained abortions, fetal or newborn deaths and infertility. In some cases they lead to persistent or recurring infection in herds resulting in long-term poor reproductive output. Reproductive losses resulting from these diseases are a threat to the long-term economic viability of such flocks. Many of these diseases are a threat to human public health, emphasizing the need to control transmission and to devise an effective means of understanding and managing their impact.

Toxoplasma gondii antibodies have been detected in naturally exposed sheep flocks worldwide. Seroprevalence rates reported also vary from as low as 3% in Pakistan (Zaki 1995) to as high as 68% (Deconinck et al. 1996) in Côte d'Ivoire and 95.7% in Turkey (Mor and Arslan 2007). There have been some reports of *T. gondii* seroprevalence in small ruminants in some parts of Africa. Hove, Lind, and Mukaratirwa 2005, reported a seroprevalence rate of 67.9% in a population of sheep and goats from different parts of Zimbabwe. They also reported a 8-fold difference in seroprevalence rate between sheep from a large commercial farm (10%) and sheep reared under a communal grazing system (80%), reporting a high concentration of domestic cats and a high household density as potential risk factors for *T. gondii* infection. In Botswana, (Binta et al. 1998) reported a seroprevalence rate of 10% in goats in 1998, whilst in 2003, (Sharma et al. 2003) reported a high seroprevalence rate of 30% also in a population of goats. In Ghana, (van der Puije et al. 2000) reported a seroprevalence rate of 33.2% in sheep and 26.8% in

goats respectively. They also reported a higher seroprevalence rate in females (35.8%) than in males (21.1%), and significant differences in seroprevalence between breeds, age groups and ecological zones from which the animals were sampled.

In Uganda, (Bisson et al. 2000) reported a seroprevalence rate of 31% in a population of goats from both urban and rural settings. They reported significantly higher seroprevalence rates in goats from urban areas than rural areas and also demonstrated a strong positive correlation between age and seroprevalence. In South Africa, (Samra et al. 2007) reported a seroprevalence rate of 5.6% via indirect fluorescent antibody test (IFA) and 4.3% via enzyme linked immunosorbent assay. The mean seroprevalence via ELISA in the Western Cape Province was 6%. They also reported a significantly higher seroprevalence in sheep from commercial farms than in sheep from rural or informal sector farms; a significant correlation between seroprevalence and the minimum average temperature; and that sheep managed in an extensive manner had a significantly lower seroprevalence (1.8%) than sheep managed in a semi-intensive or fully intensive system (5.3%).

The seroprevalence rate reported in the current study (8%) is higher than the mean provincial seroprevalence reported by Samra et al. (2007). This may be owing to the fact that the farm studied falls within the category of a commercial farm with semi-intensive management, both of these factors having been shown to contribute to a higher than average seroprevalence.

Most seropositive sheep are known to acquire an infection by 4 years of age and seroprevalence in sheep is known to increase with age, with 95% of susceptible ewes

seroconverting by 6 years of age (Dubey 2009). In our study, the highest number of seropositive sheep was in the 28 to 40 months old group and there was no significant relationship between seroprevalence and age of the sheep. These observations could be due to the fact that the sample was a convenience sample and hence the investigator had no control over the sheep sampling.

Conclusion

Transmission of *T. gondii* from food animals to humans and to non-food animals is likely to be proportional to the *T. gondii* seroprevalence within food animal populations. There is a need to actively survey at-risk populations such as feral cats, wildlife and food animals within the ecosystem in order to design appropriate interventions to manage and prevent disease transmission.

In Africa where poverty, poor hygiene and a high burden of HIV exist, the impact of *T. gondii* infection is likely to be very significant and has important implications for the health and economic well being of the people of this continent.

The reported seroprevalence rate of 8% in this study is higher than the previously reported figures of 6.0% in the Western Cape Province and 4.7% overall in South Africa in 2007. Our study was more limited than this previous study, however it does suggest that ongoing and widespread transmission of *T. gondii* in livestock continues. We recently showed that in a population of 159 feral cats, *T. gondii* anti-IgM antibody seroprevalence was 37.1% (59 out of 159) (Hammond-Aryee et al., In press) . It is therefore quite possible that farm cats, whether feral or not, continue to be major risk factors for *T. gondii* transmission to livestock. Meat consumption is very high in South

Africa and sheep are farmed not just for meat consumption, but also for the production of wool. Hence the farming of sheep has both an economic and health impact in South Africa. We therefore recommend that further and ongoing surveillance should be done in order to identify high-risk animal populations so that local control measures can be put in place to prevent inter-species disease spread.

Competing Interests

The authors declare no competing interests

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3.4 *TOXOPLASMA GONDII* IN THE CAPE PENINSULA CARACAL (*Caracal caracal*)

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This section contains a draft manuscript that should be submitted in due course. At a later stage it is anticipated that other data will be added by other researchers, such as data on environmental toxins (eg Warfarin from rat poisons used in the city), the range used by each animal, contact time with the urban edge and hence the list of authors and finalization of the manuscript will be done after this thesis is submitted. Given that this work will be submitted as an independent paper, there will be some repetition for the reader, such as the general statements about toxoplasmosis in the introduction.

Abstract

Toxoplasmosis is a disease caused by the obligate intracellular pathogen, *Toxoplasma gondii*. The disease occurs globally and the parasite has been documented to infect both mammals and birds, although felids are the only known hosts in which sexual reproduction of the pathogen occurs and therefore members of the family Felidae are of importance where transmission and infections with *T. gondii* are concerned.

Twenty three African caracals (*Caracal caracal*) were analyzed for both IgG and IgM *T. gondii* antibody seroprevalence by Indirect Immunofluorescence assay (IFA). Overall

IgG antibody seroprevalence was 69.6% (95% CI 0.3906-0.8619) and overall IgM antibody seroprevalence was 34.7% (95% CI 0.1361-0.6094). Six (26.1%) of the caracals were both IgG and IgM positive. The high seroprevalence of *T. gondi* antibodies in this group of Caracals is similar to a high rate observed in a group of feral cats in two municipal areas of Cape Town. The implications or significance of such high seroprevalence of *T. gondii* in felids investigated in South Africa, should be investigate further in order to understand the reasons for this and to design any possible intervention strategies to limit further spread.

3.4.1 INTRODUCTION

Toxoplasmosis is a zoonotic disease of significant importance due to the fact that it is both a food and water borne disease. *Toxoplasma gondii*, the protozoan parasite that causes the disease toxoplasmosis is an obligate intracellular parasite and is able to infect almost all warm blooded animals and birds, making it one of the most widespread if not the most widespread infectious agent globally (Frenkel, 1970). Human infections with *T. gondii* are acquired mainly through the ingestion of tachyzoites and/or tissue cysts containing bradyzoite forms in partially cooked or completely raw meat and also through the consumption of food and water contaminated with oocysts of *T. gondii* (Tenter, *et al*., 2000; Hill and Dubey, 2002). *Toxoplasma gondii* infections in immunocompetent humans is usually asymptomatic but in some cases may cause severe illness and therefore morbidity. Toxoplasmosis in women of childbearing age or pregnant women is considered extremely dangerous because depending on when the infection was acquired, that is, prior to or during the pregnancy, the parasite is able to establish a congenital infection in the fetus which can result in considerable and irreversible neurological

damage to the fetus (Campos *et al*., 2014). *Toxoplasma gondii* is also an opportunistic infection in immunosuppressed individuals, particularly in HIV/AIDS patients. (Luft and Remington, 1992). Felids are the only known host of *T. gondii* in which sexual reproduction is able to occur, therefore, members of the Felidae family play a significant role in the life cycle of the parasite (Dabritz and Conrad, 2010). After a primary infection with *T. gondii* either through consumption of infected prey or contaminated water, felids are normally asymptomatic and do not demonstrate any evidence of clinical toxoplasmosis, but subsequently the parasite undergoes multiplication in the gut of the felid and produces non sporulated oocysts which are shed through the feces of the felid into the environment for a period of about 10 to 21 days. The oocysts, once in the environment, sporulate when conditions are favorable and become infective to susceptible human or animal hosts (Dubey and Frenkel, 1976). Caracals (*Caracal caracal*) also called the African Lynx, are considered amongst the top predators in any ecosystem where they occur. In most ecosystems, these predators are seen as keystone predators in that changes in their population are directly related to the increase or decrease in numbers (population size) of other species within the ecosystem. They can therefore, be seen to be markers or sentinels for the biological or species stability within the ecosystems where they occur ("Urban Caracal Project - The Cape Leopard Trust" 2015). Monitoring the health status of these felids is therefore of primary significance in any ecosystem.

There is minimal data on *T. gondii* infections in caracals globally, whilst in South Africa specifically there has not been any report on the presence or absence of *T. gondii* in either

captive or wild caracals. In the USA, de Camps et al (2008) reported the detection of *T. gondii* in 2 out of 4 captive caracal*s* in a zoo in the Midwest, whilst Spencer et al (2003) also reported *T. gondii* infection in a single caracal tested in a California zoo. Dubey et al (2010) also demonstrated the presence of *T. gondii* infection in 1 out of 1 caracal *(Caracal algira)* and 5 out of 6 caracals *(Caracal schmitsi)*, in the United Arab Emirates. Although the studies are limited in number and captive caracals hardly represent what may happen in an open ecosystem, they all demonstrate a high *T. gondii* seropositivity rate in the caracal. Since *T. gondii* in *Caracal* spp has not been investigated to any significant extent, not much is known about its response to the infection. For example, we do not know whether caracals are highly susceptible to *T. gondii* infections, although as stated earlier, felids are generally susceptible to toxoplasmosis. However, for reasons not yet known, some members of the felid species are more susceptible to *T. gondii* infections than others and the infections may be quite different in their pathogenicity where the different *felid* species are concerned. We hope that investigating the *T. gondii* infection status of the Cape peninsula caracal will therefore provide us with significant insights into the epidemiological importance of the species in the transmission of *T. gondii* within the peninsula ecosystem where they inhabit and help to contribute information that will be useful to help conserve this isolated remnant population.

3.4.2 STUDY AIMS AND OBJECTIVES

The aim of the study was to determine the *T. gondii* seroprevalence in opportunistically obtained caracal serum samples from the Cape peninsula of South Africa.

The objectives of the study were,

I. To determine the *T. gondii* IgM antibody seroprevalence in the caracal serum samples through an enzyme immunofluorescence methodology
II. To determine the *T. gondii* IgG antibody seroprevalence in the caracal serum samples through an enzyme immunofluorescence methodology

3.4.3 STATISTICAL ANALYSIS

Statistical analysis was done using the Pearson Chi square test with the SAS/STAT statistical analysis software Version 9.3 (North Carolina, USA). Correlations between IgG and IgM seroprevalence and gender and age of cats were investigated. Differences were deemed statistically significant when $p \leq 0.05$.

3.4.4 ETHICAL CLEARANCE

Ethical approval was obtained from the Animal Ethics Committee of the University of Cape Town (Appendix 9).

3.4.5 MATERIAL AND METHODS

Sample size determination

Since there has been no previous study of toxoplasmosis or *T. gondii* seroprevalence rate in caracals within the Western Cape, we targeted as many samples as we could obtain. The PI on the Urban Caracal Project (Dr L Serieys) reports that the animals are extraordinarily difficult to catch and her current estimate is that the Cape Peninsula has possibly 80-100 caracals. Our sample collection therefore represents possibly a quarter of all known animals in this region.

Caracal data

A total of 23 (twenty three) African caracals (*Caracal caracal*) captured as part of an ongoing Urban Caracal project in the Cape Peninsula region of the Western Cape province of South Africa were included in this study. At capture, biodata and demographic details were collected (Table 3.1).

Sample collection

Venous blood (5 ml) was collected via venipuncture into serum separator tubes (BD), and immediately placed on ice. Blood was then taken to the laboratory and centrifuged at 3500x *g* for 5 minutes to isolate serum, the isolated serum was then stored at -80^{0}C until further analysis.

Table 3.1 Demographic details of Caracals in the study

ID	Sex	Age	Capture location	Rodenticide exposure
TMC1	Male	Adult	Kings Blockhouse	Unknown
TMC2	Female	Adult	Antelope paddock	Unknown
TMC3	Female	Adult	Noordhoek wetlands	Unknown
TMC4	Male	Adult	Orangekloof	Unknown
TMC6	Male	Adult	Orangekloof	Unknown
TMC7	Male	Juvenile	Westlake city	Unknown
TMC8	Male	Juvenile	Antelope paddock	Yes
TMC9	Female	Adult	Orangekloof	Unknown
TMC10	Male	Adult	Noordhoek wetlands	Unknown
TMC11	Male	Juvenile	Noordhoek wetland	Unknown
TMC12	Male	Adult	Silvermine	Unknown
TMC13	Female	Adult	Silvermine	Unknown
TMC 16	Male	Juvenile	Silvermine	Unknown
TMC 17	Male	Adult	Wynberg	Unknown
TMC 18	Male	Juvenile	Tygerberg	Unknown
TMC 19	Female	Adult	Cape Flats	Unknown
TMC 20	Male	Adult	Cape Flats	Unknown
TMC 21	Male	Juvenile	Noordhoek	Unknown
TMC 22	Female	Juvenile	Noordhoek	Unknown
TMC 23	Female	Adult	Cape Point	Unknown
TMC 24	Female	Adult	Cape Point	Unknown
TMC 25	Male	Adult	Cape Point	Unknown
TMC 26	Male	Adult	Hout Bay	Unknown

Note: age, sex, rodenticide exposure and capture location details of twenty-three caracals whose *Toxoplasma gondii* infection status was investigated on the study.

Serology investigations

Caracal serum samples were analyzed qualitatively for the presence or absence of antibodies to *T. gondii* by Indirect Immunofluorescence test (IFAT) using the IFAT *T. gondii* IgG and IgM antibody commercial kits (Euroimmun AG) according to the manufacturer's instructions. Briefly: 1:100 dilutions of thawed serum samples were prepared in phosphate- buffered saline (pH 7.4) containing 0.05% Tween-20 (PBS-T) to prevent non-specific binding and false positive fluorescence. The diluted sera were then incubated with *T. gondii* trophozoite smears immobilised on test slides at 37°C for 30 minutes. Slides were washed for 5 minutes in PBS-T and 5 minutes in distilled water. Bound antigen–antibody complexes were then stained with fluorescein-labelled anti-cat *T. gondii* IgM or IgG antibody. Stained slides were washed again as described above and then dried. Dried slides were covered with mounting fluid and fluorescence was visualized under a fluorescence microscope (Zeiss). Samples that showed only apical fluorescence were recorded as negative. Positive and negative controls were included in each test slide.

3.4.6 RESULTS AND DISCUSSION

There were twenty-three caracals in the study, made up of 8 (34.8%) females and 15 (65.2%) males. 16 (69.6%) were adults and 8 (30.4%) were juveniles. Seven (87.5%) of the females were adults and one (12.5%) was juvenile. The males were made up of 75.0% (12) adults and 25.0% (4) juveniles. One (4.3%) had been exposed to rodenticides and at the time of this report 95.6% had an unknown rodenticide exposure status (Table 3.1).

Table 3.2 *Toxoplasma gondii* IgM and IgG antibody seroprevalence by age of caracals (n =23)

	IgM +	IgM -	Total
IgG +	6.0 (26.1%)	10.0 (43.5%)	16.0 (69.6%)
			Adult 12.0 (75.0%)
			Juvenile 4.0 (25.0%)
IgG −	2 .0(8.6%)	5.0 (19.0%)	7.0 (30.4%)
			Adult 4.0 (57.1%)
			Juvenile 3.0 (42.9%)
Total	8 (34.7%)	15.0 (62.5%)	23
	Adult 4 (50.0%)	Adult 12.0 (70.6%)	
	Juvenile 4 (50.0%)	Juvenile 3.0(29.4%)	

Notes: free ranging caracals (n=23) were tested for their *Toxoplasma gondii* serostatus. IgG seroprevalence was 69.6%, IgM seroprevalence was 37.5% and IgG+IgM seroprevalence was 26.1%. IgG seroprevalence was higher in adult caracals than in juvenile caracals, whilst IgM seroprevalence was the same in both juvenile and adult caracals tested.

Table 3.3 *Toxoplasma gondii* IgG and IgM antibody seroprevalence by gender of caracals (n = 23)

	Female	Male	Total
IgG +	6.0 (75.0%)	10.0 (66.7%)	16.0
IgG –	2.0 (25.0%)	5.0 (33.3%)	7.0
Total	8.0	15.0	23
IgM +	1.0 (12.5%)	7.0 (46.7%)	8
IgM –	7.0 (87.5%)	8.0 (53.3%)	15
Total	8.0	15.0	23

Notes: *Toxoplasma gondii* IgG antibody seroprevalence was higher in female caracals (75%) than in male caracals (66.7%). IgM seroprevalence was higher in males (46.7%) than in females (12.5%).

Overall IgG antibody seroprevalence was 69.6% (95% CI 39.1-86.2) and overall IgM antibody seroprevalence was 34.7% (95% CI 13.6-60.9). Six (26.1%) of the caracals tested (one female adult, four male adults and one juvenile male) were both IgG and IgM positive. IgG antibody seroprevalence was higher in adult caracals (75% of adults) than in juvenile caracals (25% of juveniles) (Table 3.2). This is consistent with other studies, because normally it is expected that the level of exposure should increase with age, and a

similar pattern was observed in the study on feral cats in two municipal areas of Cape Town in the Western Cape of South Africa (Hammond-Aryee *et al*., 2015). IgM antibody seroprevalence was the same in both juvenile caracal (50.0%) and adult Caracal groups (50.0%) (Table 3.2). This suggests that both juvenile and adult caracals are being infected and therefore there is an active infection cycle ongoing within the ecosystem where these caracals are found. These newly infected felids will be shedding oocysts into the environment (Dabritz and Conrad, 2010*)*, which will then sustain the transmission cycle.

The IgM positive adult female caracals (50.0%) suggest recent infections or reactivation of existing infections. Felids are able to shed oocysts more than once, as they do not acquire lifelong immunity to *T. gondii* infection after a primary infection as was earlier believed (Dubey, 1995). Reactivation of existing infections or reinfections are of epidemiologic significance, because their occurrence implies the existence of a high infection pressure. This then results in active and repeated shedding of infectious oocysts into the environment thereby increasing the oocyst burden in the ecosystem and also the probability of onward transmission to intermediate hosts. *Toxoplasma gondii* IgG antibody seroprevalence was higher in female caracals (75%) than in male caracals (66.7%). This may reflect an older cohort of females which is also suggested by the data that shows only one (12.5%) IgM seropositive female caracal, but a 46.7% IgM seropositive rate amongst the male caracals, amongst which were juveniles (Table 3.3). In South Africa, there have been reports of high seroprevalence of *T. gondii* antibodies in some free ranging felids. For example, Cheadle et al (1999) reported 74% seroprevalence in captive and free ranging lions, leopards and cheetahs in the Kruger and Hluhluwe-Umfolozi National Parks and Penzhorn et al. (2002) also reported a seroprevalence of

100% in 12 lions and 87% in 7 leopards in the Kruger National Park, and 100% in 30 lions at the Hluhluwe-Umfolozi National Park. Hammond-Aryee *et al*., 2015 reported a seroprevalence of 37.1% in feral cats sampled in the two municipal areas in Cape Town. The implications or significance of such high seroprevalence of *T. gondii* in felids investigated in South Africa, remains to be seen or understood clearly. Currently in South Africa there is no active monitoring of humans, domestic livestock or wildlife for *T.gondii* infections.

3.4.7 CONCLUSION

With the emergence of high profile zoonotic pathogenic infections of devastating public health impact such as avian influenza, coronavirus and Ebola (Halliday *et al*., 2015),

public health systems in countries should not ignore the effects that toxoplasmosis may have on overall disease burden and should track and monitor widely occurring zoonoses such as *T. gondii*. Therefore, significant efforts and investment have to be channeled into research and active surveillance of *T. gondii* from a One Healt perspective in order to minimize the potential risk of occurrence of outbreaks.

This study is the first reported study of free ranging caracals in Africa and probably globally. These animals are free ranging, actively feed on other animals such as rodents and with such high rates of *T. gondii* exposure the likelihood of onward transmission of *T. gondii* via contamination of the environment through their feces cannot be ignored. We believe that the results from this study must be taken as exploratory in nature and therefore be used as a platform to initiate further studies into *T. gondii* in free ranging

wildlife in South Africa and specifically free ranging felids in order to gain a proper understanding of the status quo and apply it to health policy development.

3.5 GENERAL COMMENTS AND DISCUSSION

In this chapter, we investigated the seroprevalence of *T. gondii* infection in selected animal populations in defined areas of the Western Cape. The selected populations were feral cats (n=159) from the Cape Town city and Paarl areas, sheep (n=292) from one farm in Bredarsdorp in the Overberg region of Cape Town and caracals (n=23) from the Cape peninsula region of Cape Town. It must be noted that these studies were limited to defined populations in defined municipal areas in Cape Town and I did not have very large sample sizes. I am therefore cautious in the generalized conclusions that can be drawn from these findings. In the Western Cape the feral cat and the caracal studies are the only studies on *T. gondii* seroprevalence in felid species to be reported. Seroprevalence rates were high in both the feral cats (37.1%) and the caracals (69.6%), but lower in the sheep cohort (8%). Regardless of the stated study limitations, it is important to note that the seroprevalence rates in the felid *spp* investigated are high. The epidemiological significance of that is that felids are the only known definitive host for *T. gondii* and therefore play a very important role transmission of the pathogen within their habitats. Bearing in mind that both the feral cats and caracals are free roaming felids and carnivorous, we can speculate that they will be covering considerable distances and shedding oocysts of *T. gondii* in their feces over considerable geographical areas. (In fact, at least one caracal that was collared and movement monitored, ranged between the top of Table Mountain to Clovelly covering a distance of tens of kilometers in the process). The feral cats live amongst human beings and the caracals live along water sources that

provide the Cape Town populations with water and therefore contamination of water sources by these felids cannot be ruled out. South Africa is a nation known for its animal tourism and it has unique felid species such as the caracals. Human interaction with the animal species is on the increase as the population grows, tourism increases and the urban edge is pushed each year further into former wild areas and it is not surprising that felids such as the caracals and leopards are now being sighted more and more near to human settlements.

Sheep may also be important in terms of *T. gondii* epidemiologysince they are also vulnerable to *T. gondii* infections. The role of sheep in the South African setting is important. Sheep farming is an important economic driver in South Africa, is also an important employment sector and from the point of view of possible transmission of toxoplasmosis to humans, mutton consumption is also very high in South Africa. It is useful to understand and keep track of a zoonosis such as *T. gondii* and encourage advocacy on the issue at the basic level in order to equip people with enough information to help them manage any risks they might encounter.

Putting the studies in context from my perspective, if we do not investigate prevalence of *T. gondii* in an ongoing process, it will be difficult to know what to do at the basic level with regards to educating vulnerable populations. We at the very least need to establish the baseline prevalence in signature species that are vulnerable to *T. gondii* as well as those that play a role in its actual life cycle including its transmission, so that we may be able to manage our interactions with them. It is therefore important to treat the data from these studies as primary and build on it in order to make more informed decisions on the management of *T. gondii* within the region.

3.6 ADDITIONAL INFORMATION

Sample size calculation for feral cats study

Since this was the first ever study of *T. gondii* seroprevalence in feral cats in any area in the Western Cape, we did not have any reference from which to calculate an ideal sample size, therefore in discussions with the biostatisticians at the Stellenbosch University health sciences faculty we decided to target a minimum of 150 feral cats in this initial study. Our final sample size was 159 feral cats.

Sample size calculation for sheep study

The 2007 study by Samra et al, on seroprevalence of *T. gondii* in sheep in different provinces in South Africa, reported a seroprevalence rate of 6% for Cape Town and this was used as a reference to calculate the minimum sample size for our study in conjunction with the biostatics unit at the health sciences faculty of Stellenbosch University.

Given a 5% margin of error and a 95% confidence interval, from a flock of 1000 sheep, we calculated our sample size to be a minimum of 278 sheep. Hence in sampling 292 sheep we believe there is a large enough sample to be able to draw valid conclusions from the results obtained.

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CHAPTER 4 GENOTYPIC CHARACTERIZATION AND STRAIN DIVERSITY OF *TOXOPLASMA GONDII* FROM INFECTED HUMAN AND ANIMAL TISSUES FROM THE CAPE TOWN METROPOLE IN SOUTH AFRICA

4.1 INTRODUCTION

The *Toxoplasma gondii* genome is approximate 63 to 65 Mb in size. It consists of 14 chromosomes, that range in size from 1.8 Mb (chromosome Ia), to greater than 7 Mb (chromosome X) (Khan *et al*., 2005; Dalmasso *et al.*, 2014), (Figure 4.1). In addition, there are other genome elements, namely the mitochondrion and apicomplast. The nuclear genome mentioned above is mostly haploid, save for a short diploid phase in the intestine of the definitive host just before meiosis (Ajioka *et al*., 2001). *Toxoplasma gondii* possesses the ability to reproduce sexually, but sexual recombination has been shown to be rare. *Toxoplasma gondii* has an established predominantly clonal population structure and mixed infections have been shown to be rare both in the definitive host and all known intermediate hosts. Reproduction in *T. gondii* is known to be very flexible in that the different stages of the lifecycle are readily interconverted and the entire lifecycle can be regenerated from one parent. There is no geographic nor host restriction in *T. gondii* strain distribution and this ability to colonize different hosts has enabled *T. gondii* to become one of the most successful parasites known to man.

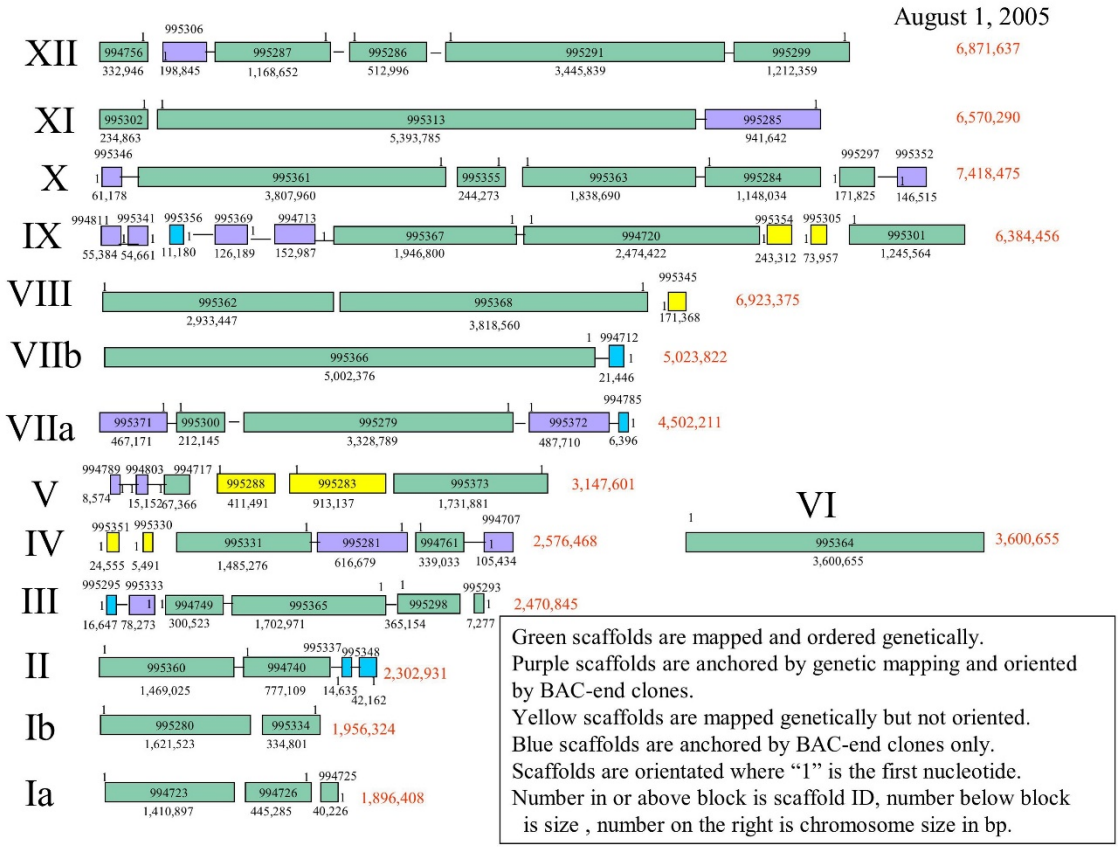


Figure 4.1 Horizontal maps of the genome scaffolds comprising each of the *Toxoplasma gondii* chromosomes. Chromosome numbers are indicated to the left, as is the total size of scaffolds in base pairs. Scaffold numbers are given within or above the colored bars (Khan et al., 2005)

4.1.1 Genetic markers

Several genetic markers have been developed for the genetic analysis of *T. gondii*. These are divided into diagnostic markers and genotyping markers. Markers for *T. gondii* diagnosis are mainly focused on the application of PCR methodology in the direct detection of *T. gondii* specific genetic material in infected host tissues. The methodology is mostly targeted at the repetitive DNA elements as this affords increased sensitivity required for such analysis, since there are more template copies in a repetitive sequence

for a given organism and hence there is increased PCR product (Su *et al*., 2010). These methods have also proven to be simple, reproducible and cost effective and have been used in diagnoses in both human and animal hosts (Herschkowitz *et al*., 2007; Su *et al.,* 2010). There are various methods based on different targets that have been employed in *T. gondii* diagnosis over the past three decades and these include those that target the 35 copy *B1* gene (Burg *et al*., 1989), the 300 copy 529 bp repeat element (Homan *et al*., 2000) and the 18S rDNA gene sequence or internal transcribed spacer (ITS-1), which is a 110 copy template (Hurtado *et al*., 2001; Jauregui *et al*., 2001; Calderaro *et al*., 2006). These methods focus on the application of conventional PCR, nested PCR and quantitative real time PCR in the amplification of repetitive DNA sequences (Su *et al.* 2010), the most sensitive of these being reported to be the quantitative real time PCR of the 529 bp repeat element (Homan *et al.,* 2000) with a reported sensitivity of 1/50 of a genome equivalent (Weiss and Kim, 2007; Su *et al*. 2010).

It is important to note that the molecular detection methods discussed above do not provide any information about the parasite apart from a positive or negative diagnosis. Although the positive identification of the parasite is important for treatment of toxoplasmosis in the clinical setting, it has to be noted that in order to obtain further information on the parasite such as genetic identity, epidemiology and population dynamics, other genetic markers need to be applied. These markers are referred to as genotyping markers earlier in this section. Genotyping markers enable genetic (type or strain) identification, epidemiological studies as well as population dynamics of *T. gondii* to be undertaken. These markers have been applied to the study of *T. gondii* for over four decades. The methods are based on high-resolution identification of isolates of *T. gondii*

and are able to differentiate between isolates of *T. gondii* to varying degrees of efficiency (Weiss and Kim, 2007). The methods are; multilocus enzyme electrophoresis typing (Darde *et al.*, 1988), random amplified polymorphic DNA-PCR typing (Williams *et al*., 1990), mobile genetic element PCR typing (Terry *et al.*, 2001), microsatellite marker typing (Ajzenberg *et al*., 2002a), multilocus DNA sequence typing (Miller *et al*., 2004) and multilocus PCR-Restriction fragment length polymorphism typing (Sibley and Boothroyd, 1992) and are discussed below.

4.1.1.1 Multilocus enzyme electrophoresis typing

Initial studies applying genotyping markers to *T. gondii* genetic analysis began with multilocus enzyme electrophoresis (Darde *et al.,* 1988). This technique is based on the differentiation of *T. gondii* strains using the genes for several polymorphic enzymes. The technique requires large amounts of purified parasite DNA, but it is able to differentiate strains with a high degree of specificity. The technique was used to characterize six main polymorphic enzyme systems in *T. gondii* namely aspartate aminotransferase, glutathione reductase, amylase, glucose phosphate isomerase, acid phosphatase and pro-pionylesterase. In a study of eighty-three isolates in France, the technique was used to describe 12 zymodemes which were then clustered into three main zymodemes, namely zymodemes Z1, Z2 and Z3 (Ajzenberg *et al*., 2002a). A fourth cluster, zymodeme Z4, was closely related to the Z2 zymodeme cluster and differed from this group by one allozyme. The main clusters Z1, Z2-Z4 and Z3 were later found to correspond to the major lineages type 1, type 2 and type 3 as described by multilocus PCR-RFLP (Howe and Sibley, 1995).

4.1.1.2 Random amplified polymorphic DNA-PCR typing

This technique involves the amplification of genomic DNA with the use of various primers of arbitrary length (Williams *et al*., 1990) and has been applied to the genetic characterization of *T. gondii* to detect DNA polymorphisms. The technique was able to resolve *T. gondii* stocks into two main groups of mouse- virulent and mouse-avirulent strains respectively (Ferreira *et al*., 2004).

4.1.1.3 Mobile genetic element PCR typing

This technique applies the phenomenon of high variability in the positioning of mobile genetic elements (MGE) in the eukaryotic genome to the study of genetic variability in *T. gondii*. The technique was able to distinguish between mouse-virulent and mouse-avirulent as well as detect atypical strains (Terry *et al*., 2001). This method is not commonly used in *T. gondii* genetic studies.

4.1.1.4 Serotyping

This technique employs the use of synthetic peptides generated from polymorphic sites of the genes coding for selected *T. gondii* antigens for differentiating between T. gondii strains. In practice antibodies generated in response to these antigenic peptides in test samples allow for the differentiation of *T. gondii* strains thereby eliminating the need for parasite isolation and DNA extraction. The technique was able to differentiate *T. gondii* strains into the three classical lineages namely types 1, 2 and 3 (Kong *et al*., 2003) based on ten empirically selected antigenic peptides. Due to the limited numbers of markers available the application of the technique to *T. gondii* strain typing has been limited to a

few studies (Weiss and Kim, 2007), the high level of similarity between *T. gondii* strains, which have most antigenic determinants in common, also limits this technique and hence it is not currently able to differentiate between types 1 and 2 accurately (Sibley, 2009).

4.1.1.5 Microsatellite marker typing

Microsatellites are short tandem repeat segments of two to six nucleotides in length that are repeated anything from two to twenty times in the genome and normally occur within non-coding regions of the genome. They are highly polymorphic, sensitive, reliable and amenable to high-throughput analysis even though they retain the ability to expand and contract at random during replication and hence are said to be prone to homoplasy (Ajzenberg *et al*., 2002a; (Sibley *et al*., 2009). They maintain the ability to mutate very fast: typically the mutation rates for microsatellites are in the order of 10^{-2} to 10^{-5} per locus per replication. This is faster than that of single nucleotide polymorphisms and hence microsatellites are well suited to individual identification of parasites and therefore very useful in molecular epidemiology studies (Weiss and Kim, 2007). The method has been applied to the differentiation of *T. gondii* into the three classical lineages based on various selected microsatellites (Blackston *et al*., 2001; Ajzenberg *et al.,* 2002b; Lehmann *et al*., 2006; Ajzenberg *et al*., 2010).

4.1.1.6 Multilocus PCR-restriction fragment length polymorphism typing

PCR-RFLP markers have been used extensively for genetic diversity and population studies in *T. gondii*, due to their ease of application and affordability. The technique is based on PCR with primers targeted at various segments of the *T. gondii* genome, followed by restriction fragment analysis on an agarose gel after digestion with restriction

endonucleases which are able to recognize single nucleotide polymorphisms within the genome and therefore cut the genome at these sites resulting in distinct bands on agarose gel electrophoreses (Howe and Sibley 1995; Grigg *et al*., 2001; Khan *et al*. 2005; Su *et al*., 2006; Sibley, 2009. The first application of this technique to *T. gondii* strain typing was reported in Sibley and Boothroyd. (1992). In this study the researchers applied a set of three RFLP markers and were able to identify two distinct groups of *T. gondii* strains based on their virulence in mice. The first group maintained the ability to be highly virulent in mice and also multiply rapidly when cultured *in vitro* whilst the second group was moderate to avirulent in the mouse model and multiplies slowly in *in vitro* cultures. Currently various modifications of the technique have been developed, each with its own unique set of RFLP markers being applied (Su *et al*., 2010).

4.1.1.7 Multilocus DNA sequencing typing

With the decreasing cost of DNA sequencing technologies, the application of multilocus DNA sequencing techniques to the investigation of genetic diversity and population structure in *T. gondii* has seen considerable increase. Currently most studies have applied sequencing at a limited number of loci to the typing of a limited numbers of *T. gondii* stocks and hence the information available on genetic diversity from the application of this technique is limited, (Miller *et al*., 2004; Frazão-Teixeira *et al*., 2011; Pan *et al.* 2012; Weiss and Kim 2007).

4.1.2 Genotype designation schemes

In addition to the conventional genotype designation scheme, there are currently three different naming schemes for *T. gondii* genotype designation. The conventional method of genotype designation assumes a clonal population structure for *T. gondii* and therefore assumes three dominant lineages namely Types I, II and III or types 1, 2 and 3 and forms the fundamental basis for the three naming schemes currently in use (Weiss and Kim, 2013). Genotypes that do not strictly belong to these three lineages are deemed to be rare and are referred to as atypical or exotic. The clonal lineages are classified based on the virulence of the strains in a mouse model, with Type I strains being extremely virulent, Type II being moderate to avirulent and Type III being completely avirulent in mice (Sibley *et al*., 2009).

The naming schemes are;

I. The ToxoDB polymerase chain reaction-restriction fragment length polymorphism naming scheme

This is based on genotyping by ten genetic markers (at ten loci) namely, *SAG1, SAG2* (*5′– 3′ SAG2* and *alt.SAG2*), *SAG3, BTUB, GRA6, L358, c22-8, c29-2* and *Apico*, this scheme further divides the Type II lineage into Type II clonal and Type II variant with the difference being that the Type II clonal bears a Type II allele at the Apico locus whilst the Type II variant genotype bears a Type I allele at the Apico locus (*Su et al*., 2010). This scheme adopts a system where each genotype is assigned a 'ToxoDB PCR-RFLP genotype' followed by a specific numeral and is used to assign genotypes to isolates characterized by the PCR-RFLP methodology (Shwab *et al*., 2014). Hence the

conventional Type I is designated as ToxoDB PCR-RFLP genotype ♯10, whilst the conventional Type II clonal is designated as ToxoDB PCR-RFLP genotype ♯1, whilst the Type II variant is designated as ToxoDB PCR-RFLP genotype ♯3.

II. The Haplogroup naming scheme

This naming scheme is founded on the sequencing of five introns in the *T. gondii* genome, namely the *UPRT, MIC, BTUB, HP* and *EF* introns, currently there are fifteen haplogroups identified and being used in genotype designation of *T. gondii* (Khan *et al*., 2007; Khan *et al.*, 2011; Su *et al*., 2012; Weiss and Kim, 2013)

III. The Toxoplasma BRC code naming scheme

This naming scheme is based on molecular typing with fifteen distinct microsatellite markers located across fourteen chromosomes of *T. gondii.* The microsatellites markers used are designated as *TUB2, W35, TgM-A, B18, B17, M33, MIV.1, MXI.1, M48, M102, N60, N82, AA, N61*, and *N83* (Ajzenberg *et al*., 2010).

4.1.3 Geographic distribution and population structure

Toxoplasma gondii exhibits a varied geographic distribution globally, with a spectrum of limited through moderate to high genetic diversity. The most recent analysis of the geographic distribution of *T. gondii* strains reveals a pattern of low diversity in Europe, and Asia, a low to moderate diversity in Africa and a moderate diversity in Australia and North America and a high diversity in Central and South America (Su *et al.,* 2012).

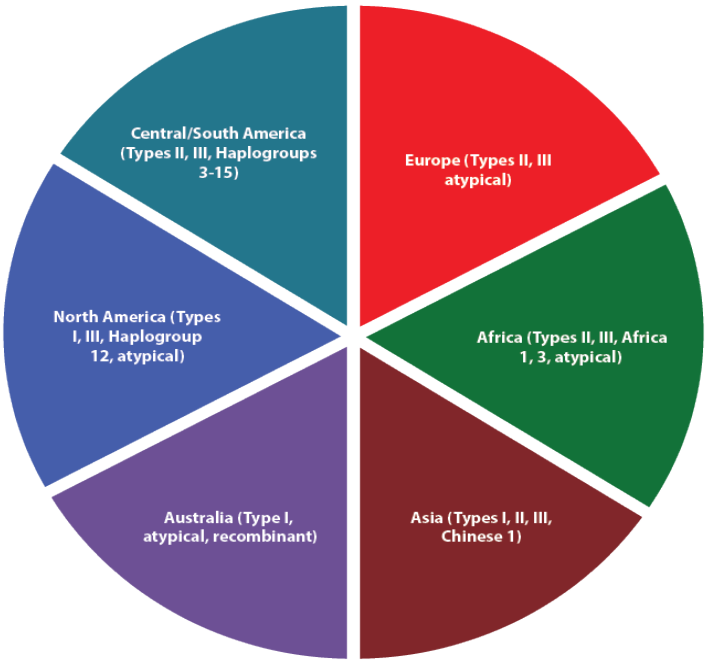


Figure 4.2 *Toxoplasma gondii* genotypes isolated in the different geographic regions (Su *et al*., 2012; Weiss and Kim 2013; Shwab *et al*., 2014).

Toxoplasma gondii is observed to exhibit limited genetic diversity in Europe (Figure 4.2) with the genotypes Types II and III being the most predominantly isolated in both human and animal studies. In an analysis of sixty-four samples from a selection of European countries, nine genotypes were identified, with the Types II and III lineages making up a majority 64.1%, (Shwab *et al*., 2014).

In Africa thirteen genotypes have been identified with most of the study samples being from Egypt. The predominant genotypes were Types II, III and *Africa* 1 and 3. It is suspected that there is a high diversity within the *T. gondii* population in Africa but studies have been limited and therefore far more data is required before any conclusive statements can be made on the diversity of *T. gondii* in Africa (Weiss and Kim, 2013). In

Asia on the other hand, there appears to be a highly uniform population of *T. gondii* genotypes, with the *Chinese* 1 being the most frequently isolated genotype, with a wide distribution within the region, whilst genotype ♯20 seems to crossover from Asia into North Africa and genotype ♯10 also having quite a high frequency in certain countries in Asia (Su *et al*., 2012, Shwab *et al.,* 2014). In Australia, Types II, atypical as well as recombinant strains have been commonly isolated, but it must be noted that studies in this region have also been limited. There appears to be limited diversity within the *T. gondii* population in Australia although this needs to be further investigated (Pan *et al.,* 2012). The northern region of Americas (USA and Canada) exhibit a total of forty genotypes with a predominance of the conventional Type II lineage, making up 220 (43.9%) of a selection of five hundred and one isolates examined while Type III accounted for 91 out of 501 (18.2%) and conventional or clonal Type 12 (now recognized as the fourth clonal lineage in North America) making up 122 out of 501 (24.4%), (Shwab *et al*., 2014). In Central and South America, *T. gondii* exhibits a highly diverse population structure with a high number of atypical or exotic strains having been identified in both human and animal studies over the years. In an analysis of a total of six hundred and six isolates that have been genotyped, one hundred and fifty six genotypes were identified with no genotype being clearly dominant. The genotypes identified include but are not limited to Types II, III, haplogroups 3-15 as well as ToxoDB genotype numbers 2, 3, 6, 7, 8, 11, 13, 19, 65 and 146 (Sibley *et al*., 2009, Su *et al.,* 2012, Weiss and Kim, 2013, Shwab *et al*., 2014).

Toxoplasma gondii genotyping can be done either directly from clinically infected tissues in which case it is referred to as direct genotyping or through isolation of viable organisms after mouse inoculation in which case it is referred to as indirect genotyping. It is generally thought that indirect genotyping introduces a selection bias in that if there was a mixed infection of two strains with one more virulent than the other, then the more virulent strain will be the one to survive multiplication in the mouse and hence the less virulent strain will not be identified. Most *T. gondii* isolates from Europe and North America belong to three clonal lineages and members of these clonal groups are known to vary in virulence, although this is also host-dependent. The members of the three clonal lines differ in a number of phenotypes, possibly the most extensively characterized being virulence in the mouse model. Most virulent are the type I strains, with a lethal dose (LD_{100}) of 1 parasite, whilst types II and III have LD_{100} of several thousand parasites and an LD_{50} of between 10^2 and 10^5 respectively (Saeij *et al*,. 2005; Khan *et al.*, 2009). Crosses between the different strains have been known to result in progeny with highly virulent phenotypes. Recent research has shown that there are a number of genes that in different ways contribute to the differences in virulence phenotypes exhibited by the three different clonal lineages (Table 4.1) (Saeij *et al*., 2006; Reese *et al*., 2011).

Table 4.1 *Toxoplasma gondii* genes and their contributions to the expressed phenotype in the mouse model (Saeij *et al*., 2006).

Strain Types	Virulence factor variants				Effect elicited in host	Phenotype
	ROP18	ROP5	ROP16	GRA15		
I	Expressed at High levels	Enhances ROP18 activity	Activates STAT3 and STAT6	Does not activate nuclear factor kB	Reduced parasite clearance Low IL-12 production	High Virulence
II	High levels	Does not enhance ROP18 activity	Does not activate STAT3 and STAT6	Activates nuclear factor kB	Parasite load clearance High IL-12 production	Intermediate Virulence
III	Very low levels	Enhance ROP18 activity	Activates STAT3 and STAT6	Does not activate nuclear factor kB	Enhanced parasite clearance Low IL-12 production	Low Virulence

The rhoptry secreted serine/threonine kinase ROP18 and its related pseudo kinase ROP5 have been shown to be the differentiating factor when it comes to differences in virulence between the three strains of *Toxoplasma gondii*. Differences in expressed levels of the *ROP18* gene have been shown to be the basis of observed differences in virulence of the three clonal *T. gondii* strain Types I, II and III (Khan *et al*., 2009).

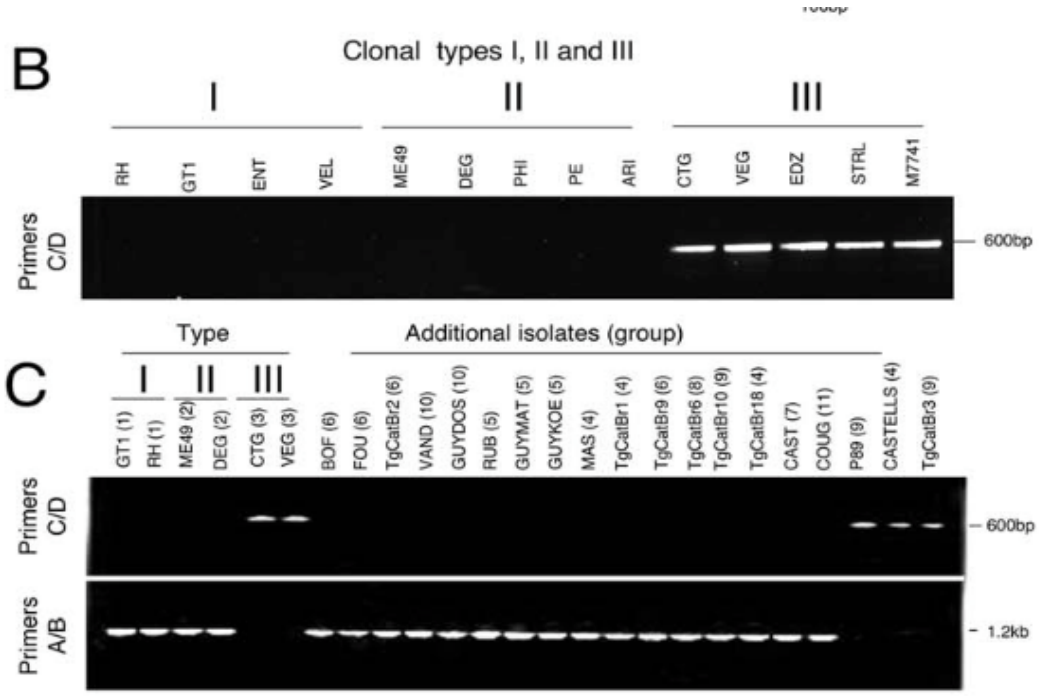


Figure 4.3 Polymerase chain reaction analysis from genomic DNA of clonal isolates

Notes: Polymerase chain reaction analysis from genomic DNA of clonal isolates demonstrating only Type III strains contain the UPS region (B), differential amplification of the upstream regions from a variety of strains demonstrates that strains lacking the UPS region resemble the clonal Types I and II whilst strains with the UPS resemble the clonal Type III (C), taken from (Khan *et al.,* 2009)

This difference in expression is reported to be due to the presence of an additional DNA segment of approximately 2 kilobase pairs in length upstream of the *ROP18* gene (referred to as the UPS region) in the Type III clonal strains that has been shown to alter transcription and is absent in *ROP18* genes of Types I and II clonal strains (Figure 4.3) (Taylor *et al*., 2006; Khan *et al*., 2009; Hunter and Sibley, 2012). This analysis therefore relates to specifically the clonal lineages and not atypical or clonal variant strains.

The relationship between the structure of the *ROP18* gene and marked differences in its expression levels during infection in laboratory mouse models has been established in

previous studies. The absence of the additional upstream segment of the gene was directly related to an increase in the expression of the gene and hence increased virulence in the mouse model (Boyle *et al*., 2008). Hence by characterizing the upstream region of the gene in different *T. gondii* isolates, inferences can be made on the possible phenotypic profile of the strain under study, by focusing on whether the upstream element is present or not followed by sequence alignment to known sequences. This is therefore an opportunity to gather some preliminary data on the virulence profile of the isolates under investigation.

4.2 STUDY AIMS AND OBJECTIVES

Study aims

1. To investigate the genotypes of *T. gondii* in infected human and animal tissues isolated in the Cape Town metropolis
2. To describe the genetic diversity and population structure of the identified genotypes
3. To characterize the upstream region of the *ROP18* gene of *T. gondii* in the genotypes identified from infected animal tissues

Study objectives

I. To genotype *T. gondii* from DNA isolated from infected human blood/CSF/Eye fluid via microsatellite marker genotyping technique

II. To compare genotyping results in human samples to those from animal samples

III. To describe genetic diversity of identified genotypes from human and animal samples

IV. To characterize the region upstream of the *ROP18* gene in *T. gondii* in locally obtained infected animal tissues by PCR amplification of the region upstream of the gene in order to determine the presence or absence of the extra DNA insert.

V. To sequence the DNA fragments obtained from the PCR analysis and compare the obtained sequences of the upstream regions of the local isolates to those of the three reference *T. gondii* strains via a BLAST sequence homology search in the NCBI database

4.3 MATERIALS AND METHODS

4.3.1 Direct genotyping of *Toxoplasma gondii* from infected human and animal tissues using 8 and 15 microsatellite markers

Materials

Human samples were from whole blood, eye fluid and cerebrospinal fluid samples opportunistically collected from patients presenting with acute *T. gondii* infection at Tygerberg Academic hospital and Groote Schuur Academic hospital in Cape Town from January 2013 to August 2014, (Table 4.2).

Table 4.2 Human sample data for genotyping analysis

Specimen ID	Age (years)	Specimen Type	Sex	Clinical Data
H1001	25	Eye Fluid	M	Panuveitis
H1002	51	CSF	M	Toxoplasmosis
H1003	38	Blood	F	Tuberculosis
H1004	36	CSF	F	Toxoplasmosis
H1005	38	Eye Fluid	F	Uveitis
H1006	33	Eye Fluid	M	Acute retinal necrosis
H1007	32	Eye Fluid	M	Acute retinal necrosis
H1008	34	CSF	F	Meningitis
H1009	50	Eye Fluid	F	Panuveitis
H1010	26	Eye Fluid	M	Pos Uveitis, Retinitis
H1011	29	Eye Fluid	F	Retinitis, CMV
H1012	46	Eye Fluid	M	Panuveitis
H1013	27	Eye Fluid	M	Retinitis
H1014	41	Eye Fluid	F	Viral retinitis
H1015	32	CSF	M	Tuberculosis
H1016	37	Eye Fluid	M	CMV retinitis
H1017	32	CSF	M	Toxoplasmosis

Animal samples consisted of tissue sections from postmortem examinations of *T. gondii* infected animals from the Western Cape Provincial veterinary services in Stellenbosch, Western Cape, collected from January 2013 to December 2014, (Table 4.3).

All samples were screened diagnostically for the presence of *T. gondii* DNA via PCR targeting the *B1* gene of *T. gondii* and only positive samples were included in the genotyping study.

Table 4.3 Animal samples for genotyping analysis

Specimen ID	Clinical Data	Specimen Type	Source
A1001a	Disseminated toxoplasmosis	Monkey Brain	Provincial vets Stellenbosch
A1001b	Disseminated toxoplasmosis	Monkey Liver	Provincial vets Stellenbosch
A1001c	Disseminated toxoplasmosis	Monkey Intestine	Provincial vets Stellenbosch
A1001d	Disseminated toxoplasmosis	Monkey Lung	Provincial vets Stellenbosch
A1002	Disseminated toxoplasmosis	Kitten Lung	Provincial vets Stellenbosch
A1003a	Disseminated toxoplasmosis	Kitten Intestine	Provincial Vets Elsenburg
A1003b	Disseminated toxoplasmosis	Kitten Lung	Provincial Vets Elsenburg
A1005a	Disseminated toxoplasmosis	Monkey Lung	Provincial Vets Elsenburg
A1006c	Disseminated toxoplasmosis	Monkey Liver	Provincial Vets Elsenburg
A1008	Disseminated toxoplasmosis	Marmoset Intestine	Provincial vets Stellenbosch
A1011	Disseminated toxoplasmosis	Marmoset Liver	Provincial vets Stellenbosch

Positive control (reference) strains

The laboratory of Dr Chunlei Su, department of microbiology, University of Tennessee, Knoxville, USA, supplied the control trains used for this study (Table 4.4). The strains are internationally agreed reference strains for *T. gondii* strain typing (Weiss and Kim 2013).

Table 4.4 *Toxoplasma gondii* genotyping positive control strains

Toxoplasma gondii reference strain	Conventional genotype designation
GT1	Type I
PTG	Type II
CTG	Type III
TgCgCal (Cougar)	Atypical
MAS	Atypical
TgCatBr5	Atypical
TgCatBr64	Atypical
TgToucan (TgRsCr1)	Atypical

The control strains were provided as extracted genomic DNA and were shipped from the USA to South Africa via cold freight. On arrival at our laboratories they were allowed to thaw and vortexed briefly and aliquoted into 50 µl aliquots in 2 ml Eppendorf tubes (Eppendorf) and stored at -80^{o}C.

Methodology

DNA extraction from human samples

DNA extraction was done using the NucliSens easyMAG automated system (BioMérieux) following the manufacturer's instructions. The DNA was eluted into a final volume of 50 µl in elution buffer. A starting volume of 200 µl each of EDTA whole blood, CSF and eye fluid was used. The eluted DNA integrity was analyzed and then

quantified on the Biodrop 80-3006-51 spectrophotometer (Isogen). The eluted DNA was then aliquoted into 25 µl aliquots and stored at -80 ^{0}C for further analysis.

DNA extraction from animal tissues

Manual DNA extraction was done using the ZR genomic DNA ^{TM} –tissue miniprep kit (Zymo Research Corporation), as outlined below.

Thawed animal tissue (40 mg) was cut and weighed in a Sartorius BP 211D scale (Sartorius AG) and placed in a 2 ml micro centrifuge tube (Eppendorff), a solution of H_2O (95 µl), 2X digestion buffer (95 µl) and 10 µl proteinase k (20 mg/ml) was then added to the digest tissue. The sample was then mixed by vortexing for 30 seconds and incubated on an Accublock^{TM} digital dry bath (Labnet International Inc) for 2 hours at 55^{o}C with brief mixing after every 30 minutes of incubation. After incubation, 700 µl of genomic lysis buffer (supplied in the kit) was then added to the tube and the contents of the tube mixed thoroughly by vortexing for 30 seconds. The tube was then centrifuged for one minute at 10,000 x *g* in a Sigma D-37520 micro-centrifuge (Sigma), to remove all insoluble debris. The supernatant (850 µl) was then gently transferred into a Zymo-Spin ^{TM} IIC column in a collection tube, without the pipette touching the sides of the tube. The column was placed in a Sigma D-37520 micro-centrifuge (Sigma) and centrifuged for one minute at 10,000 x *g*. The collection tube was replaced with a new collection tube and 200 µl of DNA pre-wash buffer added to the spin column. The spin column was then centrifuged in a Sigma D-37520 micro-centrifuge (Sigma) for one minute at 10,000 x *g*. The collection tube was replaced and 400 µl of genomic DNA wash buffer was then added to the spin column. The column was placed in a Sigma D-37520 micro-centrifuge (Sigma) and centrifuged at 10,000 x *g* for one minute. The spin column was transferred to

a clean micro centrifuge tube (Eppendorff) and 100 µl of DNA elution buffer was added to the spin column. The column was incubated at room temperature (25^{o}C) for 5 minutes. After incubation, the spin column was placed in Sigma D-37520 micro-centrifuge (Sigma) and centrifuged for 30 seconds at 16,000 x *g* to elute the DNA from the spin column. The eluted DNA's integrity was analyzed and quantified on the Biodrop 80-3006-51 spectrophotometer (Isogen). The eluted DNA was then aliquoted into 25 µl aliquots and stored at -80 ^{0}C for further analysis.

***Toxoplasma gondii* diagnostic PCR**

All human and animal DNA extracts were analyzed for the presence of *T. gondii* DNA via PCR amplification of the 35 fold repeat *B1* gene, using an adaptation of the Burg et al. (1989) protocol.

The primers used were T1 and T2 below, which amplify an approximately 196 base pair region of the *T.gondii B1* gene.

T1 5^{I} GGA ACT GCA TCC GTT CAT GAG 3^{I} (Forward primer)

T2 5^{I} TCT TTA AAG CGT TCG TGG TC 3^{I} (Reverse primer)

Each PCR consisted of 10 µl of crude DNA extract or 1 µl of control DNA, 10x PCR buffer (Mg-) (Roche), 10mM dNTP mix (2.5 mM each of dATP, dCTP, dGTP, dTTP) (Roche diagnostics), 25 mM $MgCl_2$, 25 µM each of reverse and forward primers (Integrated DNA Technologies) and faststart Taq DNA polymerase 5U/µl (Roche diagnostics) in a final volume of 50 µl. The amplification conditions were 94˚C for 2 minute, 35 cycles of (94˚C for 20 seconds, 58˚C for 30 seconds and 72˚C for 45

seconds) with a final extension at 72˚C for 7 minutes in a Veriti 96 well thermal cycler (Applied Biosystems).

Gel electrophoresis

10 µl of the PCR product was mixed with 2 µl of 6x DNA staining and visualizing dye, novel juice (Genedirex), and electrophoresed on 2% agarose gel (Sigma). The gel was then visualized and photographed in the Uvitec gel documentation system (Uvitec). Images were saved as .jpeg files for further analysis.

Microsatellite marker genotyping

In this technique, a selection of eight microsatellite markers (human and animal) and fifteen microsatellite markers (animal samples) are used to investigate *T. gondii* genetic variability by PCR amplification across fifteen different loci located on eleven chromosomes. This technique is able to achieve a two-step level of discrimination, in the first step the technique allows for differentiation of the conventional clonal lineages and in the second step the technique is able to differentiate between closely related strains belonging to the same lineage and this allows for clinical and epidemiological applications in that atypical strains causing severe disease presentation or outbreaks can be identified (Ajzenberg *et al*., 2010).

In this technique, microsatellite loci are amplified by a multiplex PCR system using fluorescently labeled forward primers and unlabeled reverse primers. Subsequently the dye-labeled products are resolved by size using automated electrophoresis and the products identified by fluorescence detection using a size standard (Table 4.5, and 4.6) (Bañuls, et al. 2002); Ajzenberg *et al*., 2010). The markers are made of eight markers

with the repeat motifs $(TA/AC)_n$ or $(TC/AG)_n$, which are applied as typing markers in the eight microsatellite typing system (Table 4.5) and eight markers with the repeat motifs $(TA/AC)_n$ or $(TC/AG)_n$ plus seven additional markers with $(TA/AT)_n$ repeat motifs applied as fingerprinting markers in the fifteen microsatellite typing system (Table 4.6)

Table 4.5 Eight microsatellite markers and tagged PCR primer sequences for *Toxoplasma gondii* genotyping (Ajzenberg et al., 2002)

Marker	Repeat Sequence	PCR primers 5^{l}-3^{l}	No. Of Alleles	Size range of Alleles (bp)
TUB 2	$(TG)_8$	(F) VIC-CCAAGTTCTTCCGTCATTTC (R) CCTCATTGTAGAACACATTGAT	3	122-126
TgM-A	$(TG)_9$	(F) NED-CATGTCCCTGTCGGTTTCTC (R) CGTAAATGCGGATGGAAACT	4	115-121
W35487	$(CT)_{10}$	(F) 6-FAM-TGCTGCGGTCTTTTCTCTTC (R) AACATGCCGTTCCCTTCC	3	95-101
N60608	$(TA)_{13}$	(F) PET-GAATCGTCGAGGTGCTATCC (R) AACGGTTGACCTGTGGCGAGT	7	131-145
N82375	$(TA)_{13}$	(F) PET-TGCGTGCTTGTCAGAGTTC (R) GCGTCCTTGACATGCACAT	10	107-131
N83021	$(TA)_{11}$	(F) NED-ACAACGACACCGCTATCTC (R) CTCTCTATACACAGACCGATTGG	10	125-161
N61191	$(TA)_{11}$	(F) 6-FAM-CCGTATCACCAGATCATGTT (R) CTCTCACCTGATGTTGATGTAA	15	120-160
AA519150	$(TA)_{13}$	(F) VIC-GTTGTCTATGCTGTCGTGCG (R) CACCCATAAACGGTTACTGGTC	16	134-170

Table 4.6 Fifteen microsatellite markers and tagged PCR primer sequences for *Toxoplasma gondii* genotyping (Ajzenberg et al. 2010)

MARKER	CHROMOSOME (POSITION)	REPEAT MOTIF	PRIMER SEQUENCE	SIZE RANGE (bp)
TUB2	IX (974608 to 974896)	[TG/AC]n	(F)5'6-FAM-GTCCGGGTGTTCCTACAAAA3' (R)5' TTGGCCAAAGACGAAGTTGT3'	287-291
W35	II (633241 to 633482)	[TC/AG]n [TG/AC]n	(F)5'HEX-GGTTCACTGGATCTTCTCCAA3' (R) 5'AATGAACGTCGCTTGTTTCC 3'	242-248
TgM-A	X (4824879 to 4825083)	[TG/AC]n	(F)5'HEX-GGCGTCGACATGAGTTTCTC3' (R)5'TGGGCATGTAAATGTAGAGATG3'	203-211
B17	XII (6474746 to 6475079)	[TC/AG]n	(F)5'HEX-AACAGACACCCGATGCCTAC3' (R)5'GGCAACAGGAGGTAGAGGAG3'	334-366
B18	VIIa (2921536 to 2921693)	[TG/AC]n	(F)5'6-FAM-TGGTCTTCACCCTTTCATCC3' (R)5'AGGGGATAAGTTTCTTCACAACGA3'	156-170
M33	IV (672591 to 672760)	[TC/AG]n	(F)5'6-FAM-TACGCTCGCATTGTACCAG3' (R)5'TCTTTTCTCCCCTTCGCTCT3'	165-173
IV.1	*IV (742419 to 742693)*	*[TC/AG]n*	*(F)5'HEX-GAAGTTCGGCCTGTTCCTC3'* *(R)5'TCTTTTCTCCCCTTCGCTCT3'*	*272-282*
XI.1	*XI (189702 to 190058)*	*[TC/AG]n*	*(F)5'6-FAM-GCGTGTGACGAGTTCTGAAAA3'* *(R)5'AAAGTCCCCTGAAAAGCCAAT3'*	*354-362*
M48	*Ia (332951 to 333166)*	*[TA/AT]n*	*(F)5'6-FAM-AACATGTCGCGTAAGATTCG3'* *(R)5' CTCTTCACTGAGCGCCTTTC3'*	*209-243*
M102	*VIIa (3093491 to 3093664)*	*[TA/AT]n*	*(F)5'NED-CAGTCCAGGCATACCTCACC3'* *(R)5'CAATCCCAAAATCCCAAACC3'*	*164-196*
N60	*Ib (1766079 to 1766221)*	*[TA/AT]n*	*(F)5'NED-GAATCGTCGAGGTGCTATCC3'* *(R)5' AACGGTGACCTGACCTGTGGCGCGAGT3'*	*132-157*
N82	*XII (1621472 to 1621585)*	*[TA/AT]n*	*(F)5'HEX-TGCGTGCGTGCTTGTCAGAGTTC3'* *(R) 5' AACGGTTGACCTGTGGCGAGT3'*	*105-145*
AA	*VIII (5836880 to 5837144)*	*[TA/AT]n*	*(F)5'NED-GATGTCCGGTCAATTTTGCT3'* *(R)5' GACGGGAAGGACAGAAACAC3'*	*251-332*
N60	*VIIb (4217145 to 4217238)*	*[TA/AT]n*	*(F)5'6-FAM-ATCGGCGGTGGTTTGTAGAT3'* *(R)5' CCTGATGTGTTGATGTAAGGATGC3'*	*79-123*
N83	*X (1772898 to 1773209*	*[TA/AT]n*	*(F)5'6-FAM-ATGGGTGAACAGCGTAGACA3'* *(R)5' GCAGGACGAAGAGGATGAGA3'*	*306-338*

Multiplex PCR with 8 and 15 microsatellite markers

The samples that tested positive for the *T. gondii* diagnostic PCR were then subjected to a multiplex PCR for 8 microsatellite markers (human and animal samples) and 15 microsatellite markers (animal samples) using the protocols of (Ajzenberg *et al*. 2002; Ajzenberg *et al*. 2010). Primer tags and sequences are listed in tables 4.5 and 4.6 above.

Each PCR consisted of 5 µl of crude DNA extract or 1ul of control DNA, 2x multiplex PCR master mix (Qiagen) which contains a hotstar Taq^{R} DNA polymerase, 25 µM of both forward and reverse tagged primer mix in a final reaction volume of 25 µl. The amplification conditions were 95˚C for 15 minute, 35 cycles of (94˚C for 30 seconds, 61˚C for 3 minutes and 72˚C for 30 seconds) with a final extension at 60˚C for 30 minutes in a Veriti 96-well thermal cycler (Applied Biosystems). PCR products were diluted 1:10 in deionized formamide (animal samples) and the 1:30 (positive control samples). 1ul of diluted product was mixed with 0.5ul of dye labeled size standard (ROX 500, Applied Biosystems) and 23.5 µl of deionized formamide, the mixture was then denatured at 95^{0}C for 5 minutes and then electrophoresed using the automatic sequencer (ABI PRISM 3130xl; Applied Biosystems). The sizes of the PCR products (alleles) in base pairs were viewed using GeneMapper analysis software (version 4.0; Applied Biosystems) and genotypes were assigned by matching to reference strain profiles.

Genotype designation

Genotype designation was done using GeneMapper analysis software (version 4.0; Applied Biosystems).

4.3.2 Clustering and phylogenetic analysis

To quantify the extent of genetic distance between the local animal and human populations and evaluate their position with respect to the reference strains from different continents, neighbor-joining trees were reconstructed from the genetic distances amongst the individual isolates using the free online software Populations 1.2.30 (1999, OlivierLangella, CNR UPR9034, http://bioinformatics.org/-tryphon/populations/.) Trees were reconstructed using the Cavalli Sforza and Edwards chord-distance estimator. The analysis was repeated for 300 bootstrap replicates (Cavalli-Sforza and Edwards, 1967; Mercier *et al*., 2010).

4.3.3 Characterization of upstream region of ROP18 gene of Toxoplasma gondii in infected animal tissues

DNA extracted from *T. gondii* infected animal tissues listed in table 4.4 above was used for the analysis.

DNA extraction

DNA extraction was done by the method described in section 4.3 above.

PCR-based analysis of the upstream region of *ROP18*

The primer set used are indicated below and were taken from the reference Khan et al. (2009)

ROP18-F CTAGCCACGCTATGCACCTCT (forward primer)

ROP18-R GCAAGTCACGCATAGTCTCATC (reverse primer)

Each reaction was carried out in 25 ul of volume containing 10 x PCR buffer (Thermo Scientific), 25 mM MgCl2, 2.5 mM dNTPs (Thermo Scientific), 50 µM each of the forward and reverse primers (Integrated DNA Technologies), 5 U/µl of DNA Faststart Taq polymerase (Roche) and 5 µl of extracted DNA from each of the infected animal tissue samples. The reaction mixture was first heated to 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 2 minutes and soaked at 15°C in a Proflex PCR system (Applied biosystems).

Gel electrophoresis

10 µl of the PCR product was mixed with 2 µl of 6x DNA staining and visualizing dye novel juice (Genedirex), and electrophoresed on 1.5% agarose gel (Sigma). The gel was then visualized and photographed on the Uvitec gel documentation system (Uvitec). Images were saved as .jpeg files for further analysis.

DNA fragment sequencing

The remaining PCR reaction products (approximately 15 µl) where then transported on ice to a central analytical facility for post PCR cleanup and sequencing. Post PCR clean up was performed using the Nucleofast 96 well PCR plate, on a Tecan EVO150 robotic workstation according to protocols supplied by the manufacturer. DNA fragment sequencing was done using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems) using the manufacturer's protocol with minor modifications.

4.4 RESULTS

4.4.1 *Toxoplasma gondii* diagnostic PCR of human and animal tissues

Toxoplasma gondii diagnostic PCR was used to screen a batch of human and animal samples in the laboratory and the samples that tested positive were used for genotyping studies (Figure 4.4 and Figure 4.5). In all, 17 human samples and 11 animal infected samples were used for this study. The human samples were from patients with different diagnoses (Table 4.2), whilst the animal samples (Table 4.3), were opportunistically obtained from the Western Cape Provincial veterinary units of Stellenbosch University and Elsenburg.

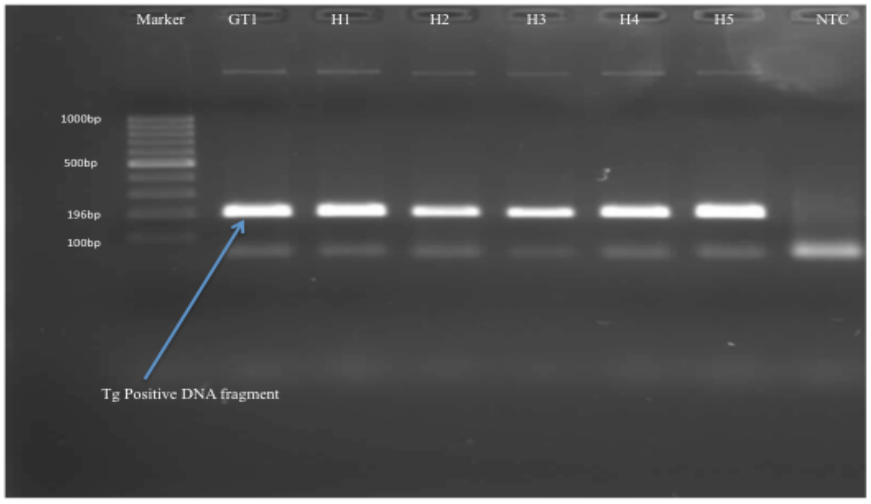


Figure 4.4 DNA fragments obtained from *Toxoplasma gondii* diagnostic PCR of human patient samples, showing results for H1 to H5 out of H1 to H17. GT1- Positive control, H1-H5 human patient samples, NTC- No template control, Marker (100base pairs, Roche)

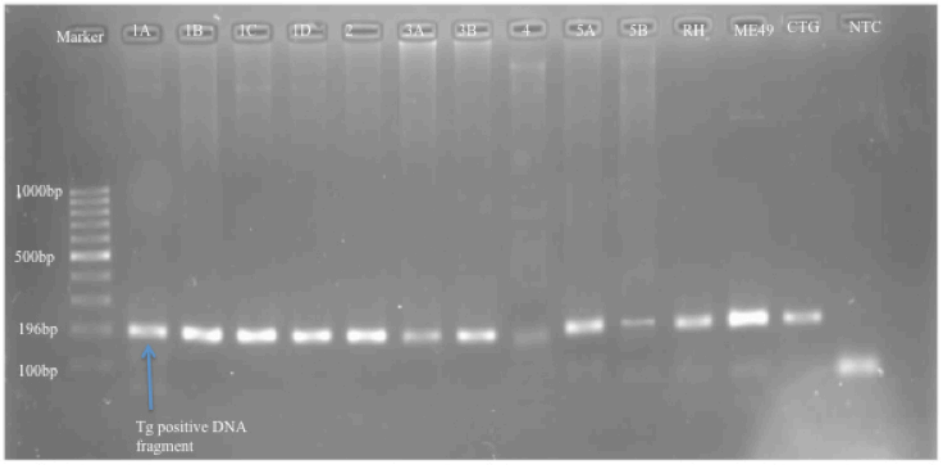


Figure 4.5 DNA fragments obtained from *Toxoplasma gondii* diagnostic PCR of animal tissue samples. RH, ME49, CTG-Positive controls, 1A, 1B, 1C, 1D, 2,3A, 3B, 4,5A and 5b animal tissue samples, NTC-No template control, Marker (100base pairs, Roche)

4.4.2 Direct *Toxoplasma gondii* genotyping of infected human and animal tissues at eight and fifteen microsatellite loci

Overall, 17 PCR positive human samples and 11 PCR positive animal samples (Tables 4.2 and 4.3) were genotyped via microsatellite marker methodology, employing eight microsatellite markers with unique dye tagged primer sequences (Table 4.5) and employing fifteen microsatellite markers also with unique dye tagged primer sequences (Table 4.6) (only PCR positive animal samples) respectively.

DNA from the animal and human tissues was amplified in a PCR amplification reaction together with standard reference strains (Table 4.4) with specific dye tagged primers targeted at eight different microsatellite loc. Amplified products were then cleaned up and processed in an automatic sequencer, generated plots were viewed using the GeneMapper^{R} software (see appendix 10 and 11 for automatic sequencer readout plots) and allele profiles at all the loci determimed for each test sample as well as the controls.

Tables 4.7 and 4.8 show allele calls (in base pairs as per automatic sequencer readout) for human and animal samples at eight microsatellite loci, whilst table 4.9 and 4.10 shows allele calls for only animal samples at 15 microsatellite loci. Standard controls were included in all the sequencing runs.

From the allele profile data obtained, genotypes were assigned to the human and animal samples by matching them to the controls. Table 4.11 shows genotype designation for human and animal samples at eight microsatellite loci and genotype designation from animal samples based on allele calls at fifteen microsatellite loci. Out of the 17 human samples genotyped, there were 8 (47.1%) Type II, 2 (11.7%) atypical, 1 (5.9%) Type III *T. gondii* genotypes whilst 6 (35.3%) were untypable despite repeated attempts, and of the 11 animal samples genotyped there were 9 (81.8%) Type II and 2 (18.2%) atypical *T. gondii* genotypes. Therefore there was a predominance of Type II genotypes causing illness in both human and animal samples (Table 4.11).

Table 4.7 Allele calls and conventional genotyping results of *Toxoplasma gondii* DNA from animal and controls samples, analyzed at eight microsatellite loci

Sample Label	Sample Type	*TUB2*	*W35*	*TgM-A*	*N60*	*N82*	*AA*	*N61*	*N83*	TYPE
CTG		128	99	121	149	115	151	128	137	III
GT1		130	105	125	147	123	147	126	131	I
PTG		128	99	123	145	115	147	130	135	II
MAS		130	99	121	145	115	213	134	164	ATYPICAL
TgCatBr5		130	99	121	143	115	147	128	139	ATYPICAL
TgCatBr64		128	99	123	138	109	145	136	NA	ATYPICAL
TgCgCa1		128	99	121	153	123	141	118	158	ATYPICAL
TOUCAN		130	105	121	143	119	145	136	129	ATYPICAL
NCT		NA	NA	NA	NA	NA	NA	NA	NA	NTC
A1001a	Animal Isolate	128	99	123	143	125	157	134	139	II
A1001b	Animal Isolate	128	99	123	143	125	157	134	139	II
A1001c	Animal Isolate	128	99	123	143	125	157	134	139	II
A1001d	Animal Isolate	128	99	123	143	125	157	134	139	II
A1002	Animal Isolate	128	99	123	143	125	157	134	139	II
A1003a	Animal Isolate	130	99	119	132	113	169	152	139	ATYPICAL
A1003b	Animal Isolate	130	99	119	132	113	169	152	139	ATYPICAL
A1005a	Animal Isolate	128	99	123	143	115	143	NA	139	II
A1006c	Animal Isolate	128	99	123	143	115	143	NA	139	II
A10008	Animal Isolate	128	99	123	143	115	143	?	139	II
A1011	Animal Isolate	128	99	123	143	125	157	134	139	II

Note: Microsatellite alleles from automatic sequencer for human (n=17) and animal (n=11) samples, at eight loci. Allelic polymorphisms were expressed as sizes of PCR products (bp), genotype designation was based on comparison with allele profile at eight loci for reference strains

Table 4.8 Allele calls and conventional genotyping results of *Toxoplasma gondii* DNA from human samples, analyzed at eight microsatellite loci

H1001	Human Isolate	128	NA	NA	NA	115	145	136	139	UNTYPABLE
H1002	Human Isolate	128	99	123	143	125	145	NA	139	II
H1003	Human Isolate	NA	NA	NA	NA	115	145	126	139	UNTYPABLE
H1004	Human Isolate	128	99	123	145	129	141	128	139	II
H1005	Human Isolate	128	99	123	143	NA	149	NA	139	II
H1006	Human Isolate	128	99	123	143	115	145	126	139	II
H1007	Human Isolate	128	99	123	143	115	153	140	139	II
H1008	Human Isolate	130	99	119	132	113	145	126	139	ATYPICAL
H1009	Human Isolate	130	99	119	NA	115	145	126	139	ATYPICAL
H1010	Human Isolate	130	NA	NA	NA	139	NA	126	139	UNTYPABLE
H1011	Human Isolate	128	99	121	149	113	149	128	139	III
H1012	Human Isolate	128	99	123	145	NA	145	126	139	II
H1013	Human Isolate	NA	NA	NA	NA	115	145	128	139	UNTYPABLE
H1014	Human Isolate	128	99	123	143	125	145	126	139	II
H1015	Human Isolate	NA	NA	NA	NA	NA	145	NA	139	UNTYPABLE
H1016	Human Isolate	NA	99	123	145	133	141	128	139	II
H1017	Human Isolate	NA	NA	NA	NA	139	145	126	139	UNTYPABLE

Note: Microsatelite alleles from automatic sequencer for human (n=17) and animal (n=11) samples, at eight loci. Allelic polymorphisms were expressed as sizes of PCR products (bp), genotype designation was based on comparison with allele profile at eight loci for reference strains

Table 4.9 Allele calls and conventional genotype results of *Toxoplasma gondii* DNA from all reference control samples and animals (Al001a and A1001b) samples, analyzed at fifteen microsatellite loci

Sample Label	Sample Type	TYPE	*TUB2*	*W35*	*TgM-A*	*B18*	*B17*	M33	MIV.1	MXI.1	*M48*	*M102*	*N60*	*N82*	*AA*	*N61*	*N83*
GT1	REF	I	**291**	**248**	**209**	**160**	**342**	**169**	**274**	**358**	**209**	**166**	**145**	**119**	**265**	**87**	**318**
ME49 (PTG)	REF	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	**215**	**174**	**142**	**111**	**265**	**91**	**310**
CTG	REF	III	**289**	**242**	**205**	**160**	**336**	**165**	**278**	**356**	**215**	**190**	**147**	**111**	**269**	**89**	**312**
MAS	REF	ATYPICAL	**291**	**242**	**205**	**162**	**362**	**169**	**272**	**358**	**221**	**166**	**142**	**111**	**332**	**95**	**339**
TgCatBr05	REF	ATYPICAL	**291**	**242**	**205**	**160**	**362**	**165**	**278**	**356**	**237**	**174**	**140**	**111**	**265**	**89**	**314**
TgCgCa1	REF	ATYPICAL	289	**242**	**205**	**158**	**336**	**169**	**274**	**354**	**219**	**174**	**151**	**119**	**259**	**79**	**333**
TgCatBr64	REF	ATYPICAL	**289**	**242**	**207**	**160**	**338**	**165**	**278**	**356**	**225**	**190**	**136**	**105**	**263**	**97**	**310**
TgToucan	REF	ATYPICAL	**291**	**248**	**205**	**160**	**364**	**165**	**274**	**356**	**209**	**192**	**140**	**115**	**263**	**97**	**304**
A1001a	Squirrel Monkey	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	225	178	140	121	275	**95**	310
A1001b	Squirrel Monkey	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	225	178	140	121	275	**95**	310

Table 4.10 Allele calls and conventional genotype results of *Toxoplasma gondii* DNA from animal samples (A1001c, A1001d, A1002, A1003a, 1003b, A1005a, a1006c, A1008 and A1011), analyzed at fifteen microsatellite loci

Sample label	Sample type	TYPE	*TUB2*	W35	TgM-A	B18	B17	M33	M1V.1	MX1.1	M48	M102	N60	N82	AA	N61	N83
A1001c	Squirrel Monkey	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	225	178	514	121	275	**95**	310
A1001d	Squirrel Monkey	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	225	178	140	121	275	**95**	310
A1002	Kitten	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	225	178	140	121	275	**95**	310
A1003a	Kitten	ATYPICAL	291	242	203	156	336	165	274	354	223	174	130	109	287	111	310
A1003b	Kitten	ATYPICAL	291	242	203	156	336	165	274	354	223	174	130	109	287	111	310
A1005a	Monkey	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	211	172	140	111	261	109	310
A1006c	Monkey	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	211	172	140	111	261	109	310
A1008	Marmoset	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	211	172	140	111	261	109	310
A1011	Marmoset	II	**289**	**242**	**207**	**158**	**336**	**169**	**274**	**356**	225	178	140	121	275	**95**	310
NTC	REF	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Note: Microsatelite alleles from automatic sequencer for animal (n=11) samples, at fifteen loci. Allelic polymorphisms were expressed as sizes of PCR products (bp), genotype designation was based on comparison with allele profile at fifteen loci for reference strains

Table 4.11 Genotype designation for human and animal samples analyzed by microsatellite methodology

Human Samples	Conventional genotype designation	Animal Samples	Conventional genotype designation
H1001	Untypable	A1001a	Type II
H1002	Type II	A1001b	Type II
H1003	Untypable	A1001c	Type II
H1004	Type II	A1001d	Type II
H1005	Type II	A1002	Type II
H1006	Type II	A1003a	Atypical
H1007	Type II	A1003b	Atypical
H1008	Atypical	A1005a	Type II
H1009	Atypical	A1006c	Type II
H1010	Untypable	A1008	Type II
H1011	Type III	A1011	Type II
H1012	Type II		
H1013	Untypable		
H1014	Type II		
H1015	Untypable		
H1016	Type II		
H1017	Untypable		

Notes: 17 PCR positive human samples and 11 PCR positive animal samples were genotyped via microsatellite marker methodology, employing eight microsatellite markers (PCR positive human and animal samples) and employing fifteen microsatellite markers (only PCR positive animal samples) respectively.

4.4.3 Clustering and phylogenetic analysis

To further investigate the population structure of the genotypes identified in our study, the phylogenetic relationship between the local *T. gondii* strains identified in both human and animal infected tissues and the eight reference strains was determined. Neighbor-joining trees were reconstructed from the genetic distances amongst the individual isolates using the free online software Populations 1.2.30 (1999, OlivierLangella, CNR UPR9034, http://bioinformatics.org/-tryphon/populations/.) The trees were reconstructed using the Cavalli Sforza and Edwards chord-distance estimator and the analysis repeated for 300 bootstrap replicates. The analysis was done for both the eight microsatellite typing scheme data for human and animal samples and the fifteen microsatellites typing scheme for only the animal samples.

In the clustering analysis for the human and animal samples typed with the eight microsatellite scheme, the genotypes A1003a, A1003b, H1008 and H1009 were observed to cluster with the reference atypical Type II genotypes MAS and TgCatBr5, whilst the genotype H1011 was observed to cluster with the Type III reference genotype CTG and the genotypes H1001, H1002, H1003, H1004, H1005, H1006, H1007, H1010, A1001a, A1001b, A1001c, A1001c, A1002, A1005a, A1006c, A1008 and A1011 were observed to cluster with the reference Type II genotype PTG (Figure 4.6). Whilst in the clustering analysis of the animal genotypes typed with the fifteen microsatellite scheme the genotypes A1003a and A1003b were observed to cluster with the reference Type II atypical genotype TgCgCa1 and the genotypes A1001a, A1001b, A1001c, A1001c,

A1002, A1005a, A1006c, A1008 and A1011 were observed to cluster with the reference Type II genotype PTG (Figure 4.7).

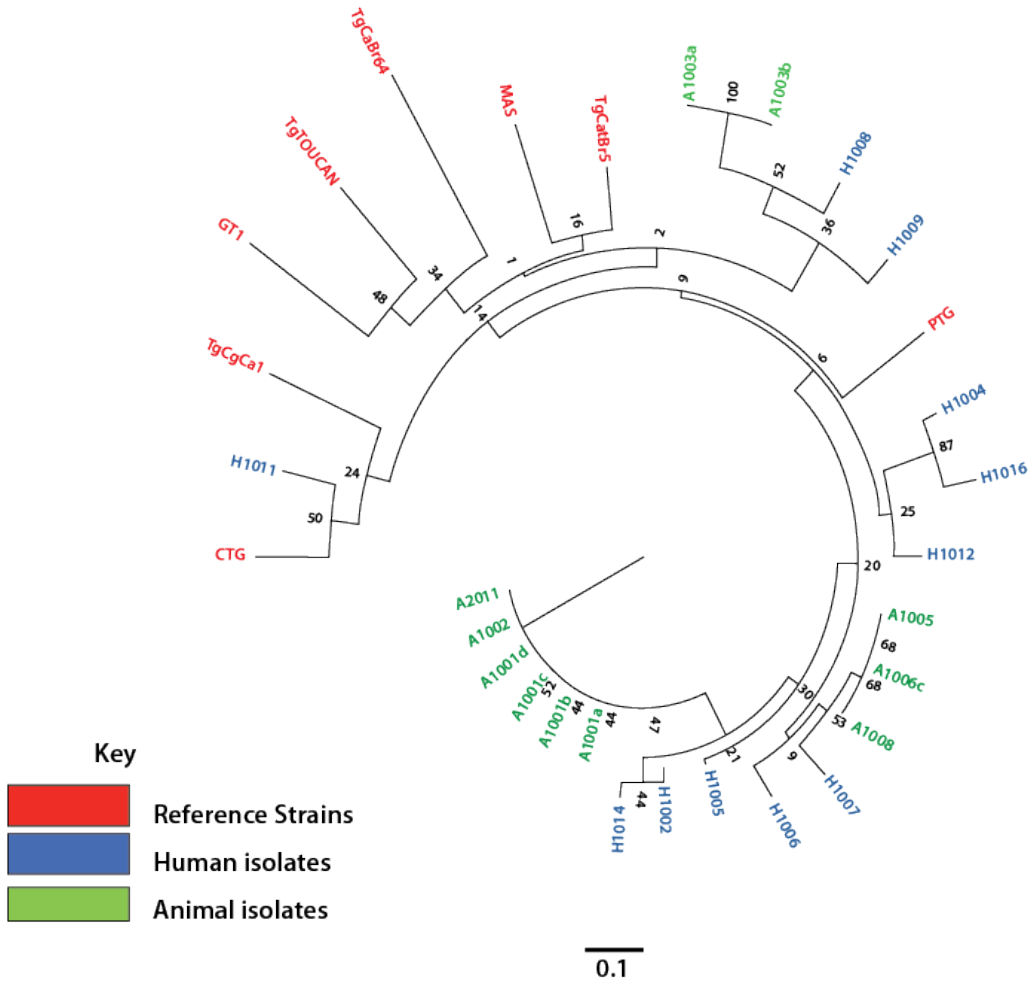


Figure 4.6 Phylogenetic relationship (Based on eight microsatellite marker methodology) among identified genotypes from infected human (n=17) and animal (n=11) samples and known reference *T. gondii* isolates.

Notes: Phylogenetic relationship (Based on eight microsatellite marker methodology) among identified genotypes from infected human (n=17) and animal (n=11) samples and known reference *Toxoplasma gondii* isolates as inferred by neighbor-joining analysis, based on distances calculated using Cavalli Sforza and Edwards chord-distance estimator.

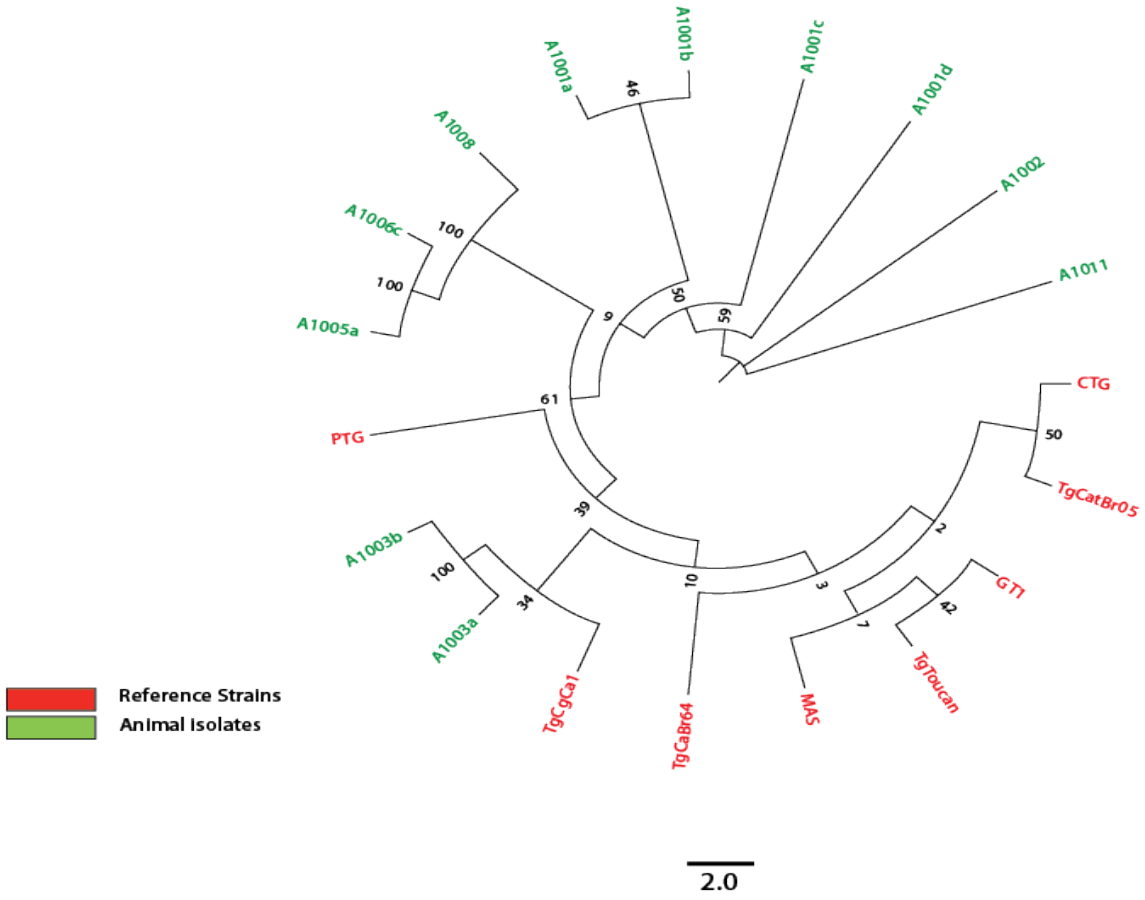


Figure 4.7 Phylogenetic relationship (Based on fifteen microsatellite marker methodology) among identified genotypes from infected animal (n=11) samples and known reference T. gondii isolates

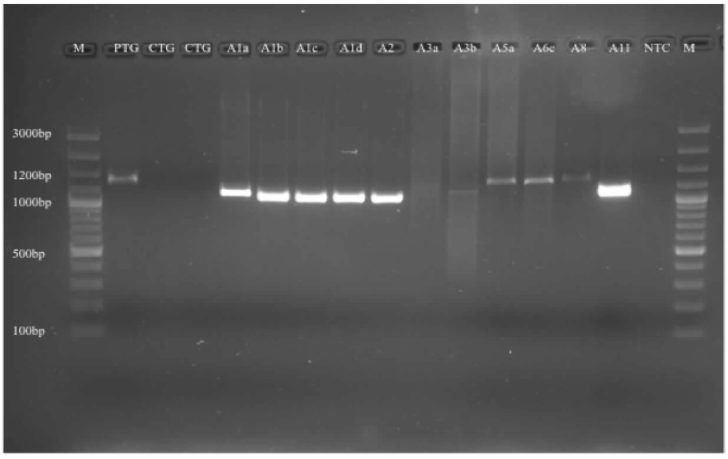
Notes: Phylogenetic relationship (Based on fifteen microsatellite marker methodology) among identified genotypes from infected animal (n=11) samples and known reference *Toxoplasma gondii* isolates as inferred by neighbor-joining analysis, based on distances calculated using Cavalli Sforza and Edwards chord-distance estimator.

4.4.4 Characterization of upstream region of ROP18 gene of Toxoplasma gondii in infected animal tissues

In order to gather preliminary information on the phenotypes of the *T. gondii* genotypes from the infected animal tissues, we investigated the upstream region of the *ROP18* gene, which has been implicated as a virulence factor in *T. gondii*. We PCR amplified a region upstream of the gene using a published set of primers, the PCR products were then sequenced and sequence alignment was done through a BLAST™ search to determine if the PCR product sequences obtained matched any known *ROP* gene sequences for *T. gondii* deposited in the database.

The samples A1003a and A1003b did not amplify in the PCR reaction implying that they may not have the target gene sequence upstream of the *ROP18* gene, whilst the samples A1001a, A1001b, A1001c, A1001d, A1002, A1005a, A1006c, A1008 and A1011 were amplified implying the presence of the target gene sequence (Figure 4.8).

The sequence alignment analysis demonstrated that the sequences for PCR products for samples (A1001a, A1001b, A1001d, A1002, A1005a, A1008 and A1011) had high homology with the *T. gondii* Type II (ME49 strain) *ROP18* gene sequence (Figures 4.9 to 4.15), whilst the sequences for samples A1001c and A1006c did not align well.



M-100 base pair plus DNA molecular marker (Thermo Scientific), PTG- Type II reference strain, CTG- Type III reference strain, A1001a to A1011– Infected animal tissue samples, NTC- no template control

Figure 4.8 DNA fragments from PCR analysis of upstream region of Toxoplasma gondii ROP18 gene from Toxoplasma gondii infected animal tissues demonstrating a 1200 base pair fragment implying the lack of a UPS region upstream of the gene.

Figures 4.9 to 4.15 below show BLAST™ search results of *ROP18* gene sequences from sequencing of PCR product from PCR analysis of upstream region of *ROP18* gene of *Toxoplasma gondii* infected animal tissues, depicting extents of sequence homology to reference match Toxoplasma gondii ME 49 (Type II) *ROP18* gene in BLAST™ database.

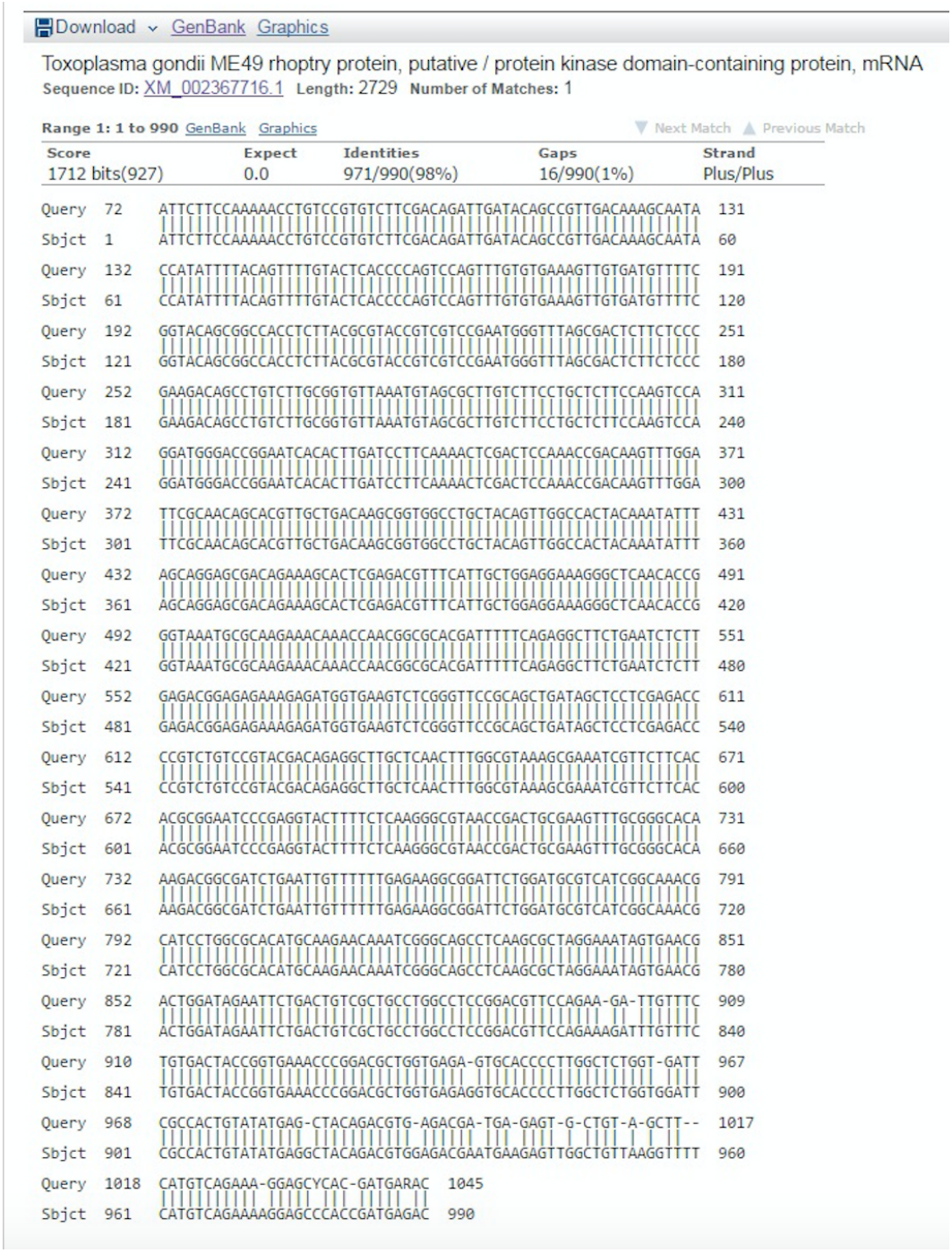
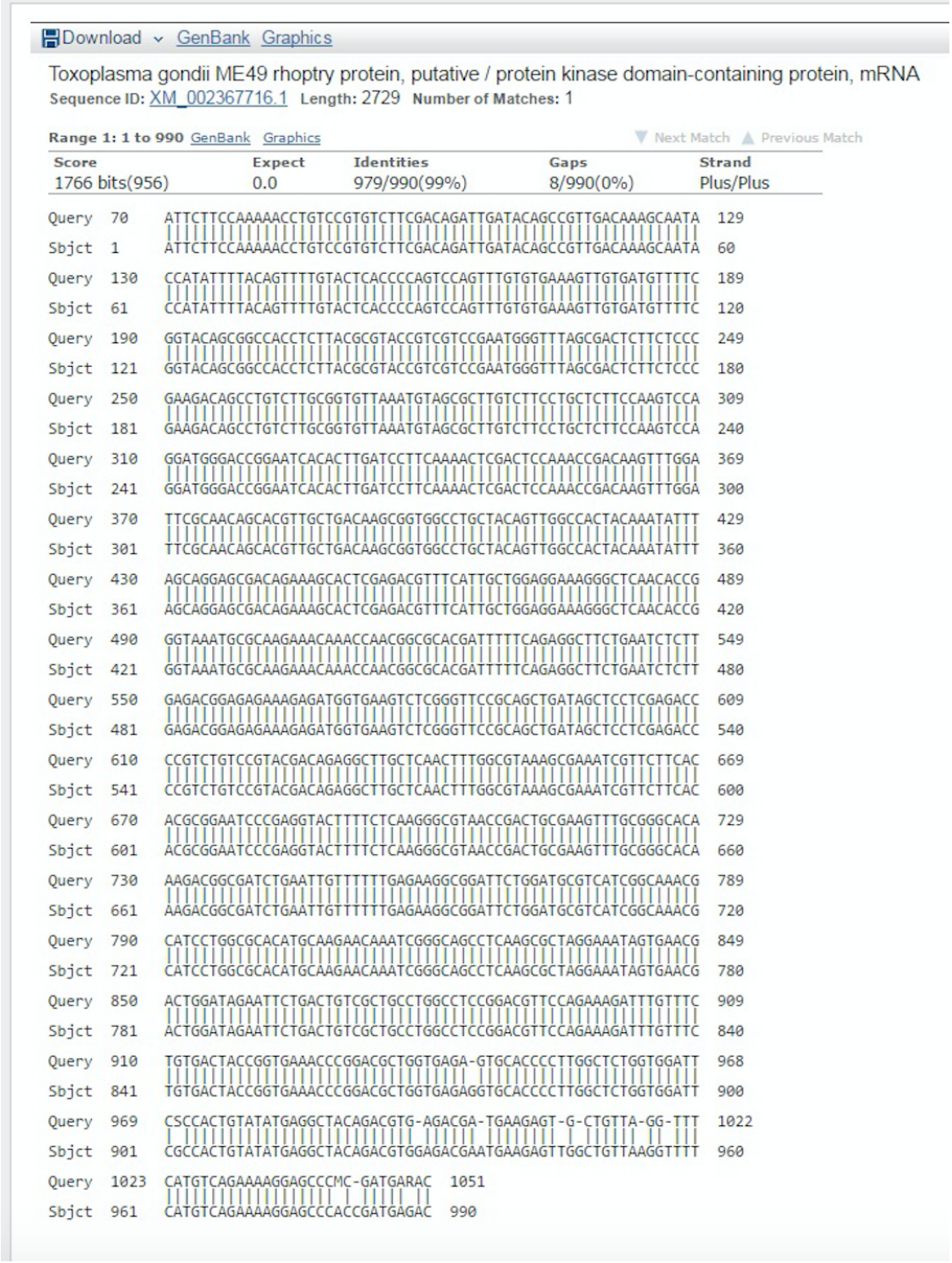


Figure 4.9 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of ROP18 gene of animal sample A1001a, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence



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Toxoplasma gondii ME49 rhoptry protein, putative / protein kinase domain-containing protein, mRNA

Sequence ID: XM_002367716.1 Length: 2729 Number of Matches: 1

Range 1: 1 to 990 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1766 bits(956)	0.0	979/990(99%)	8/990(0%)	Plus/Plus

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Figure 4.10 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of *ROP18* gene of animal sample A1001b, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence

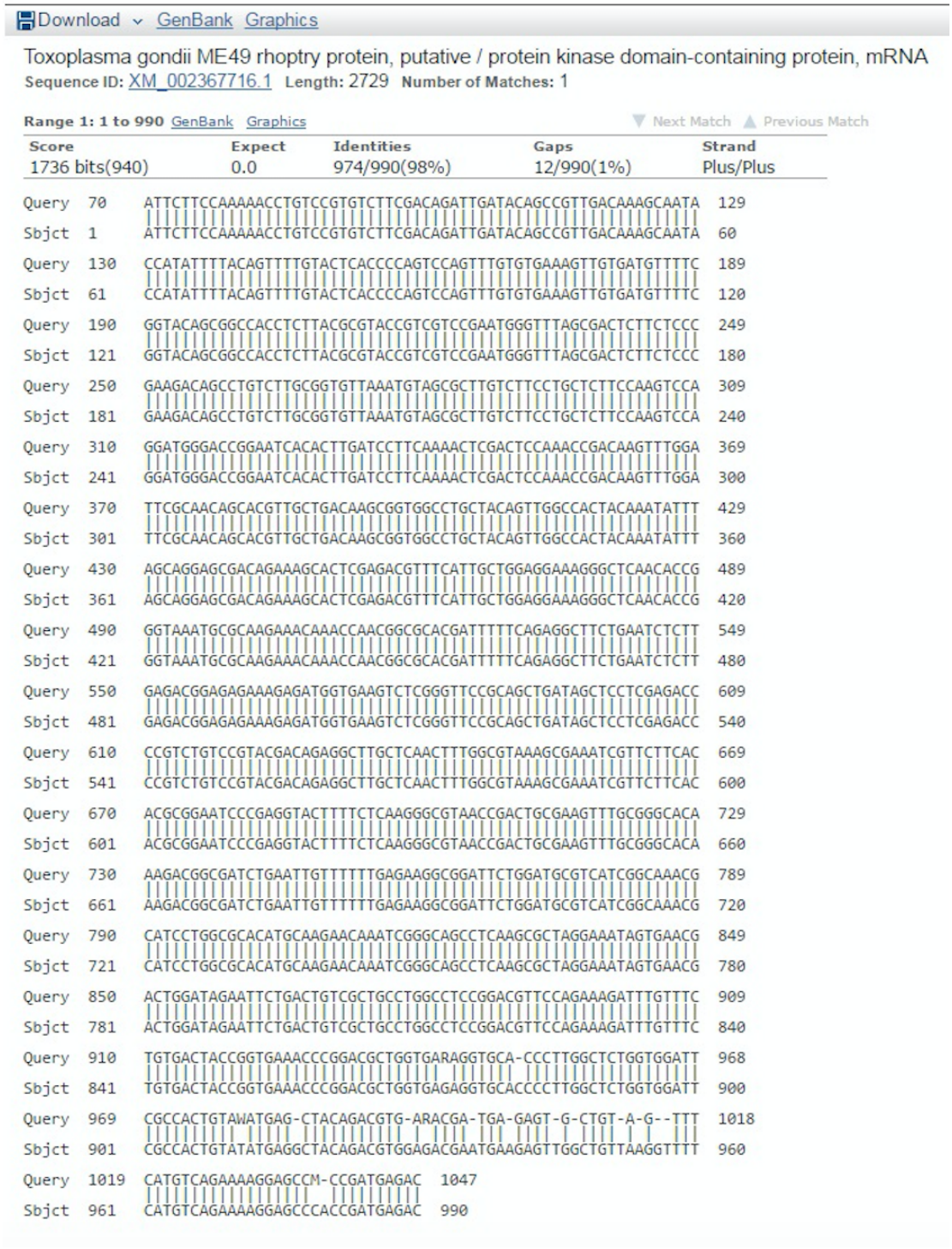


Figure 4.11 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of *ROP18* gene of animal sample A1001d, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence

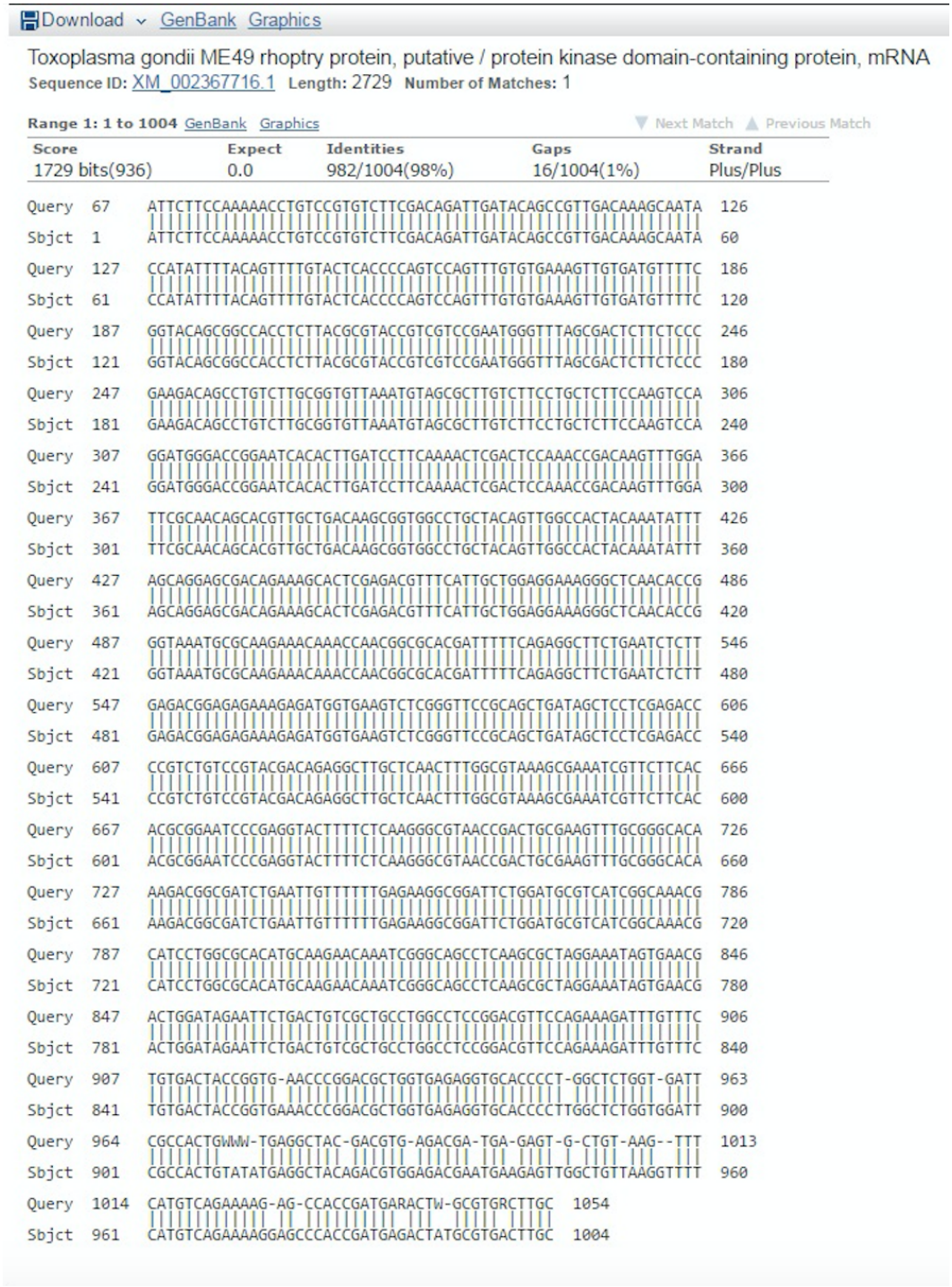
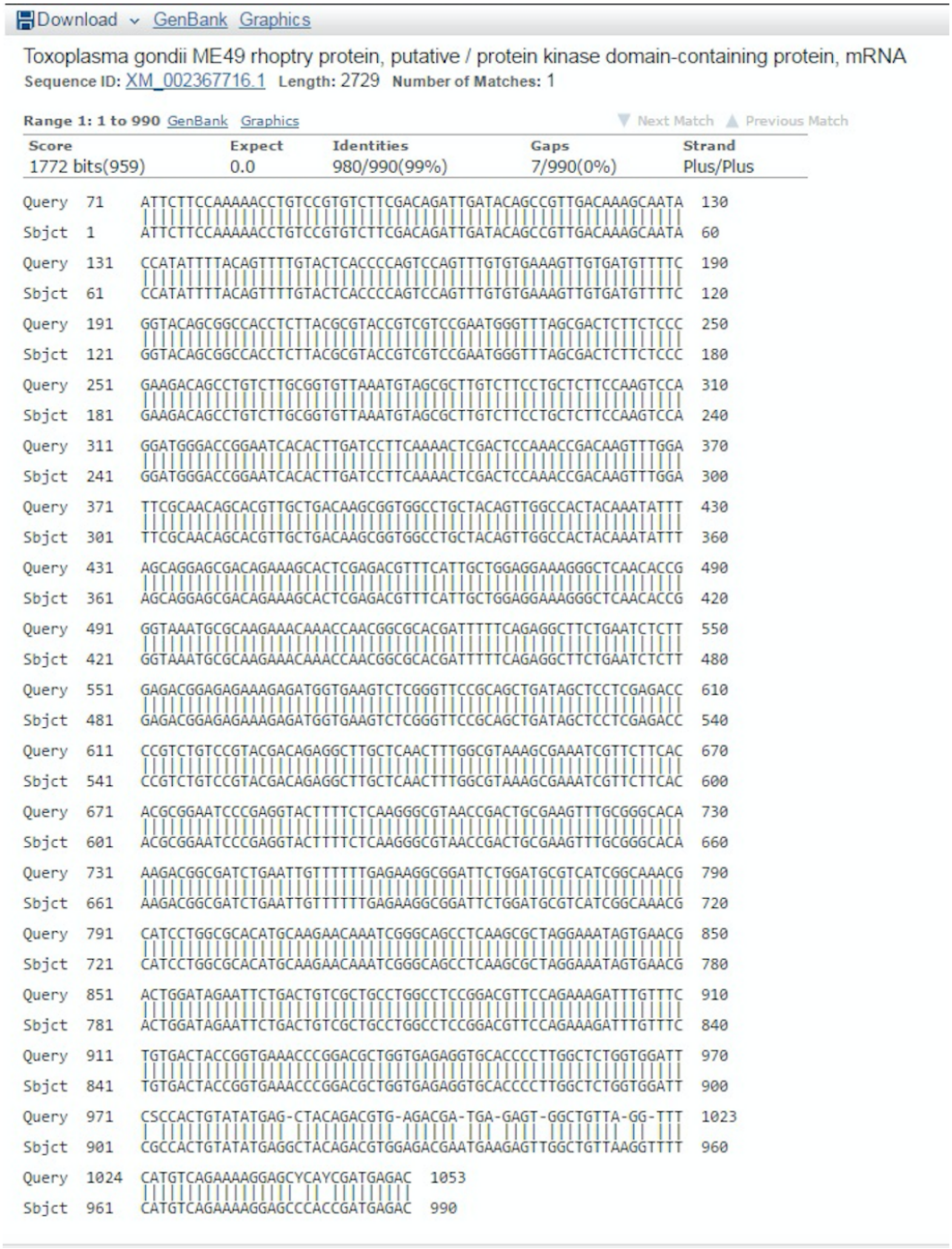


Figure 4.12 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of *ROP18* gene of animal sample A1002, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence



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Toxoplasma gondii ME49 rhoptry protein, putative / protein kinase domain-containing protein, mRNA

Sequence ID: XM_002367716.1 Length: 2729 Number of Matches: 1

Range 1: 1 to 990 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1772 bits(959)	0.0	980/990(99%)	7/990(0%)	Plus/Plus

```
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Figure 4.13 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of *ROP18* gene of animal sample A1005a, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence

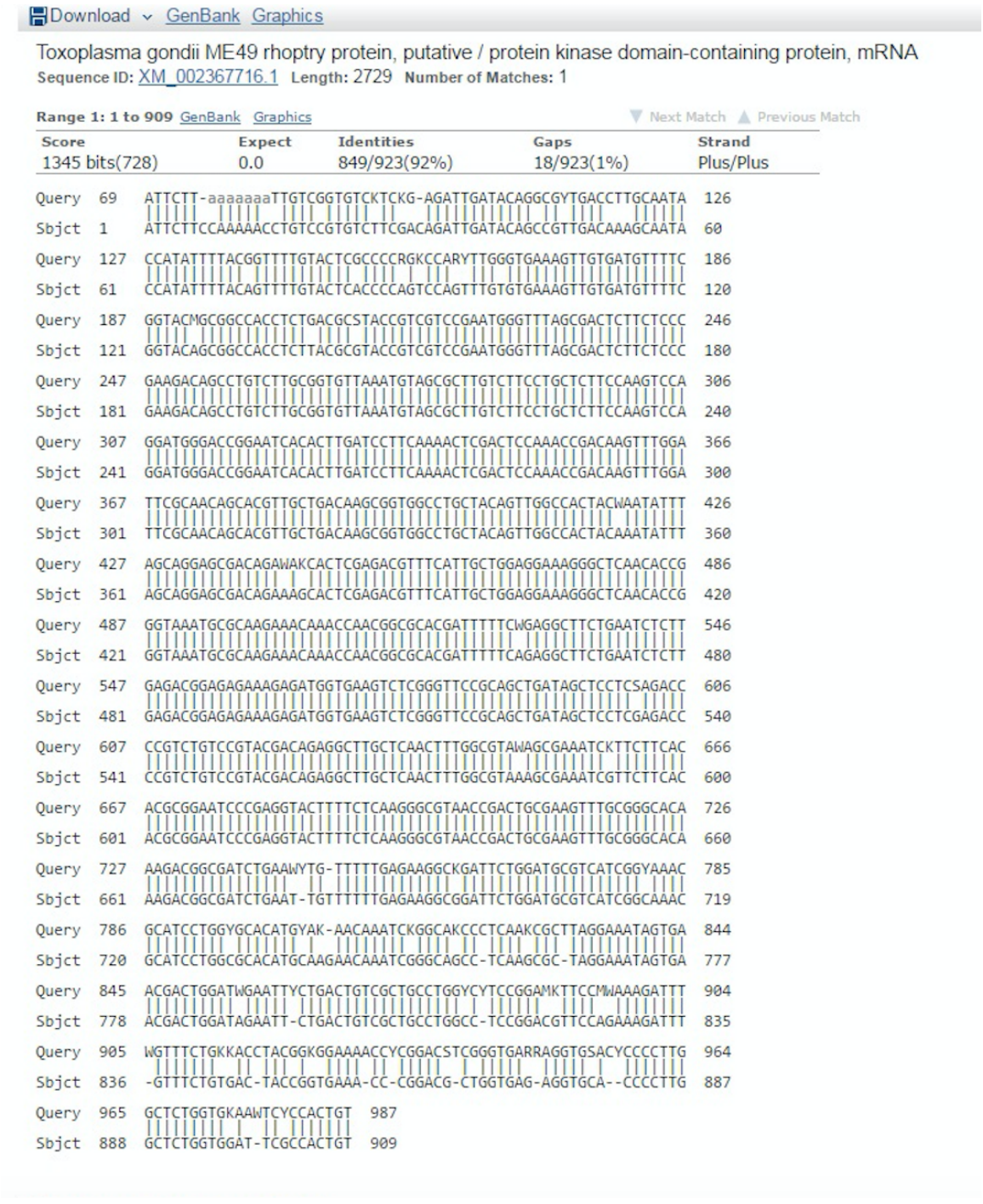
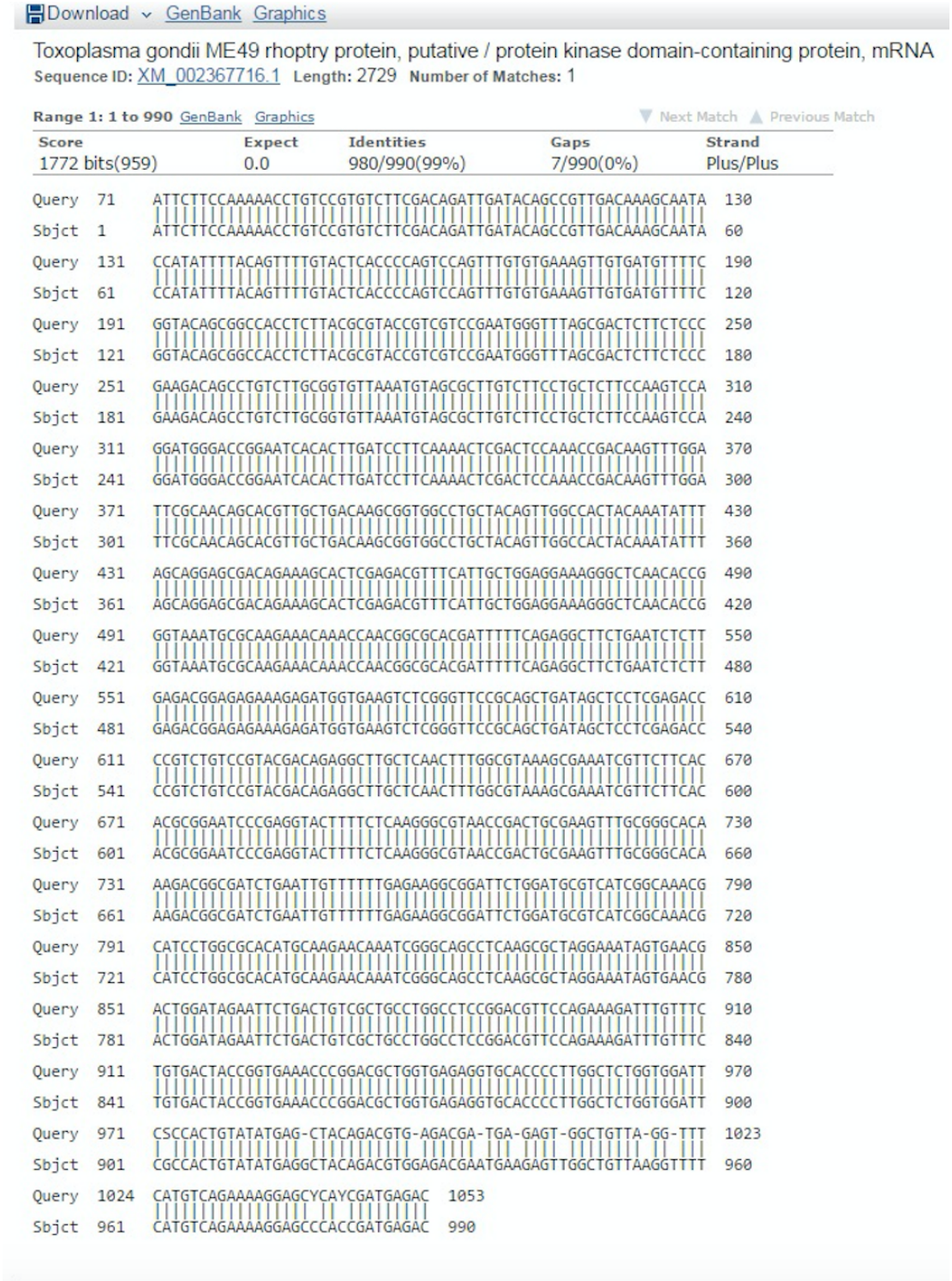


Figure 4.14 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of ROP18 gene of animal sample A1008, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence



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Toxoplasma gondii ME49 rhoptry protein, putative / protein kinase domain-containing protein, mRNA

Sequence ID: XM_002367716.1 Length: 2729 Number of Matches: 1

Range 1: 1 to 990 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1772 bits(959)	0.0	980/990(99%)	7/990(0%)	Plus/Plus

```
Query  71    ATTCTTCCAAAAACCTGTCCGTGTCTTCGACAGATTGATACAGCCGTTGACAAAGCAATA  130
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Sbjct  1     ATTCTTCCAAAAACCTGTCCGTGTCTTCGACAGATTGATACAGCCGTTGACAAAGCAATA  60

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Sbjct  61    CCATATTTTACAGTTTTGTACTCACCCCAGTCCAGTTTGTGTGAAAGTTGTGATGTTTTC  120

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Sbjct  241   GGATGGGACCGGAATCACACTTGATCCTTCAAAACTCGACTCCAAACCGACAAGTTTGGA  300

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Query  611   CCGTCTGTCCGTACGACAGAGGCTTGCTCAACTTTGGCGTAAAGCGAAATCGTTCTTCAC  670
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Sbjct  541   CCGTCTGTCCGTACGACAGAGGCTTGCTCAACTTTGGCGTAAAGCGAAATCGTTCTTCAC  600

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Sbjct  601   ACGCGGAATCCCGAGGTACTTTTCTCAAGGGCGTAACCGACTGCGAAGTTTGCGGGCACA  660

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Figure 4.15 BLAST™ alignment result for DNA fragment sequenced after PCR amplification of upstream region of *ROP18* gene of animal sample A1011, showing alignment with section of *Toxoplasma gondii* Type II ME49 ROP mRNA sequence

4.5 DISCUSSION

4.5.1 Genotypic characterization and phylogenetic analysis

This is the first known report on direct genotyping of *T. gondii* from *T. gondii* infected human and animal host tissues in South Africa. The direct genotyping results show a predominance of the classical Type II strains as well as the presence of atypical Type II strains or Type II variants and a Type III genotype. There was no Type I genotype identified. It is interesting to note that both human and animal samples were predominantly Type II, although some atypical genotypes were observed which included one new genotype from the animal isolates. The untypable samples could possibly be accounted for by the fact that direct genotyping requires high parasite loads in order to recover enough parasite DNA for identification. This can be very difficult to obtain. This is because infections in humans in most cases do not result in high enough parasite loads to allow direct genotyping procedures (Ajzenberg *et al*., 2002; Su *et al*., 2006).

Type II strains have also been commonly associated with human toxoplasmosis in North America and Europe, as opposed to Types I and III (Sibley *et al*., 2009). Whilst *Africa* 1 and *Africa* 2 atypical genotypes have been identified in human toxoplasmosis patients originating from West Africa in a study done in France (Ajzenberg *et al*., 2009), we did not detect any such types in our study. The identification of atypical genotypes as well as Type III genotype in the infected human samples does represent a deviation from the predominant pattern in North America and Europe and implies potential similarities to what is observed in South America, where there is an increased diversity in *T. gondii* strains observed in human (both systemic, congenital and ocular) as well as animal

toxoplasmosis (Khan *et al*., 2006). The atypical genotypes are particularly interesting because (genetically) it implies the presence of a distinct gene pool for *T. gondii* locally in South Africa that is distinct from any other genotypes found to date. Atypical strains are highly pathogenic and have been associated with severe life threatening toxoplasmosis in both humans and animals in the Amazon forest and Suriname and some parts of Europe (Demar *et al*., 2007; Gebremedhin *et al*., 2014). This warrants further clinical investigation in South Africa.

Currently all genotyping studies in Africa performed in animal hosts have reported a predominance of Types II and III and also the existence of atypical genotypes, as well as unique *Africa* 1 and *Africa* 2 genotypes. Most of these genotyping studies used chickens, goats and sheep as infected animal hosts, with just one report from a cat. In this study we used infected tissues from kittens, marmosets and squirrel monkeys, which were being bred in captivity, and these tissues were genotyped via microsatellite methodology (Mercier *et al*., 2010). The observation of similar predominance of Type II strains as for other studies in Africa as well as the observation of atypical Type II variant strains causing both human and animal infection suggests a situation where humans and animals live in close proximity and therefore disease causing pathogens are easily able to move from one host to the other thereby ensuring their long term survival.

The clustering together of the atypical strains A1003b and A1003a from animal samples as well as the atypical strains from human samples H1008 and H1009 is very interesting phenomenon to note and does imply that these strains may be closely related and this also goes to support the theory that the genotypes are probably able to cause disease in both human and animals and are not host specific. They cluster with atypical control strains

MAS and also TgCatBr5 from Brazil, which are known to be Type II variants and sometimes known to be highly virulent (Su *et al*., 2010). The genotype H1011 typed from eye fluid is observed to cluster together with the clonal Type III strain CTG, which is known to be an avirulent strain that causes human infections. Currently it is not clear whether the strains are partial towards any specific hosts and this is an area of *T. gondii* research that is becoming very intensive as it is thought that an understanding of the strain to host relationship will aid in drug development initiatives. From our study we propose, albeit conservatively, that the population structure of *T. gondii* in the Cape Town area investigated can be likened to that in parts of Africa and Southern America with the presence of a variety of atypical strains as well as the traditional clonal strains and this is different from what is known to exist in Europe oor North America where the existence of atypical strains is rare. We also identify the limitations of the study in that the sample size was small (17 human and 11 animal samples) and hence conclusions must be very conservative. We also propose that further studies with larger samples sizes and in different host species should be done in order to understand the strains of *T. gondii* causing infection in human and animals in Cape Town and South Africa as well as the population structure of *T. gondii* in these areas.

4.5.2 Phenotypic characterization via *ROP18* analysis

The samples A1003a and A1003b did not give PCR products and this could be because they are not of the *T. gondii* clonal Types I or II genotypes but could indeed be atypical genotypes as predicted by the genotyping results. Interestingly no single nucleotide polymorphisms were observed in matching all the sequences to the selected references in the BlAST™ database, and this could be explained by the fact that the *ROP* gene family

of *T. gondii* is known to be highly conserved with minimum variations, if any, over time (Behnke *et al*., 2012). All the remaining animal samples analyzed by PCR showed a presence of the approximately 1200 base pair PCR gel fragment, which implied the lack of the approximately 2 kilobase pair insert (UPS region) upstream of the *ROP18* gene coding region. It has been reported that the absence of this UPS region is unique to *T. gondii* strains belonging to the clonal Type I and II lineages whilst its presence is specific to members of the clonal Type III lineage. This differentiating factor has not been proven for clonal variant or atypical strains. Previous research also demonstrated that this presence or absence of this UPS region is directly related to the virulence phenotype exhibited by these clonal strain types in the mouse model, where clonal strains lacking the insert were medium to highly virulent, whilst clonal strains bearing the insert were avirulent (Khan *et al*., 2009). Furthermore clonal strains that lacked the insert were shown to have a high level of expression of the *ROP18* gene, whilst those bearing the insert expressed low levels of *ROP18* gene in qRT-PCR and expression quantitative trait locus studies (Boyle *et al*., 2008; Khan *et al*., 2009).

Sequence alignment using the BLAST™ tool for the sequenced PCR products for individual animal samples that demonstrated the absence of the insert, also demonstrated high homology with the *ROP18* gene sequences of *T. gondii* Type II strain ME 49 deposited in the NCBI database as shown in figures 4.9 to 4.15 for the samples A1001a, A1001b, A1001d, A1002, A1005a, A1008 and A1011, whilst samples A1001c and A1006c had poor sequences and did not align to a reportable extent. This we believe could be attributed to poor sequencing or poor PCR product clean up.

The *T. gondii* strains causing infection in the animal hosts specifically (A1001a, A1001b, A1001d, A1002, A1005a, A1008 and A1011) in this study can therefore be said to have mouse virulent phenotypes similar to other *T. gondii* clonal Type II strains in the study reported by Khan *et al.,* (2009) apart from the samples A1001c and A1006c which were inconclusive at the gene sequence level and also the samples A1003a and A1003b which have been shown to have atypical genotypes and therefore require further characterization in order to draw any conclusions on their genotype and mouse model phenotype.

The inference from the phenotypic study are at best only basic and preliminary and must be rigorously tested through gene expression studies before any conclusive statements can be made. Currently what is stated here is suggestive and not conclusive as the analysis holds fast for only the clonal strain Types I, II and III and therefore any inferences are only implied or suggestive and not conclusive.

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CHAPTER 5 ATTEMPTED RECOVERY OF VIABLE TOXOPLASMA GONDII OOCYSTS AND TACHYZOITES FROM CONTAMINATED WATER EFFLUENT AND INFECTED ANIMAL TISSUES THROUGH BIOASSAY IN MICE

5.1 INTRODUCTION

Toxoplasma gondii oocysts, tissue cysts and tachyzoites are the most important infective stages of the organism, and are the main forms of the pathogen that is transmitted from one host to another through the consumption of raw or undercooked meat containing tissue cysts or tachyzoites, and through ingestion of oocysts from contaminated environmental sources such as water, fruits, soil and vegetables (Dubey, 1970). In locations where consumption of meat is non-existent, *T. gondii* oocysts are probably the only known means by which the pathogen can be transmitted. Felids are known to shed *T. gondii* oocysts into the environment within a short time period of between one to three weeks and within that period they are able to shed large quantities of environmentally resistant oocysts, (from10^7 to 10^8 oocysts) per felid. Once shed into the environment, the oocysts are spread through actions of the wind, water, man, animals, manure, earthworms and arthropods and are then able to contaminate various bodies including surface water, soils, feeds, fruits, vegetables and various other surfaces (Dubey *et al*., 1970; Dubey and Frenkel, 1976). Sporulated *T. gondii* oocysts have been shown to remain infective for prolonged periods of time in water bodies. They are also able to remain infective for over fifty-four months at 4^0C and in experimental soils they have been shown to remain infective for over eighteen months under various temperature conditions, although increasing temperature has been shown to decrease survival times for *T. gondii* oocysts.

Sporulated oocysts have been known to survive under freezing conditions including constant freezing for twenty eight days at -21^{0}C (Lindsay *et al*., 1991).

Oocyst transmitted outbreaks of toxoplasmosis in humans through contamination of soil or water bodies have been reported in the USA (Teutsch *et al*., 1979; Stagno *et al*., 1980), in Panama (Benenson *et al*., 1982), in Brazil (Coutinho *et al*., 1982) and in Canada (Bowie *et al*., 1997) even though direct evidence of the contamination by oocyst was never conclusively proven, except in the Brazilian outbreak. These highlight the complexities involved in the isolation of oocysts from the environment and the lack thereof of effective methods for the isolation of *T. gondii* oocysts from the environment.

Transmission of *T. gondii* through consumption of contaminated meat products has been reported in different countries globally. Kean *et al*., (1969), Masur et al. (1978) and Cook et al. (2000) reported outbreaks of toxoplasmosis in different populations in the USA, whilst Choi et al. (1997), reported an outbreak of toxoplasmosis from consumption of contaminated meat in Korea, Sacks et al. (1983) and Ross et al. (2001) reported a presumed outbreak of clinical toxoplasmosis with associated ocular disease from the consumption of contaminated venison in the USA and Canada. Iin another instance, horsemeat originating from Canada and Brazil was linked to an outbreak of acute toxoplasmosis in France (Pomares et al. 2011). Although these reported outbreaks have been sporadic and far and few between, the fact that transmission of the pathogen did actually occur via either suspected contamination of the environment by oocysts or consumption of infected tissues emphasizes the epidemiological importance of the oocysts, tissue cysts and tachyzoites of *T. gondii* within any health system.

Detection of *T. gondii* tissue cysts in animal tissues relies on the bioassay of extracts from infected tissues in either mice or cats or other vulnerable hosts of *T. gondii*. Since the bioassay of infected tissues in cats is extremely expensive, laborious and time consuming, the bioassay of infected tissues in mice is the most commonly used technique currently (Robert-Gangneux and Dardé, 2012). *Toxoplasma gondii* propagation in mice has been used extensively to recover viable oocysts and tachyzoites from various infected sources. It is an accepted method of choice due to the fact that mice are extremely sensitive to *T. gondii* infections and are able to develop parasites in tissues cysts or ascites in days or weeks post infection depending on how virulent the infecting strain is and the dosage of the infection (Miller *et al.*,1972; Dumètre and Dardé, 2003; Hohweyer *et al*., 2013).

For this study, water effluent was collected along one coastal wetland of Cape Town and infected animal tissues were collected from the Western Cape Provincial Vetinary Services for mouse inoculation studies. Along the wetlands where the effluents were collected, the water drains into the sea at Kommetjie. Tthere are several marine mammals that are very sensitive to *T. gondii* infection, one of the most well known are otters. In the USA there are several cases of otters dying from *T. gondii* infection especially along the Californian coastline where rivers flow into the sea (Conrad *et al.*, 2005). In the Western Cape of South Africa, the Cape clawless otter (*Aonyx capensis*) was until very recently found in the sea at Kommetjie, where the wetland drains. Over a short period, these otters disappeared whilst other known otter sites appear to be doing well. No explanation has been forthcoming as yet for the disappearance of otters at this one site and no changes to the environment have been noted. However, upstream in the wetlands is an ever-growing informal settlement, which now has a large human and domestic

animal population. Since otters are vulnerable to a *T. gondii* infection we hypothesized that infective stages of *T. gondii* occurs in the wetlands that drain out to sea at Kommetjie and may affect otter health. Therefore we wished to assay for *T. gondii* in the drainage water and the otters. Live trapping of otters and collection of samples was not allowed, hence samples from dead otters collected opportunistically over some years were used for this study. Most of the animals found were victims of road accidents. We also hypothesized that the strains of *T. gondii* causing infection in the Cape Town area are genetically different from those found in other parts of the world and we therefore used *T. gondii* infected tissues from dead hosts for bioassay studies in an attempt to isolate the infective stages in order to genetically characterize them.

5.2 Study aims and objectives

The aims of the study were therefore to,

1. Investigate the presence or absence of *T. gondii* oocysts in runoff water collected from selected sites along the drainage pathway of water effluent from Masiphumelele, which is a low socio-economic settlement in Cape Town
2. Isolate viable *T. gondii* tachyzoites from potentially contaminated animal tissues collected from various locations in Cape Town through bioassay in mice.

The objectives of the study were,

1. To detect the presence or absence of *T. gondii* oocysts in collected effluent water through the use of centrifugation and concentration techniques followed by bioassay in mice

2. To inject laboratory mice intra-peritoneally with *T. gondii* infected animal tissues and monitor mice for any signs of infection
3. To harvest viable *T. gondii* tachyzoites from the infected mice and to confirm presence of *T. gondii* infection in the mice through PCR methodology
4. To store the isolated viable *T. gondii* tachyzoites through cryopreservation in liquid nitrogen

5.3 Ethical clearance

Ethical clearance was obtained from the University of Stellenbosch animal research ethics committee (Appendix 1).

5.4 MATERIALS AND METHODOLOGY

5.4.1 Attempted recovery of viable *Toxoplasma gondii* oocysts in contaminated water through mouse bioassay

Study area

Located between Noordhoek, Capri Village and Kommetjie, along the Cape Peninsula in Cape Town, Masiphumelele (Xhosa for 'we will succeed') is a township with a population of approximately 30,000 inhabitants. The hydrology of the area is such that water effluent from the township drains into furrows on the outskirts of the township and is carried into the Wildevoelvlei wastewater treatment plant via the Wildevoelvlei wetlands. In high flow periods the treated wastewater from the plant is discharged into

the sea via Klein Slangkop. Samples were also collected from the wider wetland area in the district, as shown.

Table 5.1 Longitude and latitude co-ordinates of locations from which water effluent was collected.

Coordinates for sampling (Kommetjie/Noordhoek area)

Number on map	Latitude (S)	Longitude (E)
1	34.123388	18.380727
2	34.126555	18.371809
3	34.128117	18.365917
4	34.132536	18.361085
5	34.135249	18.348403
6	34.132573	18.344902
7	34.113446	18.376567
8	34.113341	18.368992
9	34.108845	18.363324

Note: Effluent water was collected over a one -year period from drainage points along the Kommetjie and Noordhoek wetlands in Cape Town at the listed coordinates.

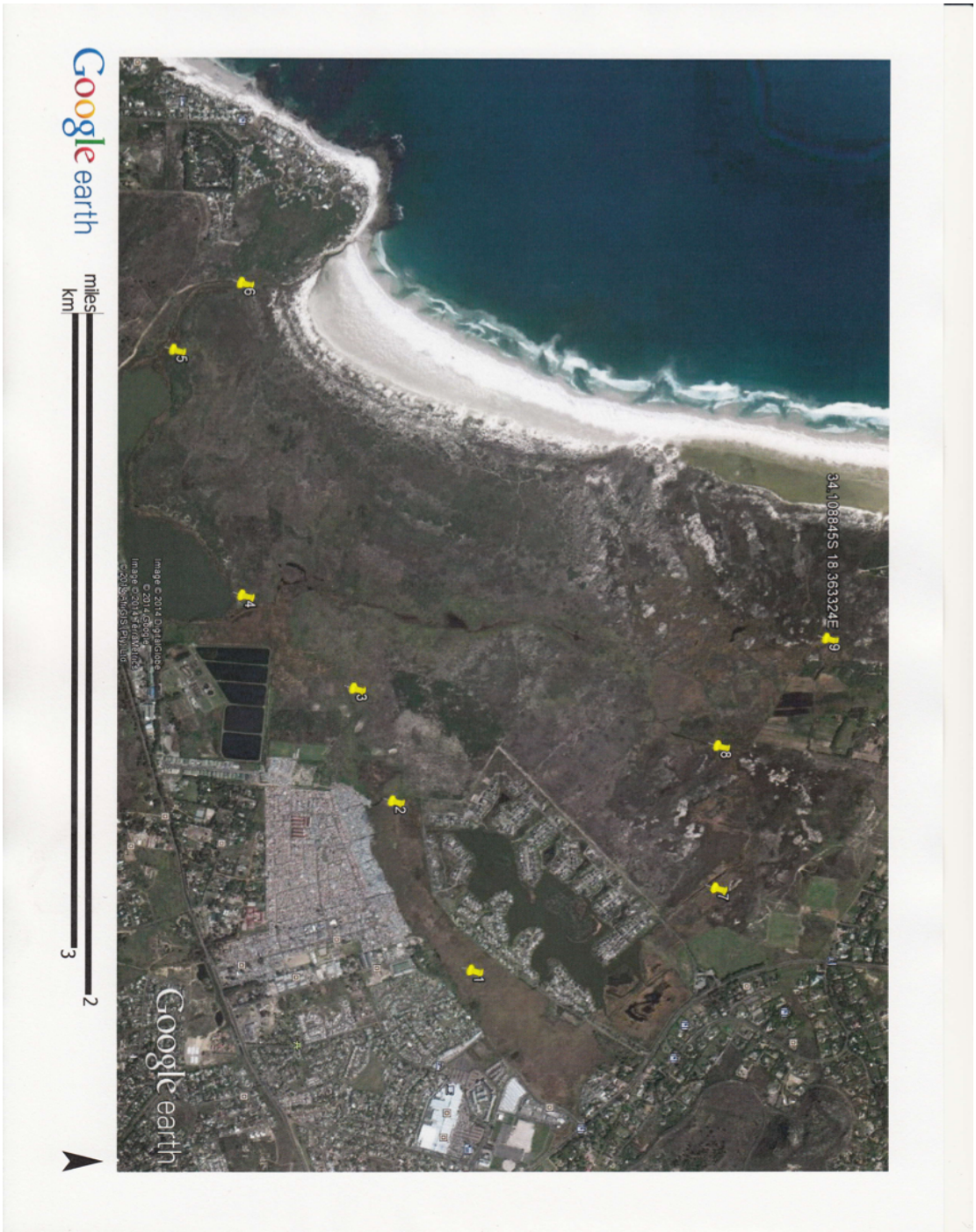

Figure 5.1 Google maps image, showing locations from which water effluent was collected. Otters used to be regularly seen at point 6. (P van Helden, personal sightings and information gathered from SANParks rangers).

Methodology

Water effluent samples (200ml per location) were collected in 50ml BD Falcon™ conical tubes (BD), and stored at 4^0C for six months before being analysed.

Centrifugation and concentration

Water effluent samples were processed for inoculation into Balb/c laboratory mice (supplied and maintained by the animal research facility at Stellenbosch University) according to a protocol adapted from (Dumètre and Dardé 2003) and (Kourenti *et al.*, 2003). The protocol was adapted to suit our particular condition see figure 5.2 for flow diagram of procedure.

The stored water effluent (200 ml) at 4^oC, was placed in the laboratory at room temperature for 7 to 10 days, on the tenth day the samples were vortexed for 30 seconds each and filtered twice through sterile gauze sponges 40 μm pore size (Lasec) into 50 ml Falcon centrifuge tubes (Eppendorf). The filtrate in 50 ml falcon tubes (Eppendorf) was then centrifuged at 4000x *g* for 10 minutes at 4^oC in an Eppendorf 5810 R centrifuge (Eppendorf) and the supernatant discarded. The resulting pellet was re-suspended in 2 ml of phosphate buffered saline pH 7.4 (Sigma) containing 10 μg/ml of Gentamycin Sulphate (Merck) in a 2 ml Eppendorf collection tube and mixed briefly by vortexing. The resulting suspension (approximately 2 ml) was then kept at room temperature for immediate inoculation into the Balb/c mice (Figure 5.2).

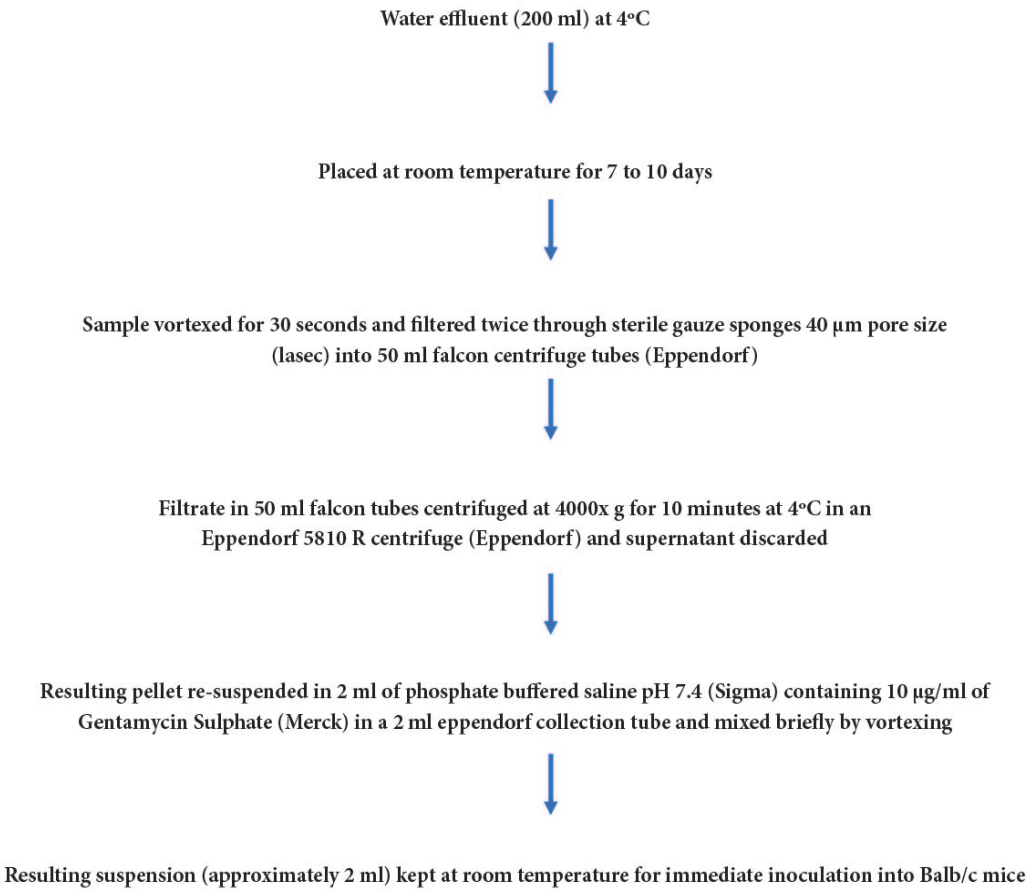

Figure 5.2 Flow chart of water effluent processing methodology

Notes: Collected water effluent 200 ml was allowed to come to room temperature and then processed through centrifugation and concentration and used as inoculation material to inoculate Balb/c mice

Mouse inoculation procedure

Six female outbred Balb/c mice at six weeks of age were used for the bioassay. Mice were supplied by and maintained at the animal research facility at Stellenbosch University, Tygerberg campus throughout the experiment. The mice were kept in cages in a biosafety level two (BSL-2) facility with free access to food and water throughout the experiment.

Table 5.2 Mice inoculation chart

Mouse Identification number	Inoculum Injected
TG-M-1	Effluent suspension 1+2
TG-M-2	Effluent suspension 3+4
TG-M-3	Effluent suspension 5+6
TG-M-4	Effluent suspension 7+8
TG-M-5	Effluent suspension 9
TG-M-6 (Negative control)	Phospate buffered saline

Notes: Table shows the inoculation that was injected into each of the Balb/c mice on the study, there was no positive control, a negative control mouse was inoculated with 2 ml of phosphate buffered saline.

The mice were housed in pairs throughout the experiment in cages. Before the inoculation with infected tissue or material, the mice were pretreated with 15 μg/ml of Dexamethasone phosphate (Sigma) in their drinking water for three days in order to suppress their immune system. The application of treated water was maintained throughout the course of the experiment. Before injection into the mice, the sample suspension in phosphate buffered saline pH 7.4 (Sigma) was vortexed for 10 seconds to mix properly, mice were inoculated according to the key shown in table 5.2. One ml of each sample was then injected intra-peritoneally into each of the six Balb/c female mice using a 21-gauge needle syringe (Lasec). Mice were examined daily for any

abnormalities or signs of infection including rough fur and sluggishness or lethargy, and observations were recorded on a chart. Normal signs were indicated by 'N' and any abnormalities if any were indicated by and 'A'. At twenty- one (21) days post infection, mice were humanely euthanized via cervical dislocation and 5ml of phosphate buffered saline (Sigma) injected into their peritoneal cavities. The contents of the abdomen were then mixed briefly and the peritoneal lavage collected into 15ml Falcon™ (Eppendorf) centrifuge tubes. The tube was then centrifuged at 1500 rpm for 10 minutes in an Eppendorf 5810 R centrifuge (Eppendorf), the supernatant was discarded and the pellet re-suspended in 2 ml of 50% fetal bovine serum in Dulbecco's modified eagle medium (Sigma) and stored at -80^{0}C until further analysis. The euthanized mice were then dissected and the heart, lung and brain tissues harvested and stored at -80^{0}C for further analysis. The mouse carcasses were disinfected with 10% bleach (JIK, SA) and placed in a 4^{0}C cold room for subsequent incineration. All processes were carried out in a biosafety level two facility under strict standard microbiological conditions.

DNA extraction from harvested mouse tissue (heart)

DNA extraction was done using the ZR genomic DNA ™ –tissue miniprep kit (Zymo research corporation), according to procedure described in chapter four materials and methods section 4.3.1.

***T. gondii* diagnostic PCR**

DNA extracts were analyzed for the presence of *T. gondii* DNA by PCR amplification of the 35 fold repeat *B1* gene, using an adaptation of the (Burg *et al*. 1989) protocol.

The starting material was 10 µl of crude DNA extract from the dissected mice tissues.

The protocol is as described previously in chapter 2 methodology section 2.3.2.

5.4.2 Attempted recovery of viable *Toxoplasma gondii* tachyzoites from *Toxoplasma gondii* infected animal tissues through bioassay in mice

Materials

Animal samples consisted of tissues sections from postmortem examinations of *T. gondii* infected animals from the Western Cape provincial veterinary services in Stellenbosch, Western Cape, collected from January 2013 to December 2014 and tissue sections from dissected otters from various locations in the Western Cape. Otters were free- living animals from Table Mountain national park that had mostly died from traffic accidents.

Table 5.3 Animal samples for mouse bioassay study

Specimen ID	Clinical Data	Specimen Type
A1001a	Disseminated toxoplasmosis	Monkey Brain
A1001b	Disseminated toxoplasmosis	Monkey Liver
A1001c	Disseminated toxoplasmosis	Monkey Intestine
A1001d	Disseminated toxoplasmosis	Monkey Lung
A1002	Disseminated toxoplasmosis	Kitten Lung
A1003a	Disseminated toxoplasmosis	Kitten Intestine
A1003b	Disseminated toxoplasmosis	Kitten Lung
A1005	Disseminated toxoplasmosis	Monkey Lung
A1006c	Disseminated toxoplasmosis	Monkey Liver
A1008	Disseminated toxoplasmosis	Marmoset Intestine
A1011	Disseminated toxoplasmosis	Marmoset Liver
OT 1-4	None	Otter tongue
OT 5-9	None	Otter tongue
OT 1-6	None	Otter heart
OT 1-4	None	Otter brain
OT 1-6	None	Otter muscle

Notes: Table depicts the list of animal tissues samples, which were used for the mice bioassay in the attempt to recover live *Toxoplasma gondii* tachyzoites

All samples were screened diagnostically for the presence of *T. gondii* DNA PCR according to the protocol described previously in chapter two section 2.3.2 and positive samples A1001a to A1011 and all negative otter samples (2 x OT 1-4, OT 5-9 and 2 x OT 1-6) were included in the mouse bioassay study.

Methodology

Sixteen female Balb/c mice of six weeks of age were housed in pairs in eight cages and labeled accordingly. The mice were kept in cages in a biosafety level two (BSL-2) facility with free access to food and water throughout the experiement. The mice were pre-treated for three days before infection with *T. gondii* infected tissue with 15 µg/ml-dexamethasone phosphate (Sigma) in their drinking water. The treatment was continued throughout the course of the study. The infected animal tissues were removed from -80^{0}C storage and placed at -20^{0}C for two days. The tissues were then removed from -20^{0}C storage and slowly allowed to thaw at room temperature. Five grams (5 g) of thawed tissue was weighed on a Sartorius BP 211D scale (Sartorius AG). The weighed tissue was then cut into pieces and placed in a 50 ml falcon tube (BD). Saline 0.85% NaCl (10 ml) (Sigma) was then added to the cut tissue and this was then homogenized with a hand held tissue homogenizer. Freshly prepared, pre-warmed (37°C) acid pepsin solution (15 ml), was then added to the homogenate and the mixture incubated at 37°C on an accublock^{TM} digital dry bath (Labnet International Inc) for 40-60 minutes with periodic shaking by hand. The homogenate was then filtered through two layers of sterile gauze (Lasec), into a 50-ml Falcon tube (BD) and centrifuged at 1500 rpm for 10 minutes on an Eppendorf 5810 R centrifuge (Eppendorf). The supernatant was then discarded. The resulting pellet was then re-suspended in 10 ml of phosphate buffered saline pH 7.4 (Sigma).

The suspension was then mixed briefly by hand and centrifuged at 1500 rpm in an Eppendorf 5810 R centrifuge (Eppendorf) for 10 minutes and the supernatant discarded. Phosphate buffered saline (5 ml) (Sigma) containing 10 µg/ml of gentamicin sulphate (Merck) was then added to the pellet and mixed vigorously. One ml of each sample was then injected intra-peritoneally into each of the six Balb/c female mice using a 21-gauge needle (Lasec) attached to a 2 ml syringe (Lasec). Mice were monitored daily for any signs of infection. Mice were humanely euthanized via cervical dislocation twenty- one days post infection.

The contents of the abdomen were then mixed briefly and the peritoneal lavage collected into 15 ml Falcon^{TM} (BD) centrifuge tubes according to the protocol below. Each mouse was euthanized humanely through cervical dislocation and then sprayed with 70% ethanol (Sigma) and mounted on a Styrofoam block on its back, the outer layer of the peritoneum was cut and gently pulled back to expose the inner lining of the peritoneum, using a pair of scissors and forceps, ice- cold phosphate buffered saline (5 ml) (PBS) pH 7.4 (Sigma) was then injected into the peritoneal cavity using a 27 G needle (Lasec) attached to a 5 ml syringe (Lasec). This was done slowly being careful not to puncture any organs after injection, the peritoneum was massaged gently in order to dislodge any attached cells into the PBS solution. A 25 gauge needle attached to a 5 ml syringe (Lasec) was carefully inserted, bevel up, into the peritoneum and the fluid collected whilst moving the tip of the needle gently to avoid clogging by the fat tissue or other organs The collected fluid or cell suspension (6 ml) was deposited into 15 ml Falcon^{TM} (BD) kept on ice after removing the needle from the syringe.

The collected peritoneal lavage in the Falcon tube (BD) was then centrifuged at 1500 rpm for 10 minutes in an Eppendorf 5810 R centrifuge (Eppendorf), the supernatant was discarded and the pellet re-suspended in 2 ml of 50% fetal bovine serum in Dulbecco's modified eagle medium (Sigma). Suspension (aliquot A) (0.5 ml) was mixed with 0.5 ml of a 20% DMSO+ DMEM solution and transferred into a 1.2 ml cryo-vial, and stored at -80^{0}C overnight. The cryo-vial was then transferred to (liquid nitrogen for long- term storage. The remaining suspension (1.5 ml) (aliquot B) was processed as follows and used for PCR analysis.

The suspension was spun down briefly and the supernatant discarded. The pellet was washed with 1 ml phosphate buffered saline pH 7.4 (Sigma) and re-suspended in 0.5 ml phosphate buffered saline pH 7.4 (Sigma). Proteinase K 10 mg/ml (Sigma) (25 μl) was added to the sample and incubated on an accublock^{TM} digital dry bath (Labnet International Inc) incubator at 55^{0}C for 1 hour (until the sample became clear). The sample was then incubated for a further 15 minutes at 95^{0}C to inactivate the proteinase K and the cell lysates were stored at -20^{0}C for further PCR analysis. The euthanized mice were then dissected and the heart, lung and brain tissues harvested, and stored at -80^{0}C for further analysis. The mouse carcasses were then disinfected with 10% bleach (JIK, SA), placed in sealed disposable plastic bags and then placed in a 4^{0}C cold room for subsequent incineration. All processes were carried out in a biosafety level two facility under strict standard microbiological conditions.

T. gondii diagnostic PCR of harvested mice peritoneal lavage

For peritoneal lavage PCR analysis, the stored cell lysates were thawed and analyzed for the presence of *T. gondii* DNA via the same protocol described in chapter two section 2.3.2.

5.5 RESULTS

5.5.1 Attempted recovery of viable *Toxoplasma gondii* tachyzoites in contaminated water through mouse bioassay

In an attempt to isolate *T. gondii* oocysts from presumably contaminated water effluent, we collected effluent water from different collection points along the Kommetjie (and neighbouring and connected Noordhoek) wetlands (Table 5.1 and Figure 5.1). We then followed an adaptation of the centrifugation and concentration method for oocyst isolation (Figure 5.2) (Dumètre and Dardé 2003; Kourenti *et al.*, 2003). Mice were then infected with concentrate from the centrifugation and concentration procedure and monitored for signs of infection. At the end of a defined infection period, DNA was extracted from tissue sections from the euthanized and dissected mice and *T. gondii* diagnostic PCR targeted at the *B1* gene was performed followed by gel electrophoresis and analysis of the resulting bands. We included a *T. gondii* PCR psotive control that was DNA extract from the *T. gondii* RH strain tachyzoites. We did not detect any positive bands in any of the tissues examined for *T. gondii* DNA (Figure 5.3).

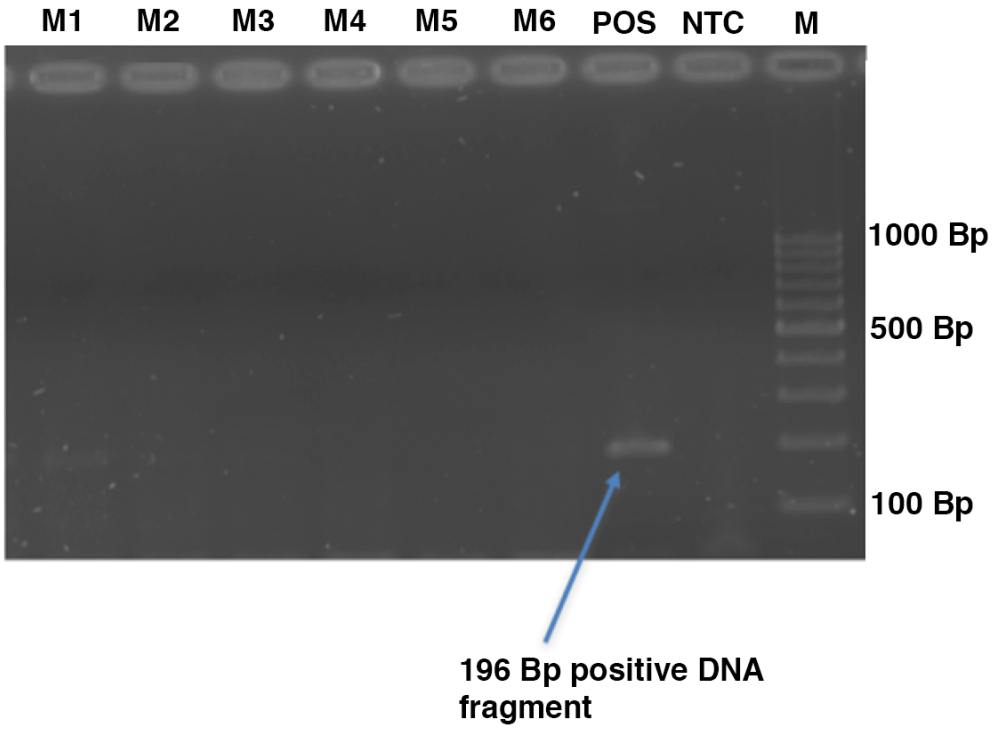

Figure 5.3 Image depicting DNA fragments from gel electrophoresis of Toxoplasma gondii diagnostic PCR of DNA extracted from mouse tissue (M1 –M6 mice samples, POS- Positive control (DNA extract from *Toxoplasma gondii* RH strain), M-100Bp DNA size marker (Roche), NTC- No template control)

5.5.2 Attempted recovery of viable *Toxoplasma gondii* tachyzoites from *Toxoplasma gondii* infected animal tissues through bioassay in mice

In an attempt to recover viable *T. gondii* tachyzoites from PCR confirmed (infected animal samples) as well as PCR negative (otter samples) *T. gondii* infected tissues (table 5.3), we performed a bioassay in Balb/c mice using processed infected and non-infected tissues as starting inoculation material. After a defined period of time, mice were humanely euthanized and tissues harvested through dissection, DNA was extracted from the harvested tissues and *T. gondii* diagnostic PCR targeted at the *B1* gene was then performed. We included *T. gondii* PCR positive control that was DNA extracted from tachyzoites of the RH strain of *T. gondii* in the PCR reaction. On gel electrophoresis and

examination of the gel image (Figure 5.4), none of the mouse and otter samples showed *T. gondii* positive bands.

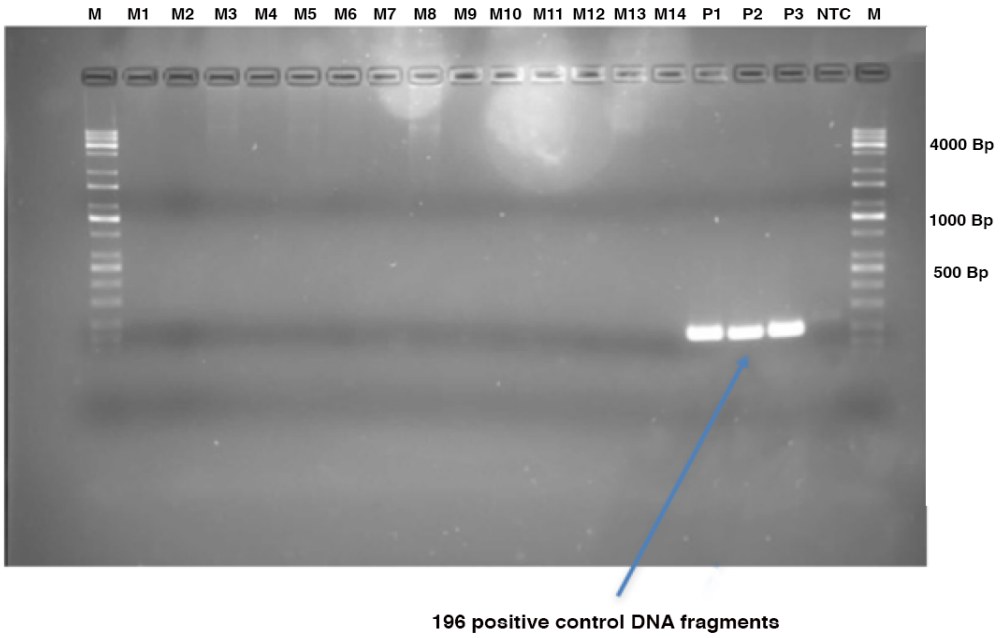

Figure 5.4 Image depicting DNA fragments from gel electrophoresis of *Toxoplasma gondii* diagnostic PCR of DNA extracted from harvested mouse tissues (M1 –M14 mice samples, P1, P2, P3 - Positive controls (Toxoplasma gondii RH, PTG, CTG strain DNA extract), M- universal DNA size marker (Kapa Scientific), NTC- No template control)

5.6 DISCUSSIONS

5.6.1 Attempted recovery of viable *Toxoplasma gondii* tachyzoites from contaminated water through bioassay in mice

None of our water effluent samples analyzed showed the presence of *T. gondii* oocysts in the mouse assay. Unfortunately we were not able to obtain live oocysts and therefore did not have a positive control mouse. Hence we do not know if the negative mouse result was from experimental problems or because there were no oocysts in the starting material.

Detecting *T. gondii* oocysts in environmental samples is complicated, with a very low success rate including the unavailability of tried, tested and sensitive methods (Dumètre and Dardé, 2003). The highly opportunistic nature of the multiplication method normally used is the bioassay in mice and for environmental detection relies on oocysts shed exclusively by felids. This shedding occurs only during a brief period of about 10 to 14 days (Dubey, 1995), during which time the oocytes may be dispersed into the environment through the actions of nature. Therefore, there is a low probability of detecting their presence at any given time in the environment even though they can survive for prolonged periods of time in the environment under varied conditions (Dubey 1998; Dumètre and Dardé, 2003).

The sampling methodology then becomes vital to improve the probability of recovery of oocysts from the environment, in that sampling must be as close to locations that are known to have a high enough number of felids which will in turn increase the probability of oocyst contamination in the particular environment or location at any given time. It has been experimentally observed that *T. gondii* oocyst recovery rates are related to the numbers of oocysts present in the water being analyzed and that high numbers of oocysts must be present in the actual sample being analyzed: for example oocyst numbers as high as 1×10^5 in 1 litre of water were required to give an appreciable recovery rate through centrifugation and / flocculation methodology and a decrease in oocyst numbers was observed to result in a direct decrease in recovery rates (Kourenti *et al*., 2003). There are currently no standardized methods for the recovery of *T. gondii* oocysts from the environment, therefore the methods available have been adapted from the methods used

for recovery of oocyst forms of other pathogens namely *Cryptosporidium* sp, *Gardia* sp and *Cyclospora* sp.

In the case of water effluent large amounts of starting material are required if there is to be any chance of recovery of oocysts and typically volumes of as high as between 50 to 100 liters have been suggested (Dumètre and Dardé, 2003). In our study we had access to opportunistic samples (starting material) as collection was not strategic and also the volumes were extremely low: we were able to obtain 200 ml of water effluent from each location and this therefore severely limited the probability of recovery of oocysts from the samples. (Note that area where samples are collected is a high violent crime risk area and thus collection by the scientists themselves was not possible. Likewise, large volumes of water could not be collected for the same reason and since none of the collection sites is anywhere near a road). Therefore, even if there was oocyst contamination in the locations sampled, the volumes of water collected were probably insufficient for any oocysts to be successfully recovered and secondly due to the unavailability of a standardized and proven method for the recovery of *T. gondii* oocysts from the environment we had to adapt a methodology which is not a tried and tested method. Therefore the study was limited in a number of aspects and a negative result cannot be accepted as definitive.

5.6.2 Attempted recovery of viable *Toxoplasma gondii* tachyzoites from *Toxoplasma gondii* infected animal tissues through bioassay in mice

There was a zero recovery rate of tachyzoites from the bioassay of *T. gondii* infected tissue from a variety of animals in mice, all samples tested PCR negative (figure 5.4). The infected animal tissues used for the bioassay were opportunistic in nature and initial storage conditions were not under the control of the investigator. We suggest that the inability to recover viable tachyzoites from the bioassay was due to the conditions of storage of the infected animal tissues at the point of collection. Isolation of viable *T. gondii* tissue cysts from infected tissues is extremely dependent on the conditions under which the tissues are stored, because under certain temperature conditions *T. gondii* tissue cysts lose their viability and hence tachyzoites cannot be recovered from such tissue cysts even though the tissues are infected with *T. gondii* and may demonstrate the presence of *T. gondii* DNA when analyzed directly by polymerase chain reaction.

The infected animal tissues were stored at -70 to -80 ^{0}C for 13 months, and this we believe resulted in killing of any viable organisms that were present in the tissue cysts. *Toxoplasma gondii* tissue cysts are not known to survive beyond four days when stored at temperatures below -7^{0}C, as observed by Jacobs et al. (1960), Dubey, (1998) and also Kuticic and Wikerhauser, (1996), even though Frenkel and Dubey (1973) did actually demonstrated that tissue cysts of *T. gondii* did survive for up sixteen days at -16^{0}C suggesting the probable existence of low temperature tolerable forms of the pathogen even though this needs further investigation.

The extent of infection with *T. gondii* in the infected tissues is also a factor in the success of the bioassay. Simply explained, the more *T. gondii* tissue cysts per unit of tissue used

for bioassay, the higher the number of tissue cysts in the applied inoculum and hence the greater the chances of the mice acquiring an infection (Ajioka *et al*., 2001). In our experiments, the mice were kept for twenty one days post infection and were all found to be *T. gondii* negative by PCR diagnosis. We acknowledge the fact that the twenty one days could be also too short for a detectable infection to be established as the multiplication or pathogenicity of the *T. gondii* in the infected samples was not known but could only be speculated based on the results of the genotyping studies on the infected tissues. From the genotyping studies, the strain types identified were Type II and atypical Type II (Chapter 4). According to literature, Type II strains are of moderate to low pathogenicity and require a parasite load of 50 to thousands of parasites in order to establish an infection, whilst atypical strains are unique in their pathogenicity. In our experiments we could only speculate on the pathogenicity of our atypical strains (Tenter *et al*., 2000). It is therefore possible that our strains are of low pathogenicity and hence both a high number of initial tachyzoites (tissue cysts concentration) as well as prolonged or extended times of incubation in the host would be required to establish any detectable infections (Miller *et al*., 1972; Hohweyer *et al*., 2013). Therefore even if the tachyzoites within the tissue cysts were to have survived freezing for prolonged times at -70 to -80^{0}C, the parasite load on the tissues as well as the pathogenicity of the strains may also prove to be barriers to the successful establishment of an infection and hence our inability to recover any live tachyzoites from this series of experiments.

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CHAPTER 6 GENERAL CONCLUSIONS AND COMMENTS

Toxoplasmosis is one of the most important and well-known food and water borne zoonotic infections of man within the global context. It is currently considered a neglected parasitic disease by the USA, Centre for Disease Control and Prevention (CDC) (Jones *et al*., 2014). In the African context, it becomes arguably even more important owing to the fact that it is transmitted through consumption and handling of contaminated food and water. Although this is not unique to Africa, much of the African human population is characterized by the day to day consumption of food and water under extremely unhygienic conditions due to high levels of poverty and food insecurity coupled with close habitation and interaction with farm animals or livestock, which may be high risk reservoirs for *T. gondii* infections.

Toxoplasmosis as an endemic zoonotic disease exerts a huge burden on human and animal health in such under-developed and developing countries especially as it remains largely under-diagnosed or misdiagnosed within such under-resourced settings (Halliday *et al*., 2015). The zoonotic nature of toxoplasmosis implies that human, animals and the environment in which they exist form an ecosystem, which facilitates the inter-movement of the causative agent *T. gondii* from one unit of the system to the other in a vicious cycle. This phenomenon therefore enables us to apply the concept of a "One Health" (van Helden *et al*., 2013; Vanderburg *et al*., 2014) approach towards a holistic investigation or analysis of "*Toxoplasma gondii* the pathogen" or "toxoplasmosis the disease" within a specific setting, by treating it as a single ecosystem. The ecosystem in this case is set or situated within the Western Cape Province of South Africa.

Rather than simply working on one isolated component of the problem, such a broader approach will ensure that we gain a better understanding of the current state of the disease, which may lead to appropriate identification of any knowledge gaps that exist and hence initiate programs to address these knowledge gaps. Ultimately, new policies and prevention work may result from this knowledge.

Our study was not without its limitations; firstly the human cohorts used, ie the post parturient mothers, the ocular *T. gondii* cohort and the infant cohorts were tenuously related if at all related, but they were all from the Cape Town area of South Africa and therefore it made sense to approach them holistically rather than as independent units. However, they are not necessarily representative of the Western Cape Province or South Africa for that matter. Secondly the animal hosts investigated were similarly unconnected. Thirdly the water effluent and some animals collected were arguably suboptimal mainly due to logistical constraints. Our conclusions therefore will be discussed in context.

In this series of studies, we investigated the seroprevalence of toxoplasmosis in selected populations of humans and animals. In the human populations we determined the seroprevalence of toxoplasmosis in a cohort of post parturient mothers two weeks after they had given birth, we then determined the seroprevalence of toxoplasmosis in their infants in order to inform on the incidence of congenital toxoplasmosis in our study area. We thereafter monitored the infants over a fifty two week period in order to determine the rate of post-natal toxoplasmosis at different timepoints, specifically, two weeks, eight weeks, sixteen weeks, twenty six weeks and fifty two weeks. We then used a questionnaire approach to investigate the possible risk factors to *T. gondii* infection in the

mother cohort as well as the effect of selected variables on their *T. gondii* serostatus. We went further to investigate the seroprevalence of toxoplasmosis in selected animal populations (feral cats, caracals and sheep). We also genotyped *T. gondii* in from infected human and animal tissues in order acquire data on the strains occurring in Cape Town and attempted to recover *T. gondii* tachyzoites from infected animal tissues and from run- off water effluent in a wetland of the Cape Peninsula. I believe that this interesting baseline information needs to be extended by further research into the complete epidemiology of *T. gondii* on a larger scale in the Western Cape of South Africa.

6.1 *Toxoplasma gondii* seroprevalence studies in selected animal populations

Animal populations selected for sampling were caracals (*Caracal caracal*), feral (domestic) cats (*Felis catus*), the cape clawless otter (*Aonyx capensis*) and sheep. The selected animal populations are epidemiologically important for a number of reasons, but in particular the pathogen is known to undergo sexual reproduction in the gut of the felid host and this results in the shedding of infective oocysts into the environment (Dubey, 1995; Dubey, 1998; Jones and Dubey, 2012). The oocysts are then dispersed into the environment through the forces of nature and subsequently end up contaminating food and water sources. The life cycle continues when the intermediate hosts, through consumption of contaminated food and/or water, ingest the infective oocysts, where they then progress to establish an infection (Elmore *et al*., 2010; Gotteland *et al*., 2014)). Therefore investigating the seroprevalence of *T. gondii* antibodies particularly in felids within an ecosystem provides valuable information regarding the epidemiology of the

disease and provides a basis for discussion of strategies for monitoring or management of the disease at the policy level.

Sheep are also vulnerable to *T. gondii* infections and infections with *T. gondii* are responsible for causing massive abortion storms in sheep globally (Dubey and Welcome, 1988). Sheep also harbor *T. gondii* tissue cysts and therefore also act as a means of infection transmission through consumption of their meat either raw or partially cooked and handling of their meat raw with unwashed hands. In the USA, consumption of undercooked or raw meat has been reported to be the primary mode of *T. gondii* infection (Cook *et al.*, 2000). In countries such as South Africa where meat consumption is very high and sheep farming is a critical economic activity (Samra *et al*., 2007), there is a public health rationale for monitoring the presence or absence of a zoonosis such as *T. gondii* in sheep. However, prior to investigating *T. gondii* in sheep, we first wished to investigate *T. gondii* in felids as a primary indicator of *T. gondii* occurrence within a particular ecosystem. To do this, we investigated two different felid species in different locations.

We report a *T. gondii* IgG antibody seroprevalence rate of 37.1%, (95% CI 29.60-44.62%) and an IgM antibody seroprevalence of 8.8%, (95% CI 4.40-13.21%), in a population of one hundred and fifty- nine feral cats sampled in two areas of greater Cape Town, with 6.3% being both IgG and IgM seropositive for *T. gondii* antibodies. The high percentage of feral cats positive for IgG antibodies (37.1%) suggests that there is a high level of exposure to *T. gondii* within the environments where these feral cats roam and also the moderately high IgM positive seroprevalence (8.8%) suggests a number of feral cats actively shedding *T. gondii* oocysts at the time of sampling. It has been previously

reported that there is about 1% of infected cats actively shedding infectious oocysts in a given environment (Dubey, 1995), whereas in this study we had 8.8% of feral cats testing positive for an acute infection and hence we speculate that they are likely to be shedding oocysts. Although we were not able to sample sheep where the feral cats live (permission not granted) , there are a number of sheep flocks that the cats can access.

In a cohort of caracals (23) in this study, *T. gondii* IgG antibody seroprevalence was 69.6%, (95% CI 39.1-86.2%) and IgM antibody seroprevalence was 34.7% (95% CI 13.6-60.9%). Although we recognize that the sample size is not representative of the whole of South Africa, caracals and large cats for that matter occupy a unique position in any ecosystem where they are found, in that they act as keystone species in their environment such that a reduction or increase in their numbers leads to a corresponding increase or decline (extinction) of other species and they are therefore more often than not seen as markers of the health of the ecosystem in which they occur ("Urban Caracal Project - The Cape Leopard Trust" 2015).

Such high levels of exposure as well as acute infection in the caracals sampled raise important questions, especially as this is the only report on *T. gondii* in free ranging caracals globally. It is to be hoped that this exploratory study will provide the basis for further research to be conducted into *T. gondii* infection in these important definitive hosts, particularly in a geographically isolated remnant population, and living on the urban edge, as occurs in Table Mountain National Park in the Cape Peninsula. The observed high levels of exposure of felids and moderate exposure of sheep in our study, in combination with the fact that the main risk factor for *T. gondii* infection in our study on the post-parturient mother cohort was that of consumption of unwashed fruits and

vegetables, likely contaminated with oocysts points to the fact that further work has to be done from the public health perspective to understand the extent of contamination of the environment by oocysts and actual risk posed by felids to the well-being of the local populations. *Toxoplasma gondii* exposure in the sheep sampled from a single farm was 8%, (95% CI 4.77-11%), out of a total of two hundred and ninety two sheep sampled. This appears to indicate an increase when compared to the 6% reported by Samra et al. (2007) for sheep in the Western Cape even though this increment was not significant and then other study looked at sheep for a number of different farms. It should be noted that the farm we used for sheep sampling had at least a dozen cats living around the farmhouse. We were not granted permission to sample them.

I believe that the probability of *T. gondii* transmission to humans and non-food animals is directly related to the levels of exposure of food animals to *T. gondii* and therefore presence of *T. gondii* in food animals at whatever levels should not be ignored. Sheep farming is a major economic activity globally and particularly important in South Africa. Unfortunately, food animal pathogens such as *T. gondii* are able to establish long-term infections in farmed sheep flocks and this may lead to substantial economic losses stemming from reproductive losses due to abortions in infected ewes and delivery of non-viable or malformed fetuses through congenital infections. The actual impact of this problem needs to be actively quantified and continuously monitored and managed in order to minimize the potential economic losses (Deconinck *et al*., 1996; Buxton *et al.*, 2007; Dubey, 2009).

6.2 Detection of *Toxoplasma gondii* oocysts in environmental samples through concentration and bioassay in mice

In order to obtain live parasites for further characterization, we attempted to detect the presence of oocysts in contaminated water effluent collected from selected locations in the Western Cape and then proceed to detect the viable oocysts through bioassay in mice (Dumètre and Dardé, 2003).

We did not detect any viable oocysts after bioassays in mice and we speculate that this is due to the fact in order to detect any oocysts we required larger amounts of contaminated water than were used. Factors such as the shedding window in felids which is 1 to 3 weeks and the number of cats shedding oocysts in a particular area within a specific period of time is estimated at about 1% (Dubey, 1995). We were not able to establish the felid density in the area where the water effluent was collected and we collected only 200 ml of water effluent per location, which is lower than the recommended 50-100 liters (Dumètre and Dardé, 2003). This almost certainly would have contributed to our inability to detect any viable oocysts in the water effluents analyzed, finally, the wetland area where we worked is seasonal and this may have implications with regards to the most opportune time to collect samples for analysis.

6.3 Isolation of viable *Toxoplasma gondii* tachyzoites from *Toxoplasma gondii* infected tissues through enzyme digestion and bioassay in mice

In an attempt to isolate live *T. gondii* tachyzoites from *T. gondii* infected animal tissues, we injected six to eight week old female Balb/c mice intraperitoneally with pepsin digested *T. gondii* infected animal tissues and monitored them for signs of infection over

a twenty-one-day period. We did not detect any viable *T. gondii* tachyzoites in the mice after twenty-one days and we speculate that this could be due to the fact that the *T. gondii* infected animal tissues were stored at -70 to -80 ^{0}C for up to eighteen months and this resulted in the destruction of any tissue cysts that would have been present in the tissues, since there is evidence that *T. gondii* tissue cysts can be destroyed after storage for prolonged time periods at low temperatures (Kuticic and Wikerhauser, 1996; Dumètre and Dardé, 2003; Dubey *et al.*, 2014; Watts *et al*., 2015).

Secondly, we monitored the mice over a period of twenty-one days only. However, after direct genotyping via microsatellite methodology of *T. gondii* from the infected tissues used to infect mice, we established that the majority of the strains causing infection were of Type II. It is possible that these strains require a longer incubation period as well as a higher inoculum dose in order to establish a detectable infection from the tissue cysts since these strains are only moderately virulent, with an LD_{50} of approximately $10^{to}\ 10^{5}$ live parasites as opposed to the rarely isolated Type I strains with an LD_{100} of as low as 1 live parasite (Ajzenberg *et al*., 2010; Dubremetz and Lebrun, 2012; Jensen *et al*., 2015).

6.4 *Toxoplasma gondii* antibody seroprevalence in selected human populations

In South Africa, toxoplasmosis is not a reportable disease and therefore data is not collected on the number of human cases diagnosed on an annual basis nor the number of animal cases diagnosed annually. This lack of surveillance data impacts directly on the ability of the existing health system to predict and respond to any outbreaks of the disease if ever that should occur, or to attempt to reduce incidence of the disease. After demonstrating the presence of *T. gondii* in animal populations in the three metropolitan

areas of Cape Town in the Western Cape, we investigated the presence of *T.gondii* in selected human populations also in the metropolitan area Cape Town in the Western Cape of South Africa. Populations were selected based on the perceived public health significance of *T. gondii* infections in the cohorts concerned.

Toxoplasma gondii infections in women of childbearing age are important, since if a primary infection is acquired before or during pregnancy, significant health implications for the unborn fetus or the newborn infant may result (Sensini, 2006; Flatt and Shetty, 2013; Mendy *et al*., 2015). Depending on when the infection is acquired and whether it is a primary infection or a reactivation of a latent infection, the effect on the fetus varies from extensive neurological complications and non viability of the fetus to a congenitally infected infant that is born apparently healthy but may go on to develop ocular complications later in life (Montoya and Remington, 2008; Rosso *et al*., 2008; Olariu *et al*., 2011).

The association between *T. gondii* infection and immunosuppression is a well-established one (Luft and Remington, 1992; Minkoff *et al*., 1997; Machala *et al*., 2013) and therefore we also investigated the interplay between HIV status in a cohort of post-parturient mothers and their *T. gondii* infection status. Within the background of a high HIV burden in South Africa, there is a growing population of infants born to HIV positive mothers but who themselves are HIV uninfected due to the current advances in strategies for the prevention of mother to child transmission of the HIV. Current research mainly from outside South Africa, suggests that such infants who have been exposed to HIV in-utero but are uninfected have a higher rate of infectious morbidity within the first year of life when compared to infants who are unexposed and uninfected that are born to HIV

uninfected mothers (Newell *et al*., 2004; Slogrove *et al*., 2012; Campos *et al.,* 2014). We also investigated the incidence of *T. gondii* infection in infants born to both HIV positive and HIV negative mothers in order to report on congenital infections as well as any differences in infectious morbidity amongst the two groups of infants. The prevalence of *T. gondii* infections in a pilot cohort of HIV positive and HIV negative patients presenting with a clinical diagnosis of uveitis of unknown causes at the Tygerberg academic hospital ophthalmology clinic was also investigated and the presence or absence of *T. gondii* infections in the eye fluid of patients was investigated via PCR tests.

Finally risk factors to *T. gondii* infection have been reported from various countries and regions. These include the consumption of undercooked to raw meat, consumption of unwashed vegetables and fruits, drinking of contaminated water as well as ethnic origins of the population sampled in some instances (Boyer *et al*., 2005; Nash *et al.*, 2005; Elsheikha *et al,*. 2009). Since there is currently no information on the risk factors for *T. gondii* infection in South Africa or specifically the Western Cape, we investigated the risk factors associated with *T. gondii* infection in our specific setting.

Toxoplasma gondii IgG antibody seroprevalence rate was 23.3% (95% CI 18.47-28.84%) and a *T. gondii* IgM seroprevalence was 18.7% (95% CI 14.29-23.86%) in our cohort of 256 post parturient mothers. The *T. gondii* IgG antibody seroprevalence was comparable between the HIV positive group (25%) and the HIV negative group (24.6%), although there was also a higher *T. gondii* IgM antibody seroprevalence in the HIV positive group (2.5%) than in the HIV negative group (0%).

There was no association between *T. gondii* IgG sero-status and HIV status in the post parturient mother cohort but we observed an association between *T. gondii* IgM sero-status and the HIV status of the post parturient mother cohort p=0.007 and an odds ratio of 2.4210, (95% CI 1.2582-4.6783%). Based on our results, we cannot confirm any association or relationship between *T. gondii* infection and HIV status. The scientific literature reports in some instances positive correlations whilst in other instances negative or no correlations have been reported (Osunkalu *et al*., 2011; Walle *et al*., 2013; Koffi *et al.,* 2015) hence the question still remains to be answered convincingly.

We did not establish any congenital *T. gondii* infections in the infant cohort, as all infants tested were negative from weeks two to sixteen. One HIV exposed but uninfected infant was *T. gondii* IgG antibody positive at week twenty six, and all HIV unexposed and uninfected infants were *T. gondii* IgG antibody negative at week twenty-six. At week fifty two there was one *T. gondii* IgG antibody positive HIV exposed but uninfected infant and also one *T. gondii* IgG antibody positive HIV unexposed and uninfected infant, cumulatively out of the 173 infants from the onset of the study the *T. gondii* incidence rate as per IgG and IgM seropositivity was 1.7% (2 IgG and 1IgM positives) over the course of the study. Due to the limited number of infants remaining on the study at week twenty six (122) and week fifty two (98) we cannot draw any substantial conclusions from these results other than to speculate that the infections acquired must have been post natal and not congenital. It is well known that the incidence of congenital *T. gondii* infections is very low in most populations screened (Pappas *et al*., 2009) and the results from our study support this observation.

From this study, the consumption of unwashed fruits and vegetables were associated with IgM seropositivity and therefore this behavior can be considered a risk factor for *T.gondii* infection within our cohort of post parturient mothers. This is inferred from the relationship between IgM antibody sero-status and the participants' washing of vegetable often or sometimes before consumption, with odds ratios of 0.0709 95% CI 0.0828-0.6072 and 2.8527 95% CI 1.3093-6.2152 respectively. The consumption of undercooked or raw meat was not observed to be a risk factor as is often reported elsewhere, for example, the USA (Bahia-Oliveira *et al*., 2003; Nash *et al*., 2005; Jones *et al*., 2014). It can therefore be speculated that within our setting, *T. gondii* transmission is probably through the spread of oocysts shed by infected felids (Dubey, 1998; Dubey and Jones, 2008; Elmore *et al,*. 2010) who in turn contaminate the environment in which they live, and this ends up contaminating fruits and vegetables meant for human consumption and hence lead to acquisition of infections.

The value of seroprevalence studies lies in the gathering of evidence to support the case for screening or surveillance of a particular disease. In this study even though the seroprevalence rates reported are an increase on previous reports for South Africa (Mason *et al.*, 1974; Jacobs and Mason, 1978; Sonnenberg *et al*., 1998; Kistiah *et al*., 2011) the selected cohorts are not representative of the general prevalence at the national level and therefore cannot be used as a basis to make a case for screening at the national level. However, in my opinion they do make a case for larger studies to be done so as to gather enough information to determine whether or not proactive education of at risk groups should be investigated as a strategy.

6.5 Molecular epidemiology of *Toxoplasma gondii* in the Western Cape of South Africa

The genetic diversity of *T. gondii* in South Africa is currently unknown. Globally, *T. gondii* infections have been reported in almost all possible locations and hence the pathogen is said to have a broad or expansive geographic distribution. *Toxoplasma gondii* has also been isolated in a wide range of hosts both intermediate and definitive and it is this ability to infect such a wide range of birds and mammals that has made *T. gondii* such a successful parasite (Shwab *et al*., 2014).

Toxoplasma gondii genetic diversity has historically been reported to be very conserved with its population structure being highly clonal in nature and with most strains previously identified belonging to one of three clonal lineages designated as Types I, II or III. These appear to have had minimal sexual recombination at the inter-lineage level and hence very minimal or limited strain diversity (Howe and Sibley, 1995; Khan *et al.*, 2005). However, this concept of limited diversity of *T. gondii* can be said to have arisen from the fact that most strains that had been studied were from Europe and North America. With the advancement in genotyping techniques and the expansion of the pool of strains studied, the global population structure of *T. gondii* appears to be somewhat more diverse than previously postulated and this has come to prominence with the genotyping of strains origination from South and Central America and to a limited extent Africa, with the discovery of numerous atypical or non-clonal strains (Ajzenberg *et al*., 2004; Ferreira *et al*., 2004; Lehmann *et al*., 2006; Mercier *et al.*, 2010; Carneiro *et al*., 2013; Shwab *et al*., 2014).

In our study we applied a well established genotyping technique, namely a direct genotyping methodology based on unique microsatellite DNA markers present in the *T. gondii* genome (Ajzenberg *et al*., 2002a; Ajzenberg *et al*., 2010) to genotype our isolates of *T. gondii* infected human and animal tissues in order to gain an understanding of the strain diversity within the South African setting where this work was done. In a total of 17 infected human samples genotyped at eight microsatellite loci, we observed 47.1% Type II genotypes, 11.7% atypical genotypes and 5.9% Type III genotype. In 11 animals infected samples we showed 81.8% Type II genotypes and 18.2% atypical genotypes (including a newly observed variant). Taken together, our results suggest a predominance of Type II genotypes as well as the presence of many atypical genotypes in both human and animal infections locally.

The predominance of Type II strains both clonal and variant, causing human and animal infections has been observed in several studies in other continents (reviewed in Su et al. (2012) and Shwab et al. (2014)), and this has been attributed to the fact that Type II strains are moderately virulent and hence able to establish infection equilibrium with the hosts, which has led to them being the most dispersed genotype in most settings. We observed a Type III genotype causing human ocular infection and this is also well established as a common genotype occurring in Africa (Mercier *et al*., 2010). We did not observe any Type I genotypes in our stocks analyzed. The presence of atypical genotypes in both our human and animal samples is interesting because this implies a deviation from the classical clonal population structure suggested for *T. gondii* originally and is rather similar to what has been observed in South and Central America and elsewhere in Africa, as reviewed in Shwab et al. (2014). The most commonly occurring genotypes

reported in Africa have been the Type III and Type II variant (atypical), whilst the Type II clonal, Type III and Type II variant (atypical) have been commonly identified worldwide.

Genetic analysis of the *ROP18* gene, which is one of the genes involved in *T. gondii* virulence confirmed that our infected animal stocks were of two different groups, one group with a *ROP18* gene sequence consistent with the clonal Type II *ROP18* gene sequence as deposited in ToxoDB database (Kissinger *et al*., 2003) and a second group with a *ROP18* gene sequence inconsistent with any *ROP18* gene sequence in the Toxodb database belonging to the two atypical strains A1003a and A1003b. Genotypes A1003a and A1003b were identified as atypical *T. gondii* genotypes from animal infections whilst isolates H1008 and H1009 were identified as atypical genotypes causing human infections. These need to be further characterized so they are properly identified and labeled in line with internationally accepted nomenclature.

Currently in South Africa, human toxoplasmosis is not a notifiable disease, hence the health system is not responsible for or is not mandated to screen any populations for signs of the infection. This implies that the onus is on the medical practitioner who is faced with a patient to decide whether to include a test for toxoplasmosis in the battery of tests that is being required to make a diagnosis. A patient who is well informed may request or insist on a test if, for example, they are planning to start a family. Our recommendation is therefore to generate interest and public information where the disease is concerned so that medical practitioners will be inclined to look out for it more often and that at-risk groups such as infants, AIDs patients and pregnant women will ask to be tested

voluntarily without a medical practioner being responsible for deciding if a test should be done or not.

In the event where the person tests negative, there is a need for simple hygiene precautions to be taken so that the person stays sero-negative. Some preventive measures include;

- Do not consume undercooked or raw meat or eggs, and unpasteurised milk.
- Washing your hands before eating or handling food, as well as after touching raw meat and outdoor activities such as gardening.
- Washing fruits and vegetables well before eating them raw.
- If you own a cat then:
    - Make sure the litter tray is cleaned regularly, and keep it outside or away from the main living areas. Ask someone who is not infected with HIV and is not pregnant to change the litter box daily. If you must clean the box yourself, wear gloves and wash your hands well with soap and water afterwards.
    - Keep your cat indoors to prevent it from hunting.
    - Feed your cat only cat food or cook all meat thoroughly before giving it to your cat. Do not give your cat raw or undercooked meat.

Animal toxoplasmosis on the other hand is a matter that the animal breeding industry likes to discuss. In the case of exclusive felid breeders as well as captive animal facilities,

we were refused entry into a number of facilities whenever we sent out letters that said we were from the University and that we wished to study toxoplasmosis using their facility. In their world, there appears to be a stigma attached to the disease because prospective buyers will not patronize a breeder or a captive animal facility if it is made known that any animal in that facility had died of toxoplasmosis.

6.6 GENERAL COMMENTS

This type of holistic thinking used in this study has enabled us to establish a comprehensive baseline for further studies into both the seroprevalence of *T. gondii* as well as its molecular epidemiology in the Western Cape of South Africa and in South Africa as a whole. In order to tackle such endemic infectious diseases, we need as much information as possible on the complete ecosystem where the zoonosis (van Helden, *et al*., 2013) occurs, and this should comprise information on the pathogen itself, the environment, the human host and the animal host both wild and domesticated. Through such a holistic approach we will be able to determine the weakest link in the system and therefore be able to effectively design interventions to monitor and manage the disease, in question by targeting these weak links.

Noting that our populations studied were actually not intrinsically linked to one another, we applied the ‘one health one world’ philosophy as a guide or framework to enable us contextualize the parameters we were investigating into defined and easy to analyze compartments, this further allowed us to draw conclusions and make recommendations for future investigations.

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APPENDICES

APPENDIX 1

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
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Protocol Approval

Date: 23-May-2014

PI Name: Hammond-aryee, Kenneth KNO

Protocol #: SU-ACUM14-00017

Title: Isolation of visable T.gondii tachyzoites from mice after intra-peritoneal inoculation

Dear Kenneth Hammond-aryee, the Initial Application, was reviewed on 15-May-2014 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number, SU-ACUM14-00017 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU secretariat at WABEUKES@SUN.AC.ZA or 218089003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

APPENDIX 2

Western Cape Government
Agriculture

Dr Gininda Msiza
Chairperson: DECRA
Email: GinindaM@elsenburg.com
tel: +27 21 808 5001 fax: +27 21 808 7619

Reference: DECRA R14/97

Dear Dr van Helden

PROJECT ON SAMPLING OF ELSENBURG EWES FOR TOXOPLASMA RESEARCH

The DECRA evaluated the project proposal on *Sampling of Elsenburg ewes for Toxoplasma research* submitted on 28 February 2014 for ethical review:

The project was approved with DECRA reference nr R14/97 on 4 March 2014.

Please note that annual reports should be submitted by the researcher to the DECRA secretariat.

Kind regards

for Dr Gininda Msiza
DECRA Chairperson

Date: 24 March 2014

APPENDIX 3

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Approval Notice
Response to Modifications- (New Application)

28-Jan-2014
Smit, Derrick DP

Ethics Reference #: N13/10/146

Title: **The causes of intraocular inflammation in HIV-positive and HIV-negative patients in the Western Cape Province, South Africa**

Dear Doctor Derrick Smit,

The **Response to Modifications - *(New Application)*** received on , was reviewed by members of **Health Research Ethics Committee 2** via Expedited review procedures on **22-Jan-2014** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **22-Jan-2014 -22-Jan-2015**

Please remember to use your **protocol number** (**N13/10/146**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389207.

Included Documents:
Application Form
Declaration - de Groot
Declaration - Koegelenberg

APPENDIX 4

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Ethics Letter

26-Jun-2012

Ethics Reference #: S12/01/009
Clinical Trial Reference #:
Title: MOTHER INFANT HEALTH STUDY

Dear Dr Esser Monika,

The Health Research Ethics Committee approved the following laboratory amendment (substudy) on 26 June 2012:

Seroprevalence of Toxoplasma Gondii Infection in a Mother-Infant Cohort in The Western Cape South Africa.

The updated Participant Information Leaflet and Consent Form is also approved.

If you have any queries or need further help, please contact the REC Office 0219389657.

Sincerely,

REC Coordinator
Franklin Weber
Health Research Ethics Committee 1

APPENDIX 5

Western Cape Government
Agriculture

Dr Gininda Msiza
Chairperson: DECRA
Email: GinindaM@elsenburg.com
tel: +27 21 808 5001 fax: +27 21 808 7619

Reference: DECRA G13/89

Dear Dr van Helden

PROJECT ON POPULATION CONTROL OF FERAL CAT COLONIES AT ELSENBURG

The DECRA evaluated the project proposal on *Population control of feral cat colonies at Elsenburg* submitted on 11 November 2013 for ethical review:

The project was approved with DECRA reference nr G13/89 on 14 Jan 2014.

Please note that annual reports should be submitted by the researcher to the DECRA secretariat.

Kind regards

Dr Gininda Msiza
DECRA Chairperson

Date: 24 March 2014

APPENDIX 6

PID

SID

MIHS01
MOTHER INFANT HEALTH STUDY
CLINICAL NOTES: Visit 0

Date of Visit 0:
……
_____

DD MON YYYY

Section A: Review of Inclusion – Exclusion Criteria

INCLUSION CRITERIA

Read the statements below with the mother and record whether or not she agrees

	Yes	No
1) Consent Process:		
a) Mother agrees that adequate time has been provided to review the Information and Consent Form and that all questions have been answered …….	☐	
b) Mother has read and signed the Information and Consent Form prior to any study procedures and no edits have been made ……	☐	
c) Mother has been provided with a copy of the signed Information and Consent	☐	

Form which identifies that the child is involved in this research project ……

2) Mother delivered baby at Kraaifontein MOU/Karl Bremer Hospital …….. ☐

3) The mother's HIV status is known and documented……. ☐

EXCLUSION CRITERIA

	Yes	No
1) Study team and mother unable to communicate in a common language……..		☐
2) Mother resides in one of the following suburbs: Country Places, Eversdal, Goedemoed, Goodwood, Kenridge, Loevenstein, Protea Hoogte, Protea Valley, Rosendal, Stellenridge, Stellenryk, Uitzicht, Valmary Park, Vierlanden, Vygeboom, Welgemoed or another recognized high-income area……		☐
3) Mother delivered before arrival at Kraaifontein MOU/ Karl Bremer Hospital……..		☐
4) Mother did not receive any antenatal care prior to admission in labour……		☐
5) Mother's HIV status is unknown……..		☐
	Yes	**No**
6) HIV infected mother with untraceable antenatal CD_4 count……..		☐
7) HIV infected mother on 2nd line cART……..		☐
8) Mother has eclampsia or pre-eclampsia……		☐

9) Mother with multiple pregnancy……. ☐

10) Mother's pregnancy terminated in an intrauterine death or stillbirth……. ☐

11) Baby with gestational age less than 34 weeks or birth weight less than 2000g……. ☐

12) Mother and/or newborn referred to tertiary care…… ☐

13) Baby with severe terminal congenital defects or genetic abnormality…… ☐

14) Mother participating in another research study…….. ☐

15) Mother plans to move away from Cape Town during the next 12 months……. ☐

16) Mother less than 18 years of age…… ☐

Study Staff Signature:

_____

Date: _____ Time: _____:_____

DD MON YYYY HH MM

Section B: Maternal Interview 1 (Staff Nurse)

1=Positive 2=Negative
Unknown

B1) **On the Maternity Case Record** mother's HIV status is

*	If HIV status unknown on MCR continue with B2)
*	If HIV

1=Positive 2=Negative

B2) What is your HIV status?.....

*	If mom answers 'positive', confirm with her "This means that you have HIV, is that correct?" Continue with B3)

*	If more than one CD4 during pregnancy, record the FIRST CD4 count DURING pregnancy
*	Record date the sample was TAKEN

B3) Record mom's antenatal CD4 count as reported by the NHLS

CD4 absolute count: _____cells/µl

CD4 % (of total lymphocyte count):_____%

Date: _____ DD MON YYYY

1=Yes 2= No ☐ ☐

1) Are you currently taking triple anti-retroviral therapy (cART)?.....

*	If mom does not know then refer to MCR. If not indicated on MCR assume "No"
*	If answer is "No" to 1) skip to 2)

1a) Which of the following ARV meds are you taking? (tick all that apply)

☐

1=TDF…… ☐

2=3TC…… ☐

3=FTC…… ☐

4=NVP…….. ☐

5=EFV…… ☐

6=d4T…… ☐

7=AZT…….. ☐

8=LPV/r…….. ☐

9=Don't know/Declined to answer…….

1b) When did you start taking cART? (month & year acceptable).....

_____

DD MON YYYY

2) Did you receive any medicine for vaginal discharge during this pregnancy?...

3) Did you receive TB treatment during this pregnancy?.....

4) Is this the first baby you have given birth to? (excluding miscarriages)……..

Study staff signature: _____

Date:_____

DD MON YYYY

Section C: Sample Collection (Staff Nurse)

Yes **No**

C1) Was blood obtained from the mom?..... ☐ ☐

Date blood sample was collected……_____

DD MON YYYY

Time blood sample was collected……_____:_____

HH MM

Were the following blood samples obtained?:

Yes **No** **NA**

- CD4 – ☐ ☐ ☐

3ml……

……..

- Viral Load –

4ml……

……

- CMV – ☐

  4ml……

  ……..

- Toxoplasma –

  4ml……

  ……☐

	Yes	No
C2) Was a maternal buccal swab collected?.....	☐	
C3) Was the placenta collected?.....	☐	

If blood, buccal swab or placenta not collected, explain why:

_____

_____

_____

Study staff signature: _____Date:_____

DD MON YYYY

Section D: Maternal Interview 2 (Counsellor)

Ask the mother the following questions:

5) Which racial group do you belong to? (choose only 1)

1=Black African…….. ☐

2=Colored……..... ☐

3=Indian/Asian……..... ☐

4=White……..... ☐

5=Other(Specify)_____ ☐

6=Don't know/Declined to answer……..... ☐

6) What language do you speak most often at home? (choose only 1)

1=Afrikaans……. ☐

2=English…… ☐

3=Xhosa…… ☐

4=Zulu…… ☐

5=Other African…….. ☐

6=Other Non-African…….. ☐

7=Don't know/Declined to answer…… ☐

7) Choose one of the following to describe your current marital status? (choose only 1)

1=Never married……..... ☐

2=Married…….. ☐

3=Widowed…….. ☐

4=Divorced/Separated…… ☐

5=Don't know/Declined to answer…….. ☐

8) Do you consider yourself to be in a stable relationship?

1=Yes..... ☐

2=No..... ☐

3=Don't know/Declined ..... ☐

9) Did you receive any of the following support services during this pregnancy (choose all that apply)?

1=Community health worker (e.g. lay health workers from the community)..... ☐

2=Mother support groups (e.g. breastfeeding support groups, formula feeding support groups or support groups for HIV infected mothers in the community)..... ☐

3=Social worker..... ☐

4=Psychologist..... ☐

5=None of the above..... ☐

6=Don't know/Declined to answer..... ☐

10) Have you ever attended school ☐

1=Yes……..... ☐

2=No…….....…

3= Don't know/Declined to answer.....

* *If "No" to 10) skip to 11)*

10a) If yes, what is the highest level completed?

0=less than 1 year.....

1=sub A/grade 1.....

2=sub B/grade 2.....

3=standard 1/grade 3.....

4=standard 2/grade 4.....

5=standard 3/grade 5.....

6=standard 4/grade 6.....

7=standard 5/grade 7.....

8=standard 6/grade 8.....

9=standard 7/grade 9.....

10=standard 8/grade 10.....

11=standard 9/grade 11.....

12=standard 10/grade 12.....

13=Further studies incomplete.....

14=Diploma or other post-school complete.....

15 = Further degree complete.....

16=Don't know/Declined to answer.....

11) In the last 12 months did you work?

1=Yes.....

2=No.....

3=Don't know/Declined to answer.....

12) Which racial group does your baby's father belong to: (choose only 1)

1=Black African.....

2=Colored.....

3=Asian/Indian.....

4=White.....

5=Other (specify)_____

6= Don't know/Declined to answer....

13) Is the baby's father alive?

1=Yes – he is alive.....

2=No – he passed away.....

3=Don't know/Declined to answer.....

14) How do you intend to feed your baby for the first 6 months?

1=Only breast milk……

2=Only formula milk……

3=Combination of breast milk, formula milk or solids……

4=Don't know/Declined to answer……

* *If answers "only breastmilk" to 14), skip to D1)*

14a) If you are planning on using formula, do you always have R400 extra per month to pay for sterilizing liquid, transport to the clinic and more formula in case the clinic runs out?

1=Yes……

2=No……

3=Don't know/Declined to answer….

D1) Are you willing to give the study invitation letter to your baby's father?

Yes.....

No.....

Review the following with the mother:

Yes

a) Mother has been reminded we will call in 1 week to book Visit 1 appointment

b) Mother has been provided with KID-CRU contact card……

c) Mother has been provided with Airtime voucher……

d) Mother has been provided with fathers invitation letter……..

e) Mother has been provided with stool specimen container & instructions……..

Study staff signature: _____Date:_____

DD MON YYYY

Section F: Maternity Case Record Review (done after interview by Staff Nurse)

Enter the following data from the MCR

15) Site of delivery

1=Kraaifontein MOU.....

2=Karl Bremer Hosp.....

16) Mother's date of birth (page 1)……

_____

DD MON YYYY

17) Syphilis status during pregnancy (page 2)

1=Positive.....

2=Negative.....

3=Indeterminate.....

99=Not indicated/unknown.....

* *If HIV-uninfected skip to 21)*

18) Date of diagnosis with HIV (page2)……..

_____

DD MON YYYY

19) Mothers WHO clinical stage during pregnancy (page 2)

1=Stage 1……

2=Stage 2…….

3=Stage 3…….

4=Stage 4…….

99= Not indicated/Unknown

*	*If mom on cART skip to 21)*

20) AZT start date if mom not on cART (page 2)…….....…_____

DD MON YYYY

21) Gestational age at booking in completed weeks (by midwifes estimate on page 3)_____weeks

22) Number of antenatal visits prior to delivery (count visit entries on page 3)_____visits

23) Obstetric complications (choose all that apply; page 3)

1=Gestational hypertension……

2=Gestational diabetes……

3=Placenta praevia……

4=Other(specify)_____

5=None……

99=Not indicated/Unknown……..

24) Gestational age at delivery in completed weeks (page 10)
……_____weeks

*	*If HIV-uninfected or HIV-infected not on cART skip to 26)*

25) ARV's received during labour and delivery if mom not on cART (tick all received; page 13)

1=sdNVP…….....

2=TDF/FTC…….....

3=3 hourly AZT…….....…

99=Not indicated / Unknown.....

26) Prolonged rupture of membranes >18 hrs (page 22)

1=Yes…….....

2=No…….....…..

99=Not indicated/Unknown.....

27) Mode of delivery (page 22)

1=Normal vaginal……

2=Assisted vaginal (forceps/vacuum)…….

3=Vaginal breech……..

4=Caesarean section……..

99=Not indicated/unknown……...

28a) Apgar score 1 min (page 27)……...

28b) Apgar score 5 min (page 27)…….....

29) Baby's date of birth (page 31)……._____

DD MON YYYY

30) Baby's gender (page 31)

1=Male…….....

2=Female…….....

31) Birth weight (page 31)…….....
_____g

32) Birth head circumference (page 31)…… _____cm

33) Birth length (page 31)……_ _____cm

34) Has the baby been started on NVP? (from drug register)

1=Yes…….

2=No…….

99=Not indicated / unknown……

Study staff signature: _____Date:_____

DD MON YYYY

*	**End of Visit 0 (Enrolment)**

Section A: Mother's Personal Income, employment and educational attainment

1. Do you receive any type of grant? ☐

1=Yes…..…… ☐

2=No

If 'no', skip to question 5

2. If yes, what grant(s) do you receive? (Tick all that apply)

1=Old age…….. ☐

2=Disability…… ☐

3=War veteran.…… ☐

4=Care dependency…..…….. ☐

5=Foster child…….…….. ☐

6=Child support…… ☐

7=Social relief of distress..... ☐

98=Don't know…….. ☐

3. If child supports grants received, specify how many....._____

4. If foster care grant received, specify how many.....

_____

5. In the 12 months before you gave birth to (CHILD IN STUDY), did you work?

1=Yes……..... ☐

2=No……..... ☐

If mom worked during the 12 months before delivery continue; if no, skip to question 6

5.a) Do you usually work throughout the year, or do you work seasonally, or only once in a while, or never had a job?

1=Throughout the year……. ☐

2=Seasonally/only part of the year…..… ☐

3=Once in a while.……. ☐

4=Not applicable.....……. ☐

5.b)In your job were you 1)working for someone else for pay (including paid domestic workers, gardener or security guards), 2) an employer (employing one or more employees), or 3)working for yourself (not employing any employees)

1=Working for someone else for pay….. ☐

2=An employer.....……. ☐

3=Working for herself..... ☐

If yes to option 1 above (working for someone else for pay) continue; If no, skip to question 6

5bi) Were you employed on the basis of 1) a written contract, or 2) A verbal agreement

1=Written contract..…..…….. ☐

2=Verbal agreement.…….. ☐

5.c) In the 12 months before you gave birth to (CHILD), how many days a week did you usually work? Days per week:_____

6. In the 12 months before you gave birth to (CHILD), how much money did you **personally** get each month from all sources added together? (Including wages, rent, grants, informal income)?

Rand per month:_____

7. In the past 7 days did you work for money?

1=Yes..... ☐

2=No..... ☐

99=Don't know..... ☐

**If" yes" continue, if "no", skip to question 8*

7a) In the last 7 days, how many days did you work? (Pick numbers 1-7)

Number of days:_____

7b) Who usually takes care of (CHILD) when you are working?

1=Respondent..... ☐

2=Husband/partner..... ☐

3=Child's father..... ☐

4=Older female child..... ☐

5=Older male child..... ☐

6=Other relatives..... ☐

7=Neighbours..... ☐

8=Friends..... ☐

9=Servants/hired help…….. ☐

10=Institutional child care/daycare..…… ☐

98=Other.....…… ☐

99=Don't know...…… ☐

Questionnaire continues on next page. Please turn over.

8. For most of the time until you were 12 years old, did you live in a city, in a town, on a farm or in rural areas, or an informal settlement?

1=City…… ☐

2=Town…… ☐

3=Rural/farm…… ☐

4=Informal Settlement…….. ☐

9. How many years have you been living continuously in the area where you currently live?

Number of years: _____

10. Are you currently attending school?

1=Yes…….…… ☐

2=No…...……..…… ☐

Section B: House Structure

Tell mother: I would now like to ask you some questions about **the house where (CHILD) normally lives.** If (CHILD) normally lives in a different house from where you live, please think about the house where (CHILD) lives instead of the house where you live.

11. What type of structure is the house?

1=Stand alone.…… ☐

2=Flat in block of flats…… ☐

3=House/flat/room in backyard…… ☐

4=Informal dwelling/shack in backyard. ☐

5=Informal dwelling/shack not in backyard……. ☐

6=Room/flatlet on shared property..... ☐

7= Caravan or tent..... ☐

8=Other.....…….. ☐

12. What is the main source of drinking water for members of the household?

1=Water piped into dwelling..…… ☐

2=Water piped into site/yard…… ☐

3=Public tap.....…… ☐

4=Surface water (such as from river/stream, pond/lake, pool/stagnant water, or dam. ☐

5=Rainwater……. ☐

6=Water supplier/carrier/tanker..... ☐

7= Other..... ☐

13. What kind of toilet facilities does the household have?

1=Flush toilet (connected to sewage)…. ☐

2=Flush toilet (with septic tank)..…… ☐

3=Ventiled improved pit latrine…… ☐

4=Traditional pit toilet..... ☐

5=Portable toilets…… ☐

6=No facility/bush/field..... ☐

7= Other..... ☐

14. Do the members of the household share these (toilet) facilities with other households?

1=Yes…… ☐

2=No..... ☐

15. What type of fuel do the member of the household mainly use for cooking?

1=Electricity…….. ☐

2=Gas..... ☐

3=Parafin..... ☐

4=Coal..... ☐

5=Firewood/Straw…… ☐

6=Other..... ☐

16. What type of fuel does the household mainly use for heating?

1=Electricity…….. ☐

2=Gas..... ☐

3=Parafin.....……. ☐

4=Coal..... ☐

5=Firewood/Straw…… ☐

6=Other..... ☐

17. What type of fuel does the household mainly use for lighting?

1=Electricity……... ☐

2=Gas.....……. ☐

3=Parafin.....……. ☐

4=Coal..... ☐

5=Firewood/Straw…… ☐

6=Other..... ☐

18. How many rooms does the household have, including kitchen or cooking area? (Exclude bathrooms, sheds, garages, stables, or any other rooms unless people live in them)

Number of rooms: _____

**If household has only one room; If household has more than one room, skip to question 19*

18a) Is this room shared with other households? ☐

1=Yes……..…

2=No.....……

19. What is the main material of the floor in this dwelling?

1=Earth/sand/dung……...

2=Bare wood planks.....……

3=Vinyl/asphalt strips.....……

4=Ceramic tiles.....

5=Cement……..…….

6=Other.....

20. What is the main material of the walls in this dwelling?

1=Plastic/cardboard……...

2=Mud.....……

3=Mud and cement.....……

4=Corrugated iron/zinc.....

5=Prefab……..…….

6=Bare brick or cement blocks..... ☐

7=Plaster/finished..... ☐

8=Other.....

C: Household Asset Ownership

21. Does the household have any of the following items in working condition?

a. A radio

1=Yes..... ☐

2=No..... ☐

b. A television

1=Yes..... ☐

2=No..... ☐

c. A computer

1=Yes..... ☐

2=No..... ☐

d. A refrigerator

1=Yes..... ☐

2=No..... ☐

e. A landline telephone ☐

1=Yes……..…

☐

2=No.....……

f. A cellphone

☐

1=Yes……..…

☐

2=No.....……

22. Does any member of the household own:

g. A bicycle?

☐

1=Yes……..…

☐

2=No.....……

h. A motorcycle or motor scooter?

☐

1=Yes……..…

☐

2=No.....……

i. A car or truck?

☐

1=Yes……..…

☐

2=No.....……

j. A donkey or a horse?

☐

1=Yes……..…

☐

2=No.....……

k. Sheep, goat, or cattle?

1=Yes……..…. ☐

2=No.....…… ☐

23. How long does it take you to walk from your house to the clinic where (CHILD) goes for "well-baby visits"? (In minutes)

Distance:

_____minutes

24. Have you been tested for toxoplasmosis?

1=Yes…… ☐

2=No…… ☐

3=Don't know/refused to answer…… ☐

……

……

If no, skip to question 27

25. What was the outcome?

1=Positive…… ☐

2=Negative…… ☐

3=Don't know/refused to answer…… ☐

……

……..

26. Did you receive treatment?

1=Yes…… ☐

2=No…… ☐

3=Don't know/refused to answer…… ☐

……

……

Section D: Household Occupancy and Kinship Structure

27. How many people usually live in the household? (By "usually live" I mean they spend most nights of the week sleeping in the house.)

Number of people:_____

28. Has any member of the household been tested for toxoplasmosis?

1 = Yes..... ☐

2 = No..... ☐

3 = Don't know/Refused to answer..... ☐

**If no, skip to question 31*

29. Were any of the results positive?

1 = Yes..... ☐

2 = No..... ☐

3 = Don't know/Refused to answer..... ☐

30. Did anyone receive treatment?

1 = Yes..... ☐

2 = No..... ☐

3 = Don't know/Refused to answer..... ☐

31. Who is the head of the household? ☐

1 = Husband.....

2 = Partner ..... ☐

3 = Son or daughter ..... ☐

4 = Son-in-law or daughter-in-law ..... ☐

5 = Parent..... ☐

6 = Parent-in-law ..... ☐

7 = Brother or sister..... ☐

9 = Nephew/niece ..... ☐

10=Other adult relative..... ☐

11=Not related adult..... ☐

☐

Codes for Relationship to Mother (Q. 33)

01=Husband
02=Partner
03=Son or daughter
04=Son/daughter in-law
05= Grandchild
06=Parent
07=Parent-in-law
08=Brother or Sister
09=Nephew/niece
10=Other relative
11=Adopted/foster/stepchild
12=Not related
98=Don't know

Codes for highest level of school completed (Q. 37)

00=less than 1 year completed
01=Sub A/Grade 1
02=Sub B/Grade 2
03=Standard 1/Grade 3
04=Standard 2/Grade 4
05=Standard3/Grade 5
06=Standard4/Grade 6
07=Standard 5/Grade 7
08=Standard 6/Grade 8
09=Standard 7/Grade 9
10=Standard 8/Grade 10
11=Standard 9/Grade 11
12=Standard 10/Grade 12
12=Further studies incomplete
14=Diploma or other post-school completed
15=Further degree completed
98=Don't know

Now I would like to ask you some q[illegible]bout the people who usuall[illegible]e household.

		1			2			
	Please give me the names of the people who are **16 years old or older** who usually liv in your house	is [illegible]E)'s [illegible]onship [illegible]ou?	Is (NAME) male or female?	as [illegible]n [illegible]st	Has (NAME) ever attended school? 1=Male……. 2=Female…	**If attend** What is t level of s complete

3	_____	☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes..... ☐ 2=No ..... ☐ 3=D.K. .... ☐	

APPENDIX 7

(Continue adding household members 16 years old or older below)

	32.	33.	34.	35.	36.	
	Please give me the names of the people who are **16 years old or older** who usually live in your house	What is (NAME)'s relationship with you?	Is (NAME) male or female?	How old was (NAME) on (her/his) last birthday?	Has (NAME) ever attended school?	**I** What is t level of sch
4	_____ _	☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes..... ☐ 2=No ..... ☐ 3=D.K. .... ☐	
5	_____ _	☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes..... ☐ 2=No ..... ☐ 3=D.K. .... ☐	
6	_____ _	☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes..... ☐ 2=No ..... ☐ 3=D.K. .... ☐	
7	_____ _	☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes..... ☐ 2=No ..... ☐ 3=D.K. .... ☐	
8	_____ _	☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes..... ☐ 2=No ..... ☐ 3=D.K. .... ☐	

Codes for types of grants (Q. 40)

01=Old age

02=Disability

03=War veteran

04=Care dependency

05=Foster child

06=Child support

07=Social relief of distress

98=Don’t know

(Place beside Questions 26-32 to complete row for each person)

	Does (NAME) receive any type of grant?	**If receives a grant** What type of grant does (NAME) receive?	Since (CHILD) was born, has (NAME) regularly given you money to buy things for yourself or (CHILD)?	Since (CHILD) was born, has (NAME) regularly bought things like nappies, wet-wipes or toys for (CHILD)?	Has (NAME) tested positive for TB?	**If TB test positive** Is (NAME) receiving daily medication for TB infection?	Has (NAME) been diagnosed to be HIV-positive?	**If HIV-positive** Is (NAME) receiving daily medication for their HIV infection?
1	1=Yes .. 2=No.... 3=D.K..		1=Yes ..... 2=No ..... 3=D.K.....	1=Yes 2=No 3=D.K.	1=Yes... 2=No.... 3=D.K..	1=Yes ..... 2=No..... 3=D.K.....	1=Yes..... 2=No ..... 3=D.K.	1=Yes 2=No 3 = D.K.
2	1=Yes .. 2=No.... 3=D.K..		1=Yes ..... 2=No ..... 3=D.K.....	1=Yes 2=No 3=D.K.	1=Yes... 2=No.... 3=D.K..	1=Yes ..... 2=No..... 3=D.K.....	1=Yes..... 2=No ..... 3=D.K.	1=Yes 2=No 3= D.K.
3	1=Yes .. 2=No.... 3=D.K..		1=Yes ..... 2=No ..... 3=D.K.....	1=Yes 2=No 3=D.K.	1=Yes... 2=No.... 3=D.K..	1=Yes ..... 2=No..... 3=D.K.....	1=Yes..... 2=No ..... 3=D.K.	1=Yes 2=No 3= D.K.

(Place beside Questions 26-32 to complete row for each person)

	39.	40.	41.	42.	43.	44.	45.	46.
	Does (NAME) receive any type of grant?	**If receives a grant** What type of grant does (NAME) receive?	Since (CHILD) was born, has (NAME) regularly given you money to buy things for yourself or (CHILD)?	Since (CHILD) was born, has (NAME) regularly bought things like nappies, wet-wipes or toys for (CHILD)?	Has (NAME) tested positive for TB?	**If TB test positive** Is (NAME) receiving daily medication for TB infection?	Has (NAME) been diagnosed to be HIV-positive?	**If HIV-positive** Is (NAME) receiving daily medication for their HIV infection?
4	1=Yes ☐ 2=No ☐ 3=D.K. ☐	☐☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ..... ☐ 2=No ..... ☐ 3 = D.K. . ☐
5	1=Yes ☐ 2=No ☐ 3=D.K. ☐	☐☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ..... ☐ 2=No ..... ☐ 3= D.K. .. ☐
6	1=Yes ☐ 2=No ☐ 3=D.K. ☐	☐☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ..... ☐ 2=No ..... ☐ 3= D.K. .. ☐
7	1=Yes ☐ 2=No ☐ 3=D.K. ☐	☐☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ..... ☐ 2=No ..... ☐ 3= D.K. .. ☐

8	1=Yes ☐ 2=No ☐ 3=D.K. ☐	☐☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ..... ☐ 2=No..... ☐ 3=D.K..... ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes ☐ 2=No ☐ 3=D.K. ☐	1=Yes..... ☐ 2=No ..... ☐ 3= D.K. .. ☐

What is (NAME)'s relationship with you?	Is (NAME) male or female?	How old was (NAME) on (her/his) last birthday?	Has (NAME) ever attended school?	**If attended school** What is the highest standard/grade/ level of school (NAME) has completed?	Has (NAM been diagı to be HIV positive?
☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes…….. ☐ 2=No…… ☐ 3=D.K…….. ☐	☐☐	1=Yes…. ☐ 2=No….. ☐ 3=D.K…. ☐
☐☐	1=Male……. ☐ 2=Female… ☐	_____years	1=Yes…….. ☐ 2=No…… ☐ 3=D.K…….. ☐	☐☐	1=Yes…. ☐ 2=No….. ☐ 3=D.K…. ☐
☐☐	1=Male….. ☐ 2=Female… ☐	_____years	1=Yes…….. ☐ 2=No…… ☐ 3=D.K…….. ☐	☐☐	1=Yes…. ☐ 2=No….. ☐ 3=D.K…. ☐

☐ ☐	1=Male….. ☐ 2=Female… ☐	_________years	1=Yes…….. ☐ 2=No……… ☐ 3=D.K…….. ☐	☐ ☐	1=Yes…. ☐ 2=No….. ☐ 3=D.K…. ☐
☐ ☐	1=Male….. ☐ 2=Female… ☐	_________years	1=Yes…….. ☐ 2=No……… ☐ 3=D.K…….. ☐	☐ ☐	1=Yes…. ☐ 2=No….. ☐ 3=D.K…. ☐
☐ ☐	1=Male….. ☐ 2=Female… ☐	_________years	1=Yes…….. ☐ 2=No……… ☐ 3=D.K…….. ☐	☐ ☐	1=Yes…. ☐ 2=No….. ☐ 3=D.K…. ☐

Appendix 7

Details of variables investigated

Variable name	Abbreviation	Question	Response categories
Mother's racial group	momrace	Which racial group do you belong to?	1=Black African, 2=Coloured, 3=Asian or Indian,4=White, 5=Other, 77 = don't know/declined to answer, 99 = missing data
Mother's educational level attained	meducoth	What is the highest level of education you completed?	00 = less than 1 year completed, 01 = Grade 1, 02 = Grade 2, 03 = Grade 3,04 = Grade 4, 05 = Grade 5, 06 = Grade 6, 07 = Grade 7, 08 = Grade 8, 09 = Grade 9, 10 = Grade 10, 11 = Grade 11, 12 = Grade 12, 13 = Further studies incomplete, 14 = Diploma or other post-school completed, 15 = Further degree completed, 77 = don't know/declined to answer, 99 = missing data
Household members Tested for *T. gondii*	hhtoxtest	Has anyone in your household been tested for toxoplasmosis?	1 = yes, 2 = no, 77 = don't know/declined to answer, 99 = missing data
Mother's meat eating preference	mdietmeat	How do you and the members of your household prefer to eat your meat: well cooked or partially cooked?	1 = Well cooked, 2 = Partially cooked,3 = N/A (doesn't eat meat), 77 = Don't know/declined to answer, 99 = Missing data
Mother's vegetable eating preference	mcleanVeg	How often do you and the members of your household clean your fruits and vegetables when preparing them?	1 = Always, 2 = Often,3 = Sometimes, 4 = Never, 77 = Don't know/declined to answer, 99 = Missing data
Presence of pets in the house (cats)	hhpets	Does the household have pets (cats)?	1 = yes, 2 = no, 77 = don't know/declined to answer, 99 = missing data

Type of household	hhhouse	What type of structure is the house? (The following household questions refer to the house that the child is living in)	1 = stand alone house, 2= flat in block of flats, 3 = house/flat/room in backyard, 4 = shack in backyard, 5 = shack NOT in back yard, 6 = room/flatlet on shared property, 7 = caravan or tent, 8 = other, 77 = don't know/declined to answer, 99 = missing data
Source of household water	hhwater	What is the main source of drinking water for members of the household?	1 = water piped into dwelling, 2 = water piped into site/yard, 3 = public tap, 4 = surface water such as from a river/stream\\, pond/lake\\, pool/stagnant water or dam, 5 = rainwater, 6 = water supplier/carrier/tanker, 7 = other, 77 = don't know/declined to answer, 99 = missing data
Household toilet type	hhtoilet	What kind of toilet facilities does the household have?	1 = flush toilet (connected to sewage), 2 = flush toilet (with septic tank), 3 = ventilated improved pit latrine, 4 = traditional pit toilet, 5 = portable toilets, 6 = no facility/bush/field, 7 = other, 77 = don't know/declined to answer, 99 = missing data
Household toilet usage	hhtoiletYN	Do the members of the household share these (toilet) facilities with other households?	1 = yes, 2 = no, 77 = don't know/declined to answer, 99 = missing data
Mother’s previous toxoplasmosis status	mtoxtest	Have you (mother) been tested for toxoplasmosis	1 = yes, 2 = no, 77 = don't know/declined to answer, 99 = missing data

Appendix 8

1. **Investigation into the prevalence of *Toxoplasma gondii* antibodies in serum of HIV-positive and HIV-negative individuals presenting with intraocular inflammation at Tygerberg academic hospital ophthalmology clinic**
2. **Detection Of *Toxoplasma gondii* DNA in DNA from ocular (Aqueous) fluid of patients presenting with intraocular inflammation via polymerase chain reaction amplification methodology**

Inclusion criteria:

Clinical diagnosis of uveitis (new or last episode > 1 year ago)

Chronic uveitis of unknown cause

Age ≥ 18 years and ≤ 60 years

Patient consents to HIV test with appropriate counseling

Exclusion criteria:

Recurrence of uveitis < 1 year since previous episode

Uveitis with known cause

I. New cases with obvious cause (eg trauma or lens-induced)

II. Age <18 years or >60 years

III. Patient does not consent to HIV test after appropriate counseling

Appendix 9

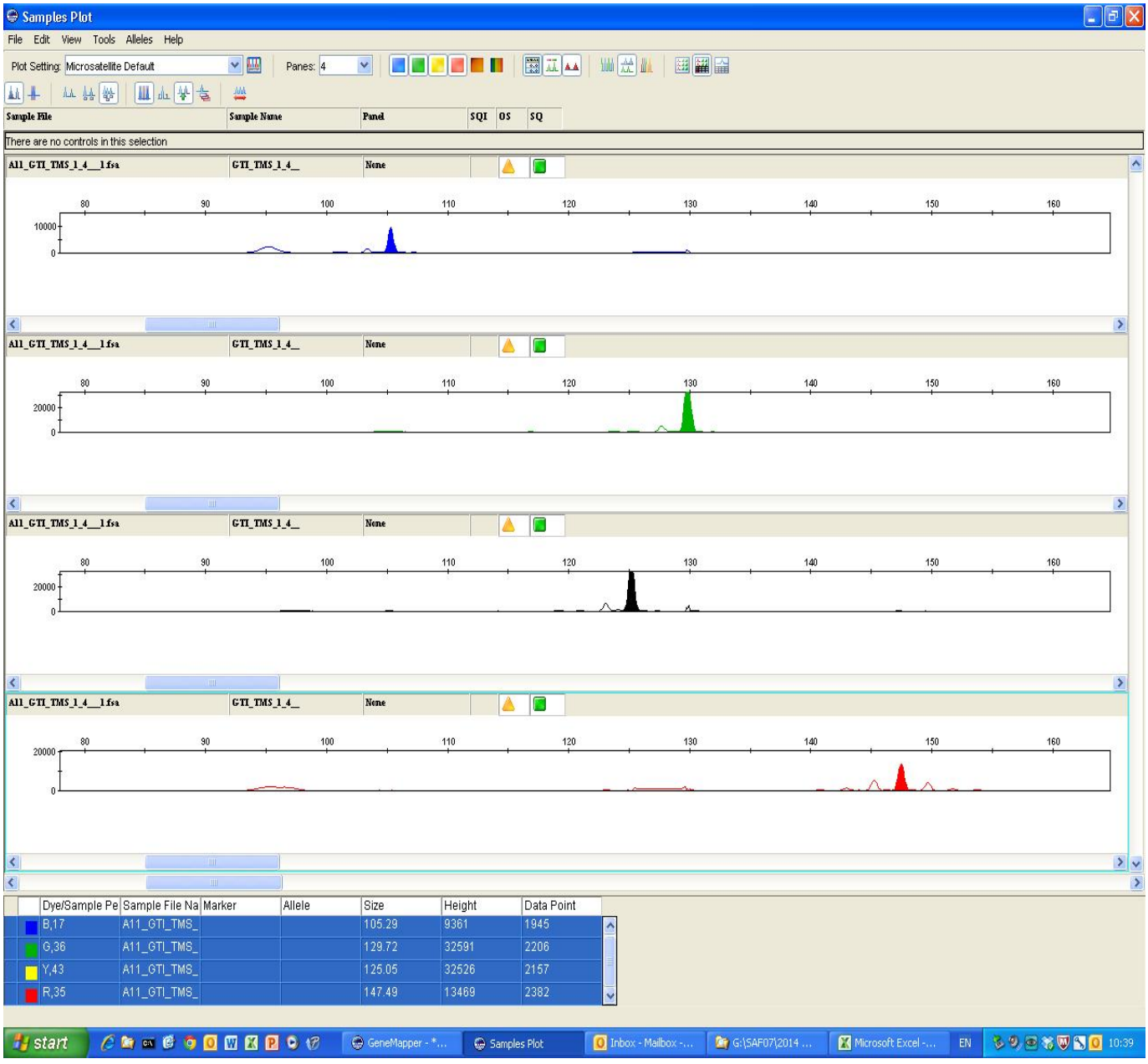
Ethics clearance certificate number for the Cape peninsula Urban Leopard identification project

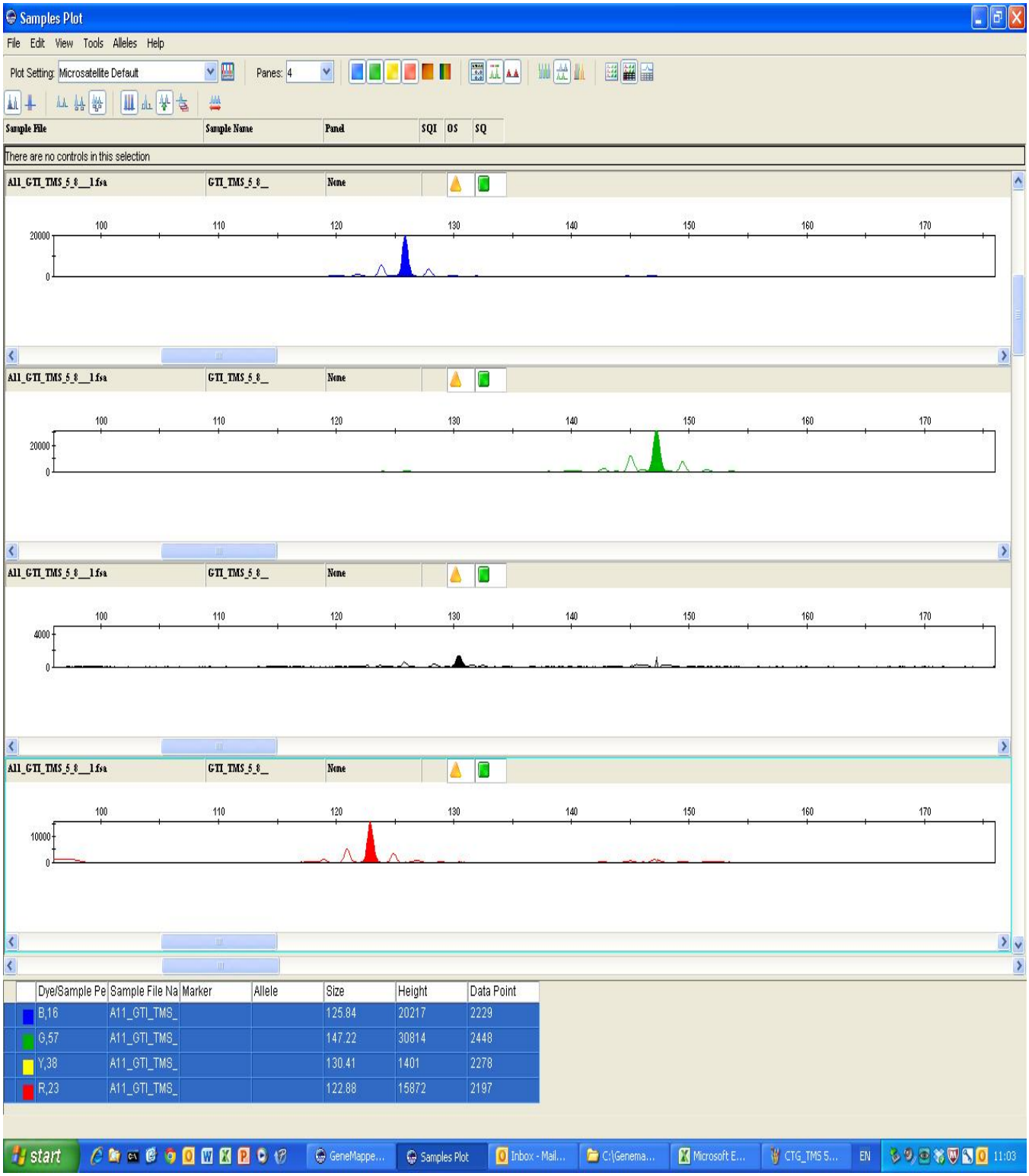
Certificate number 2014/V20/LS

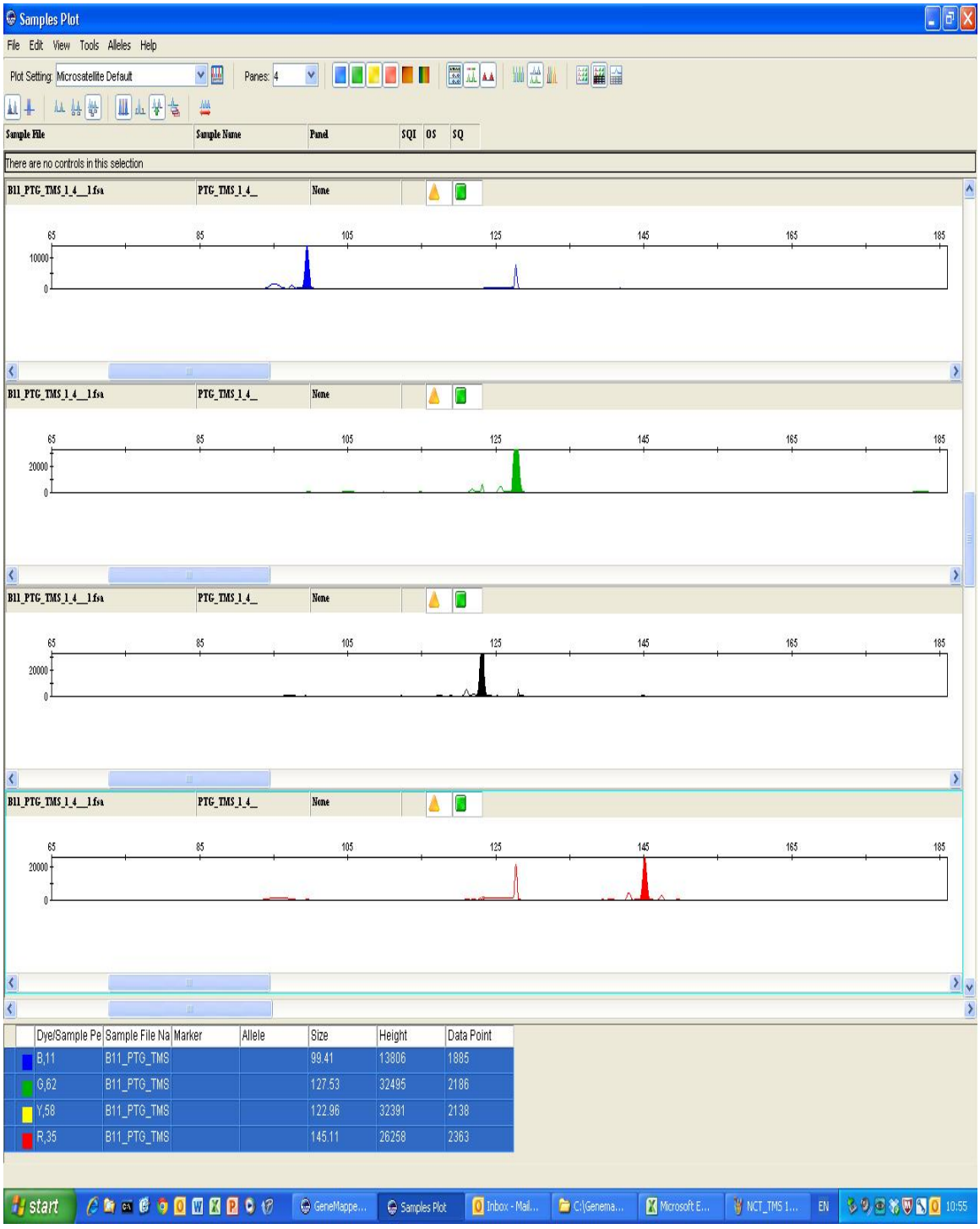
Appendix 10

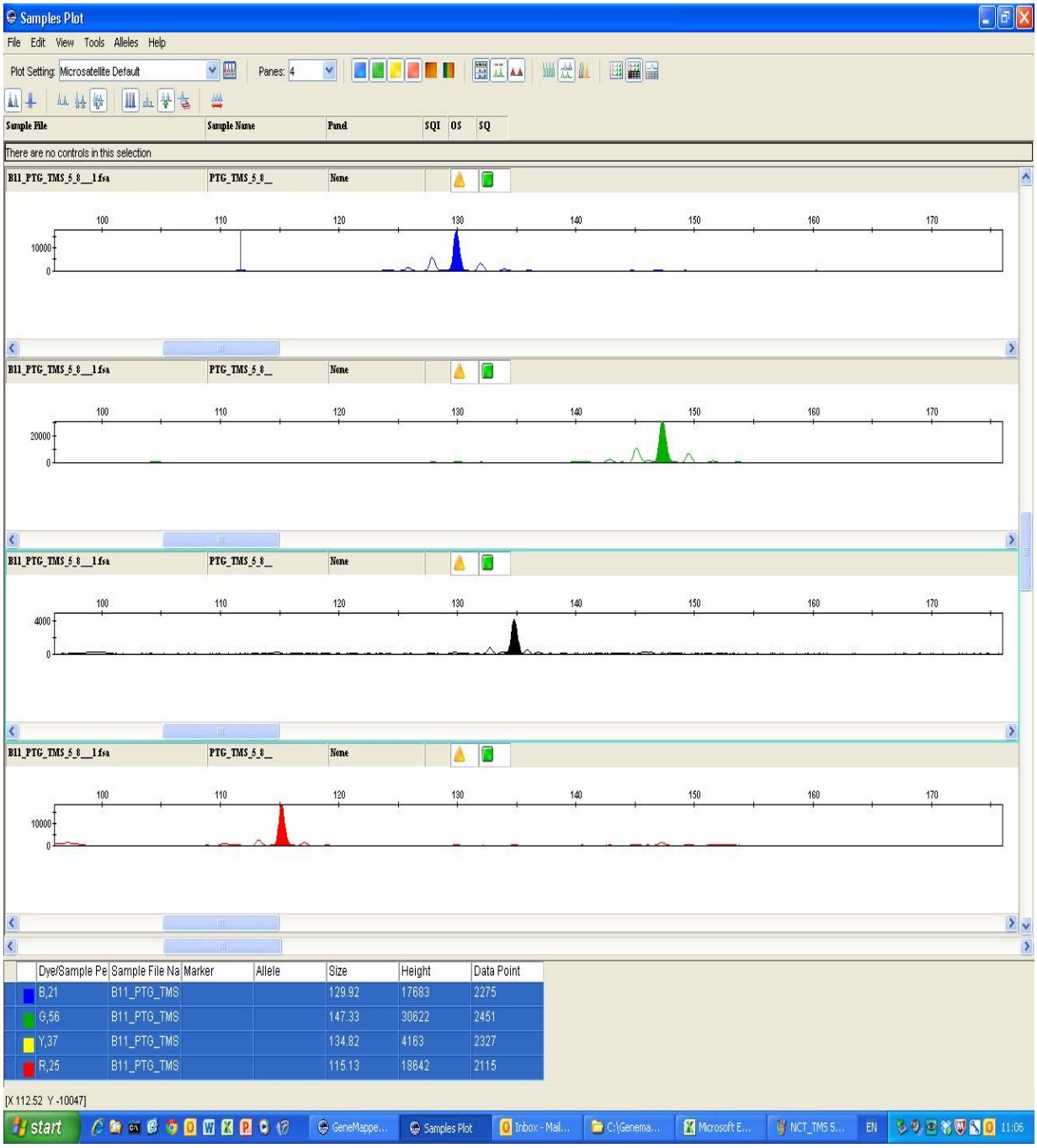
GeneMapper[R] plots for control reference strains, animal and human samples genotyped at eight microsatellite loci

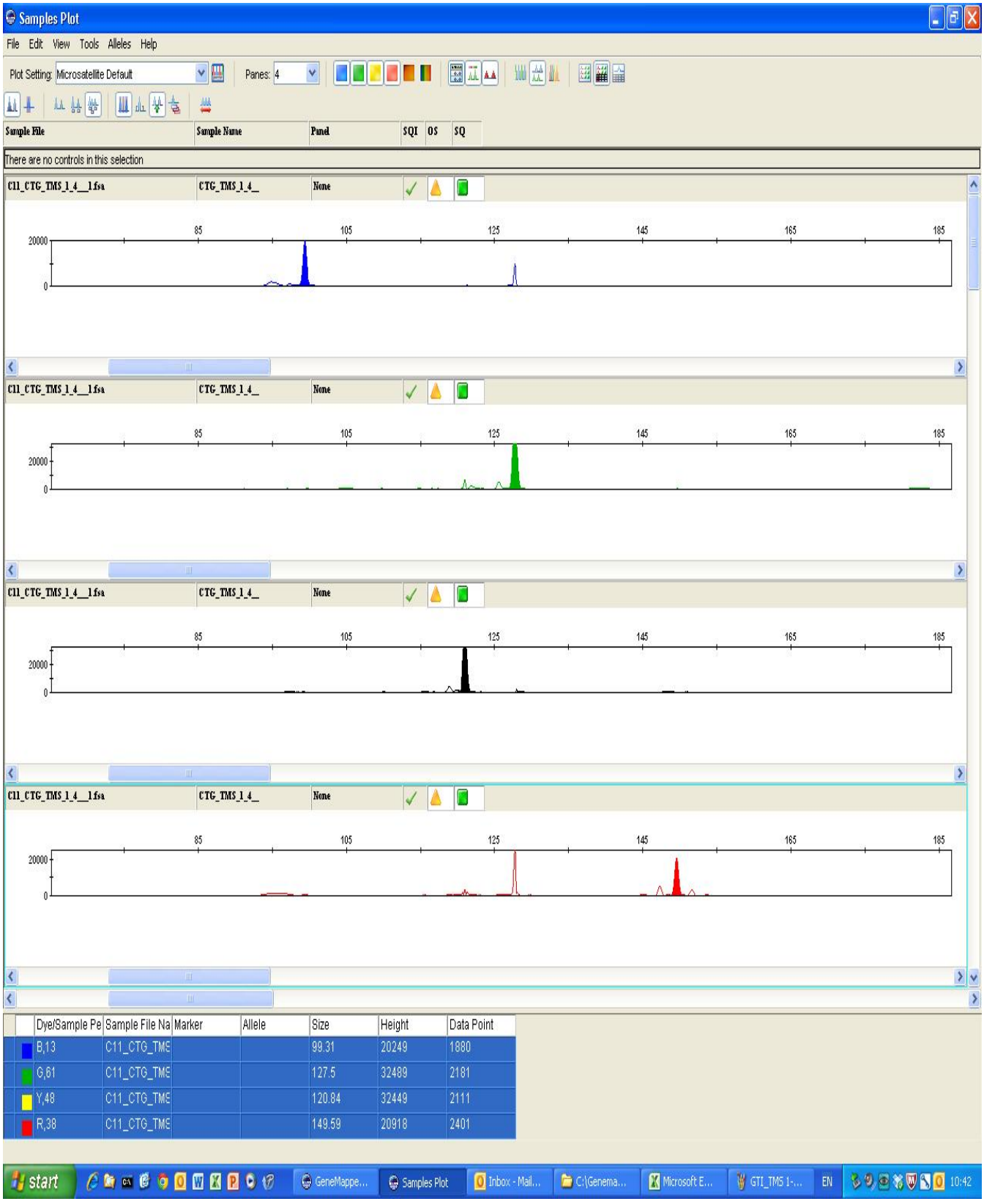
Reference strains

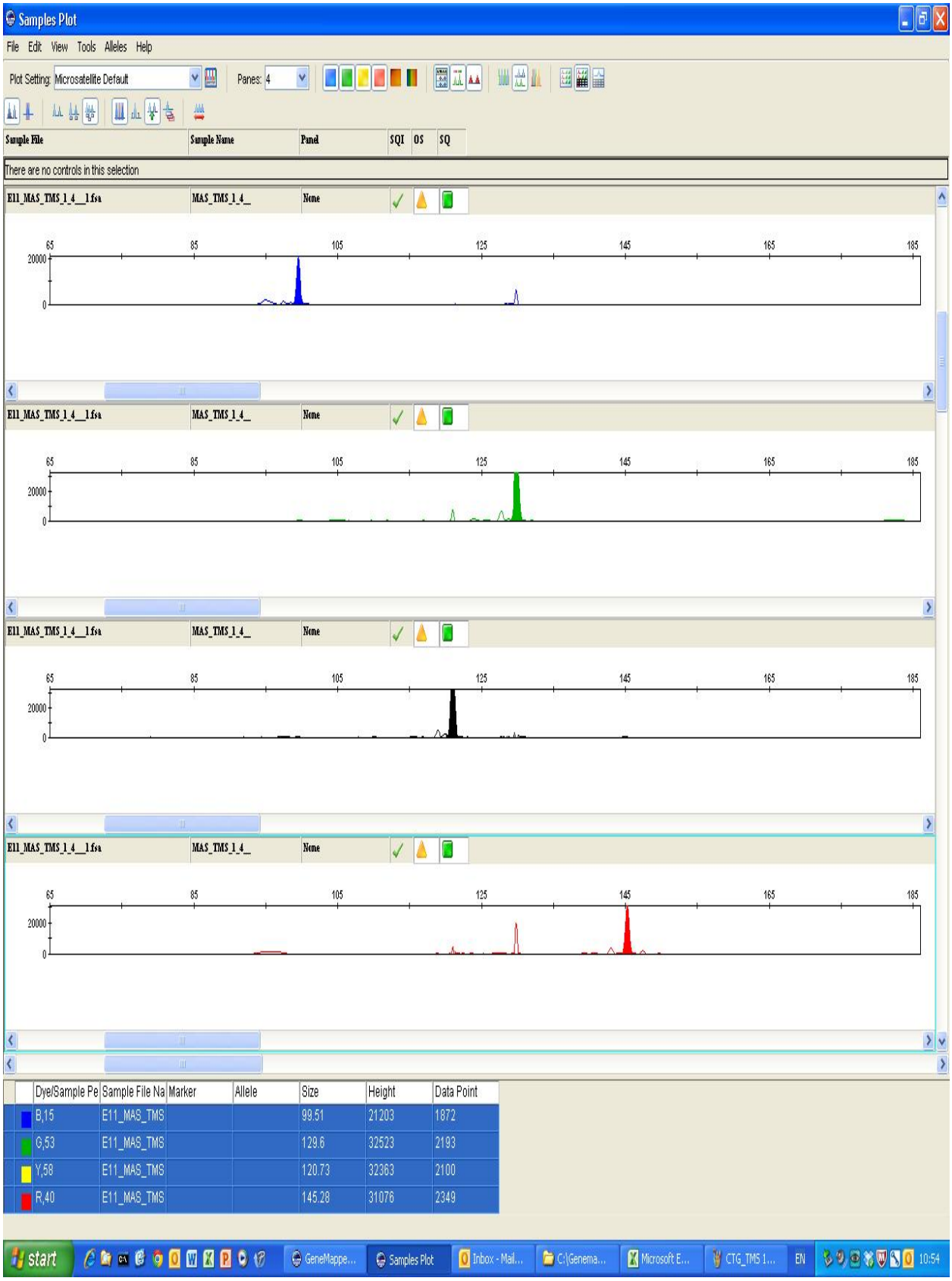


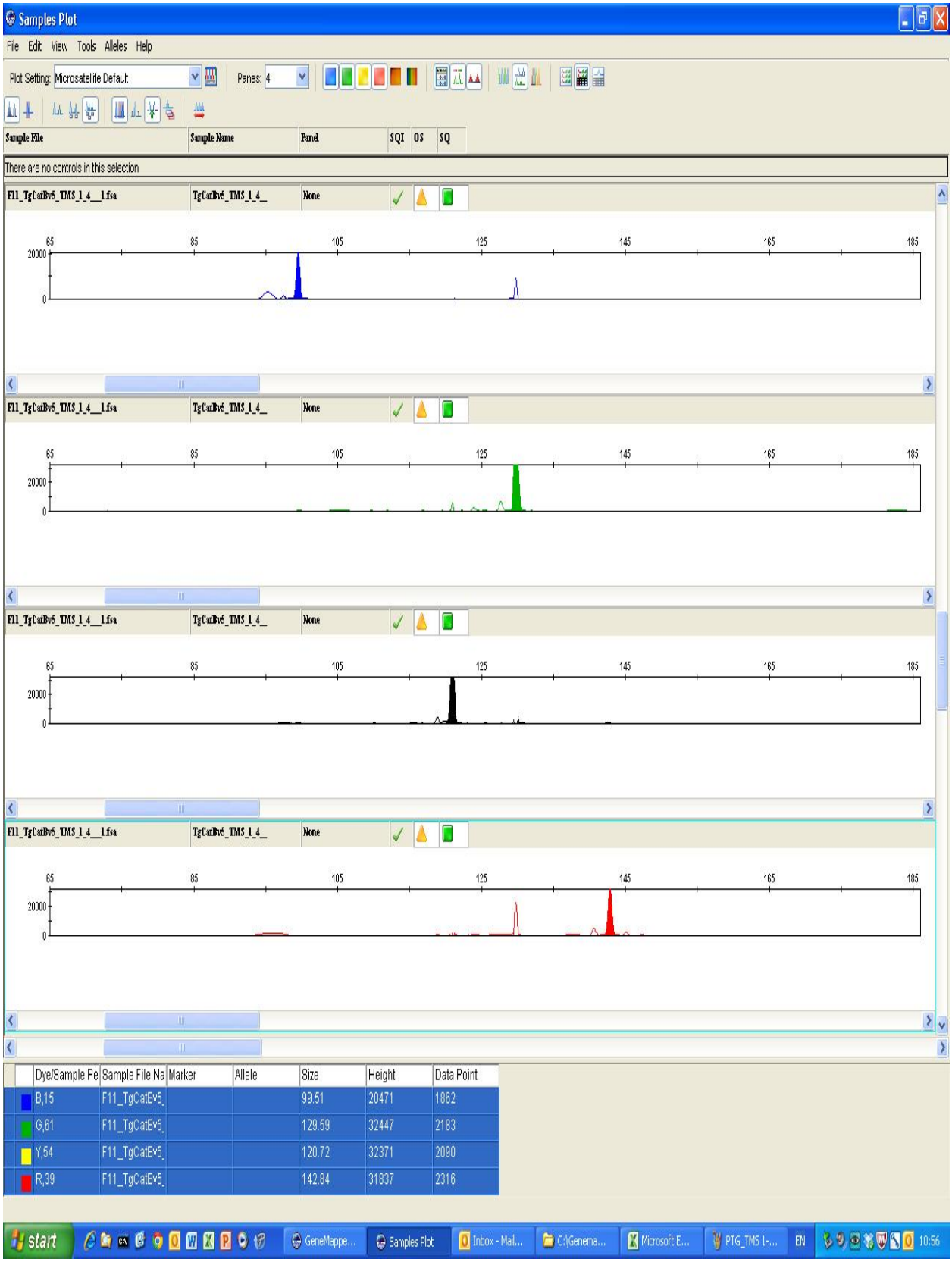


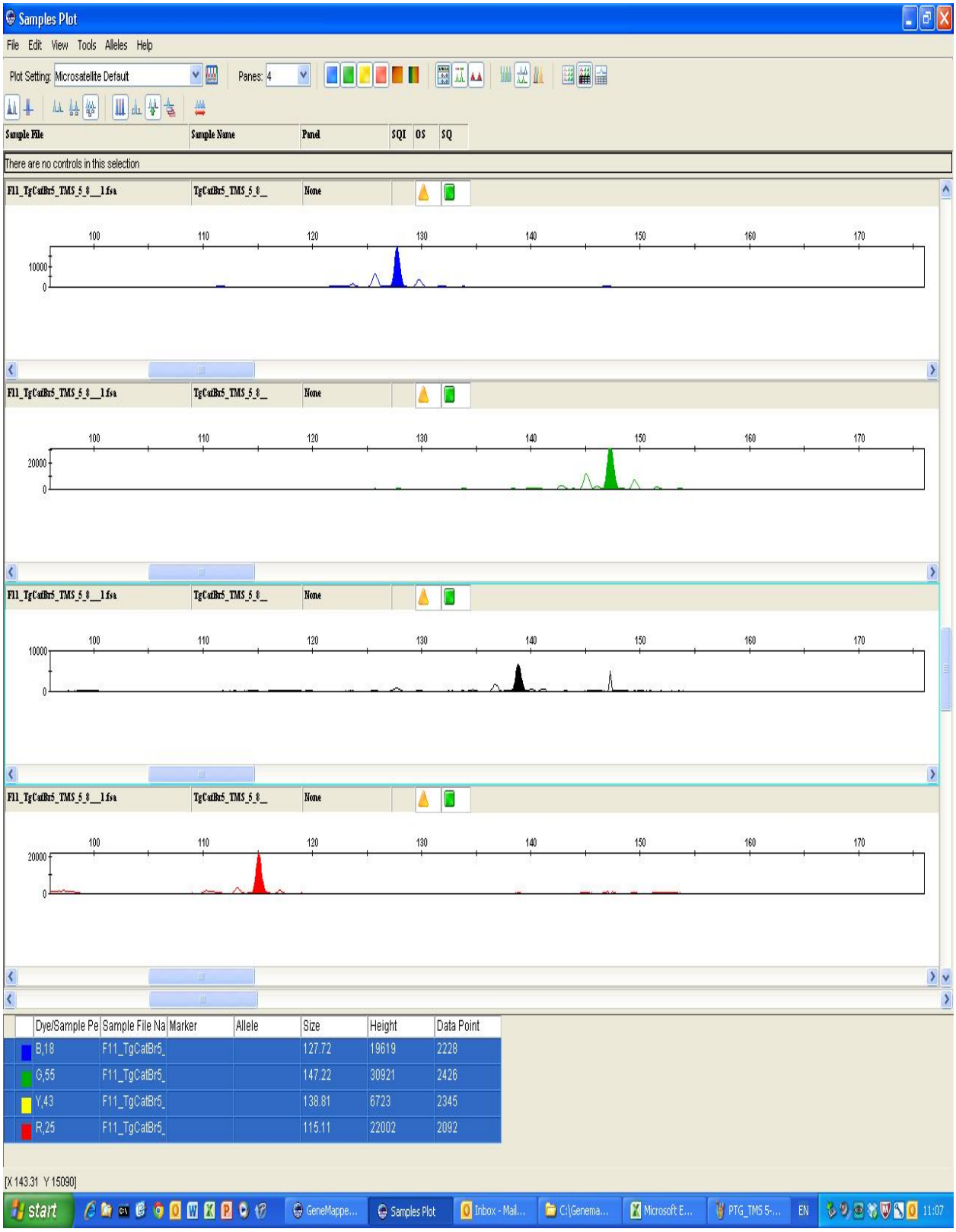


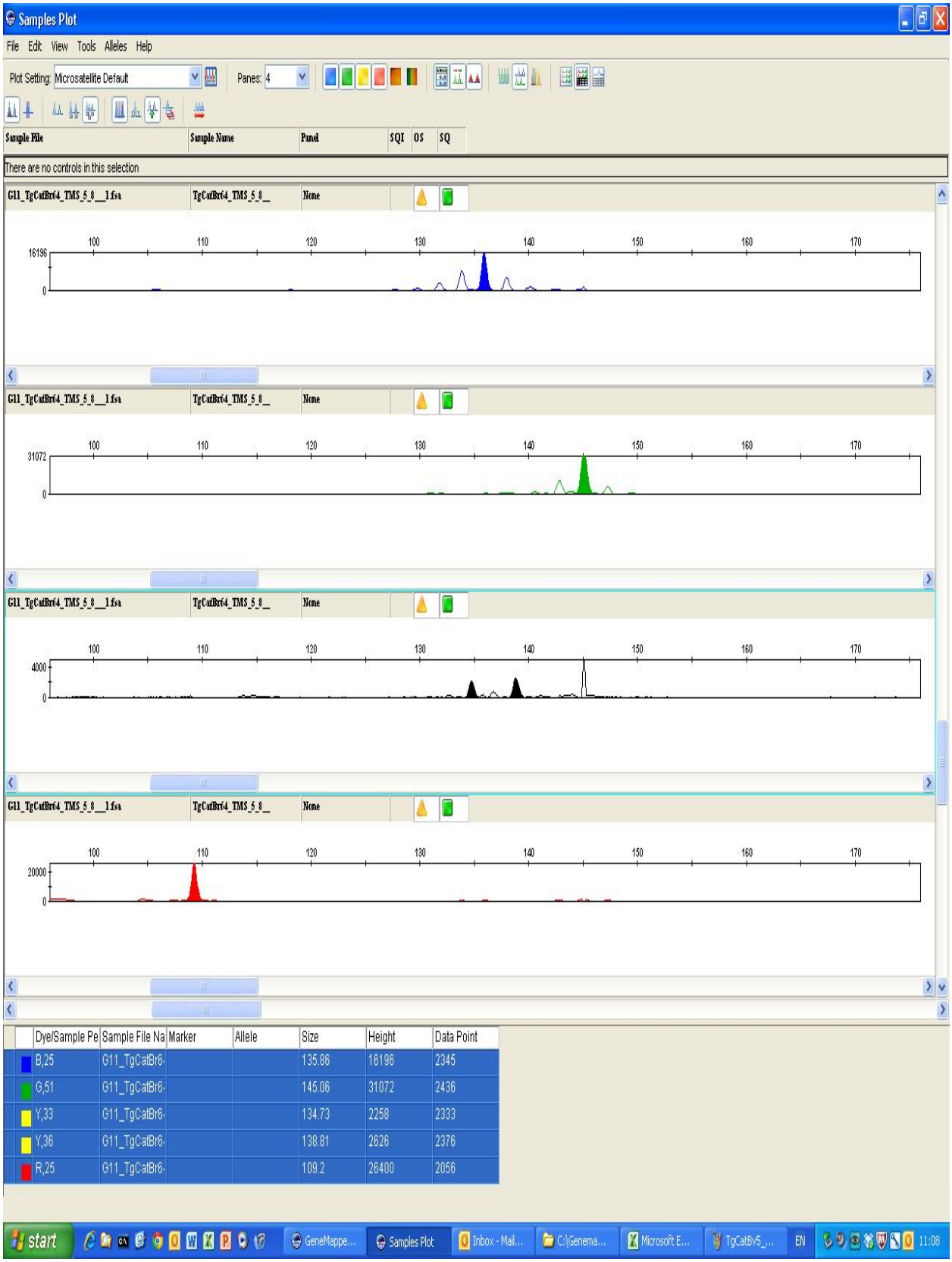


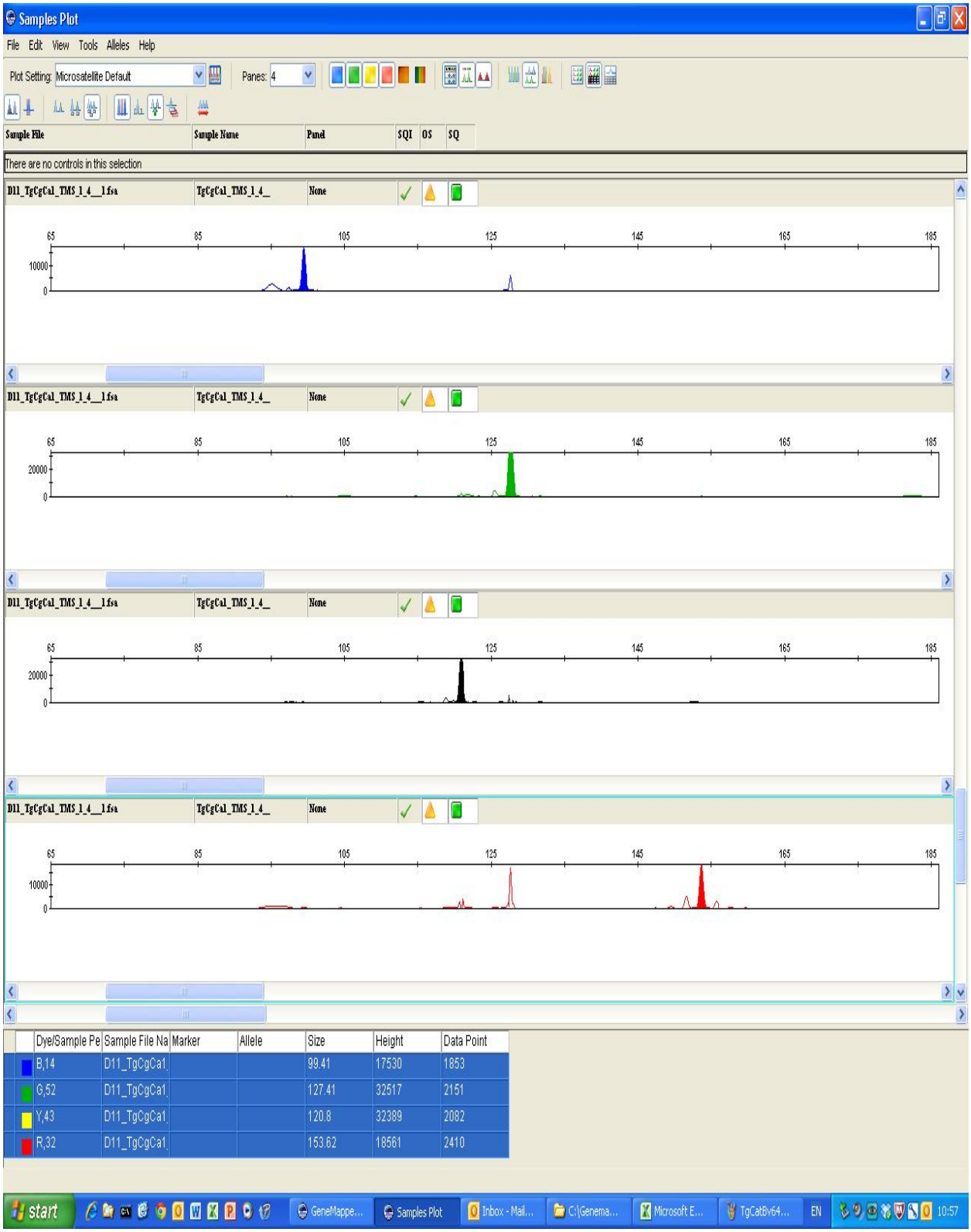


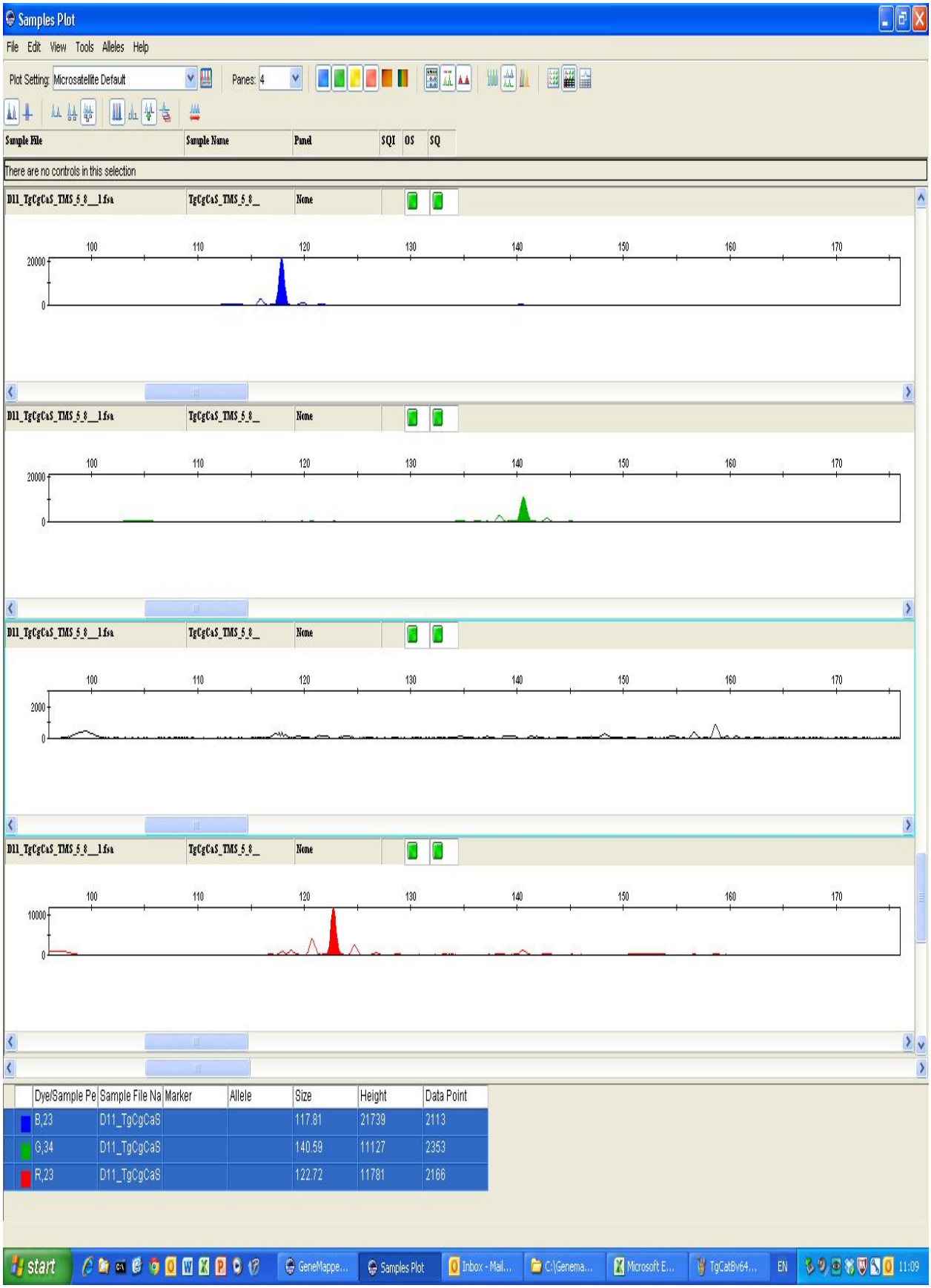


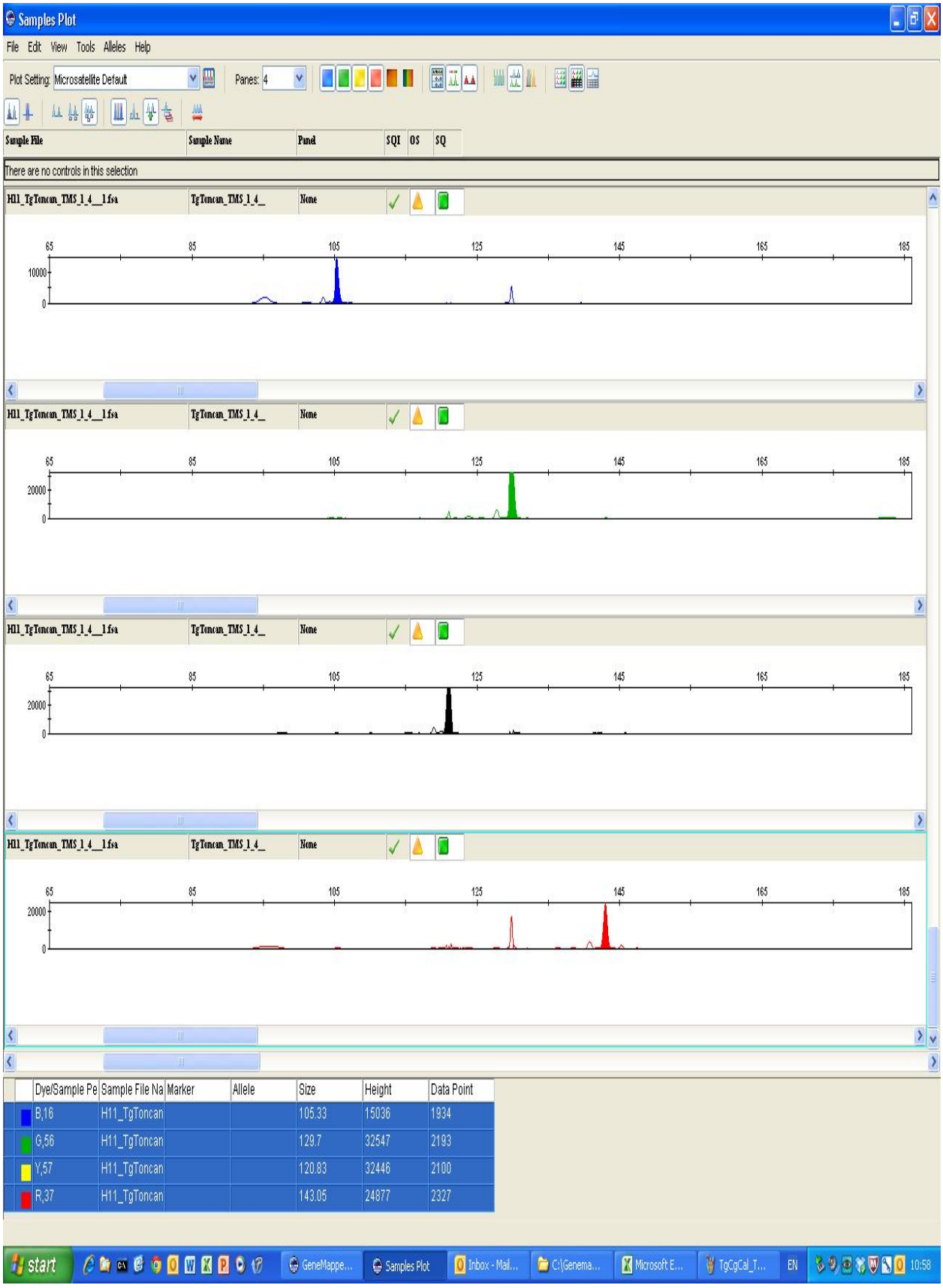


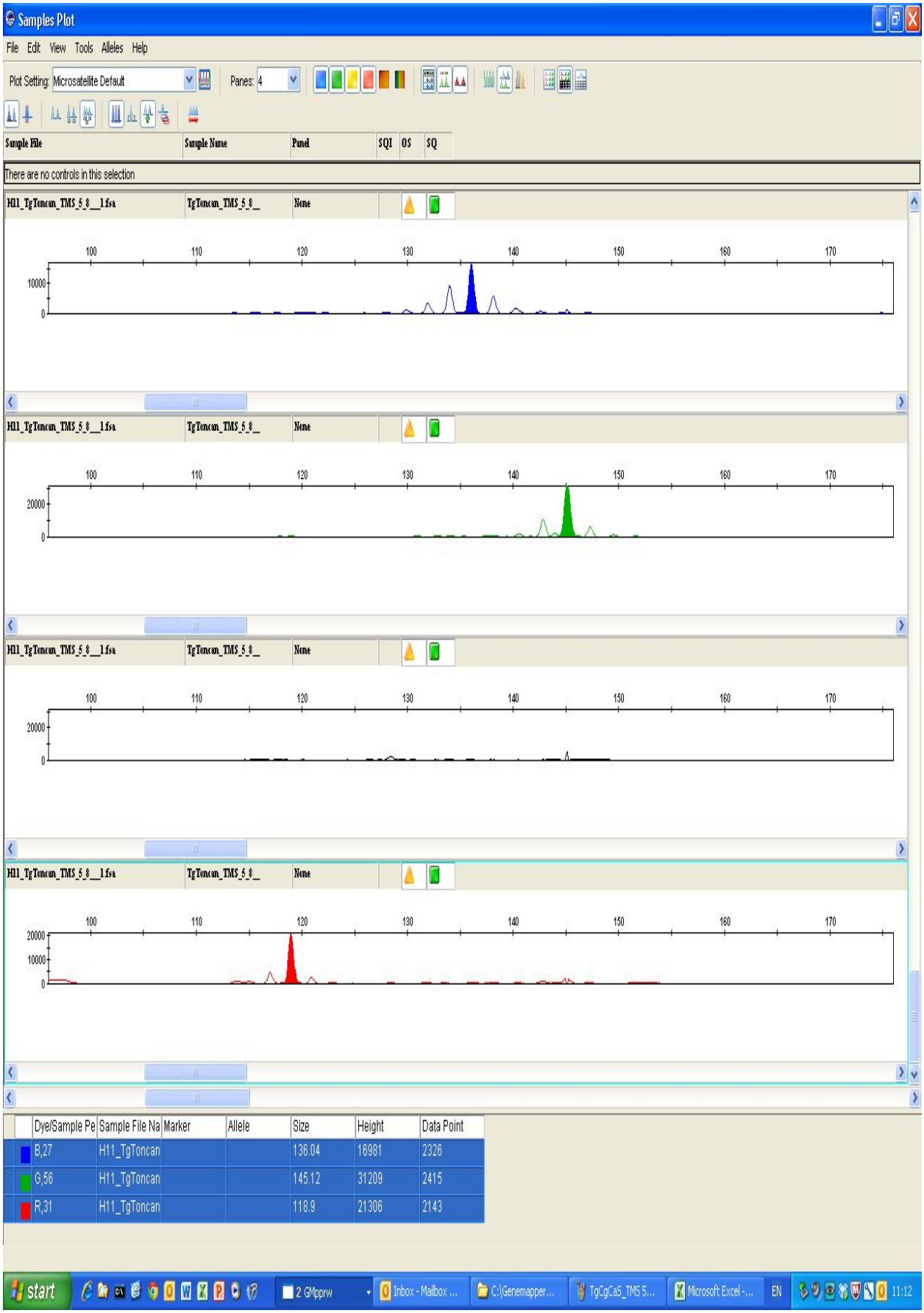


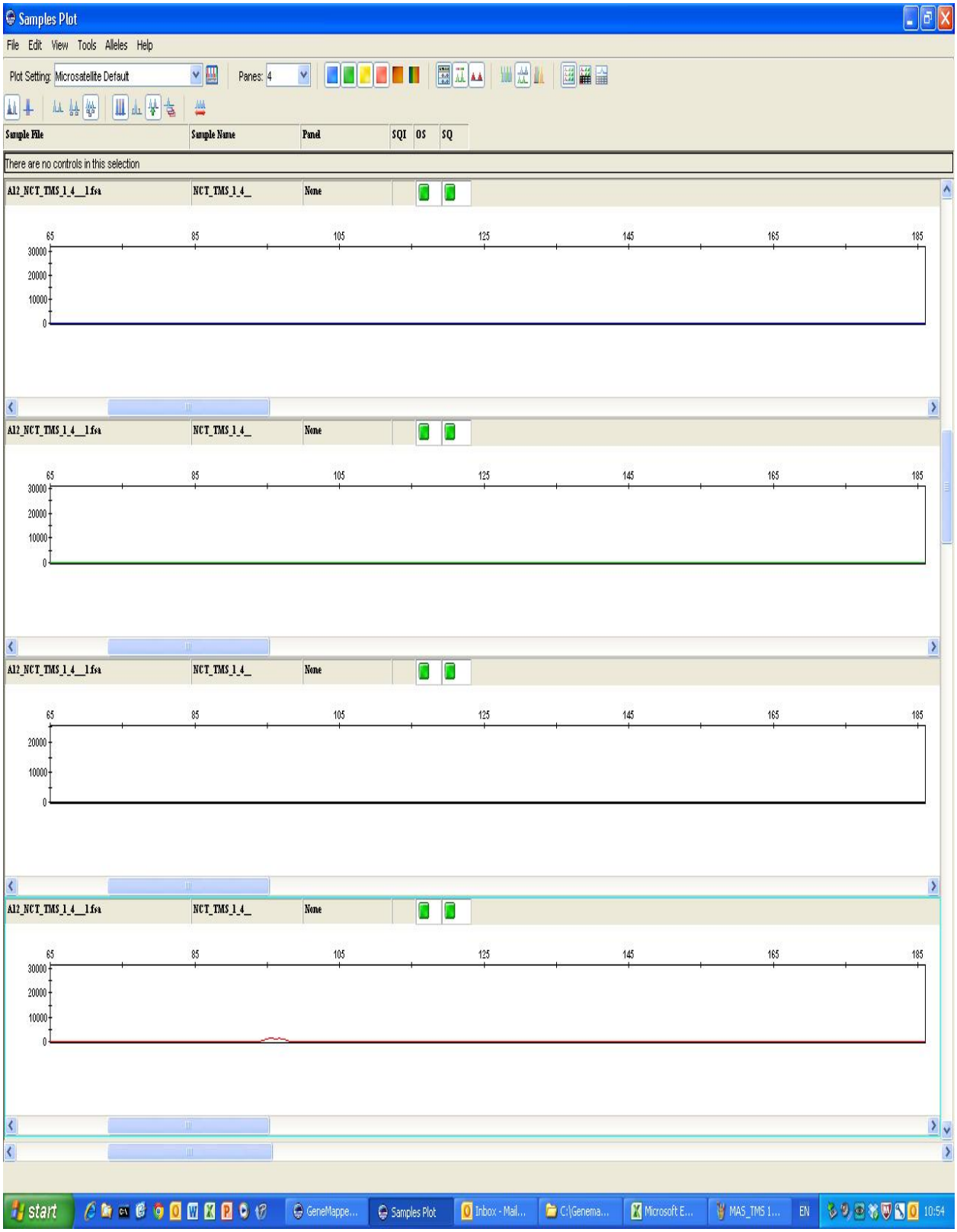












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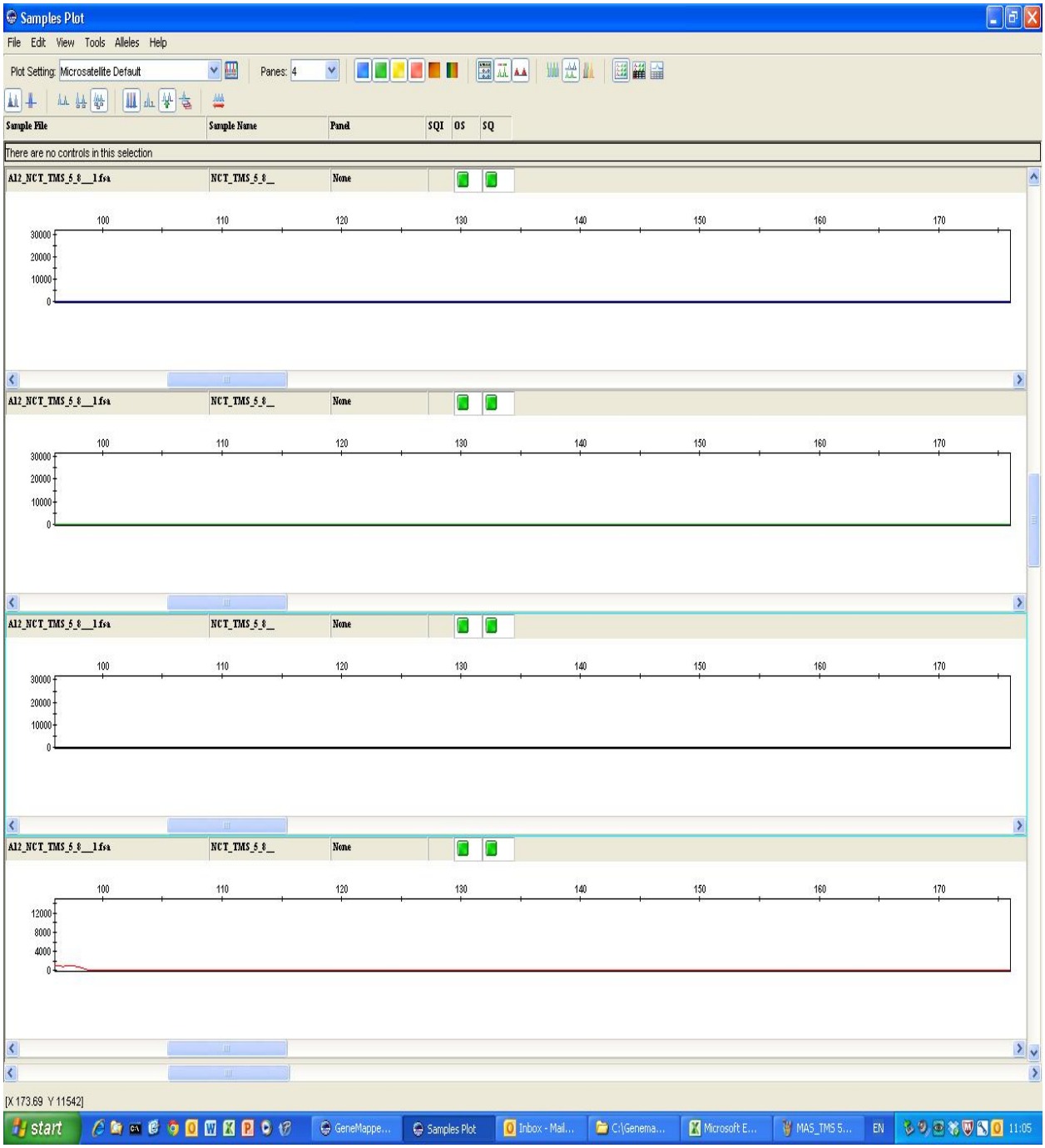
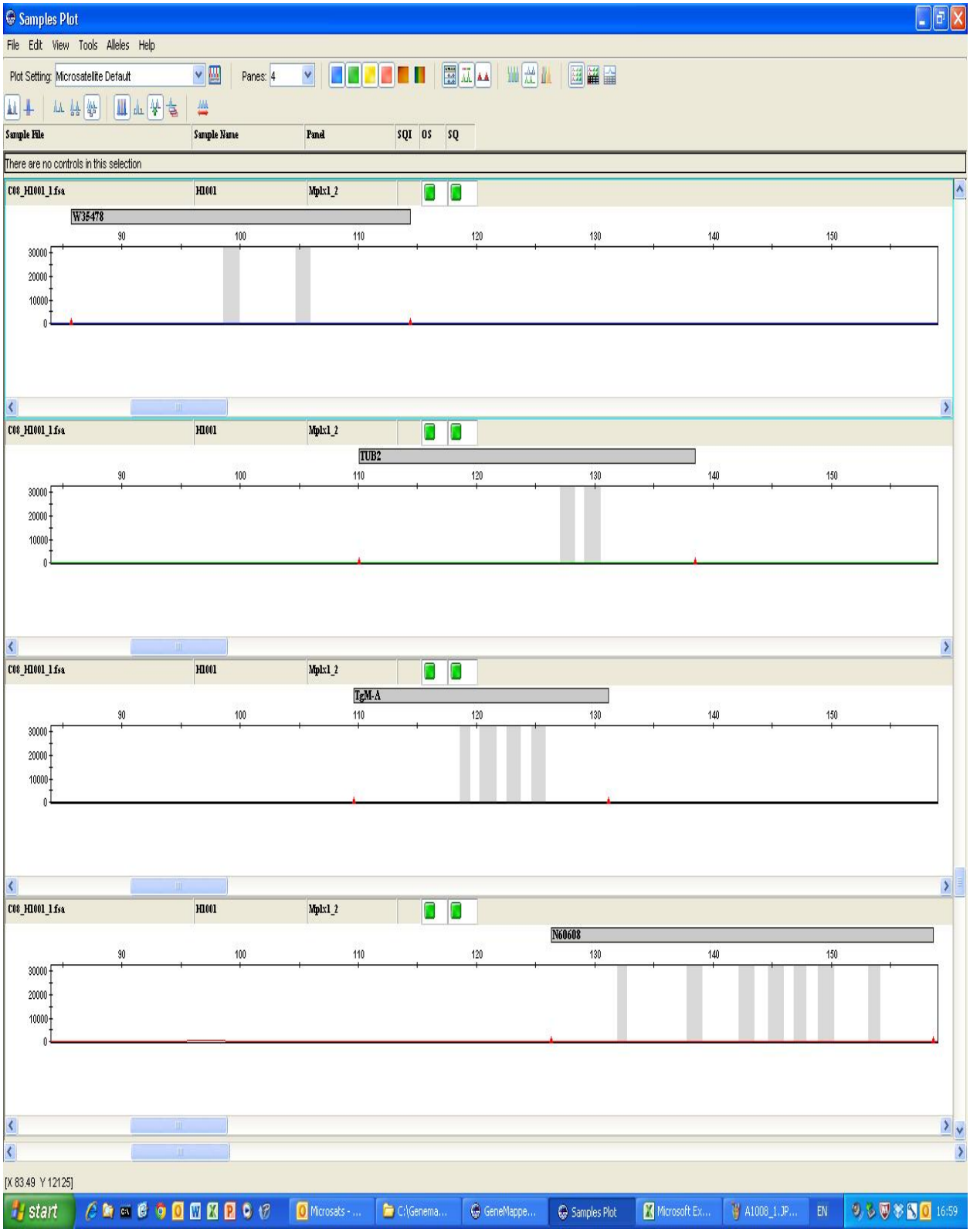
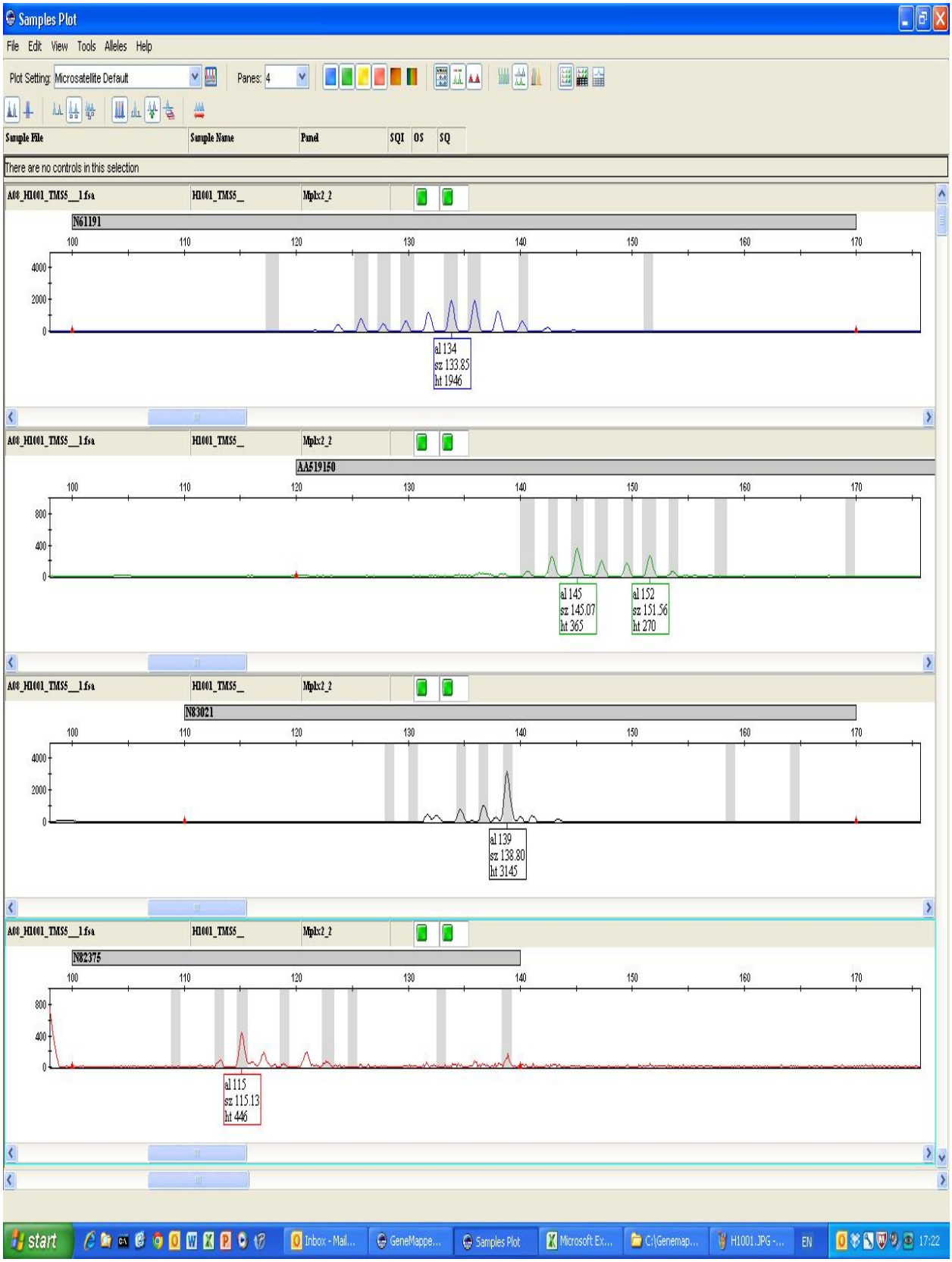
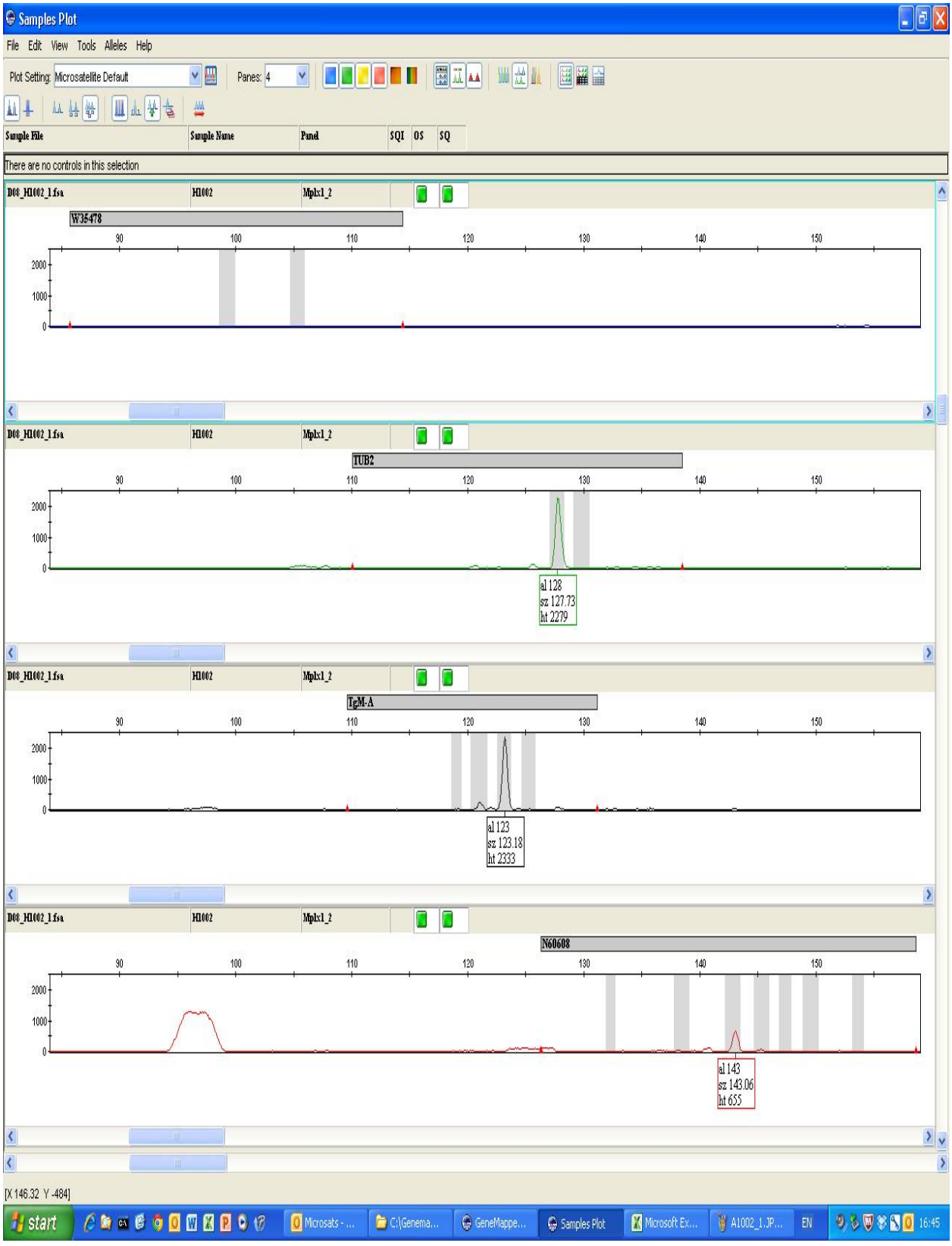
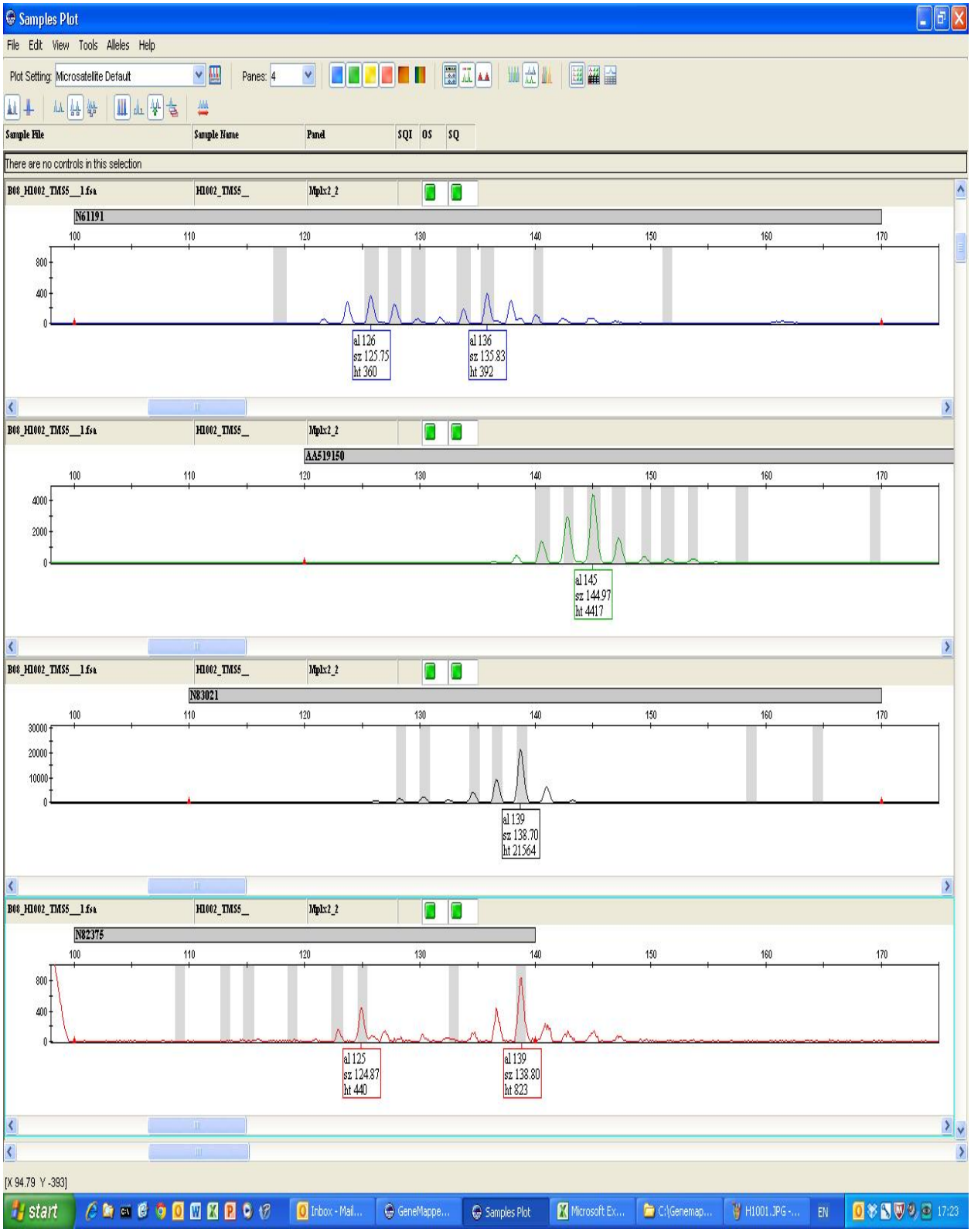


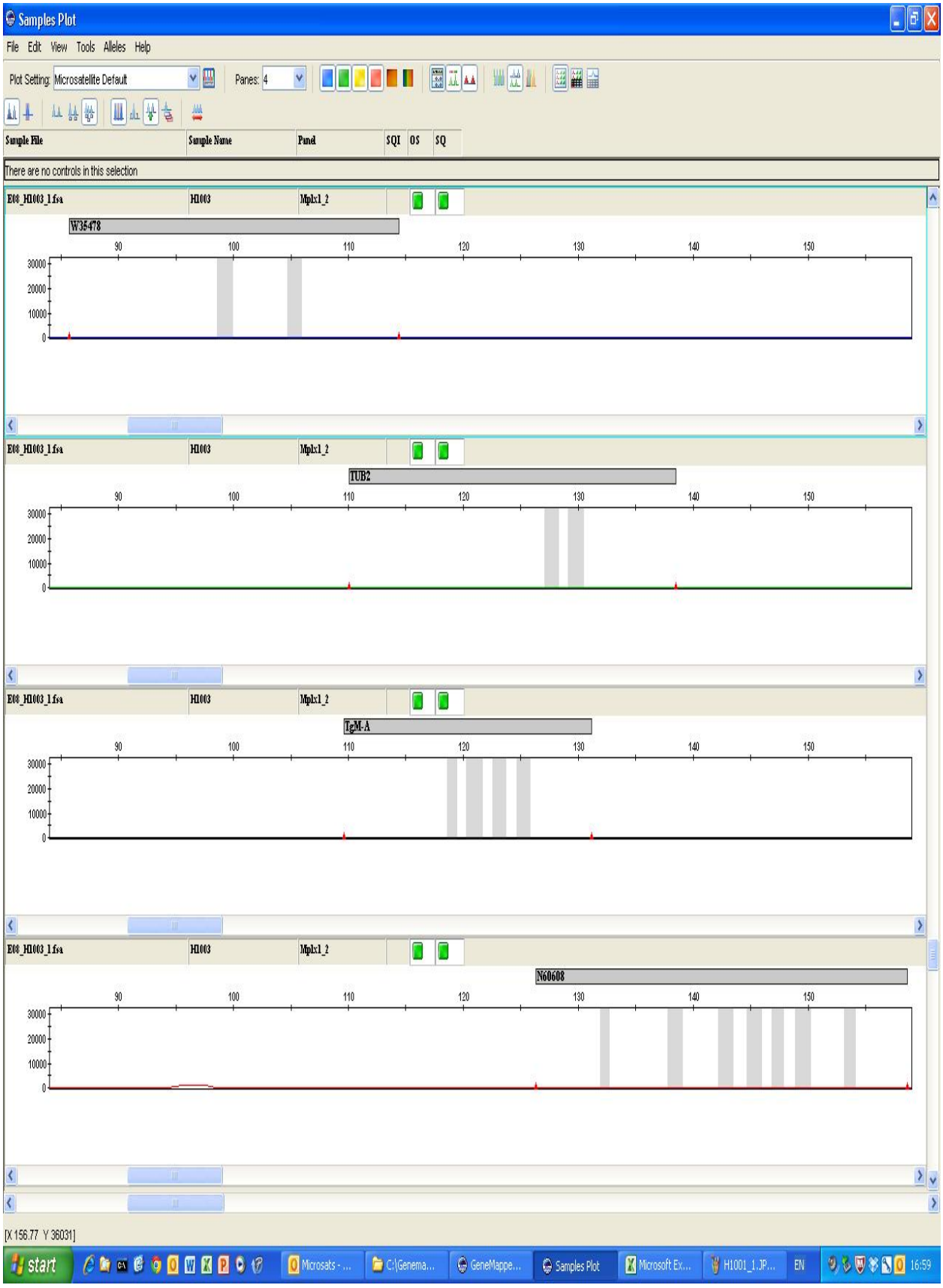
Human sample plots

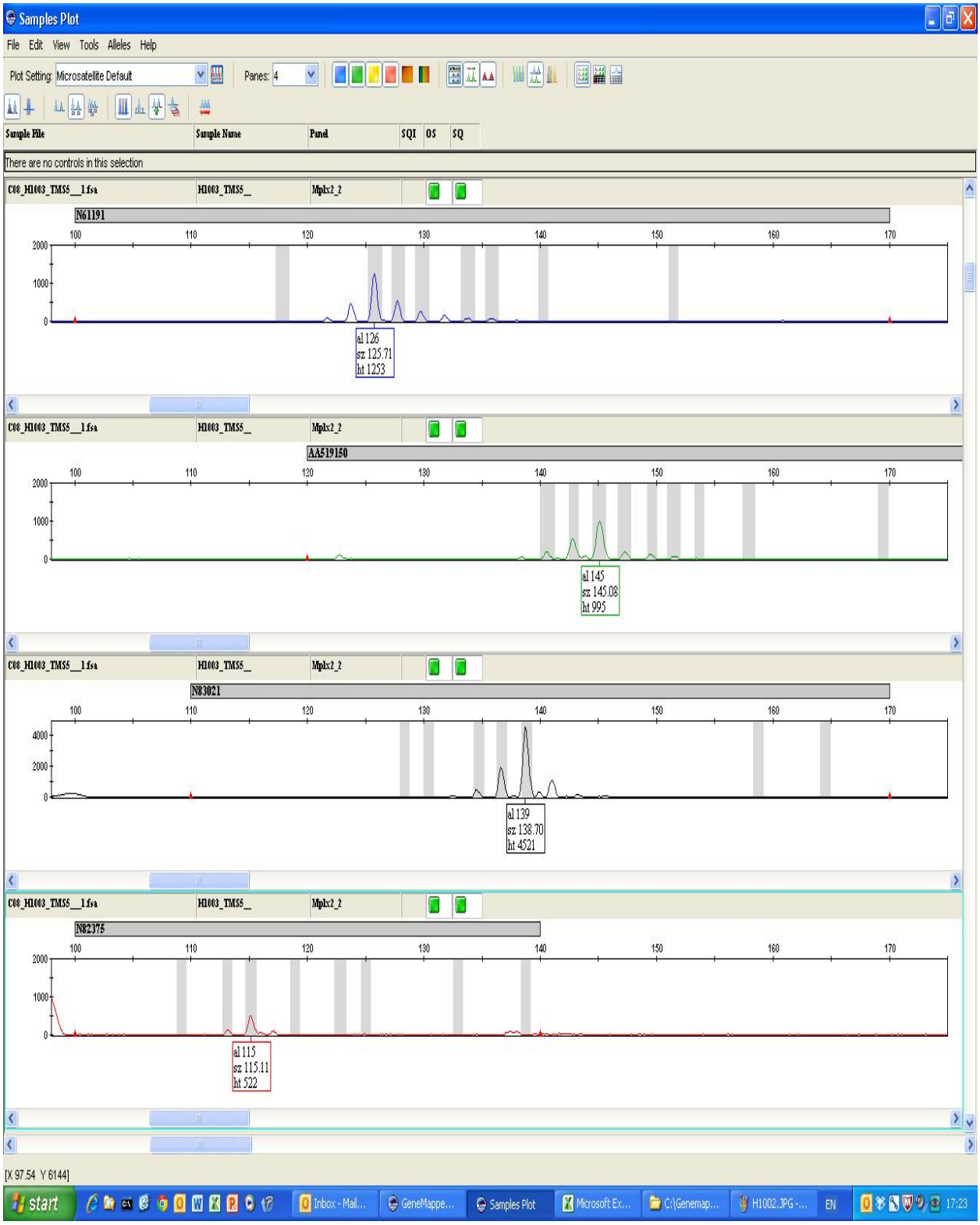


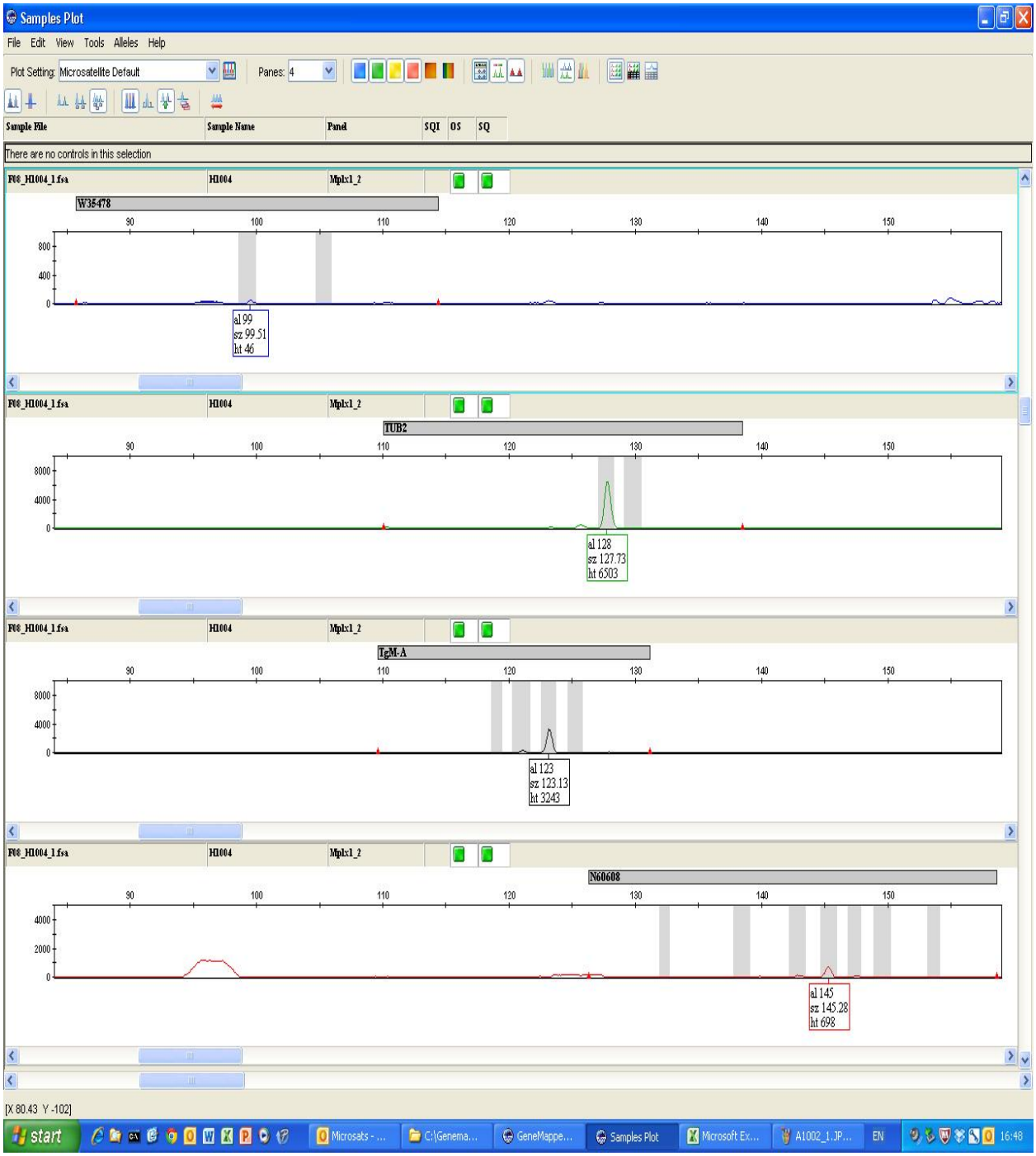


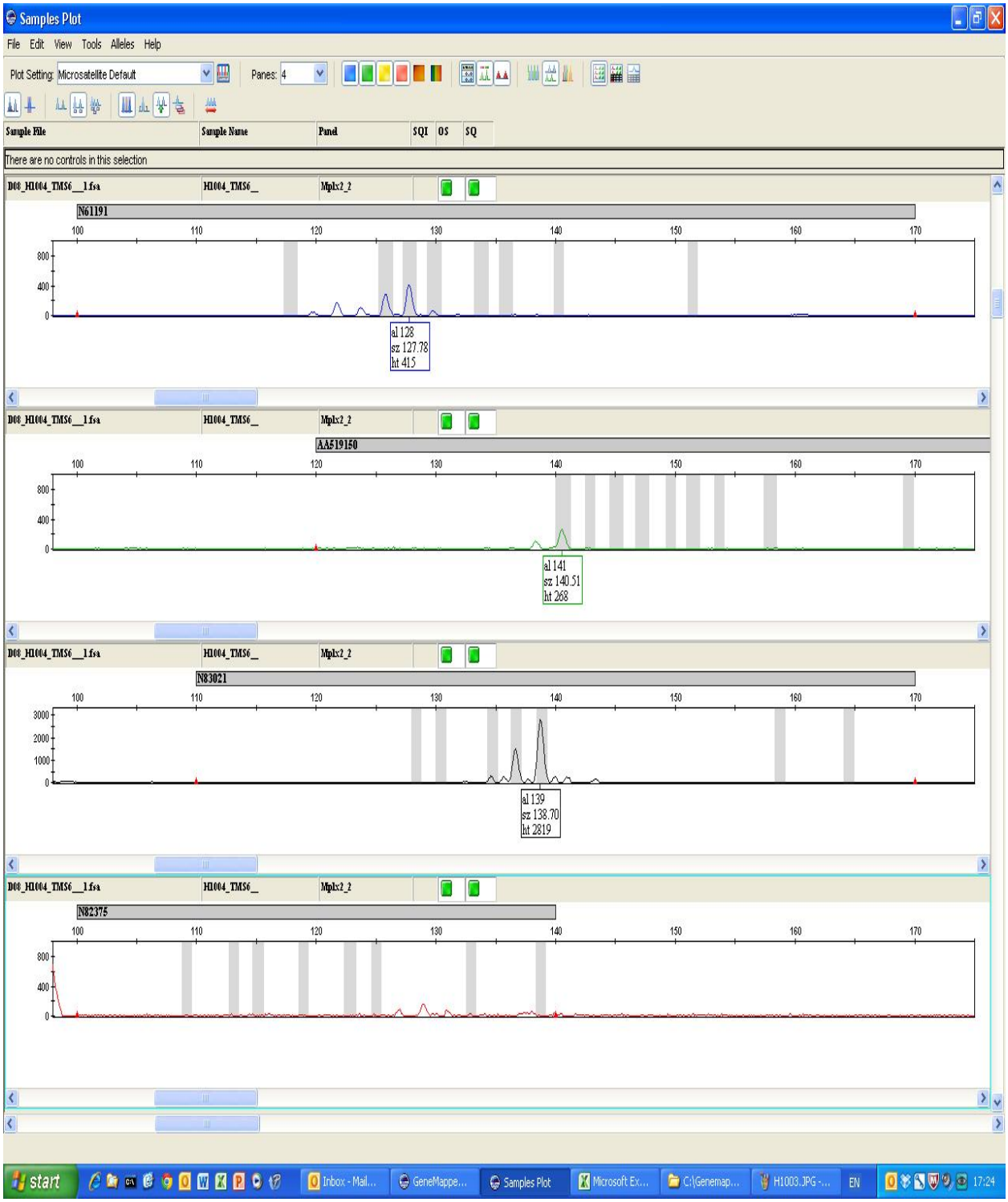


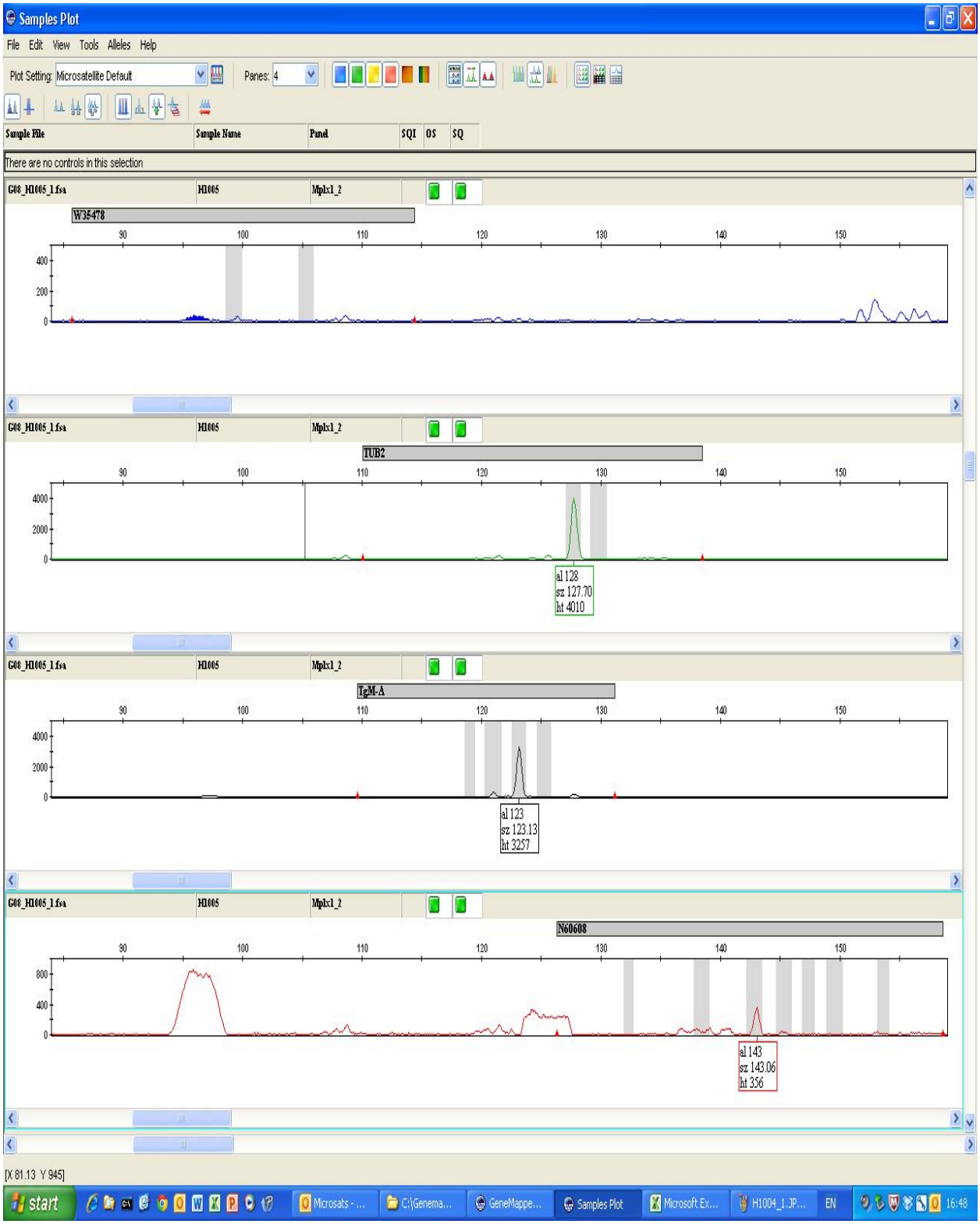


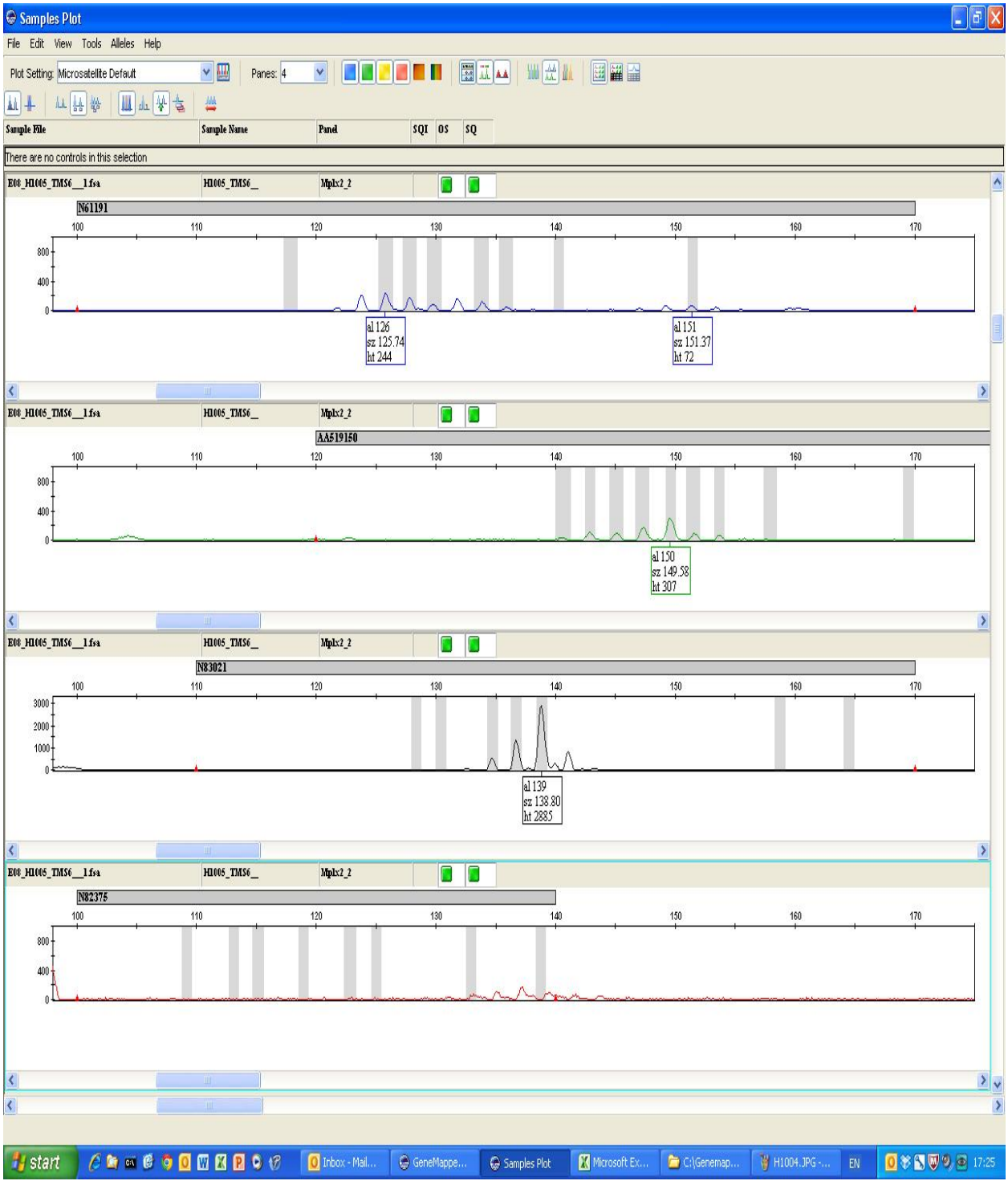


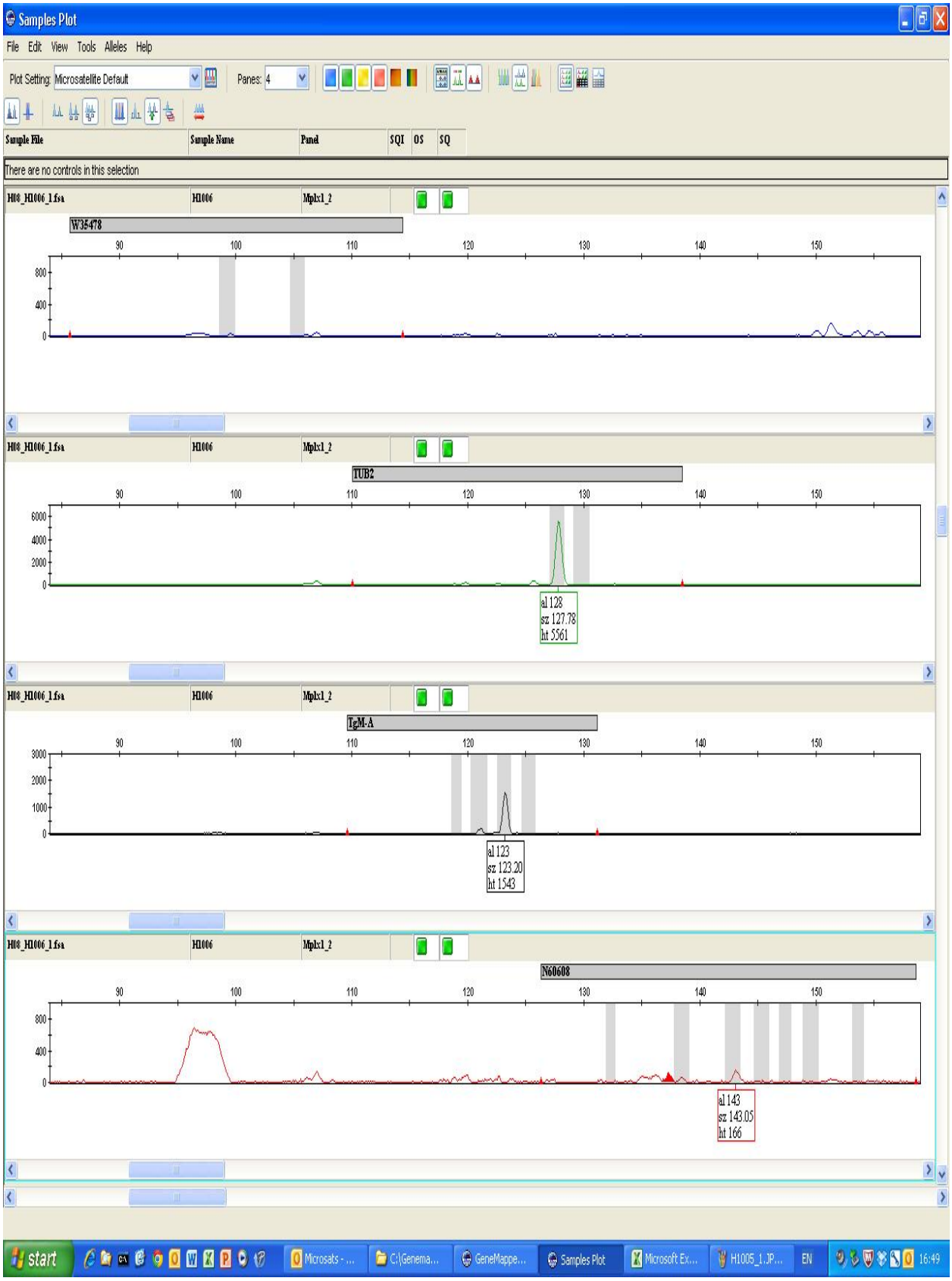


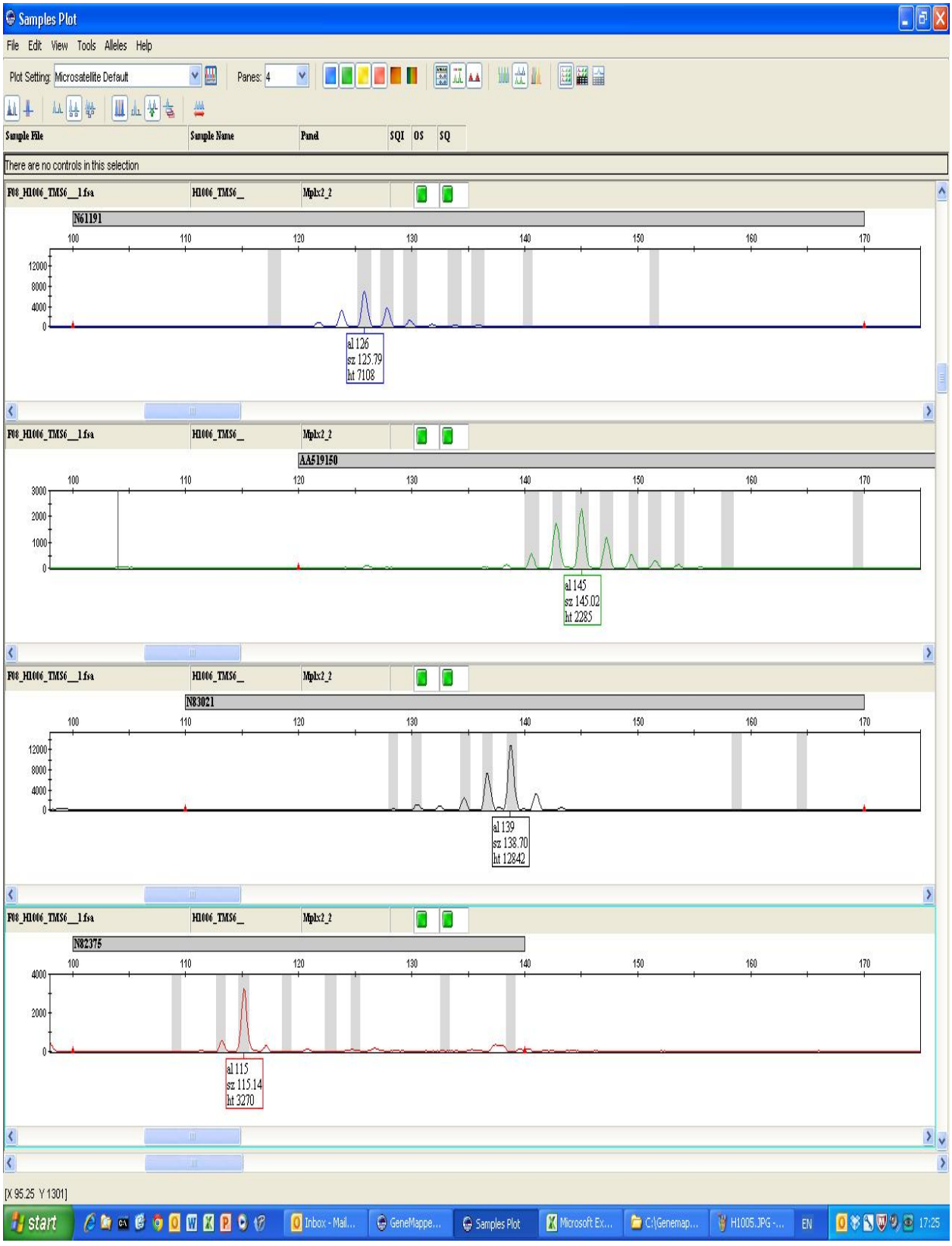


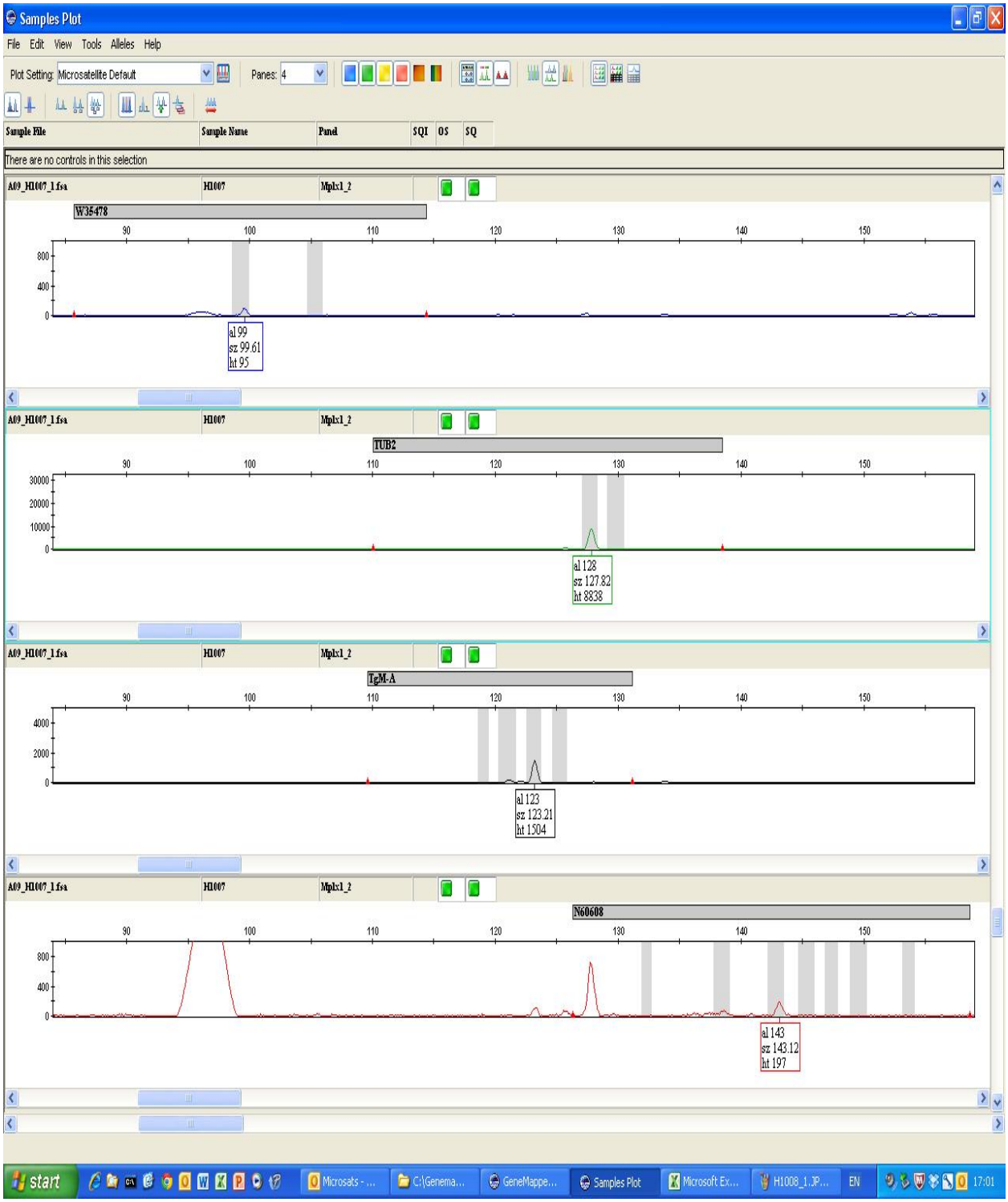


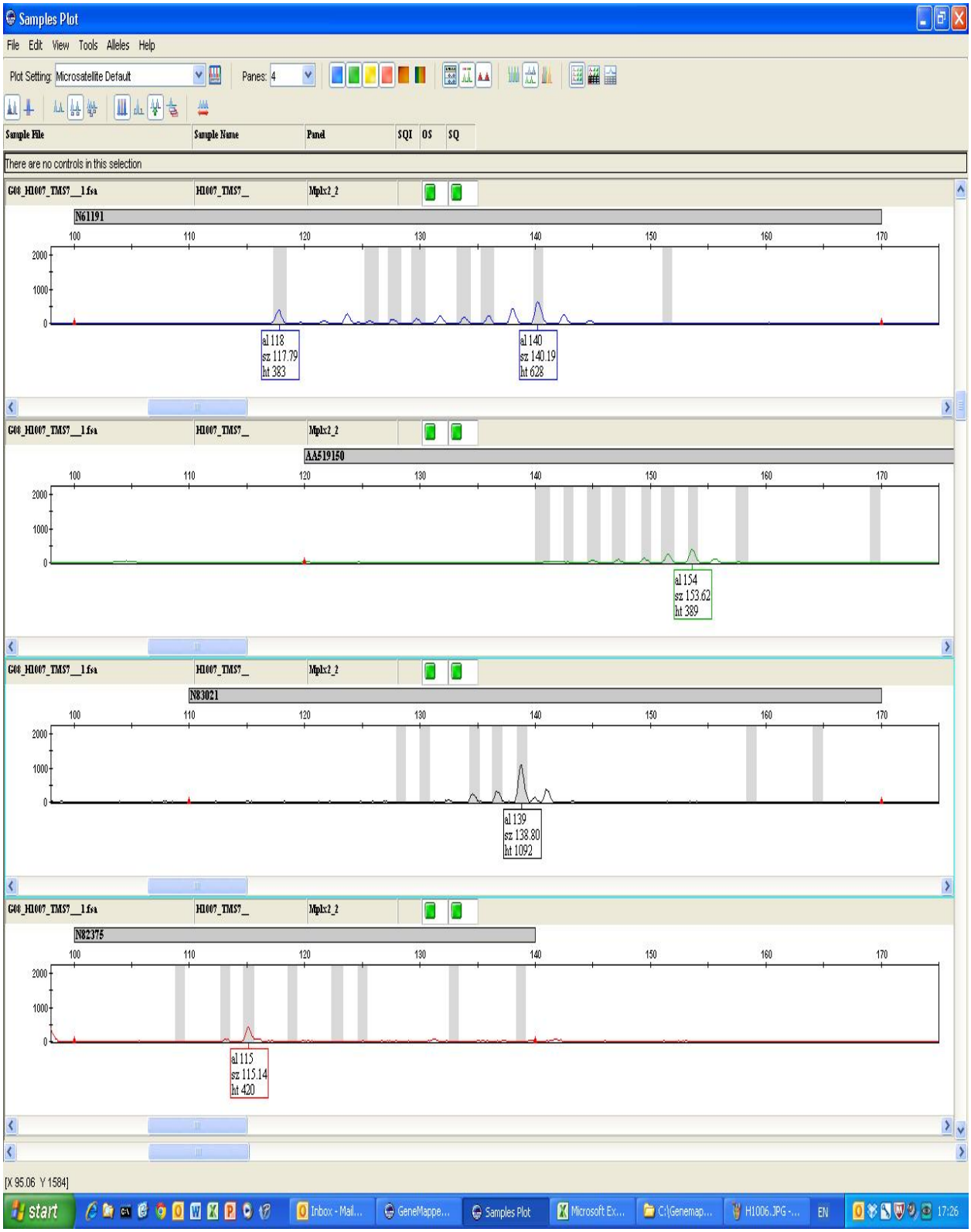


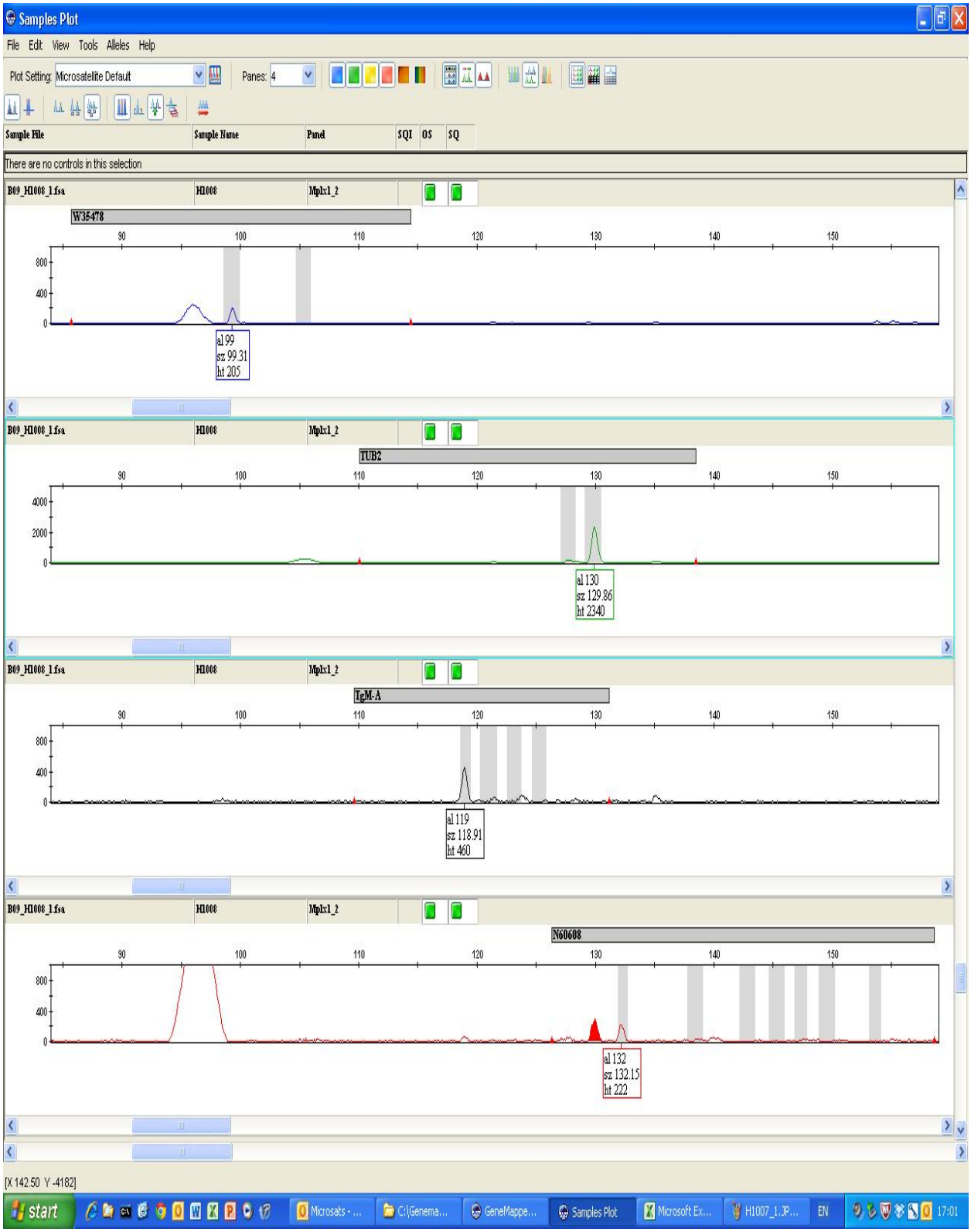


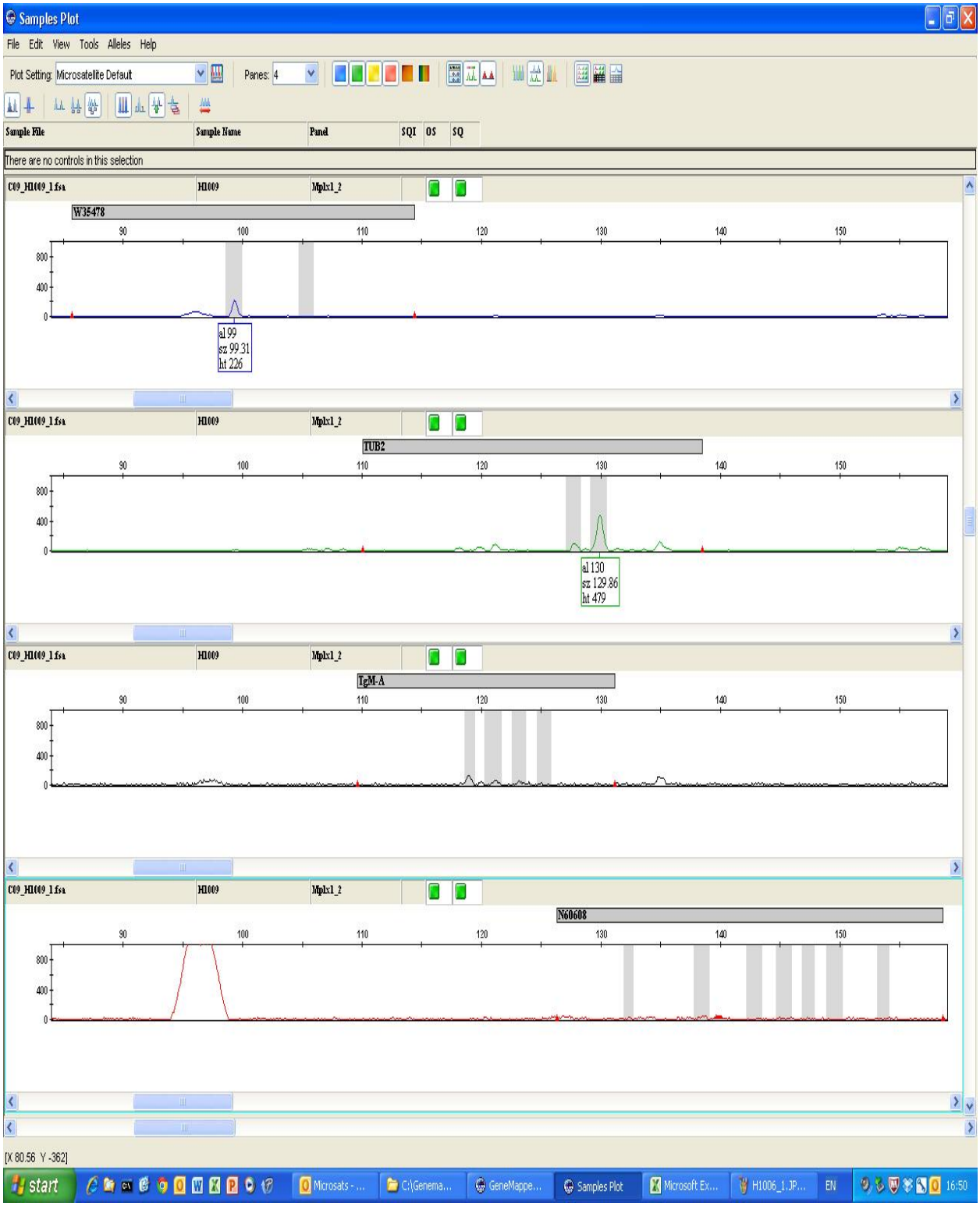


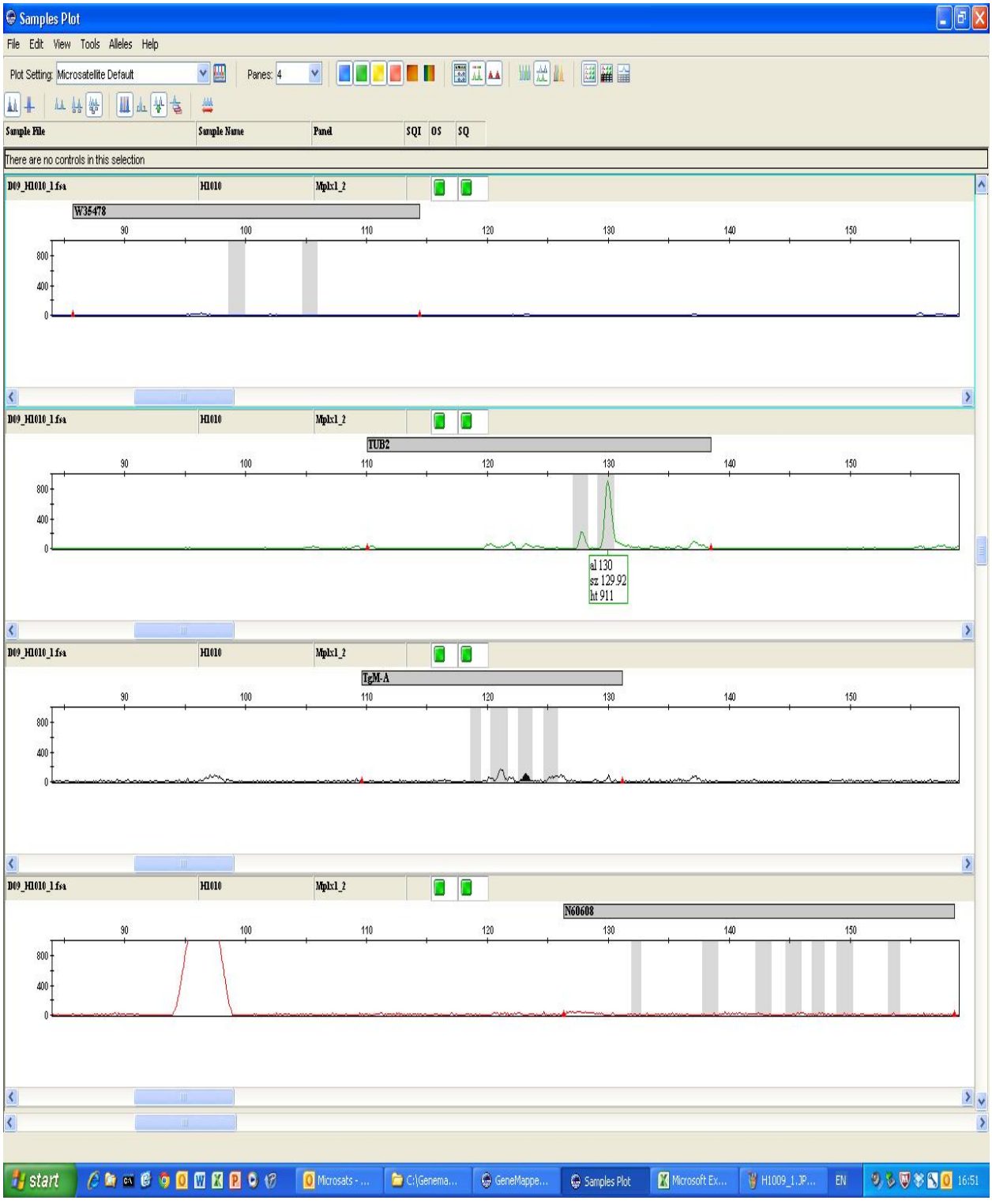


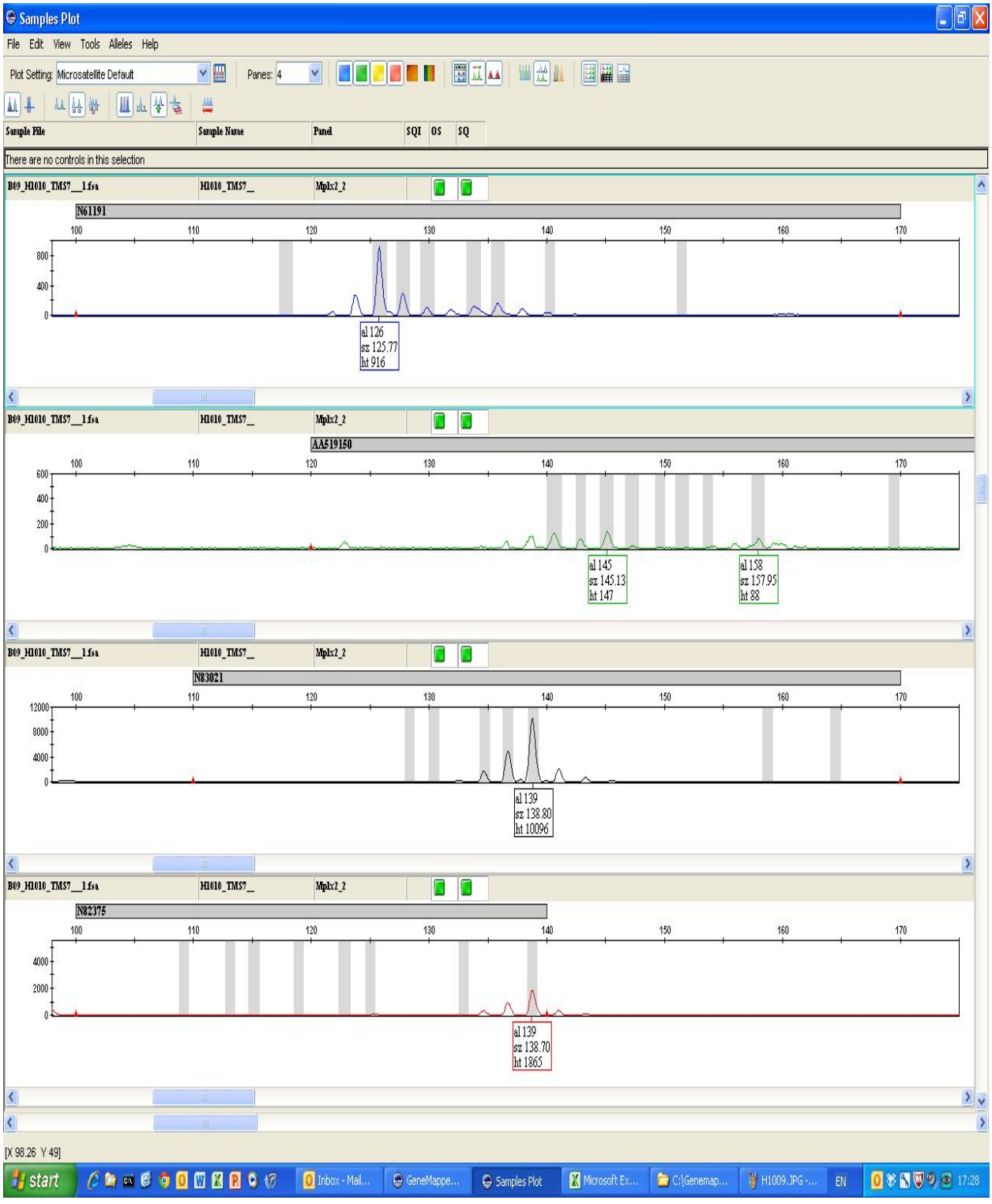


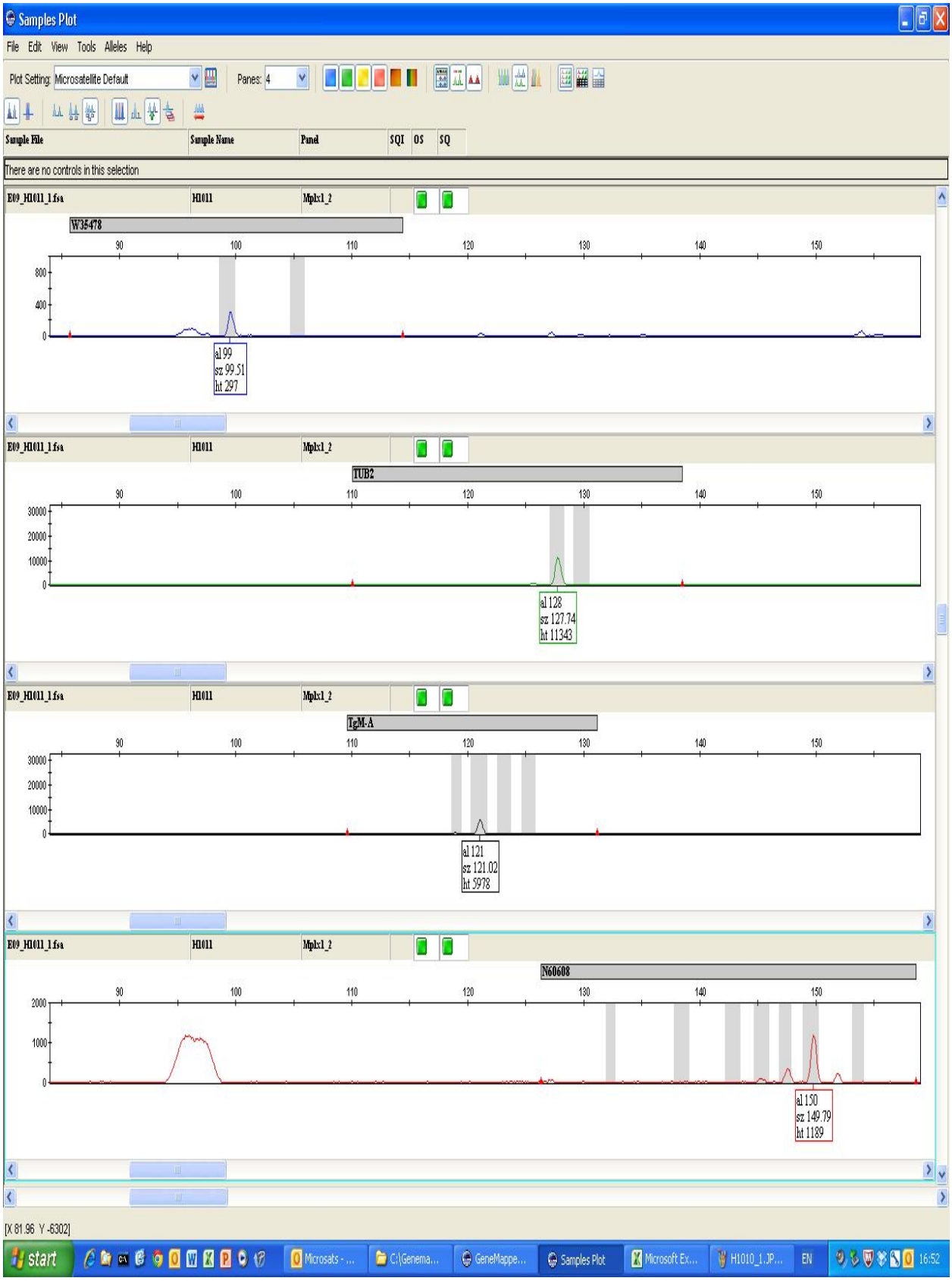


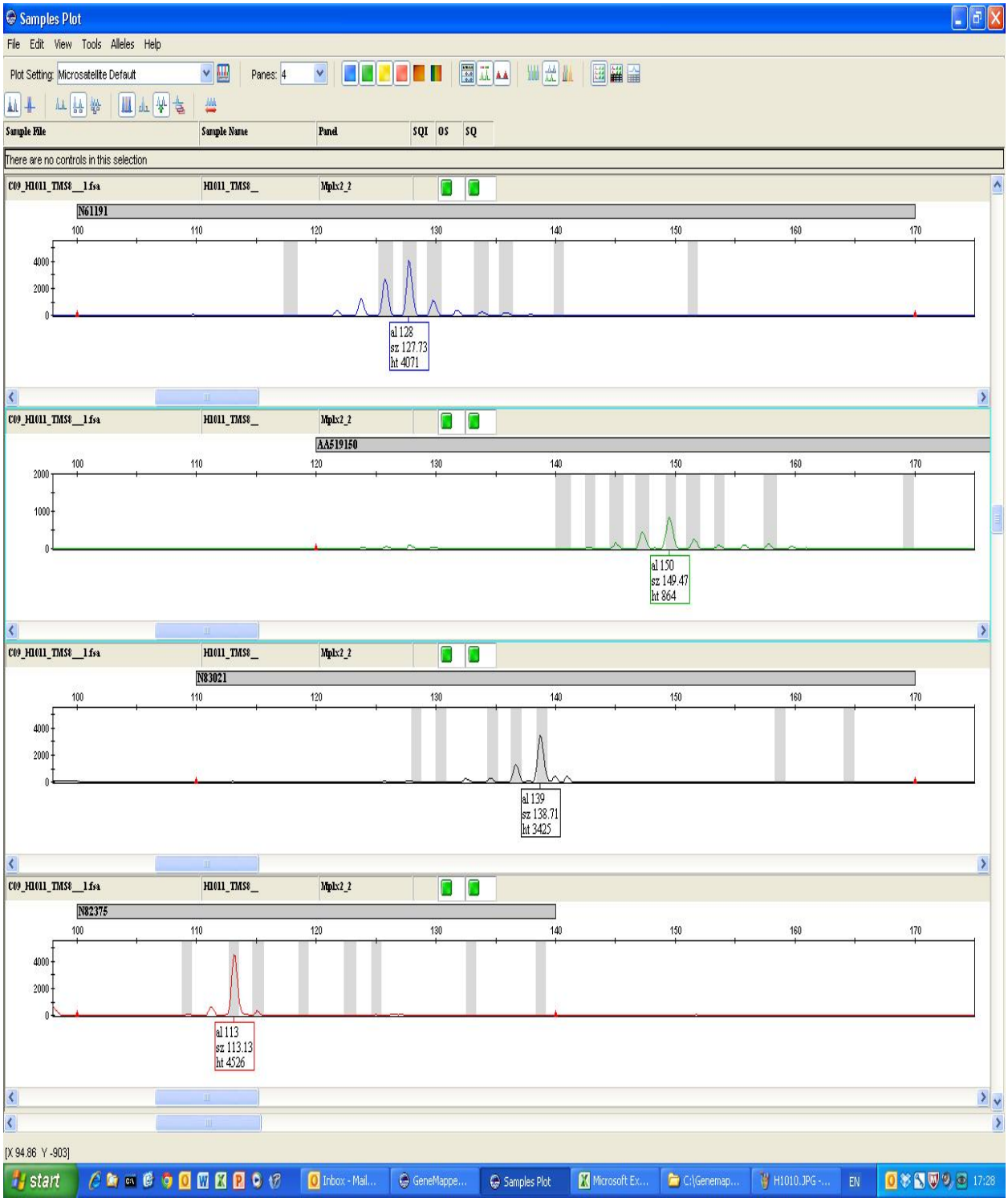


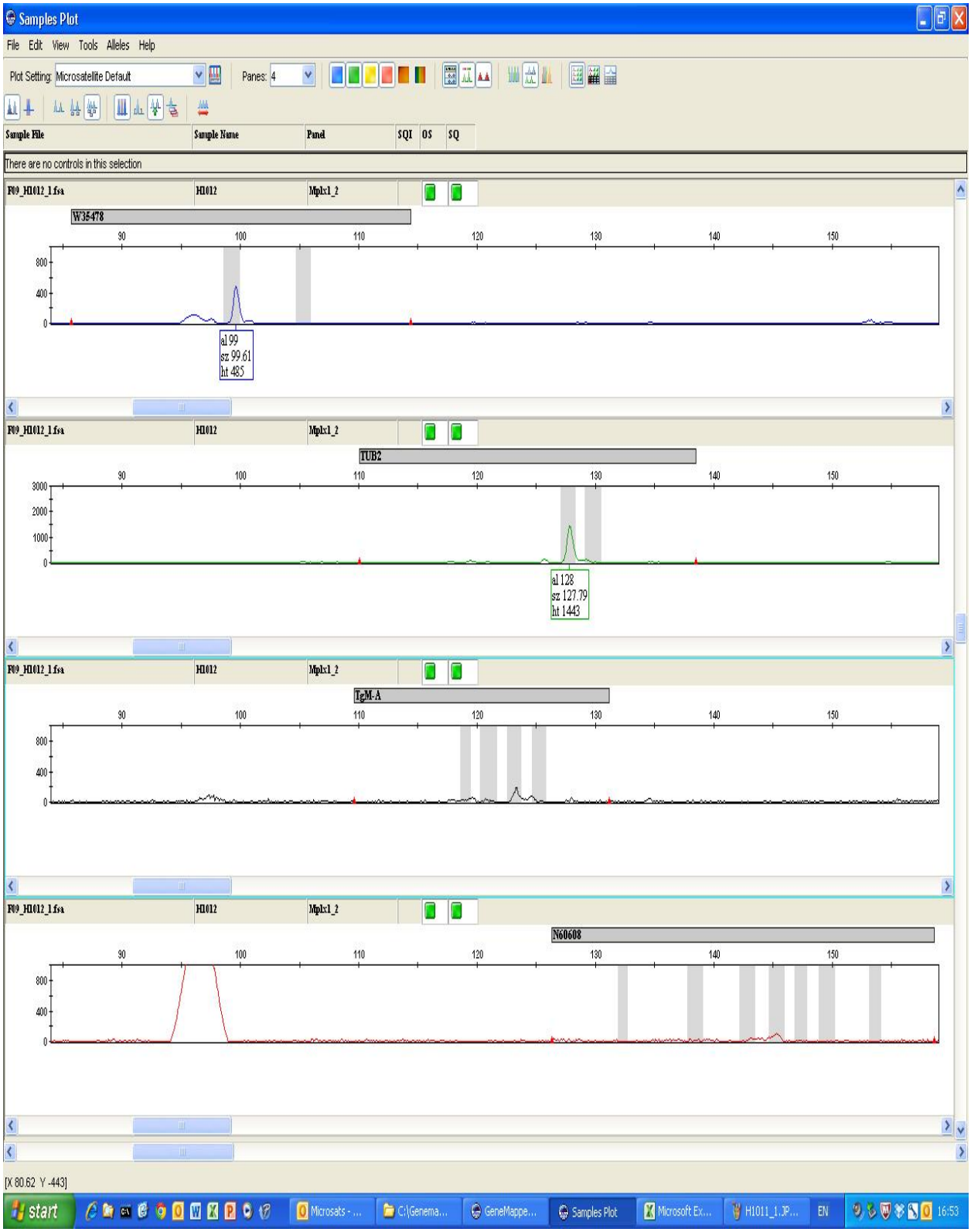


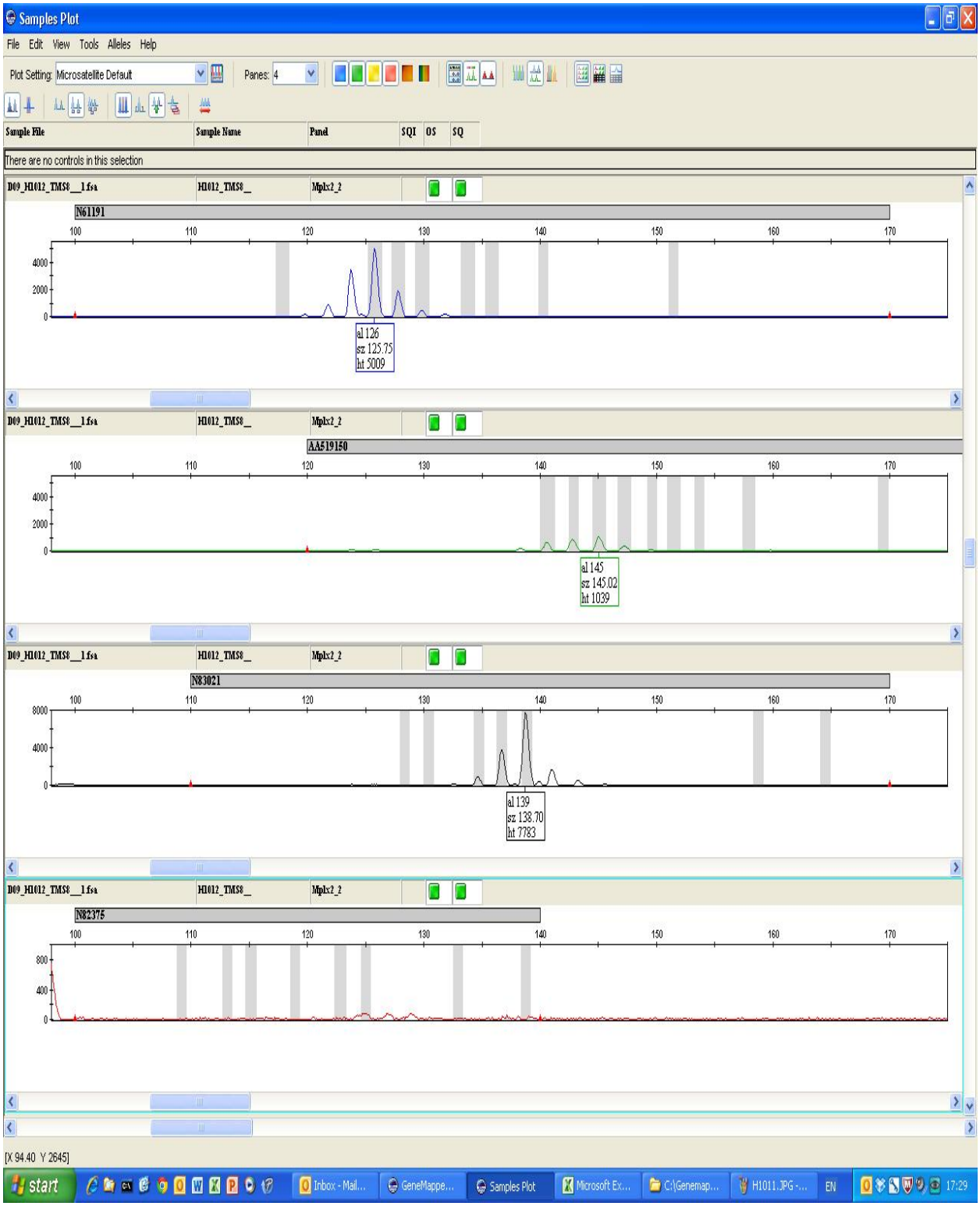


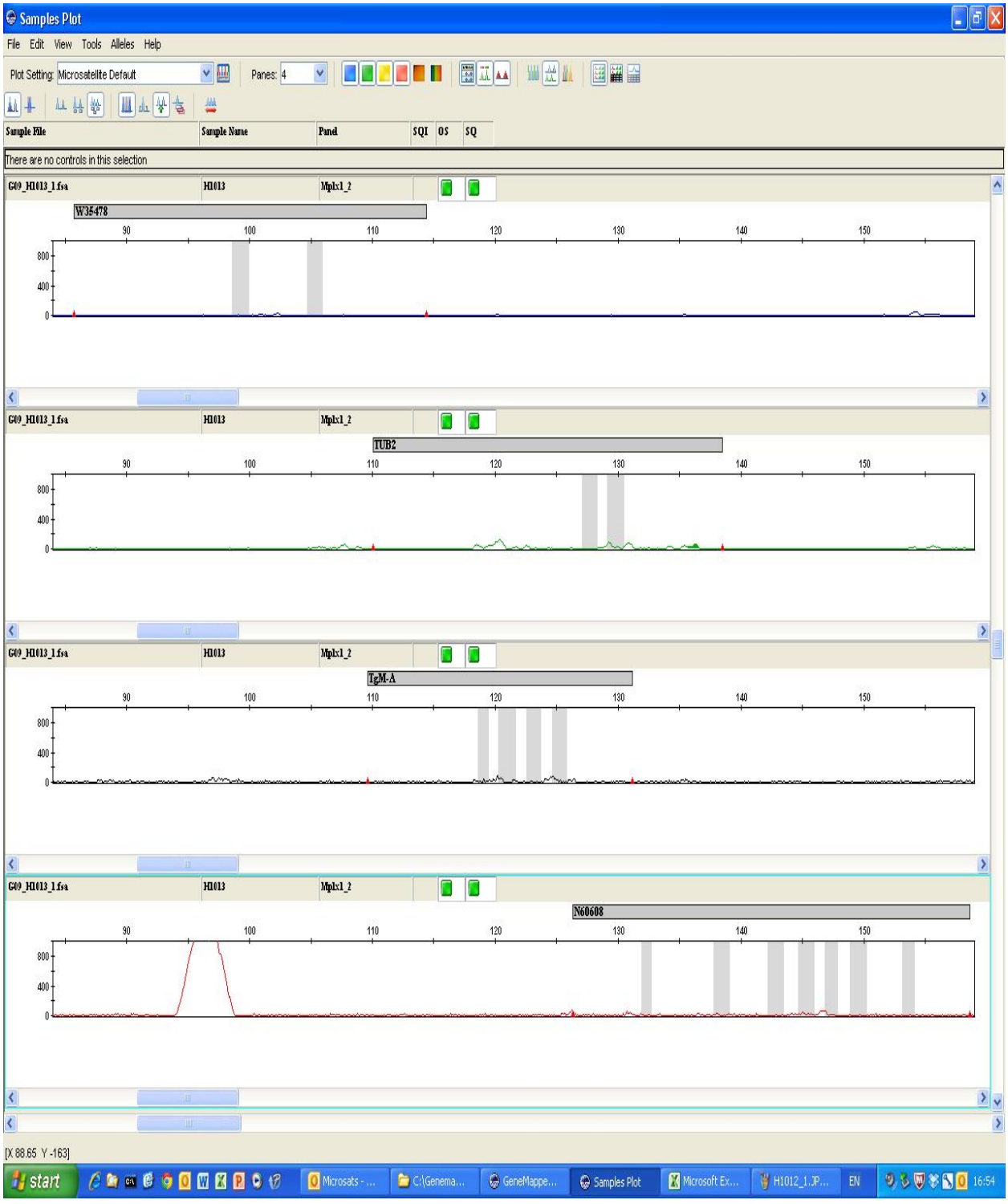


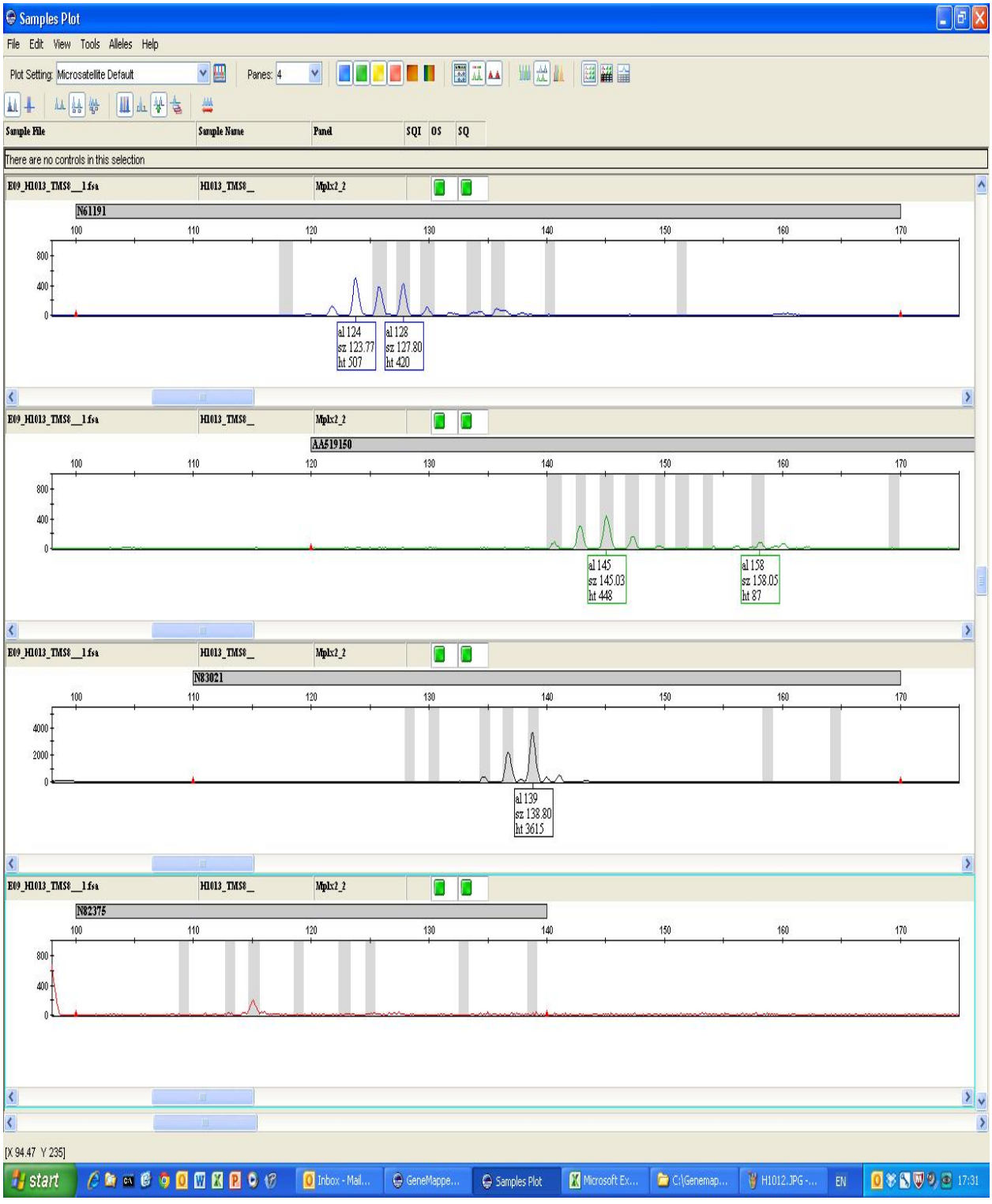


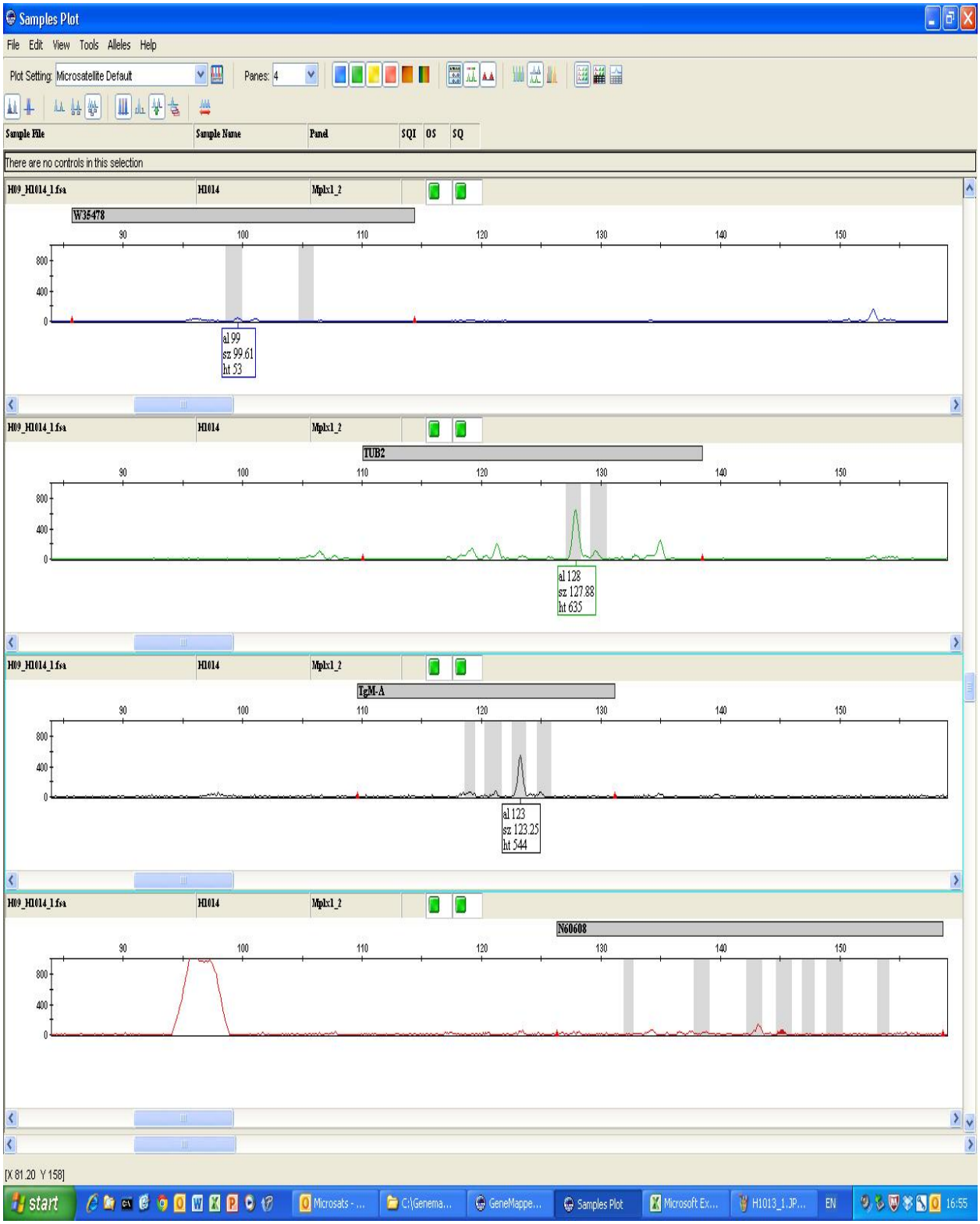


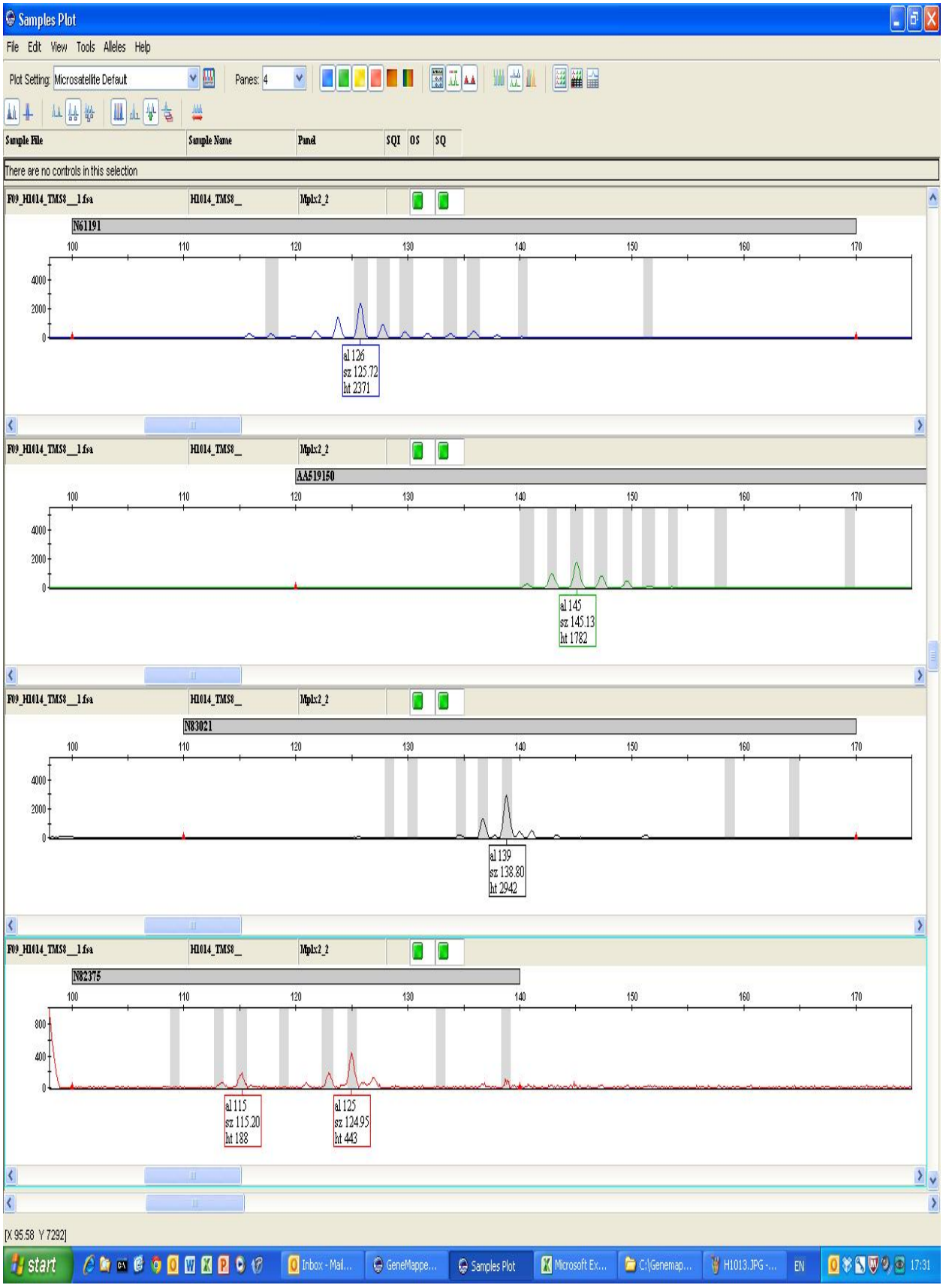


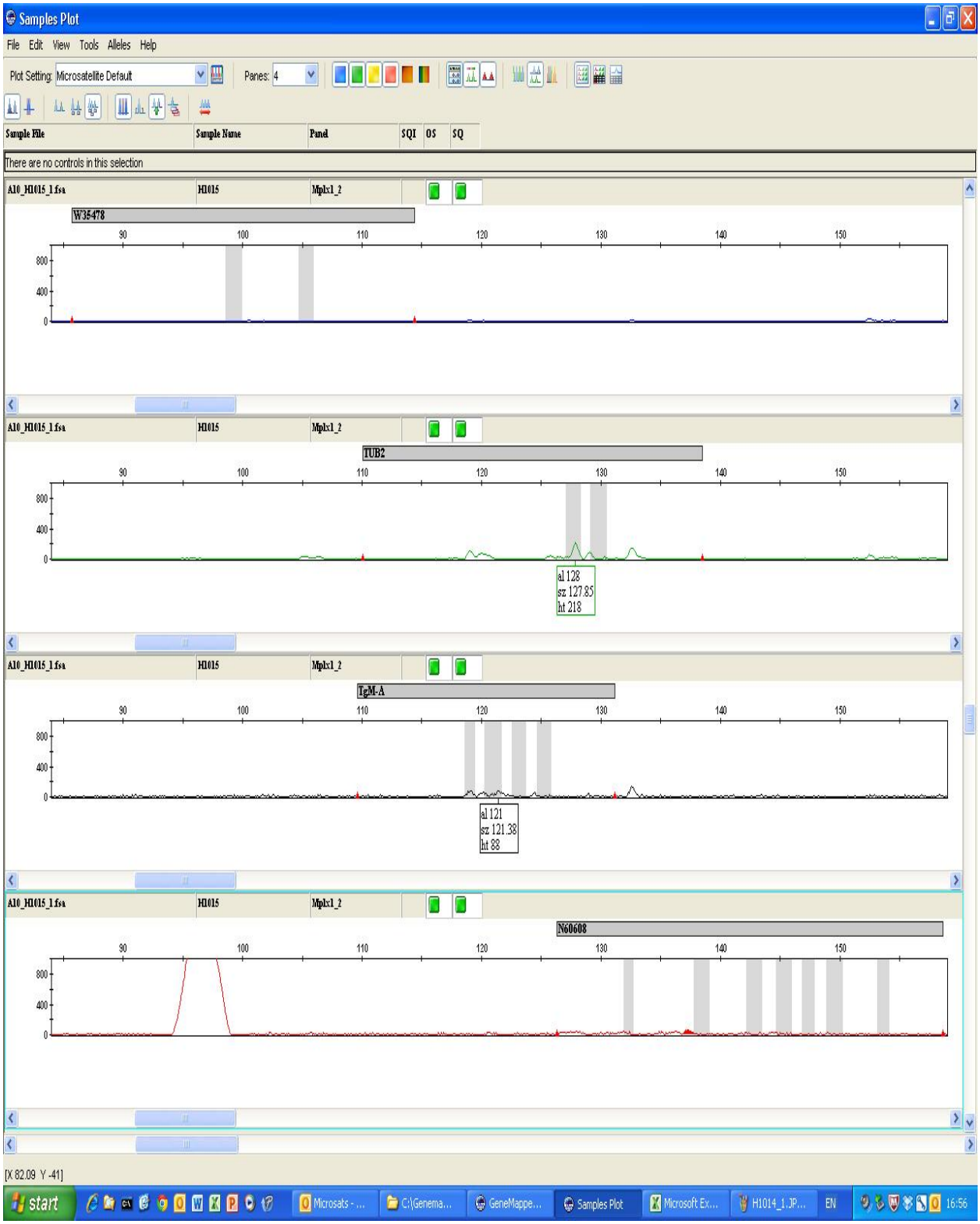


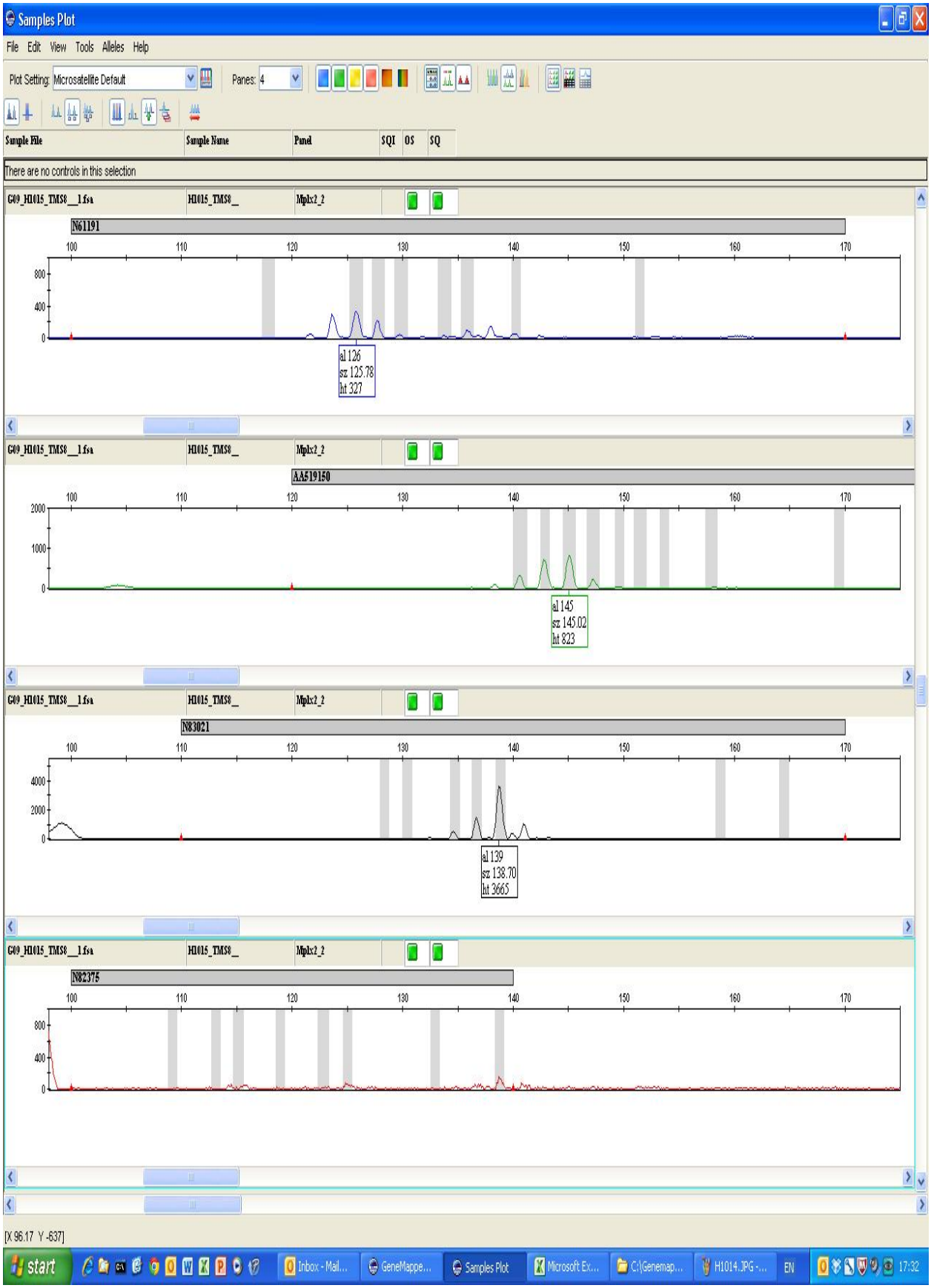


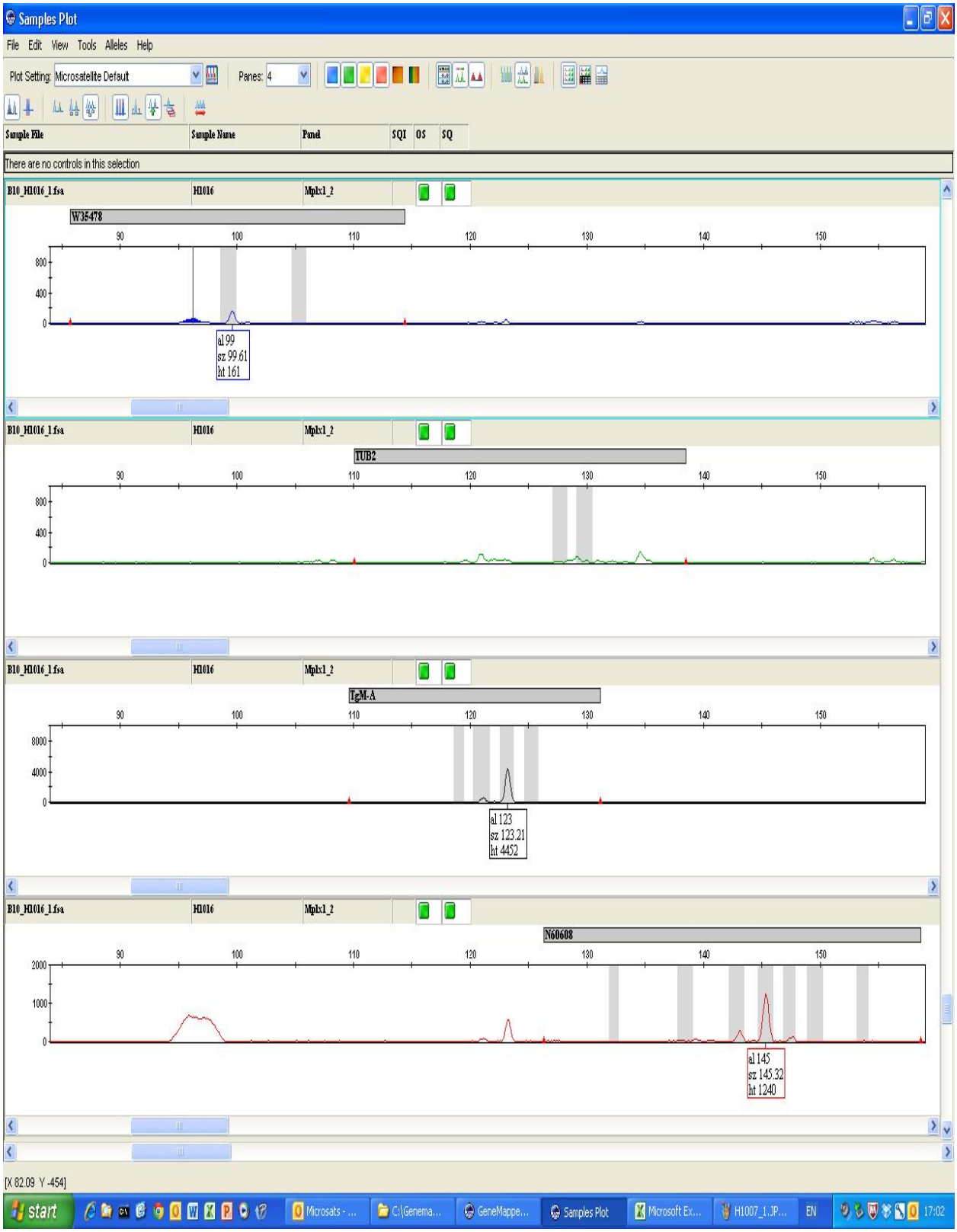


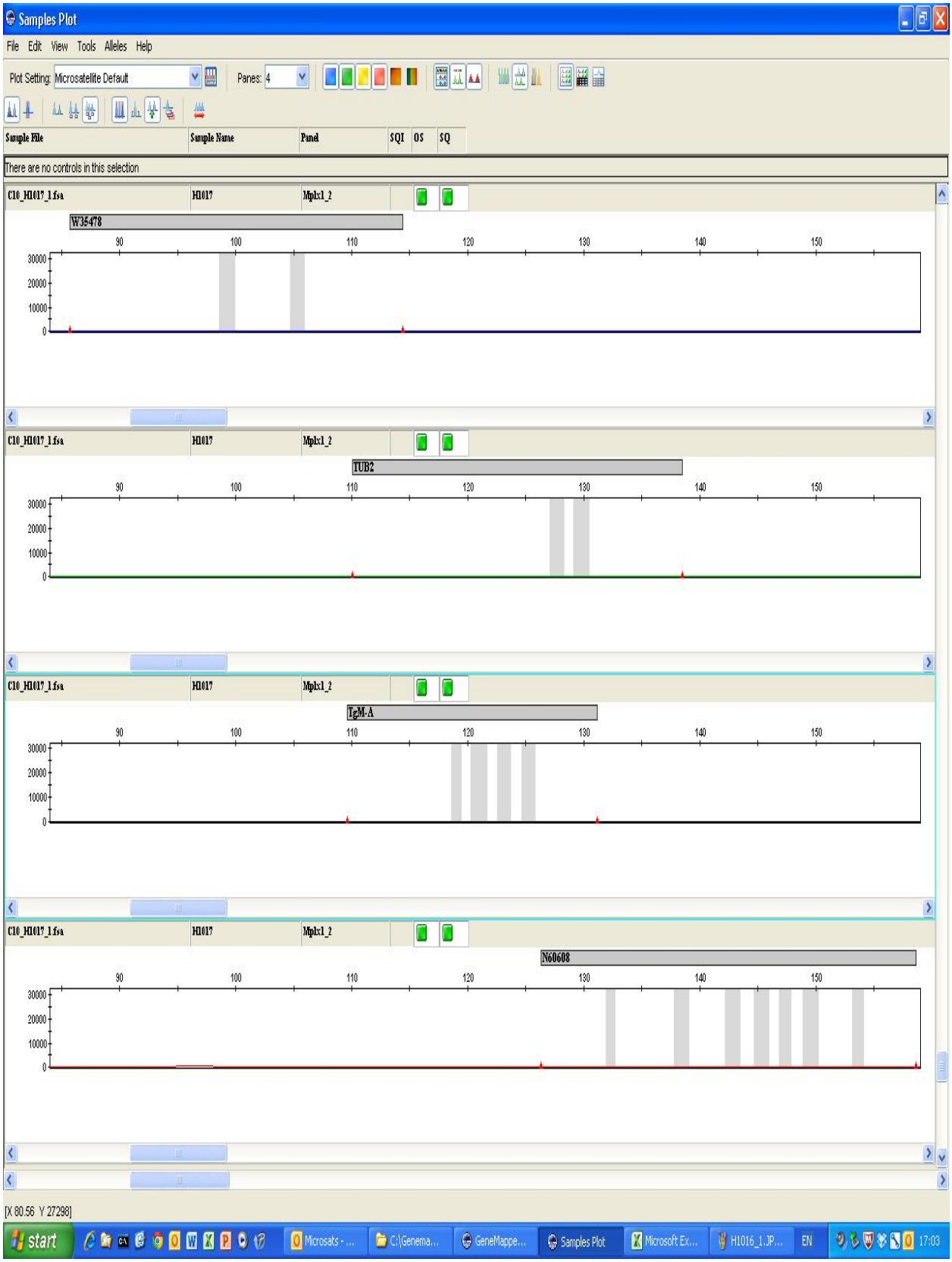


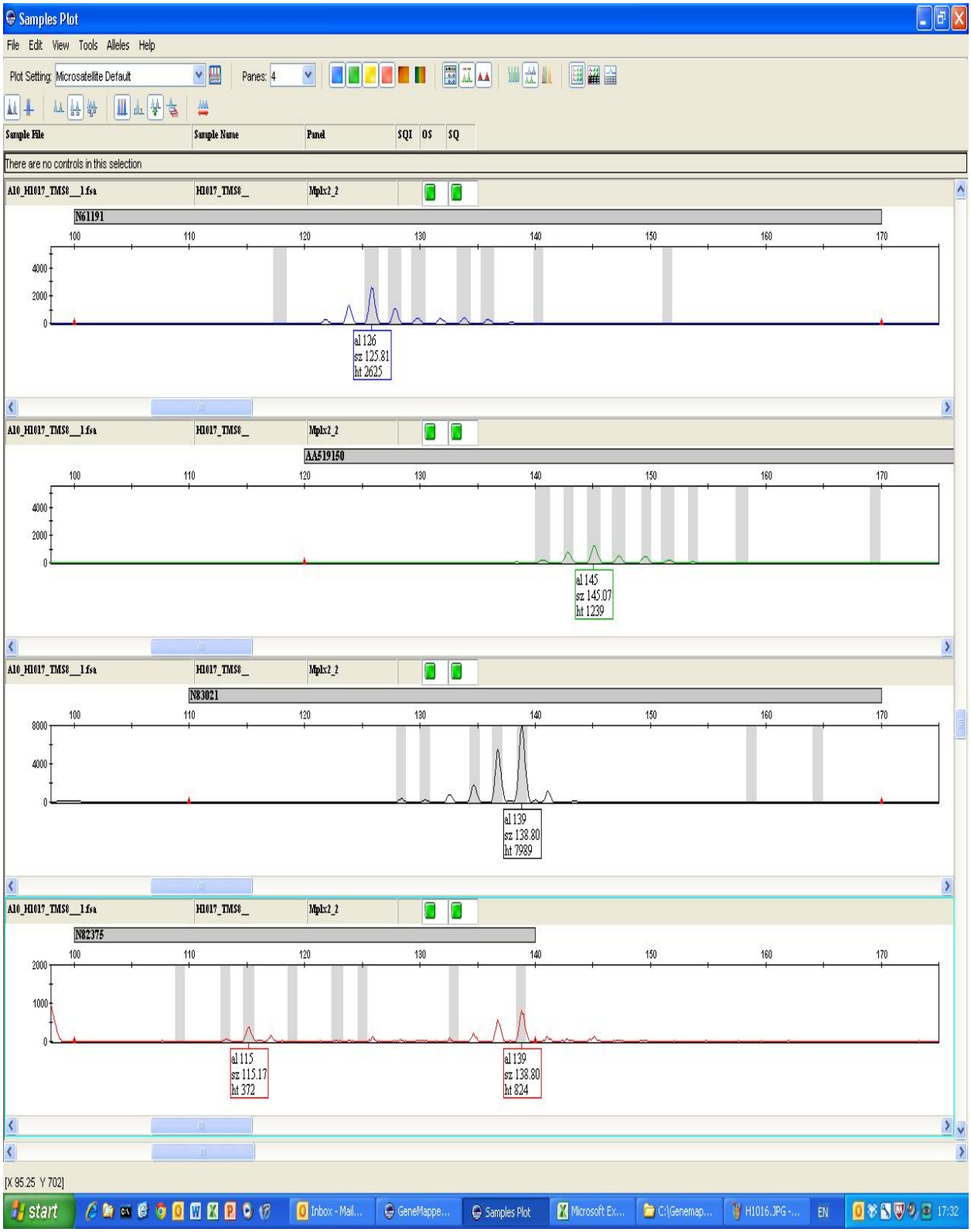




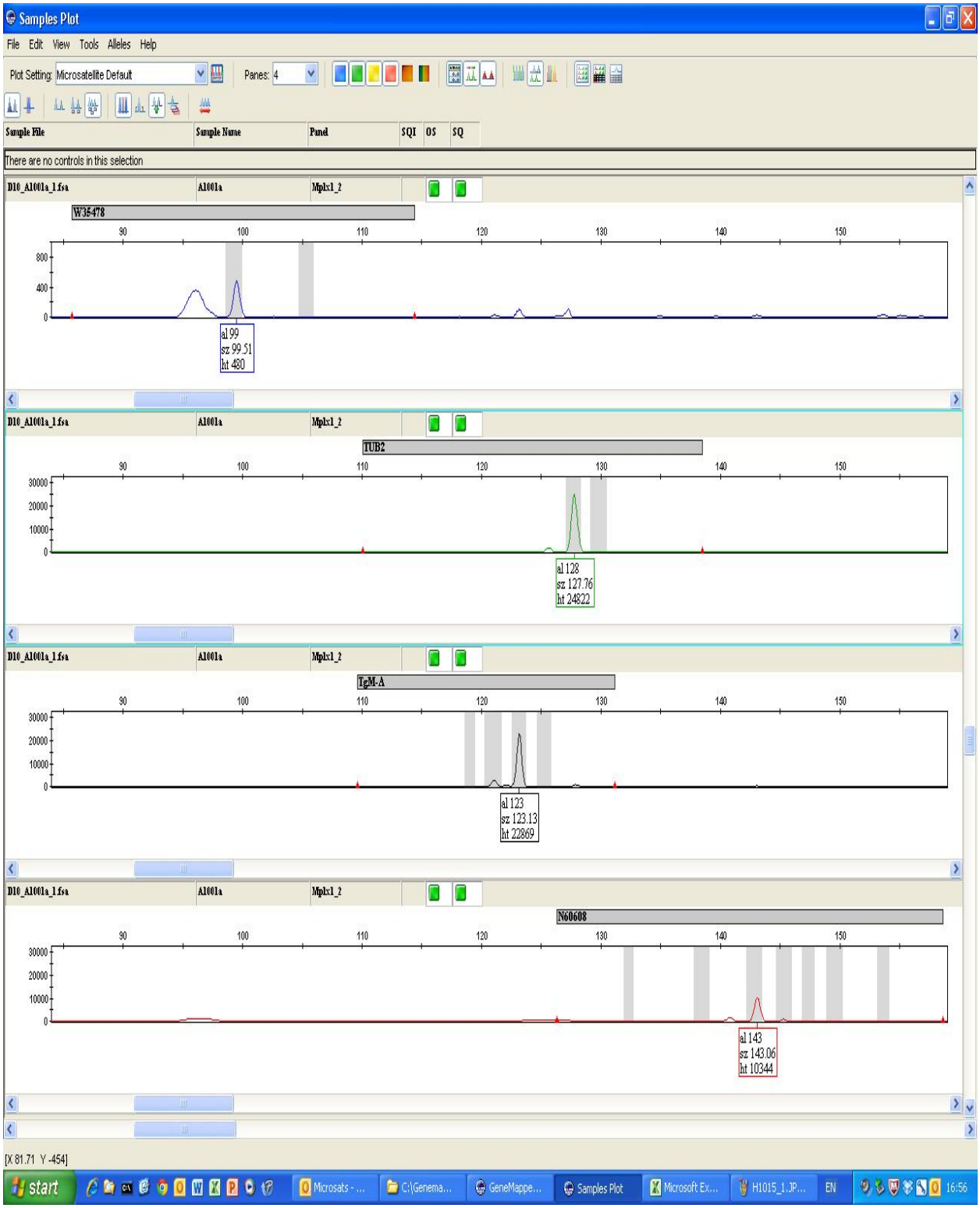


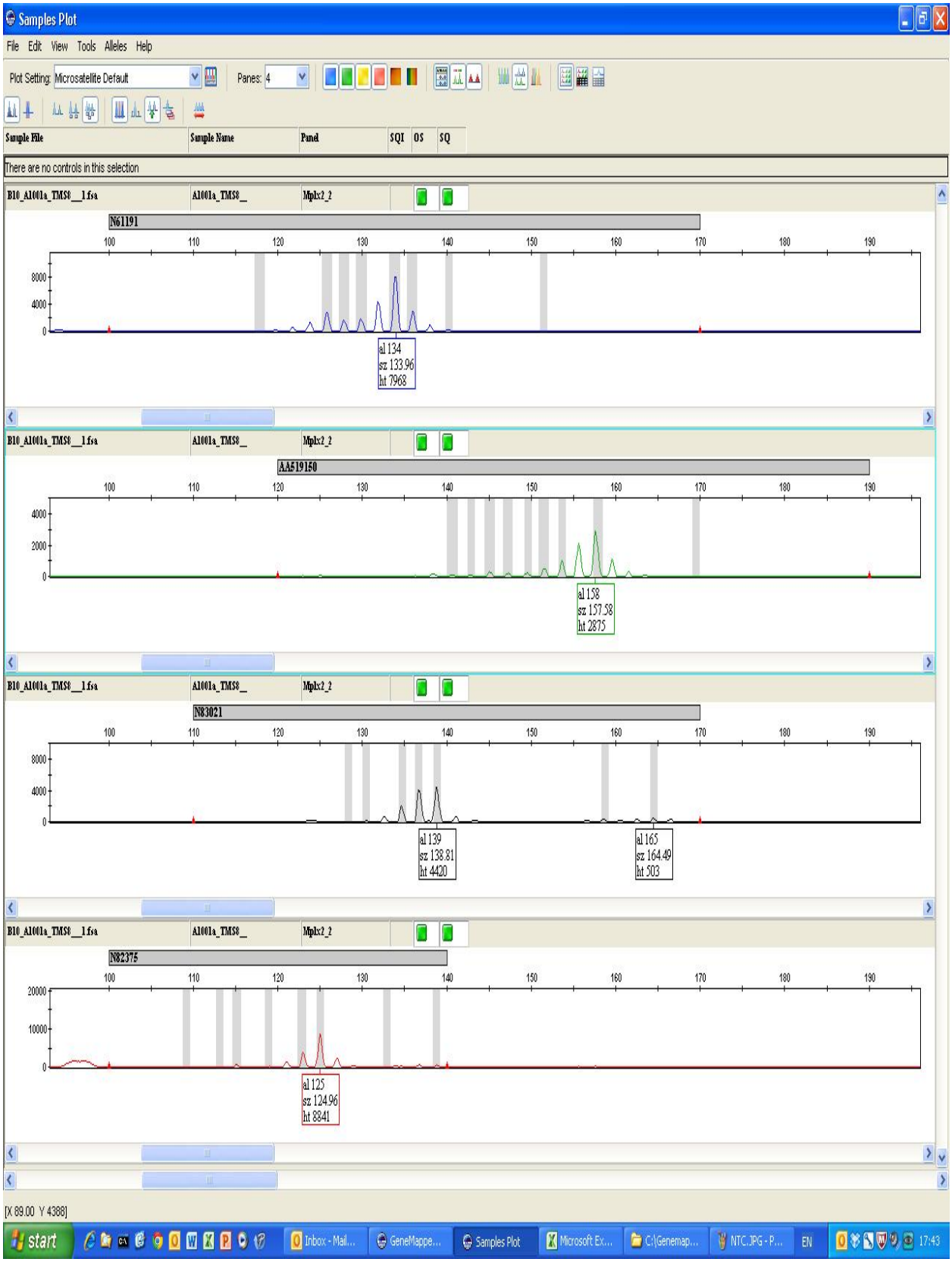


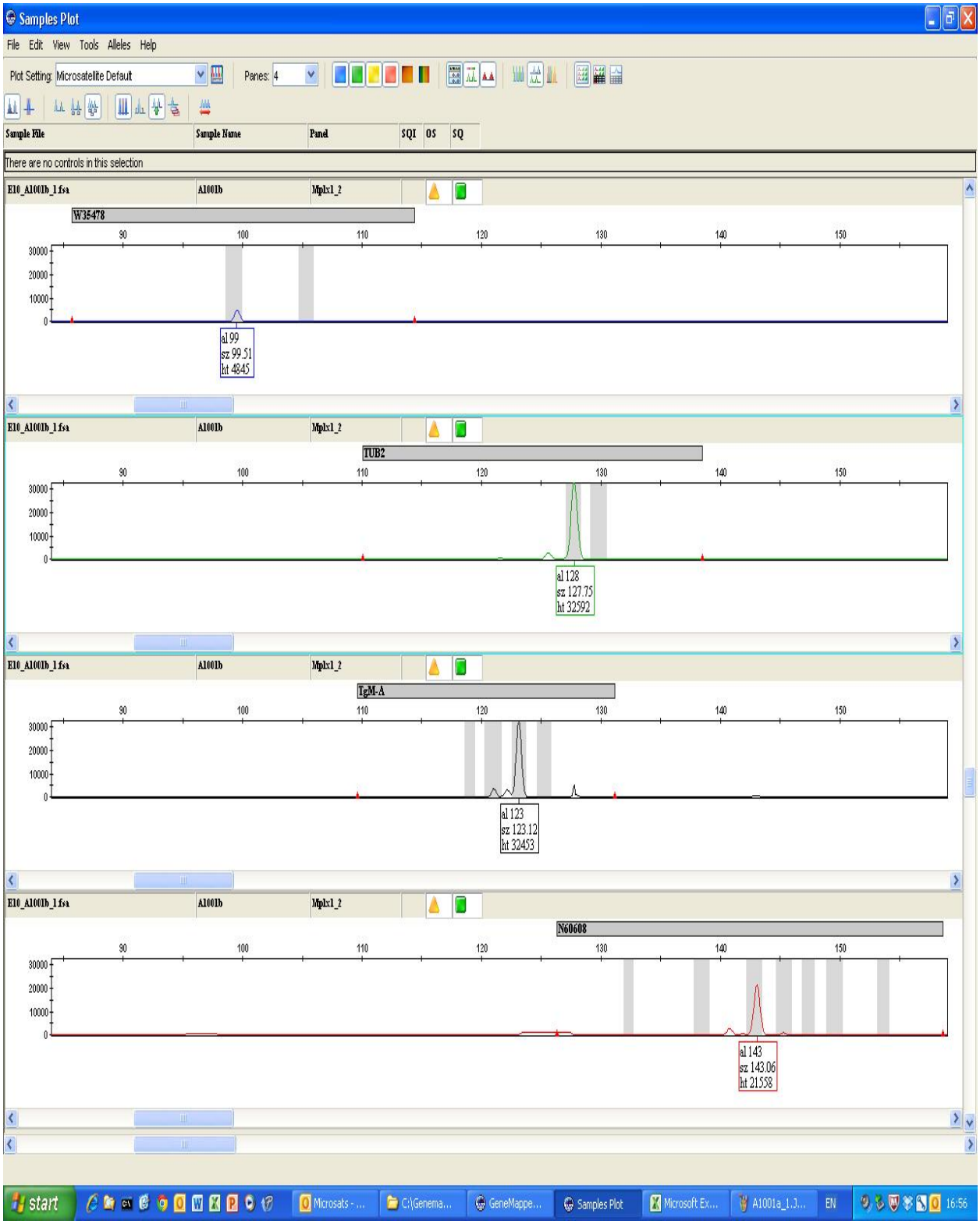


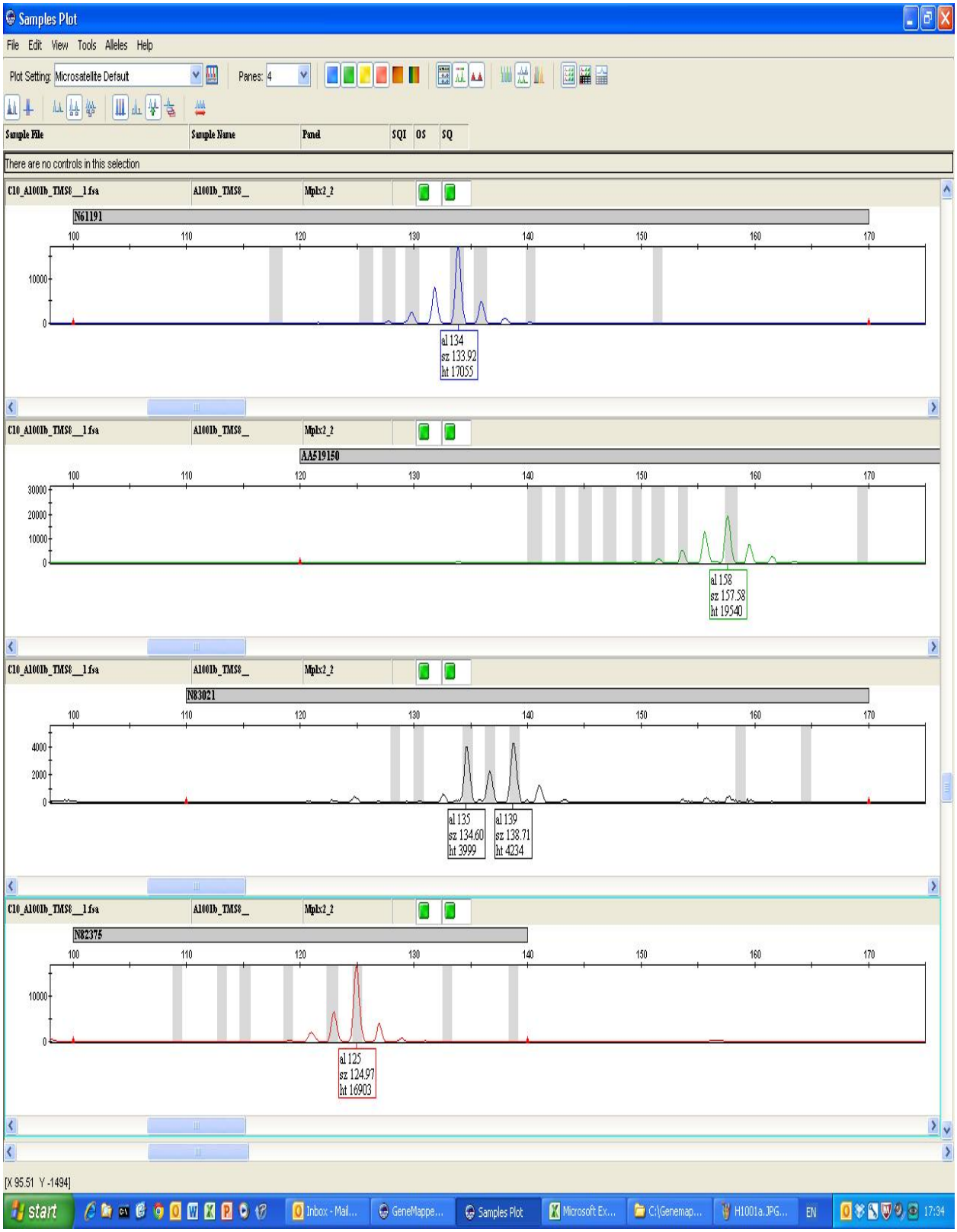


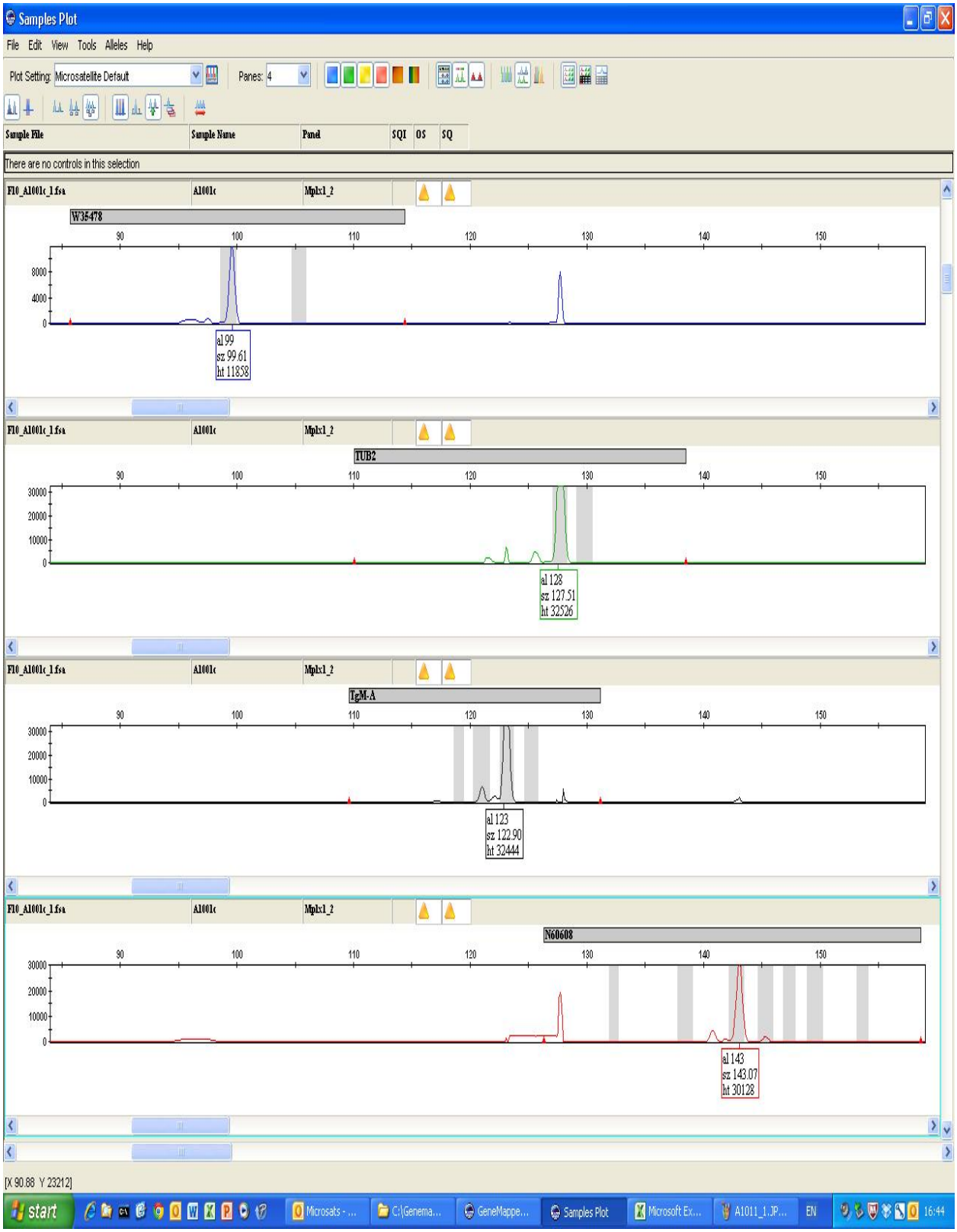
Animal sample plots

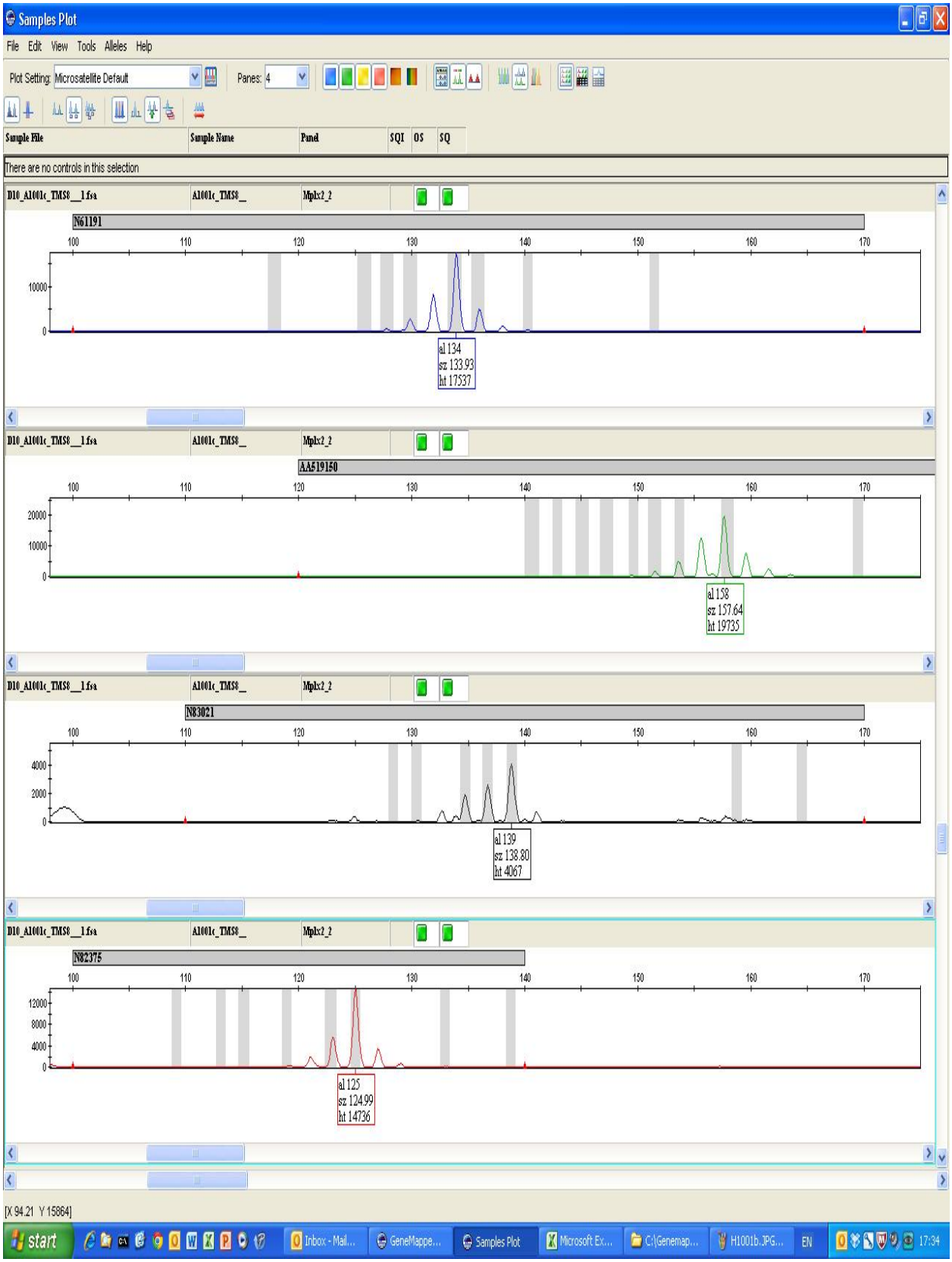


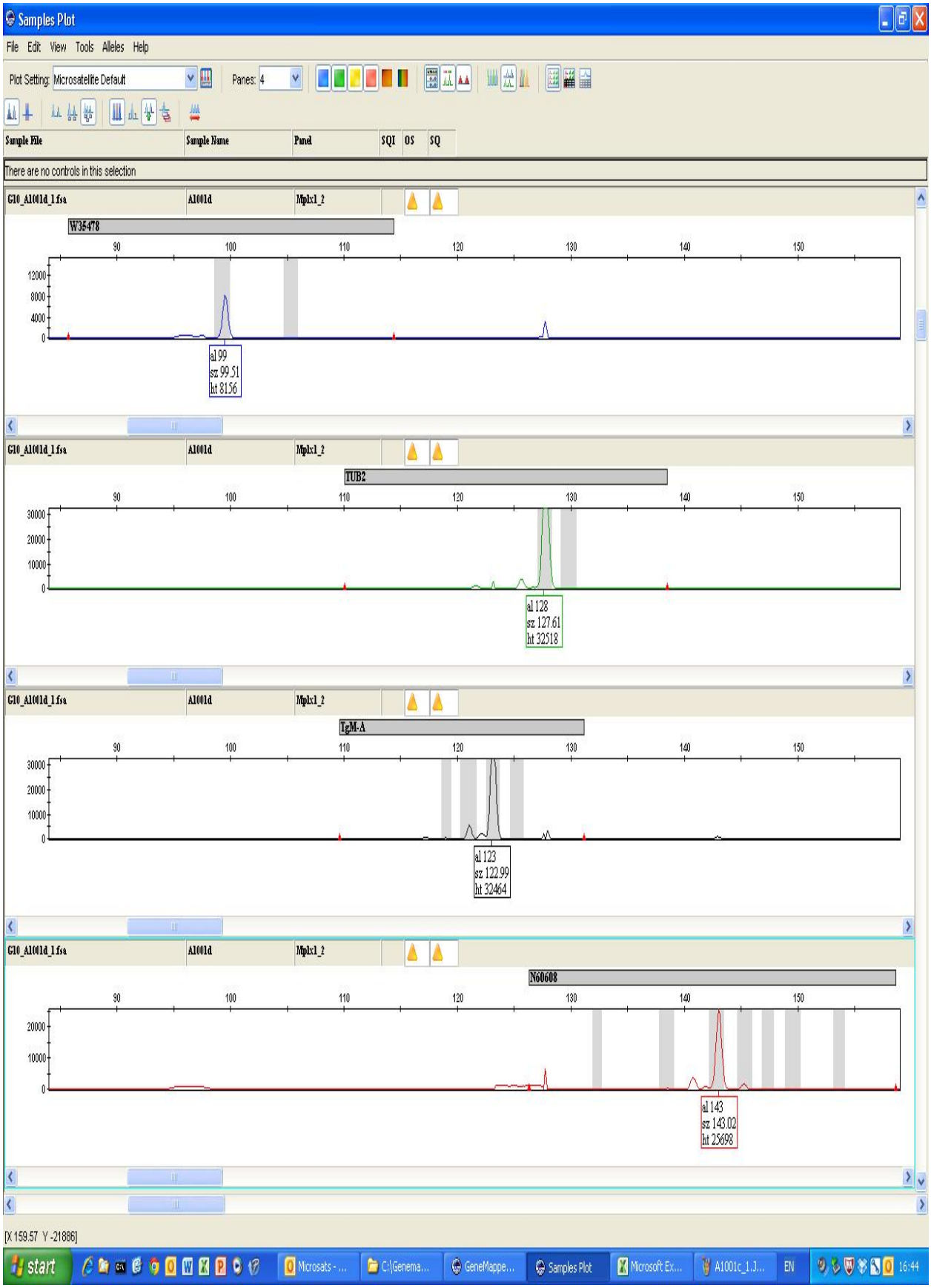


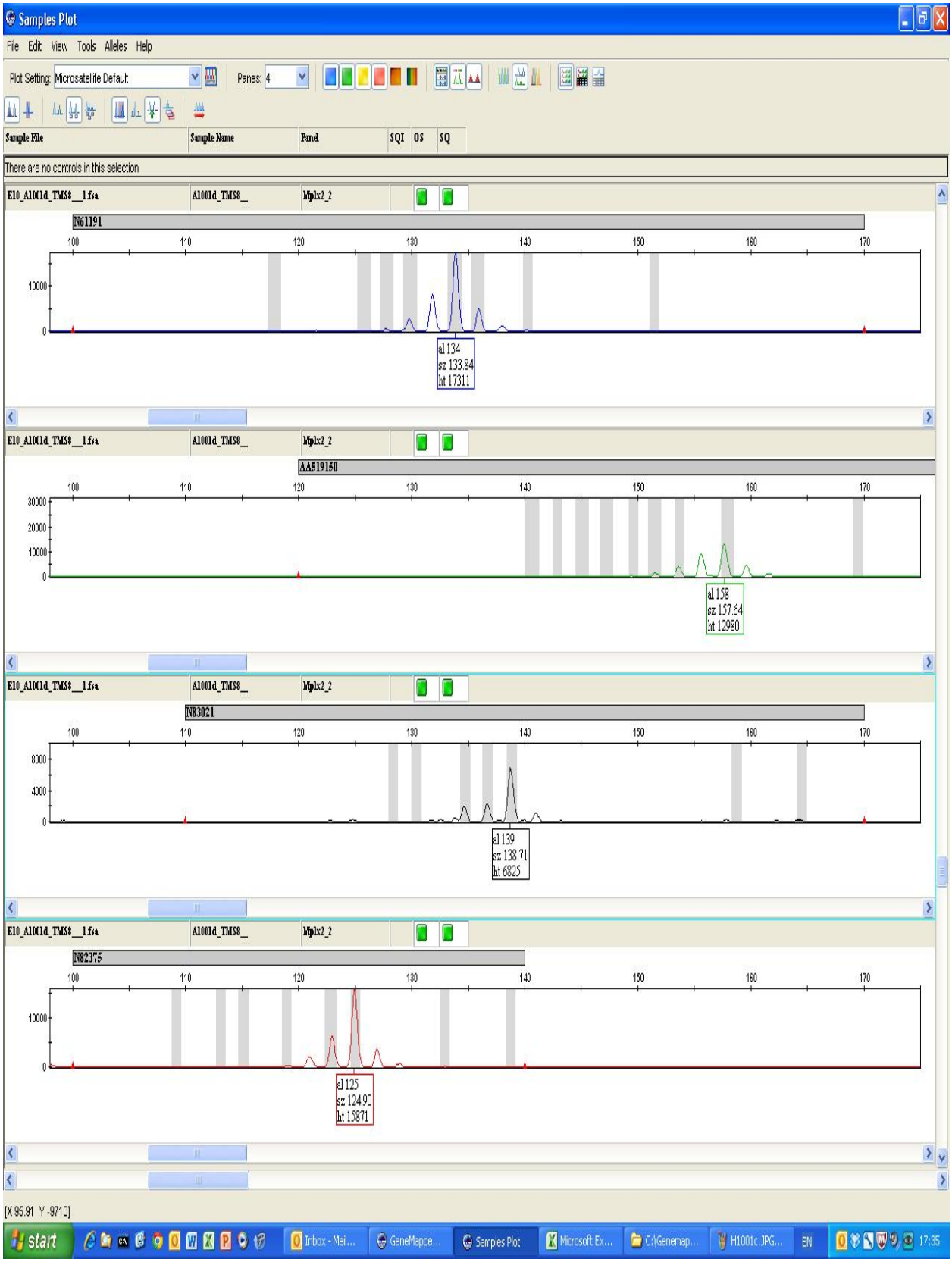


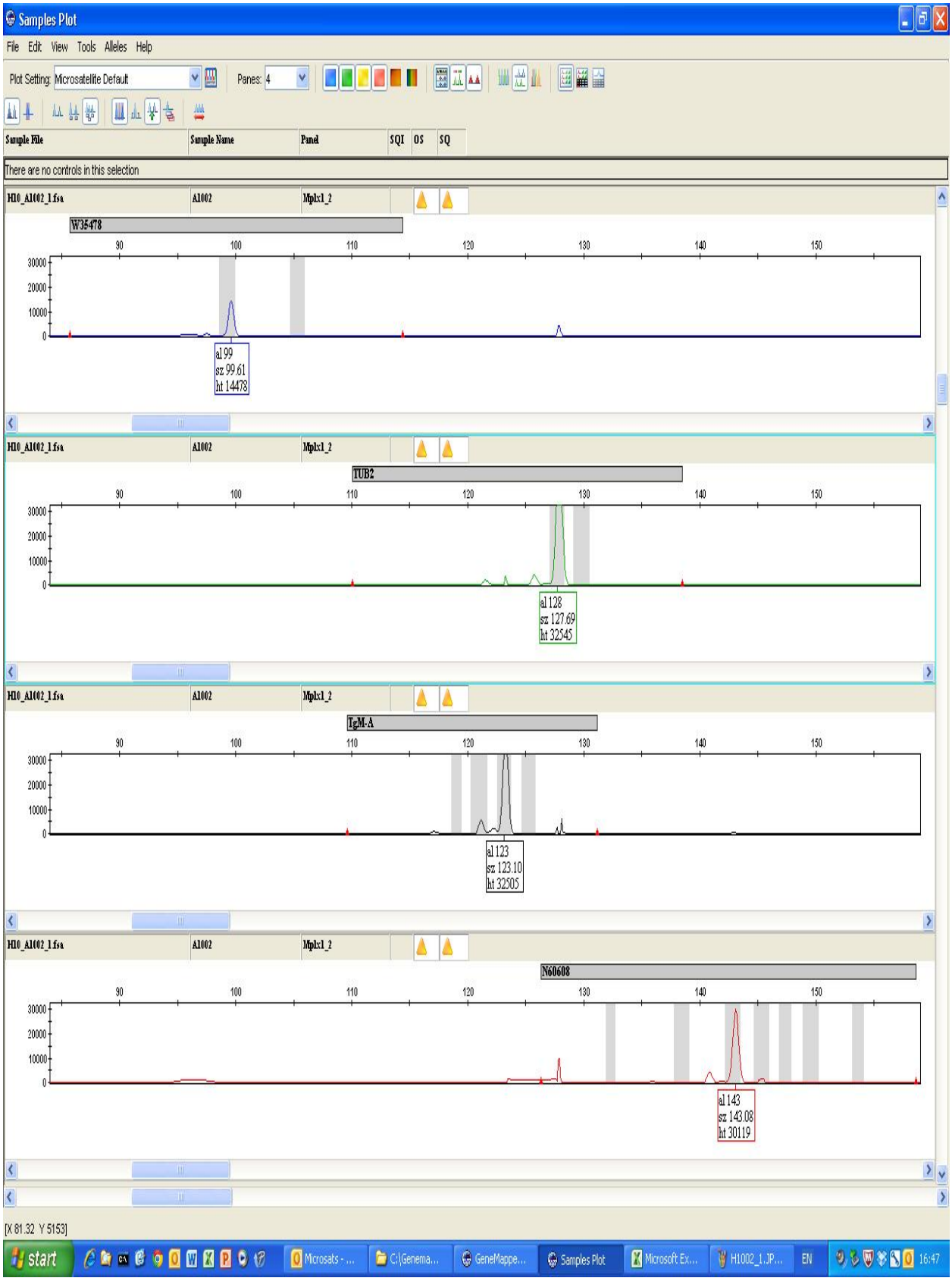


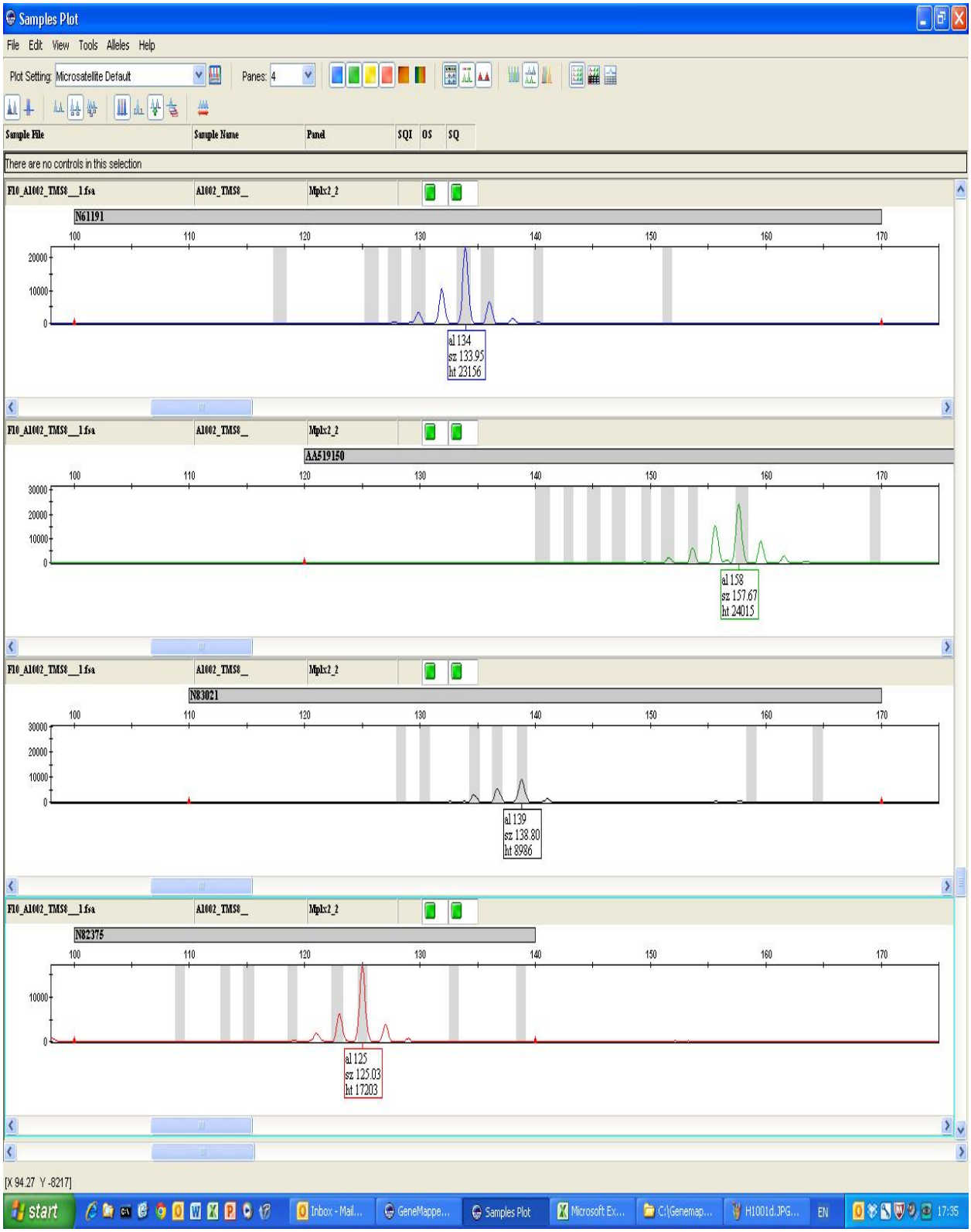


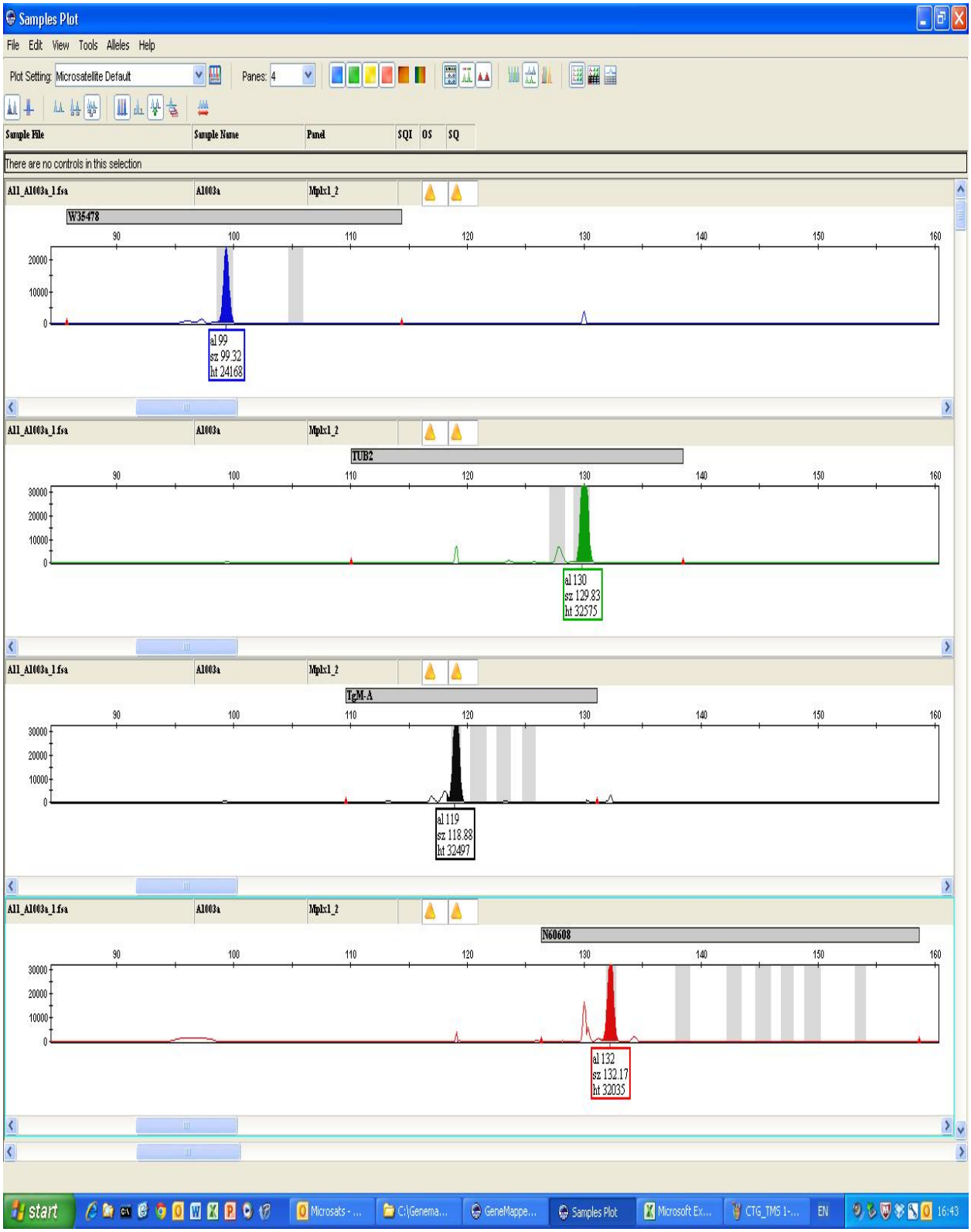


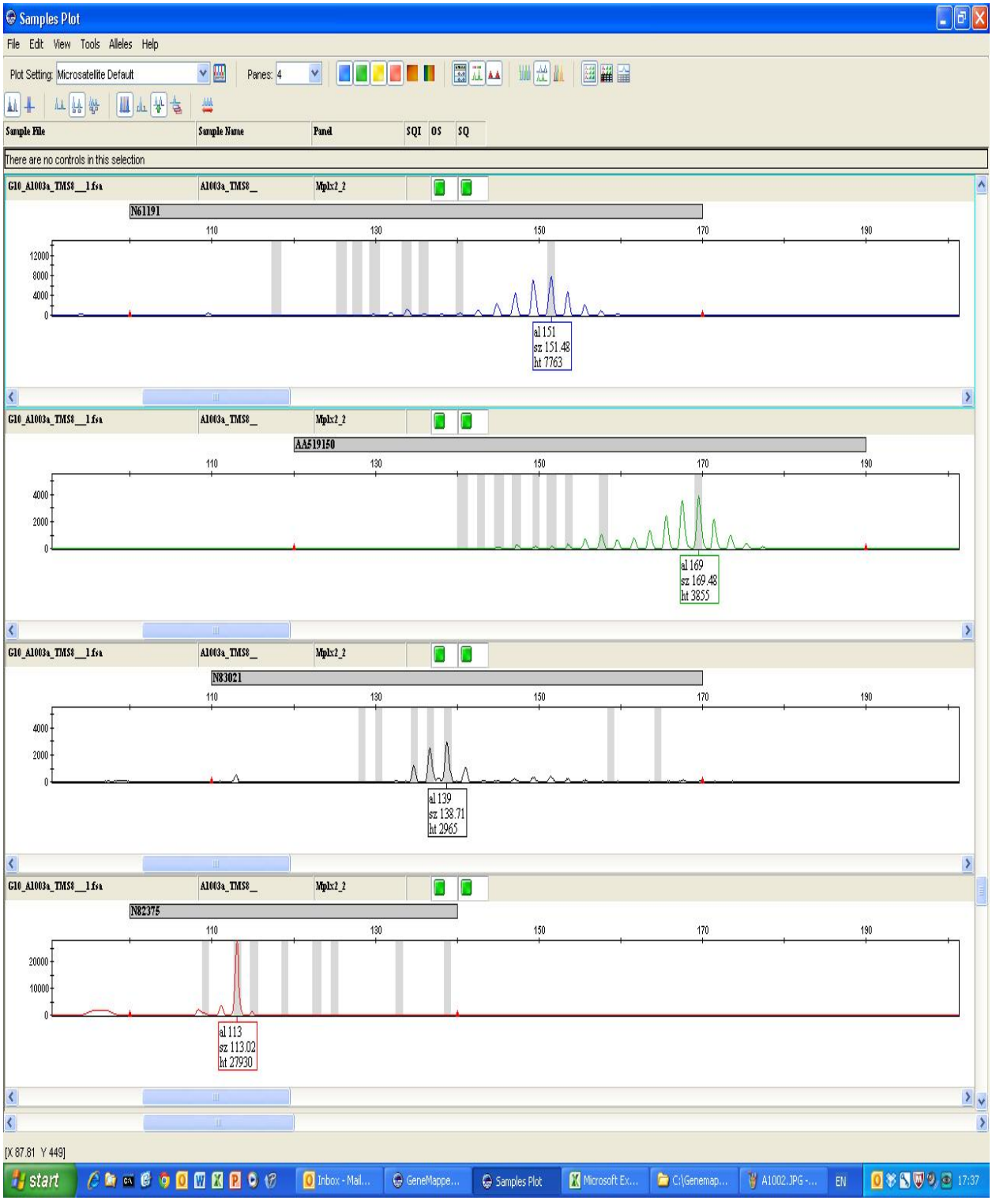


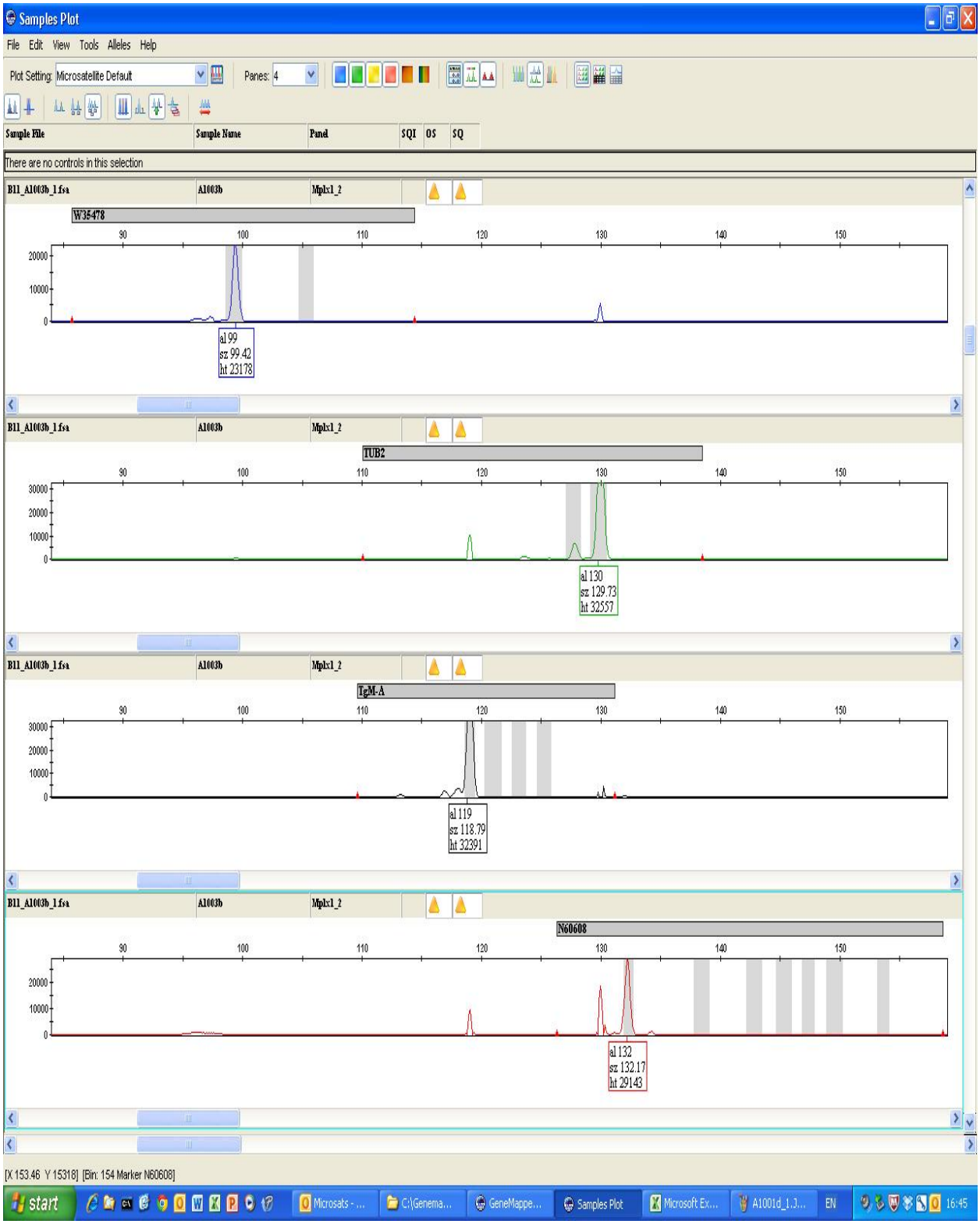


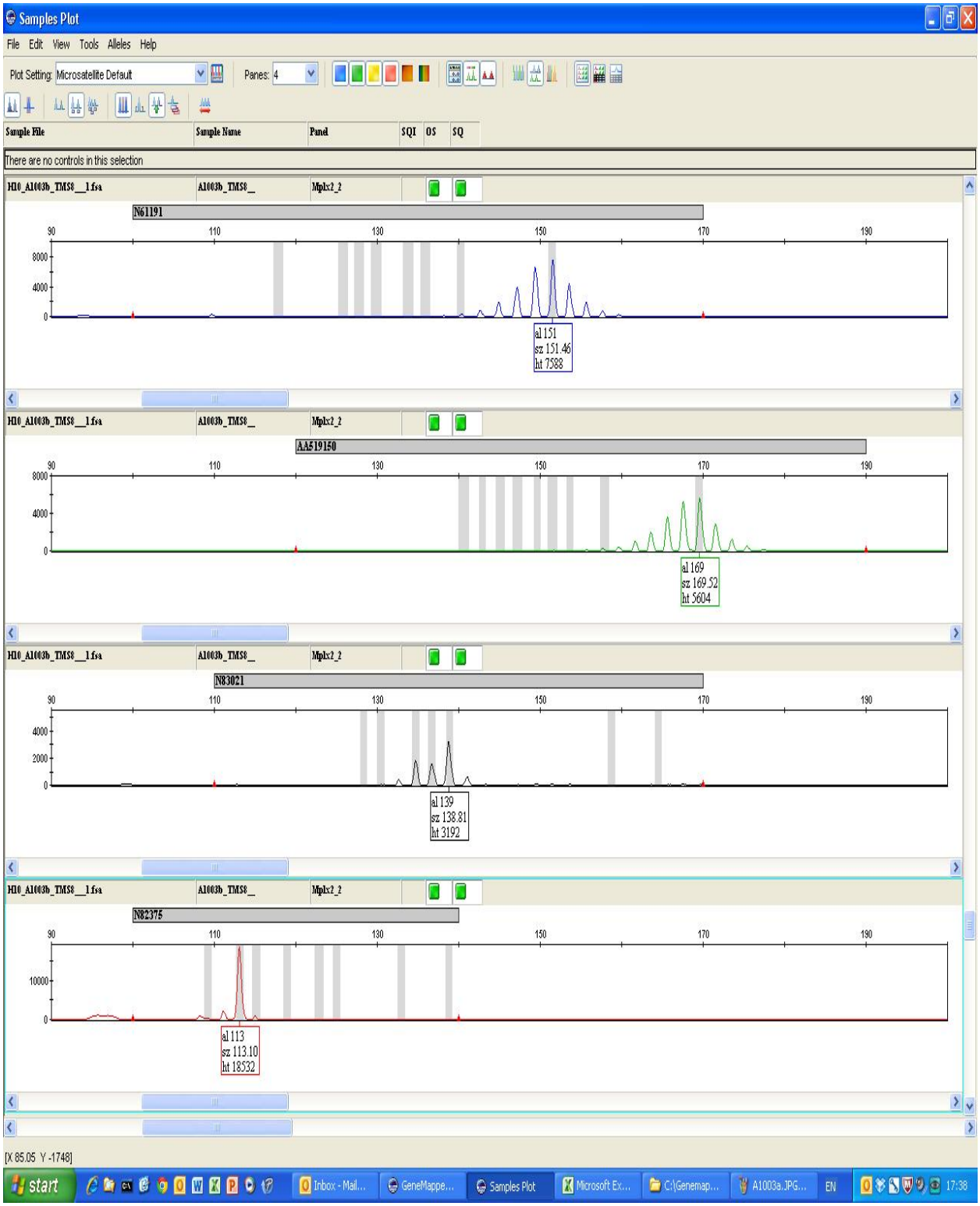


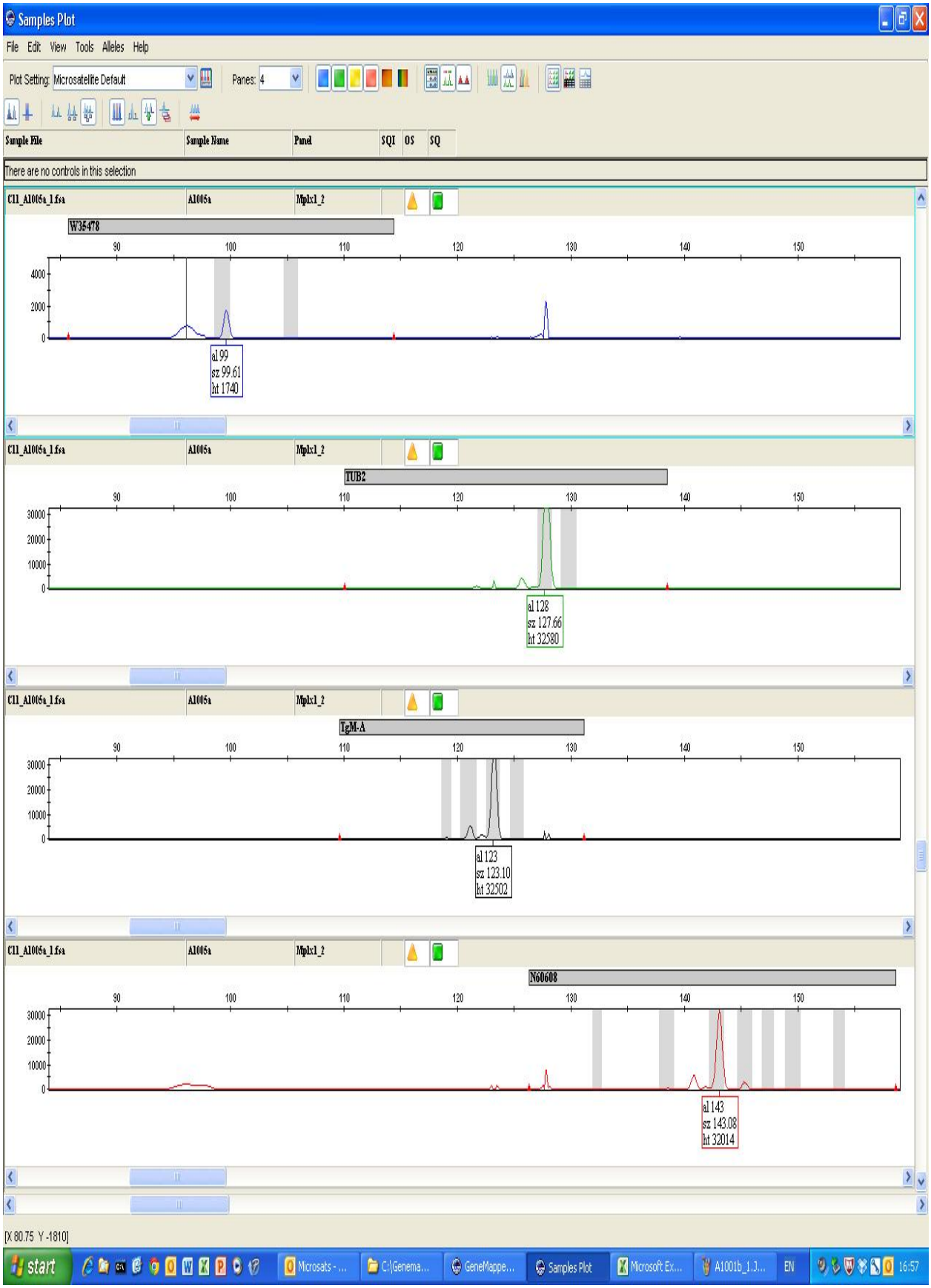


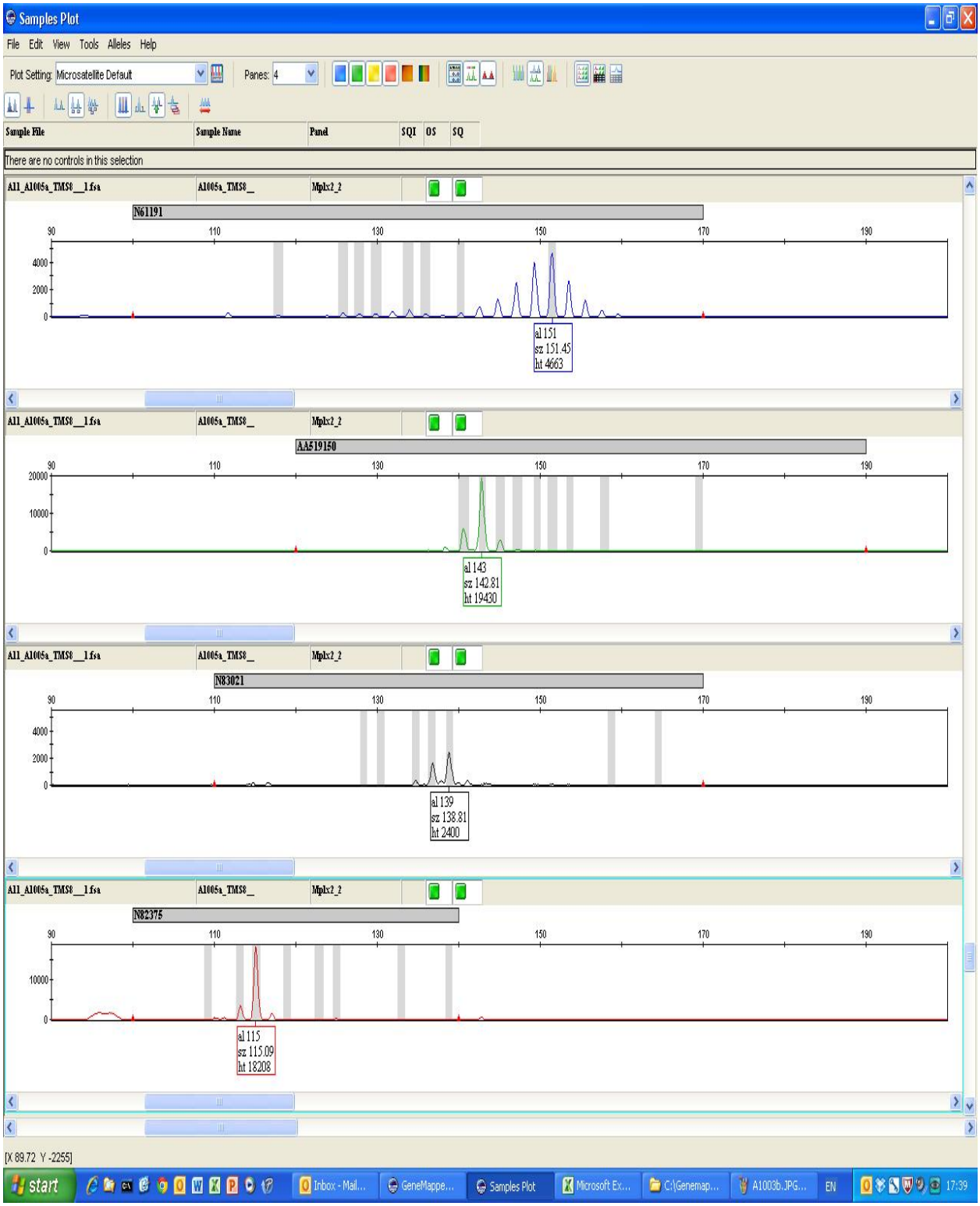


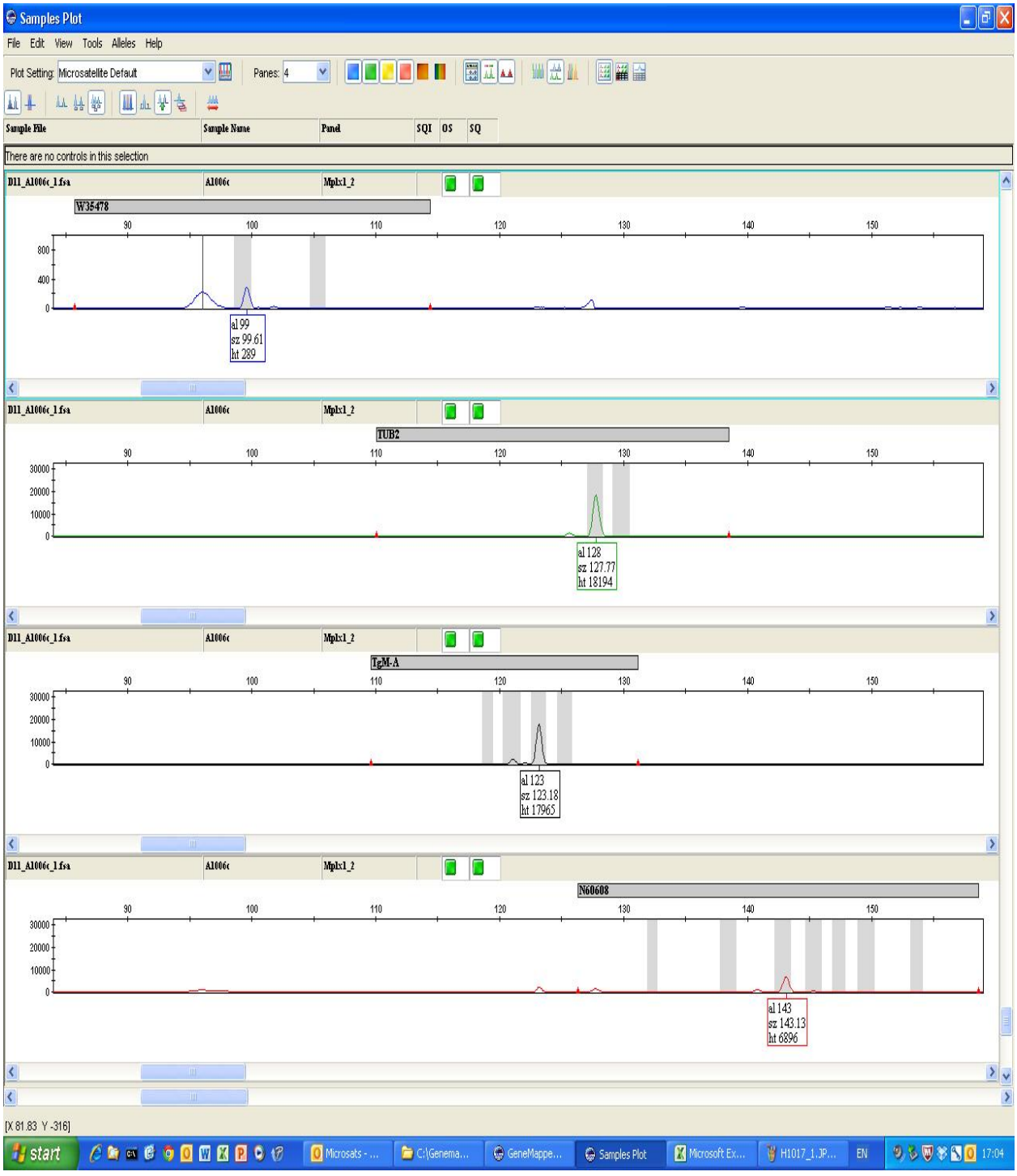


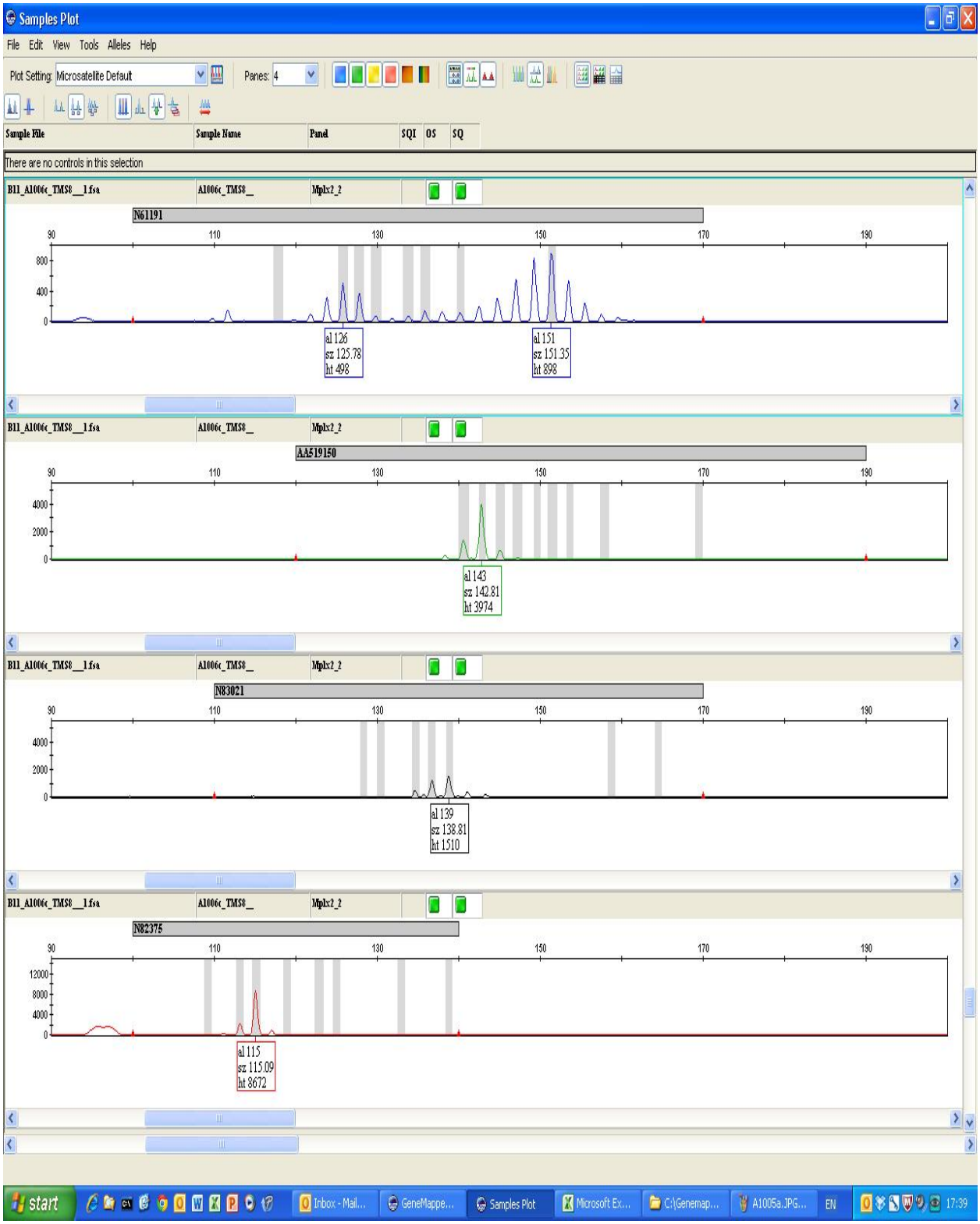


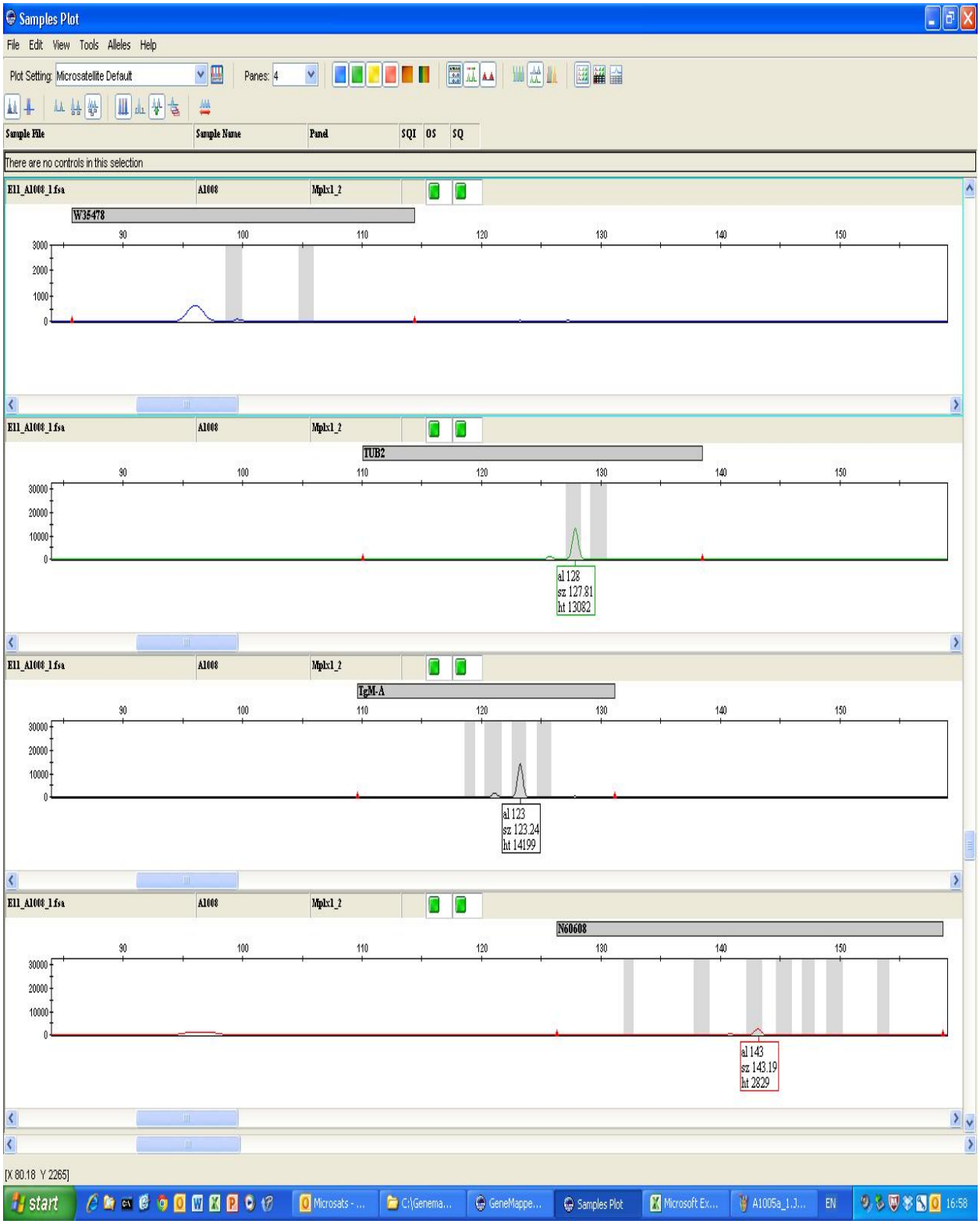


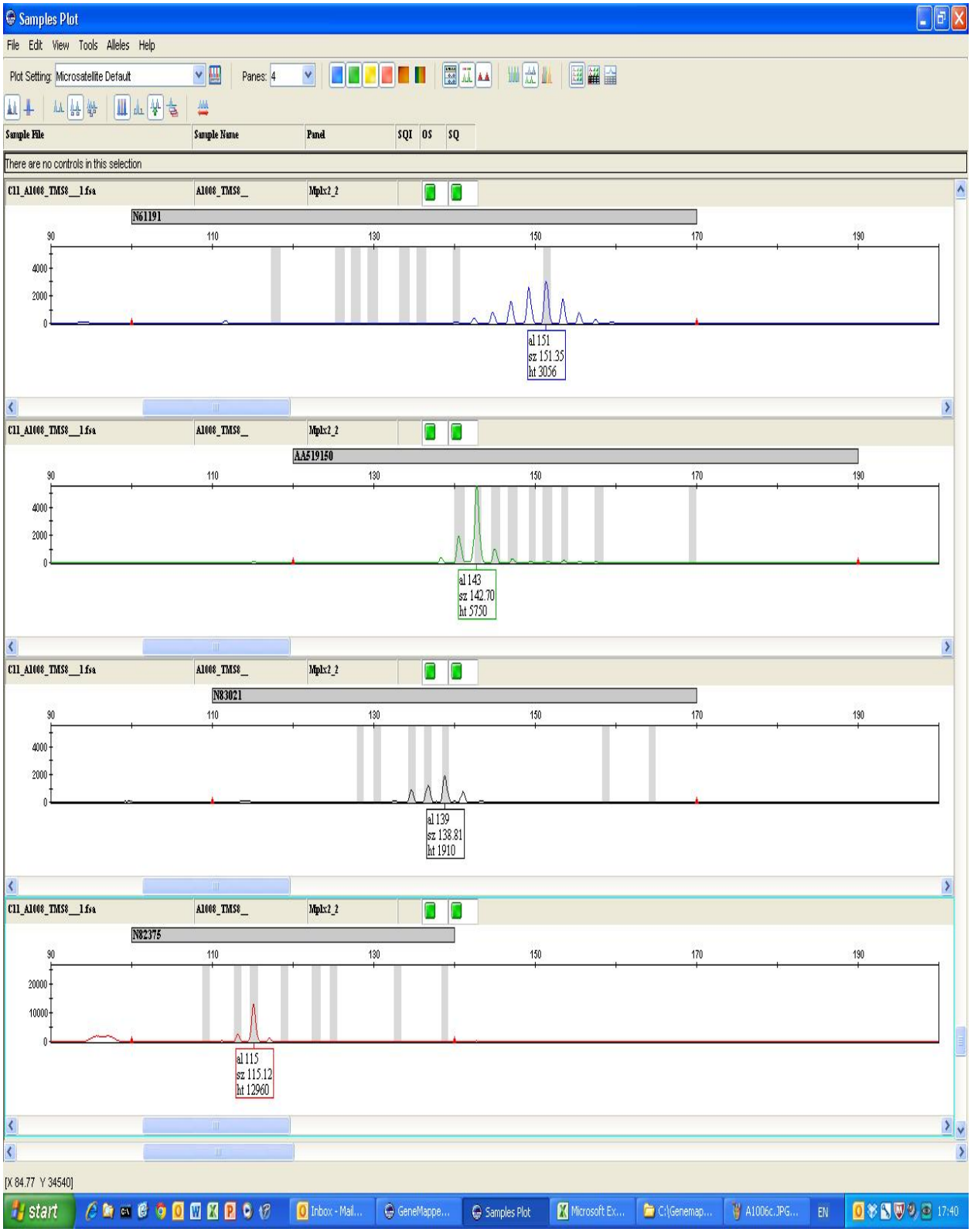


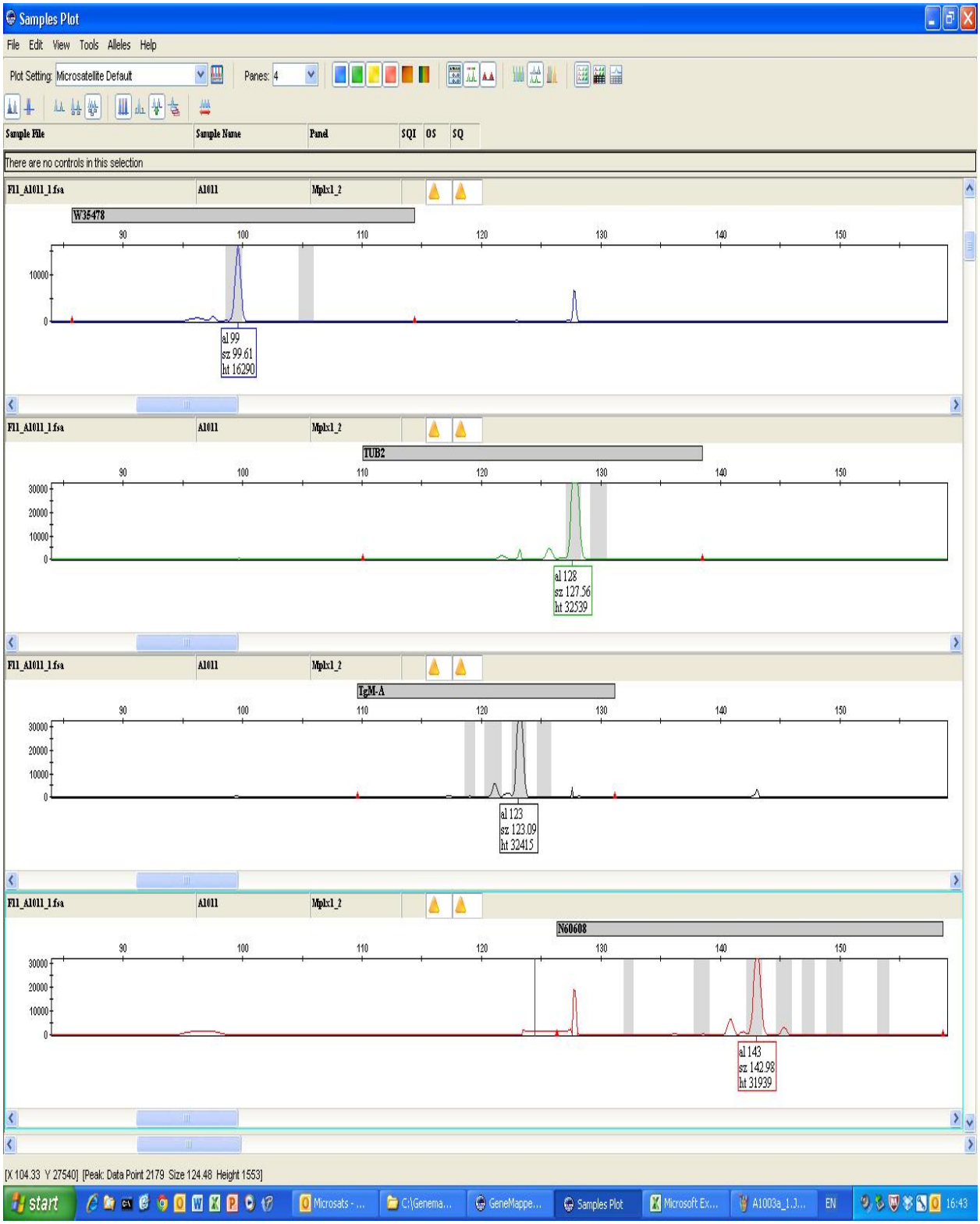


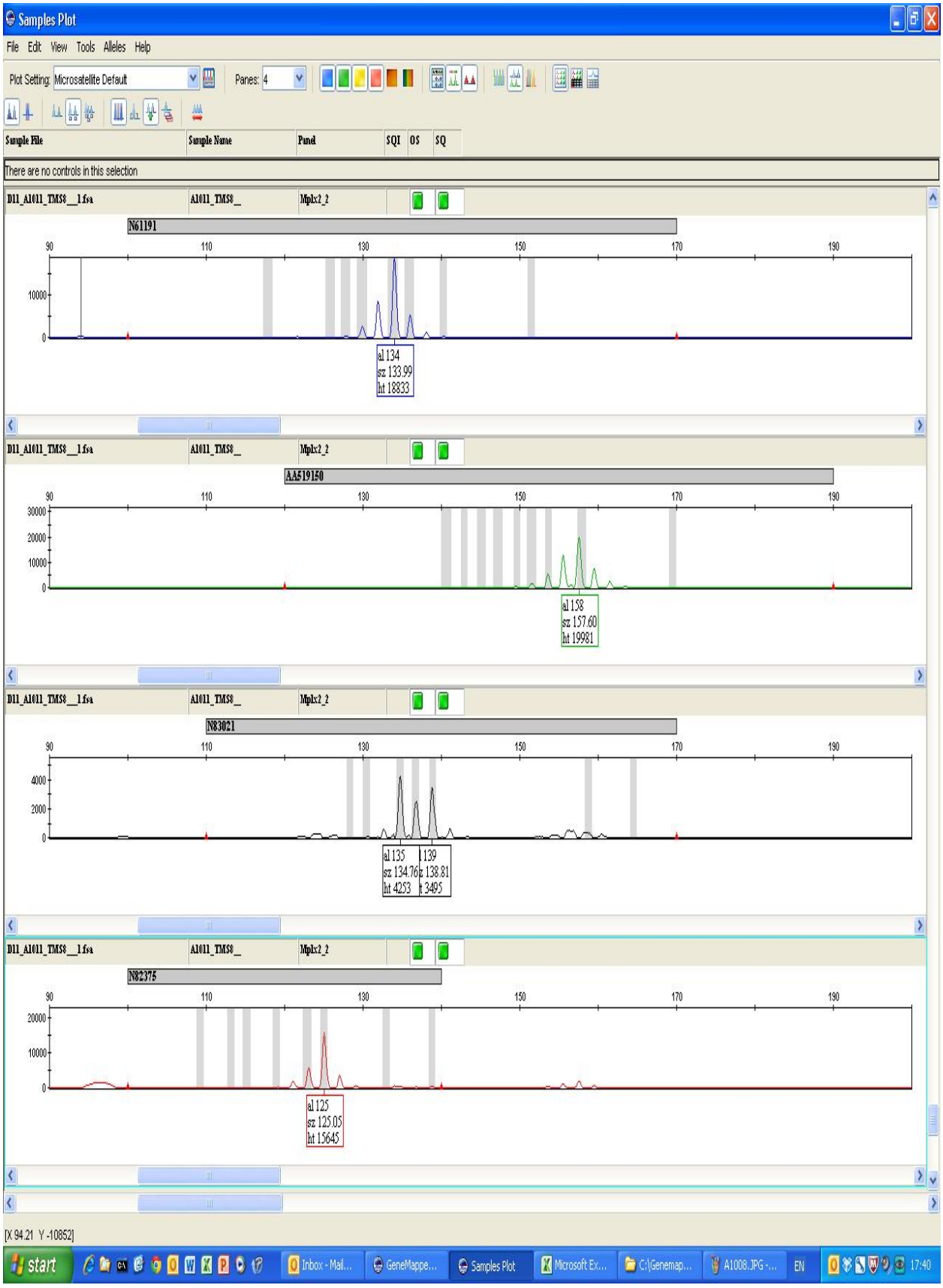








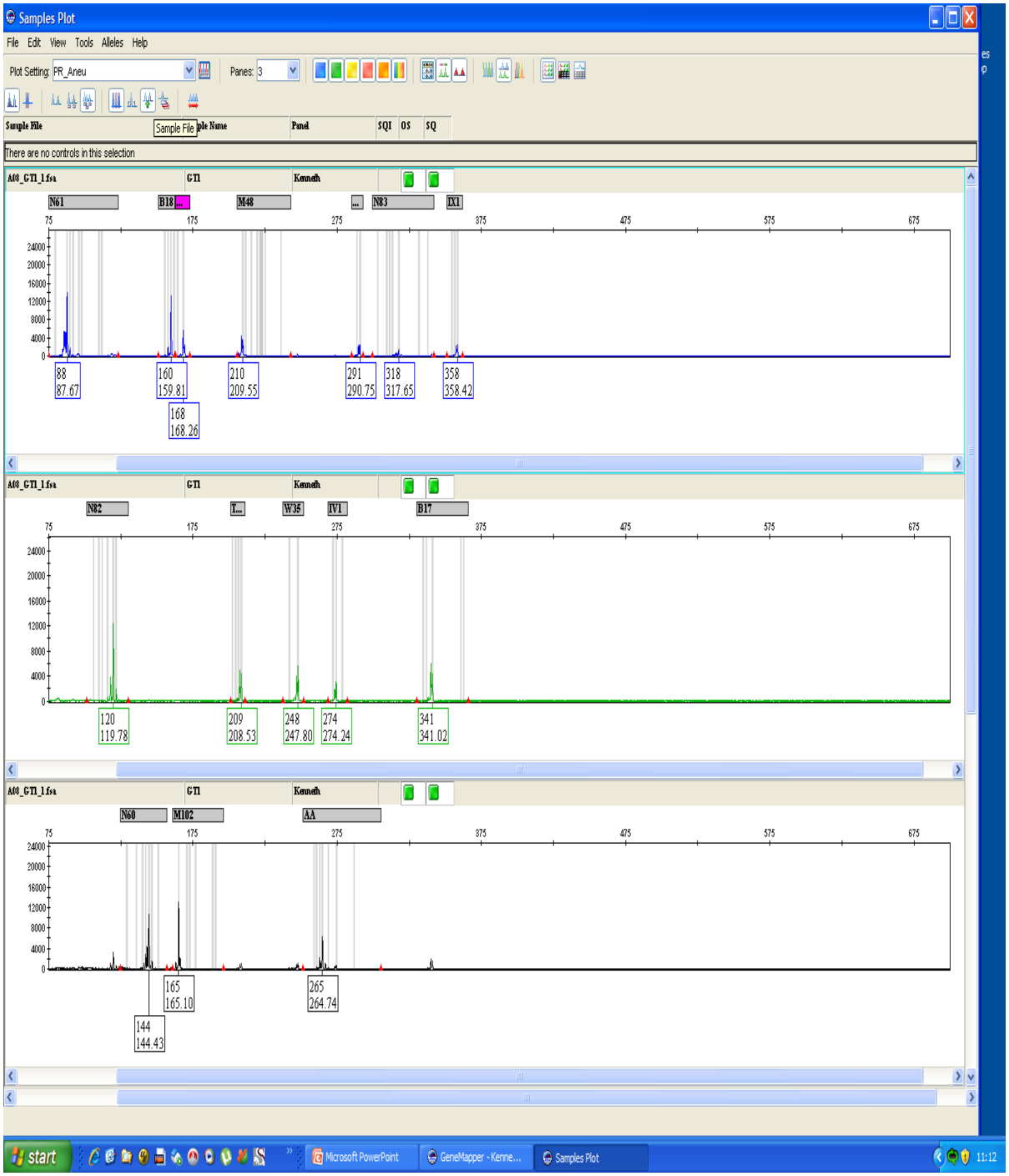




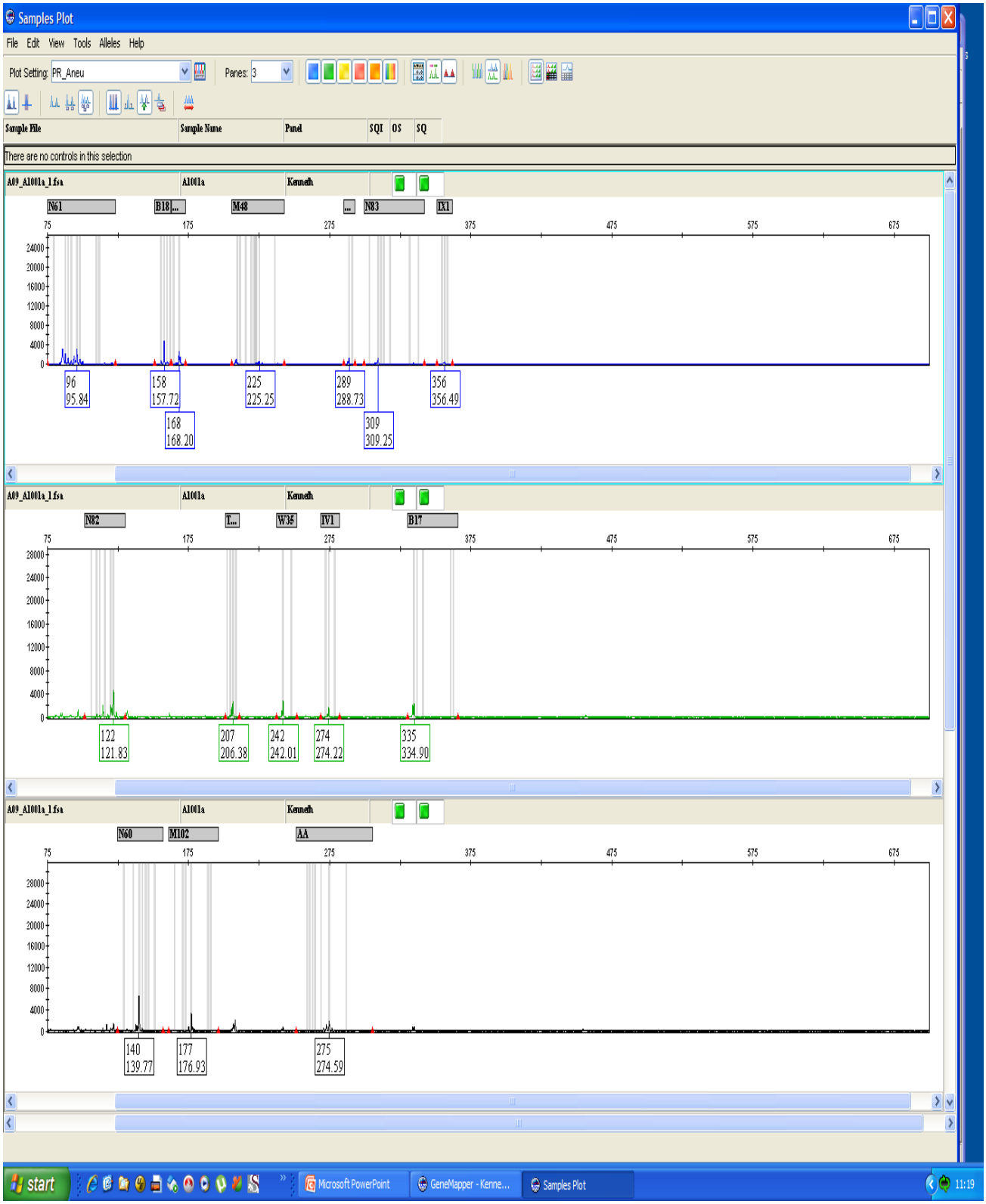
Appendix 11

GeneMapper[R] plots for animal samples genotyped at fifteen microsatellite loci

Reference strain GT1



Sample A1001a



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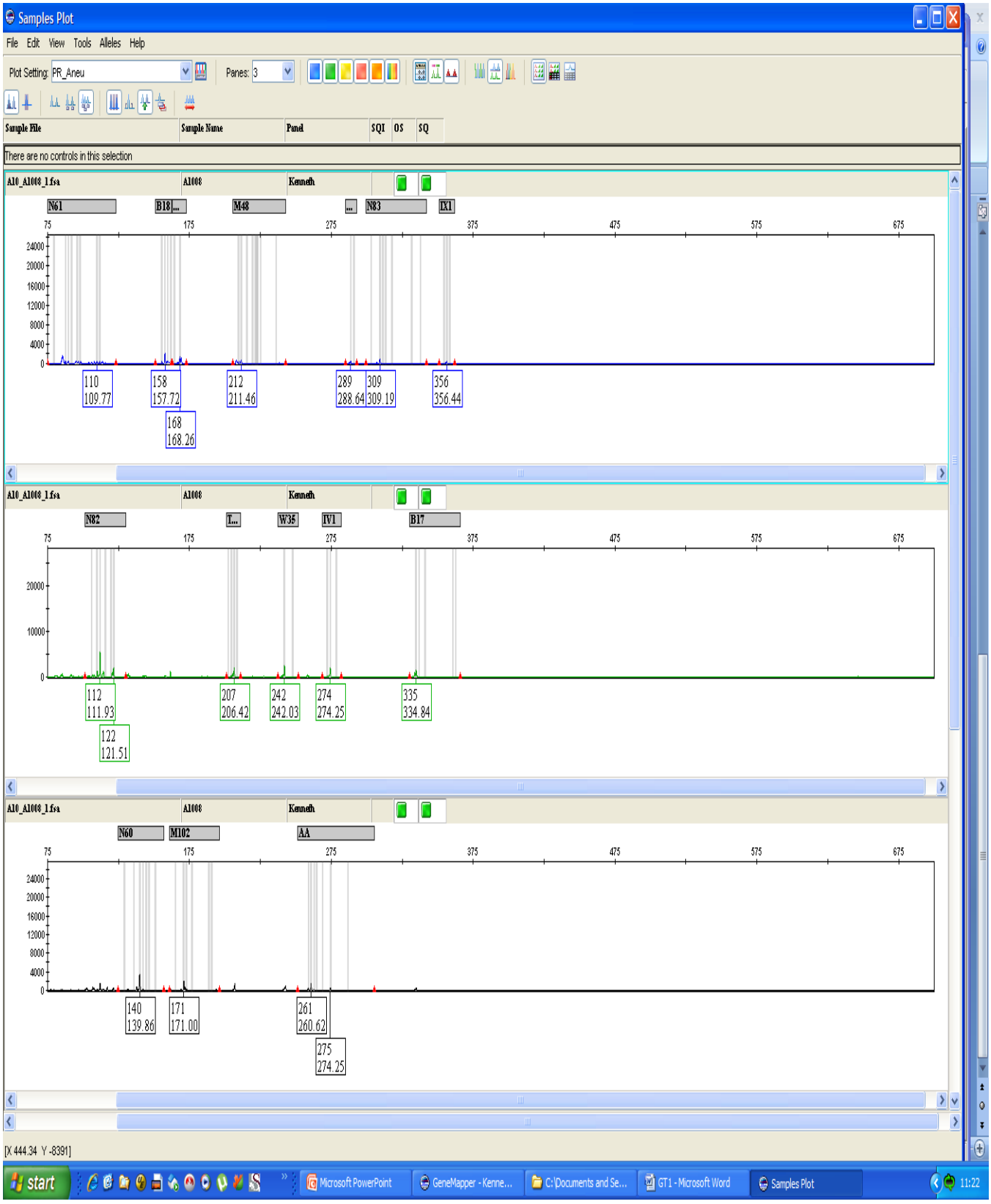
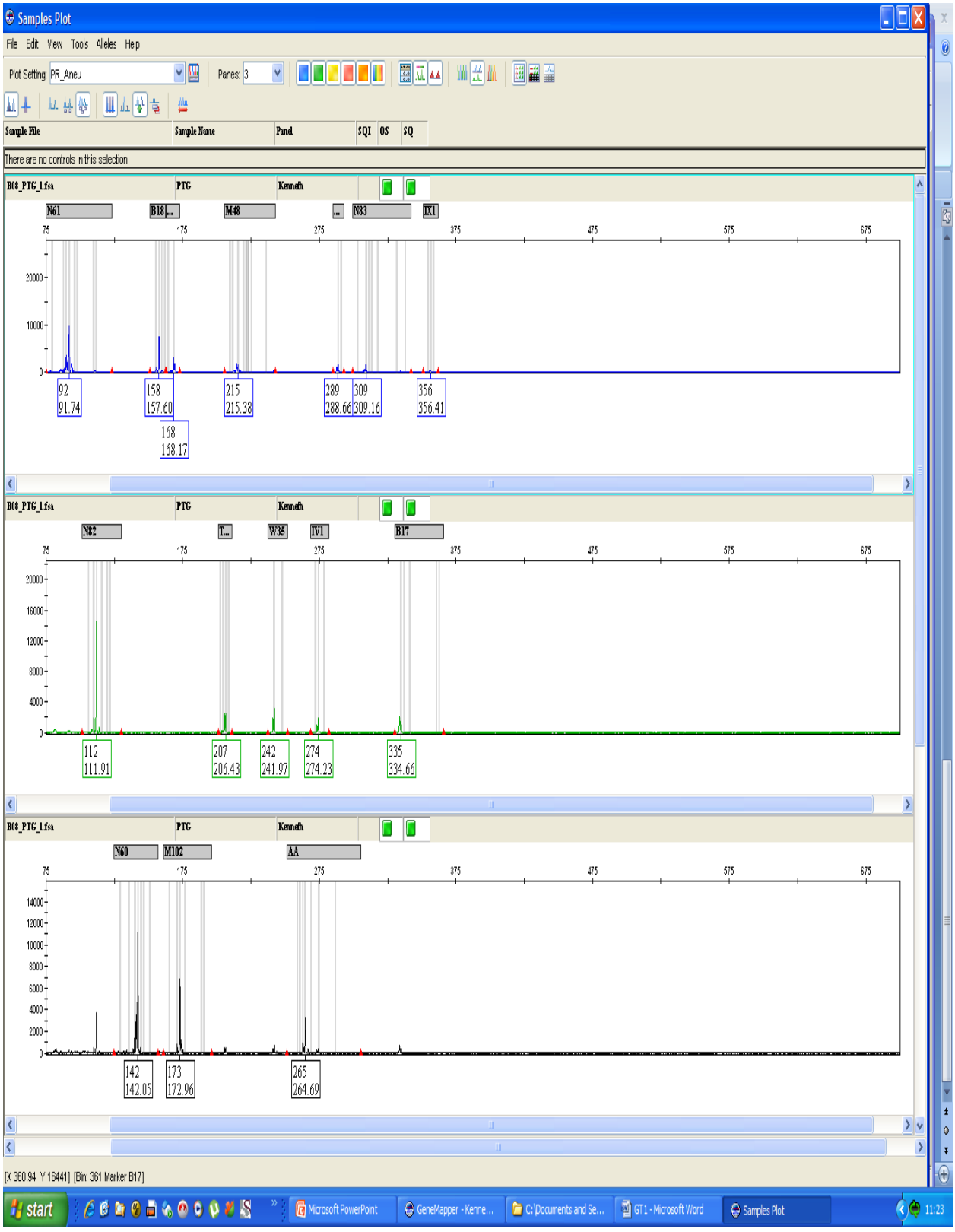


Reference strain PTG



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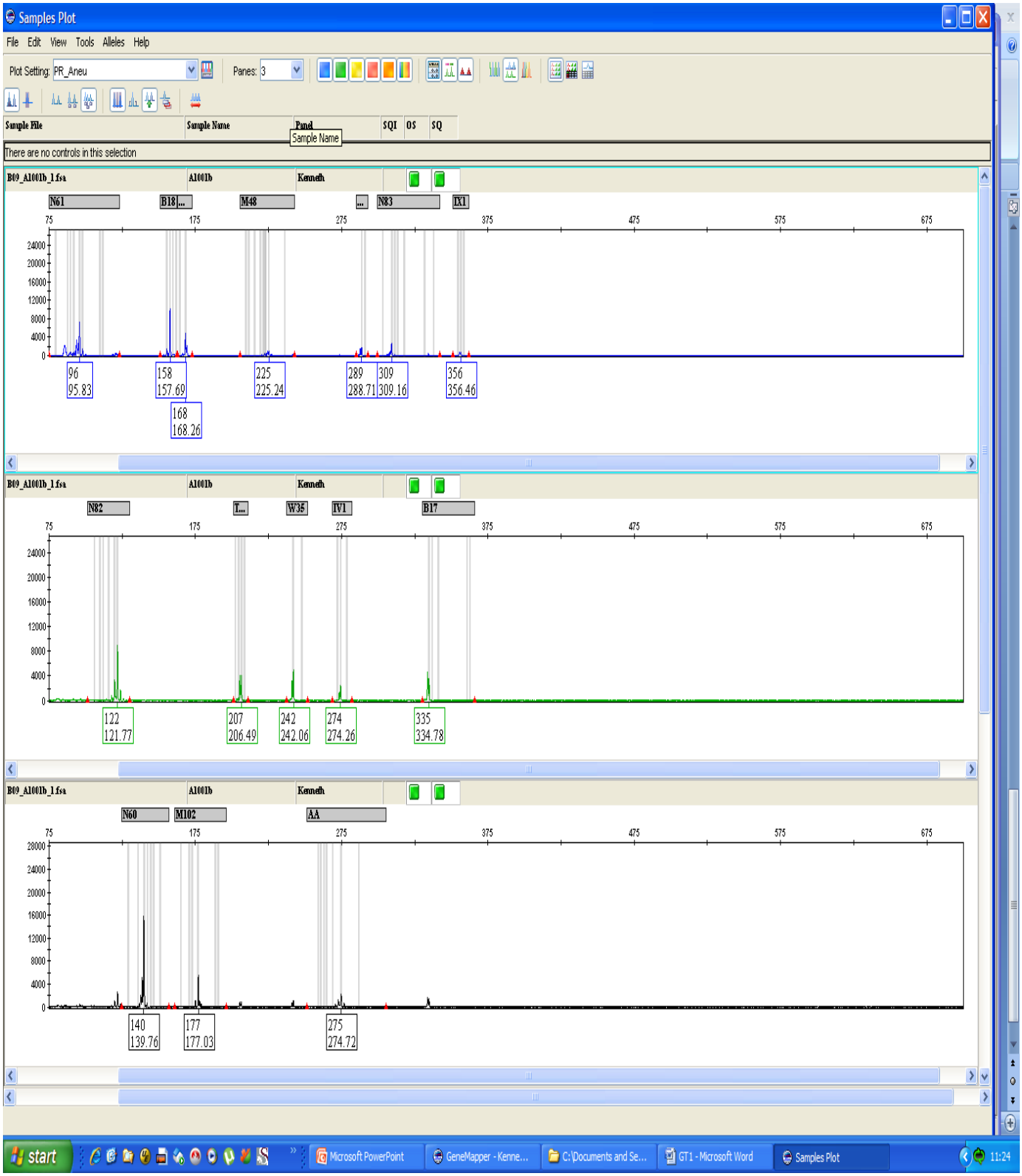

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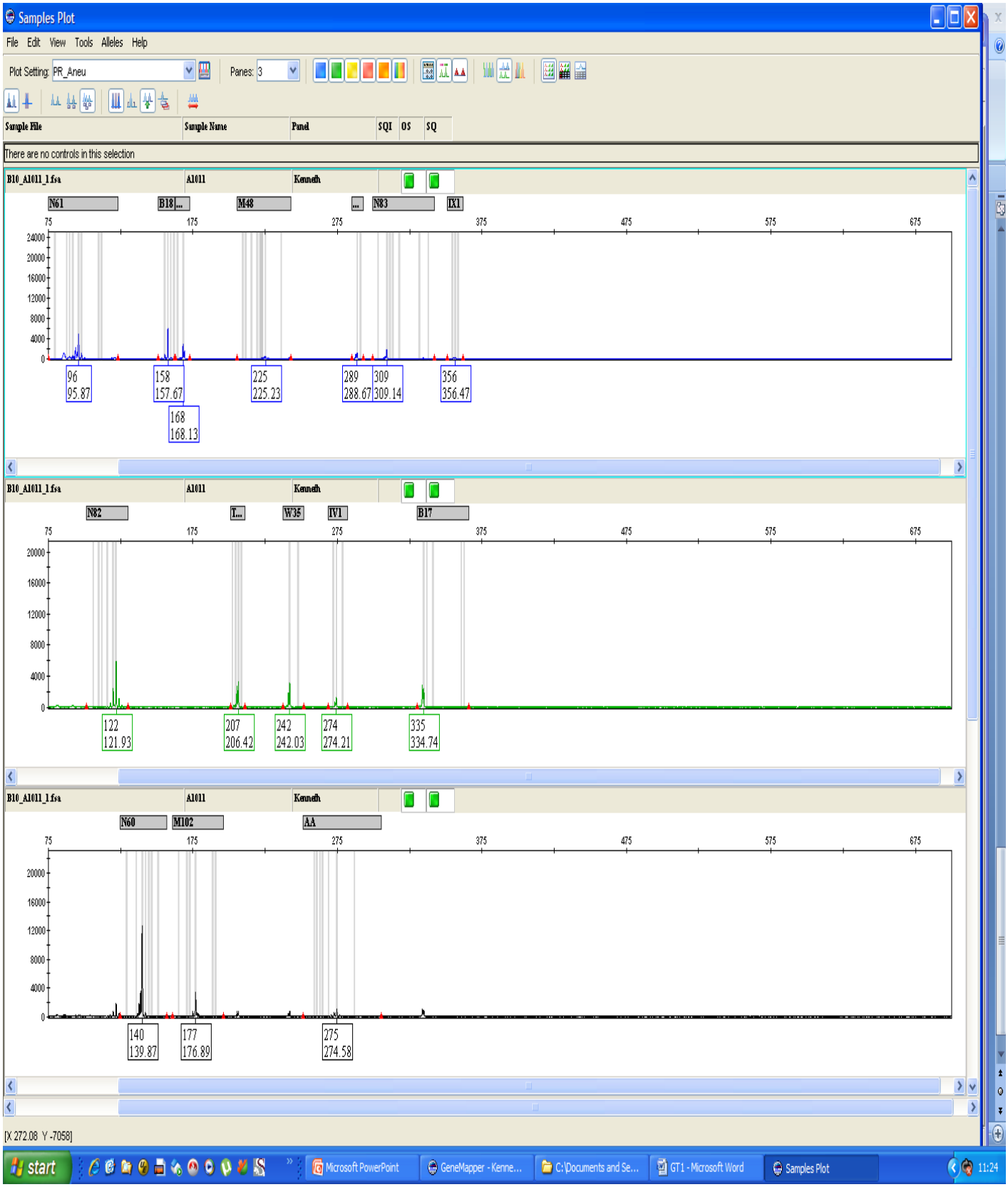
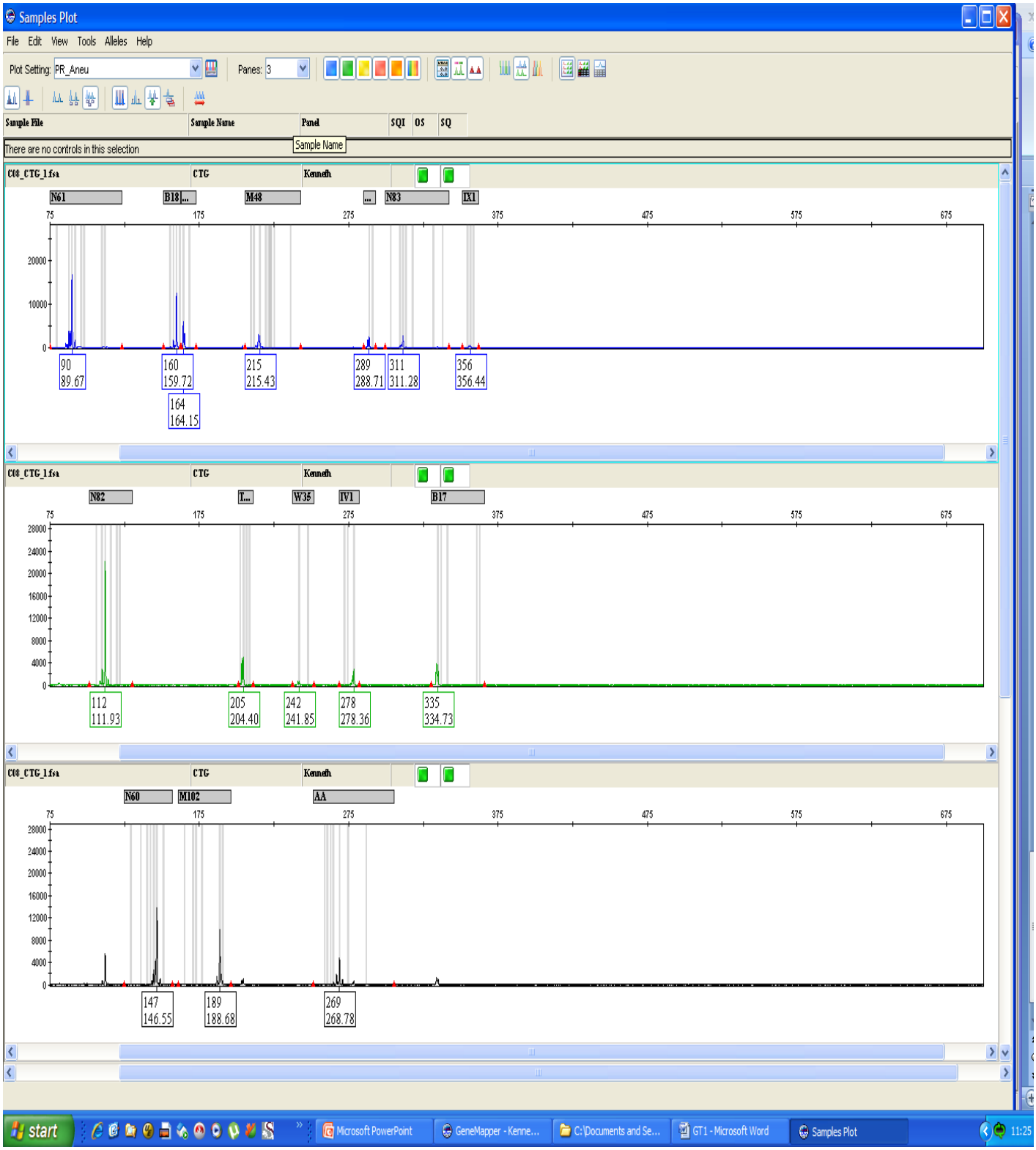


Reference strain CTG



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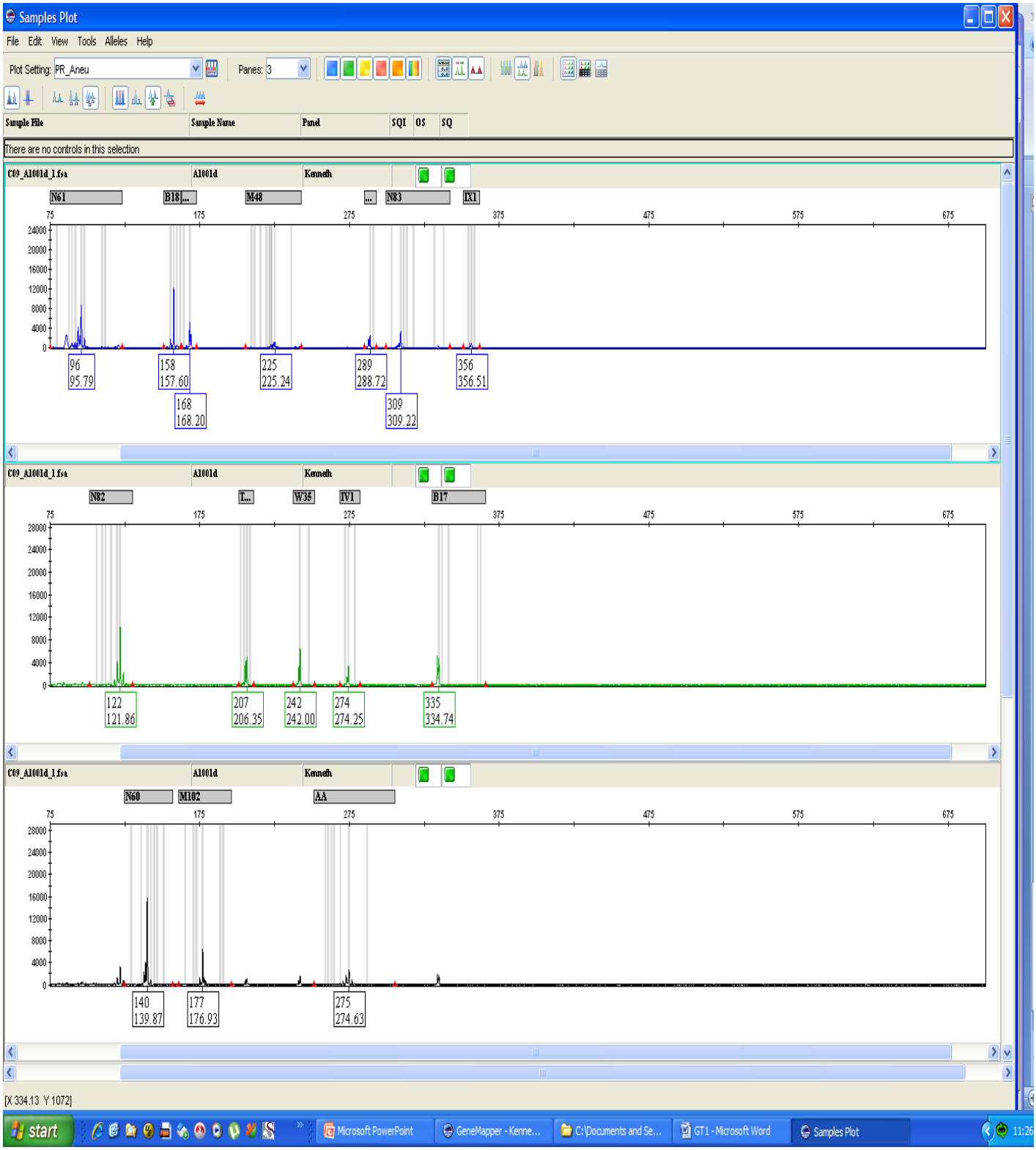

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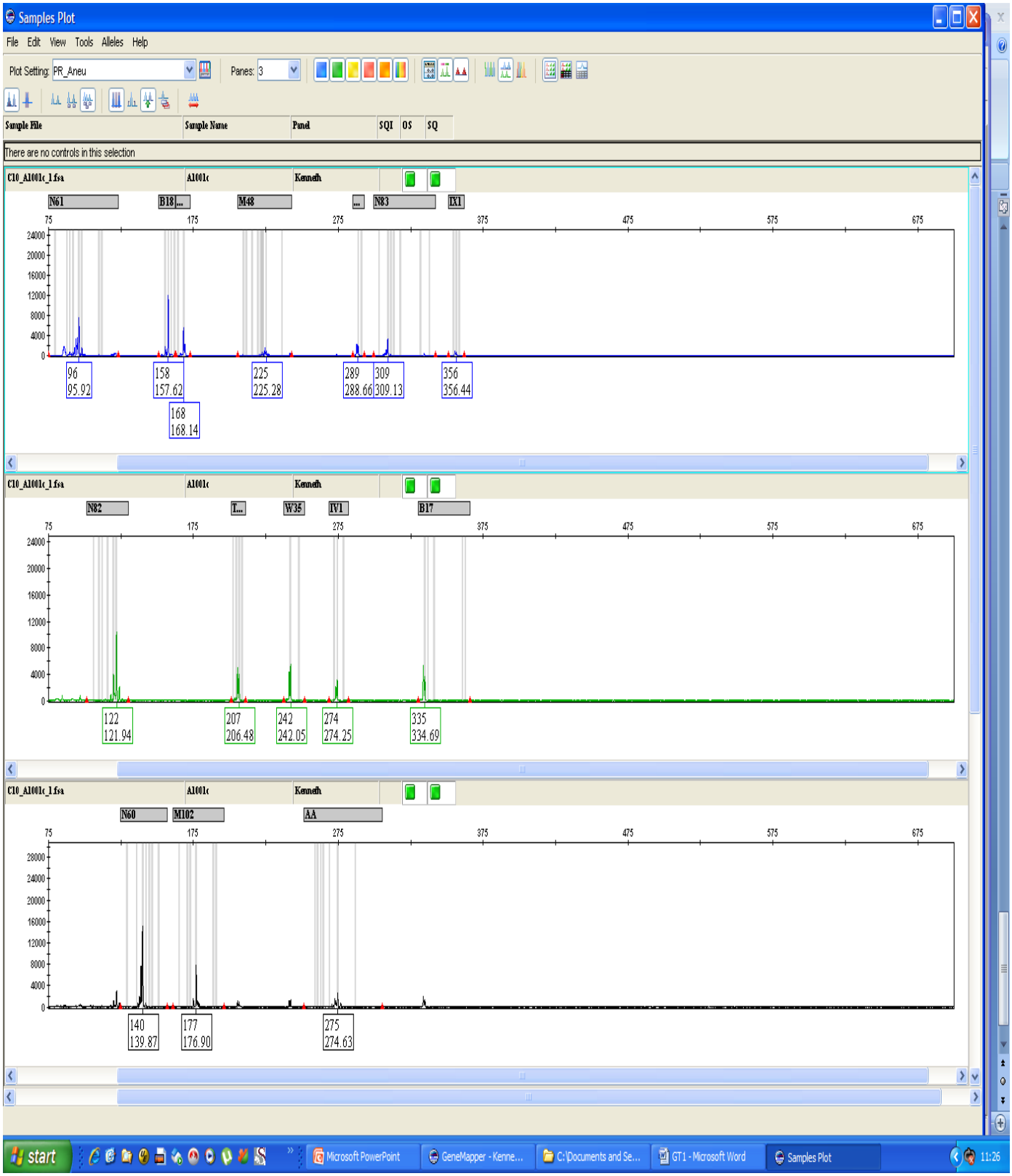

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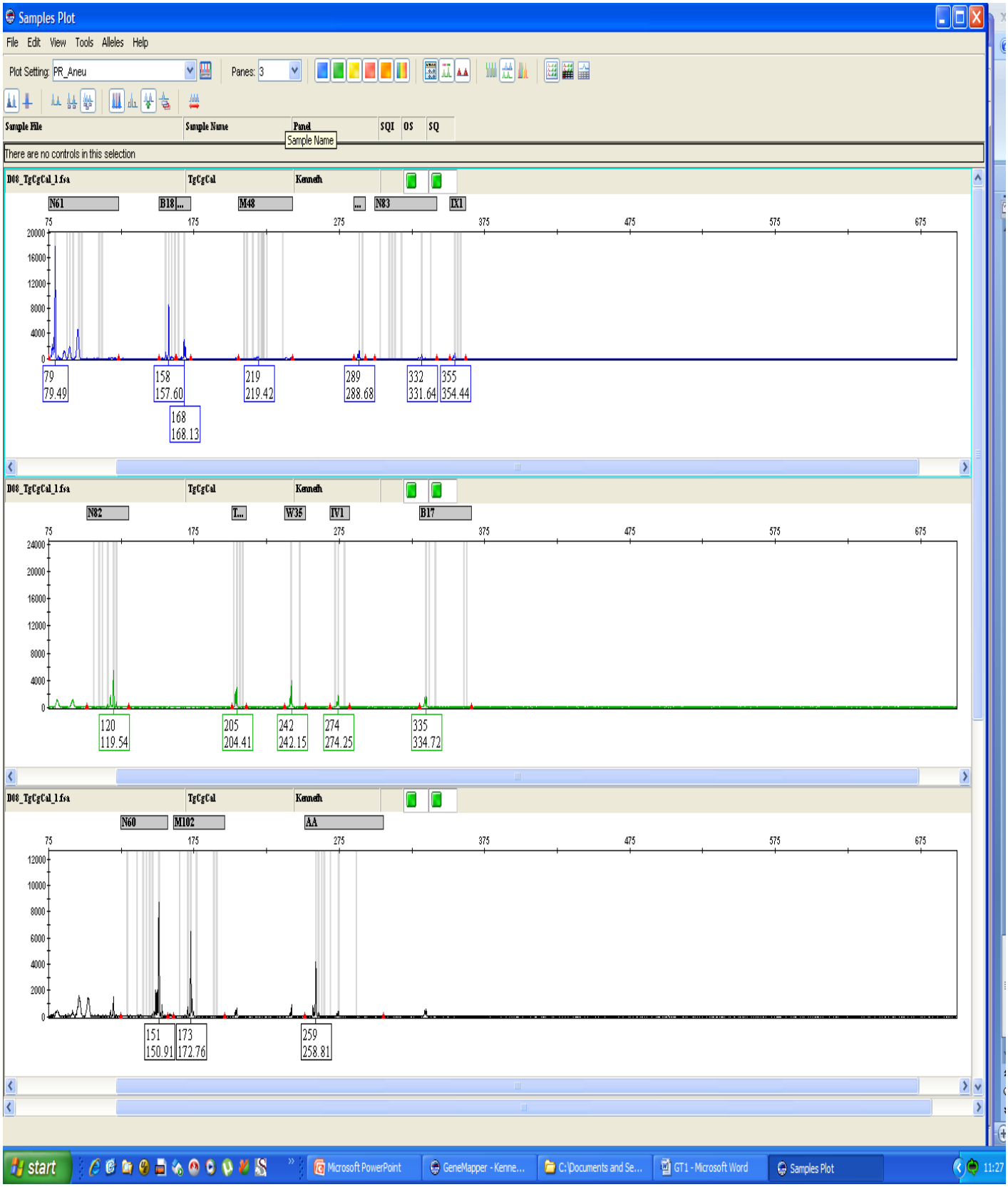
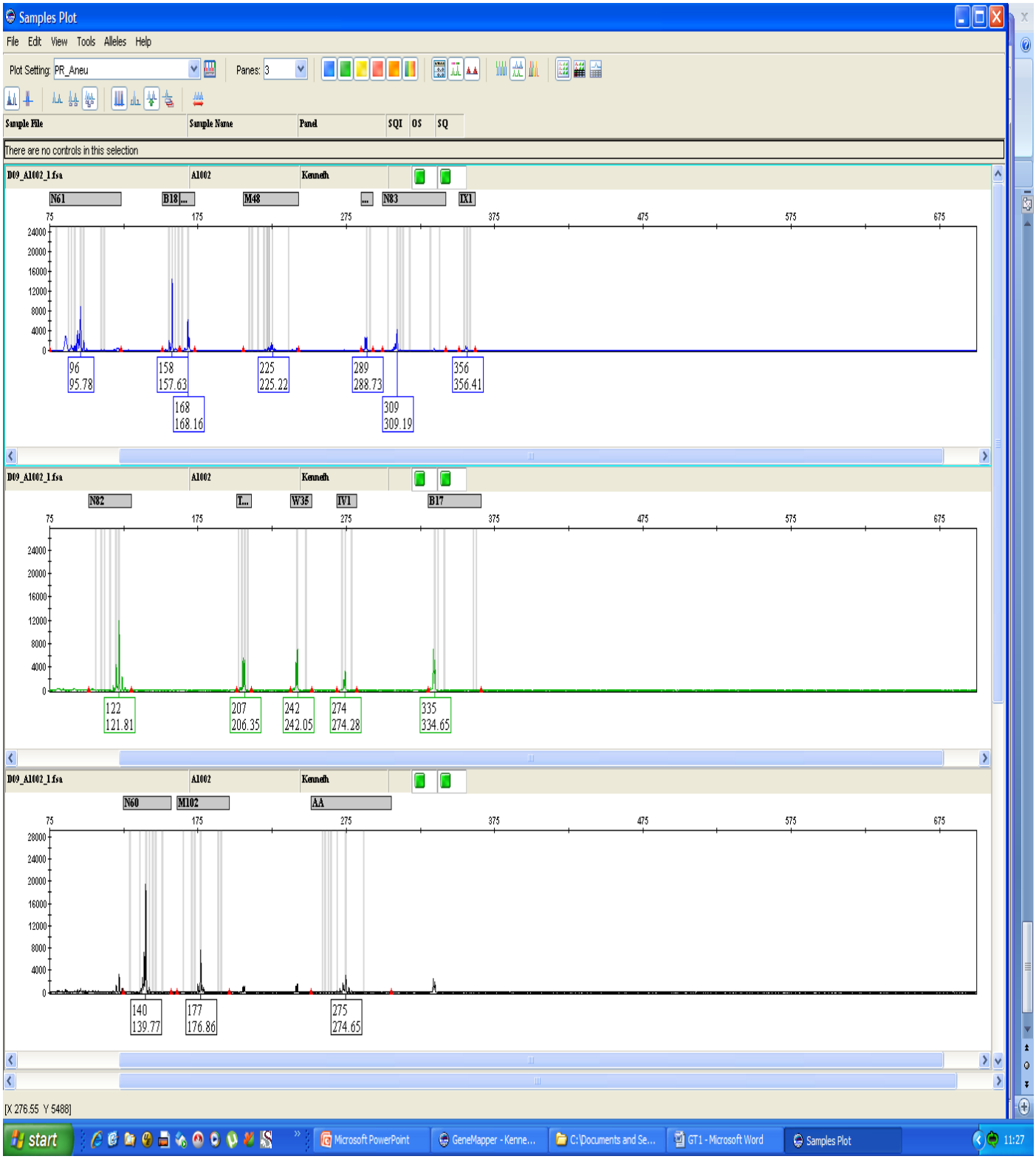


Sample A1002



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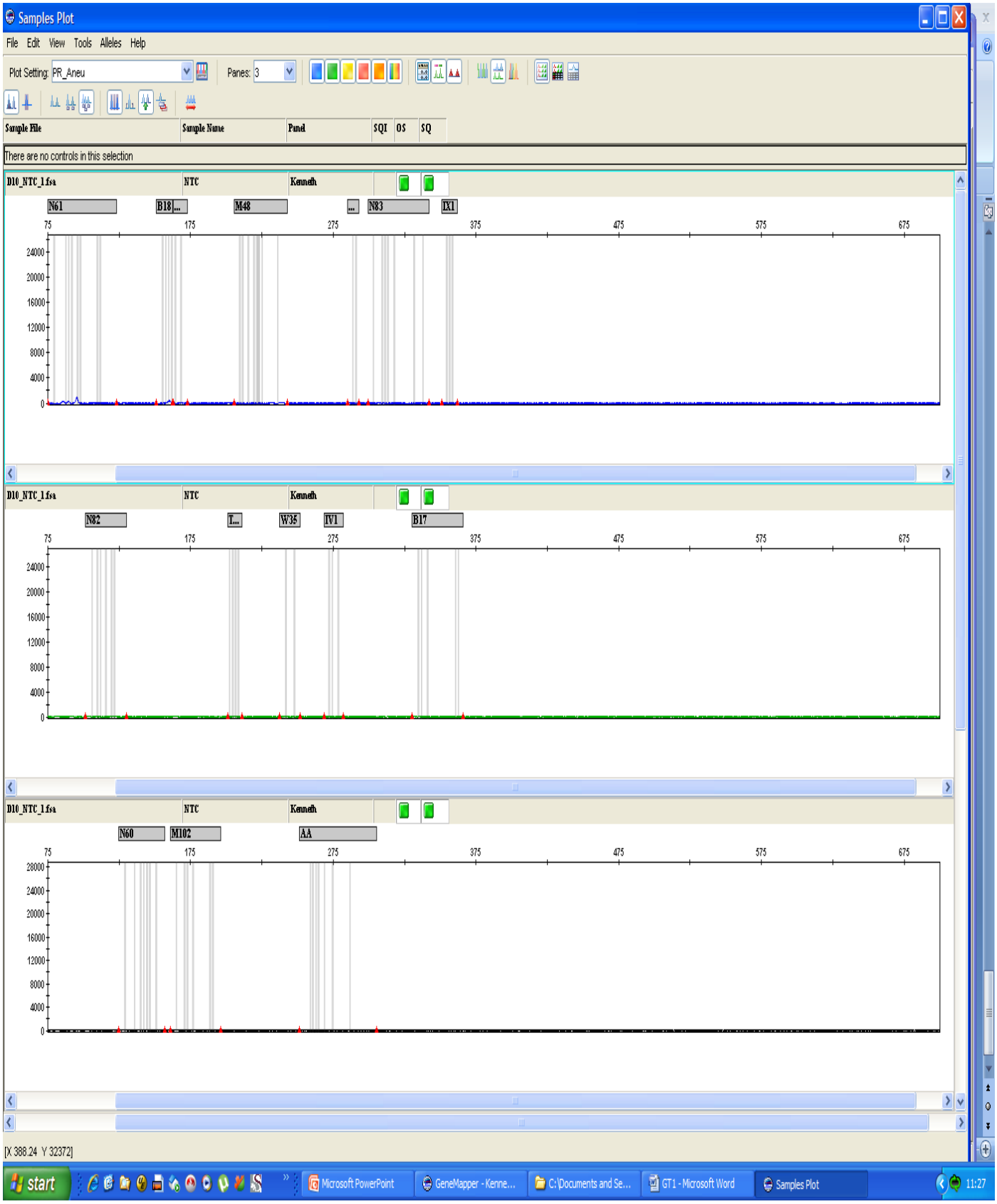
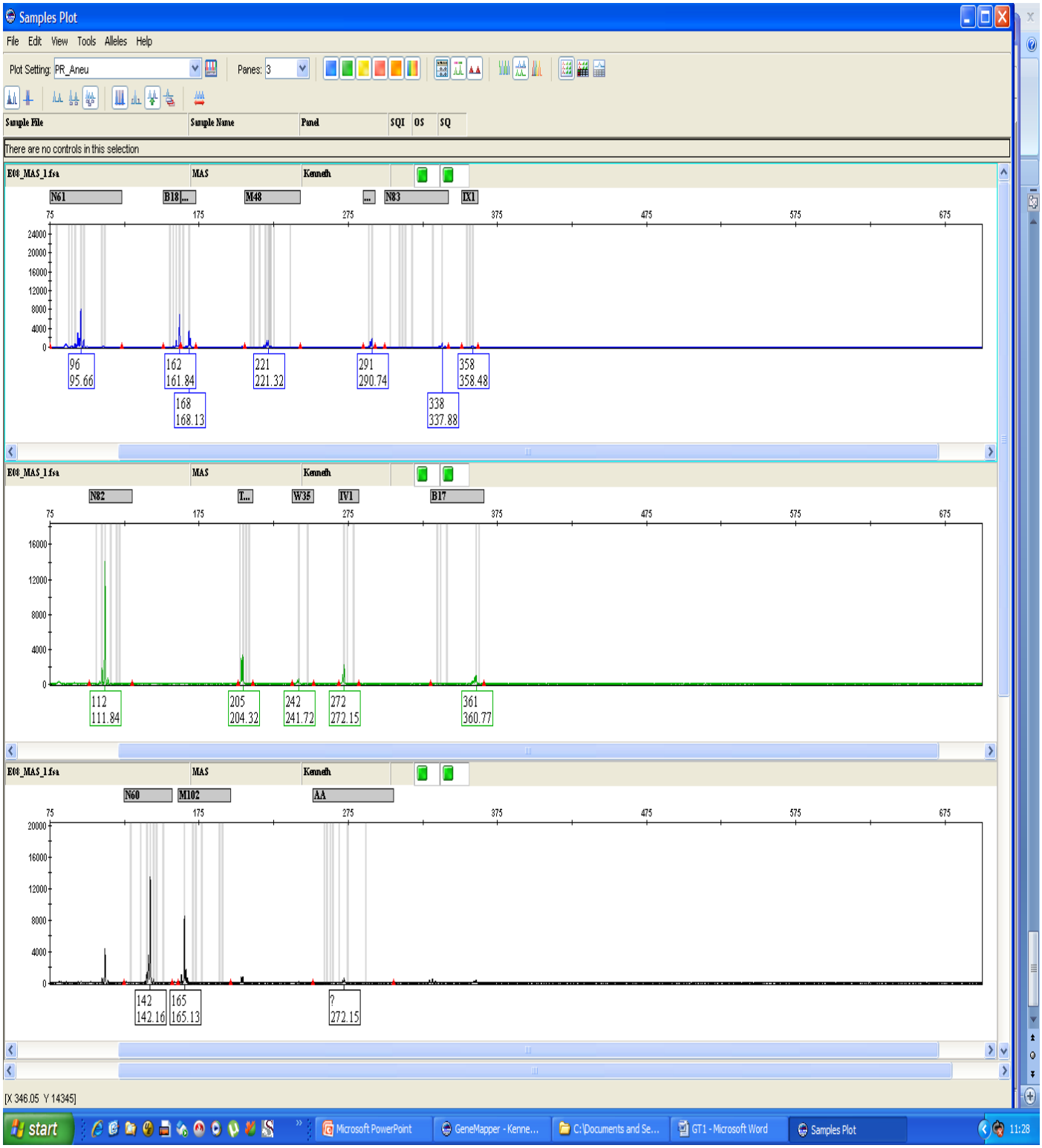


Reference strain MAS



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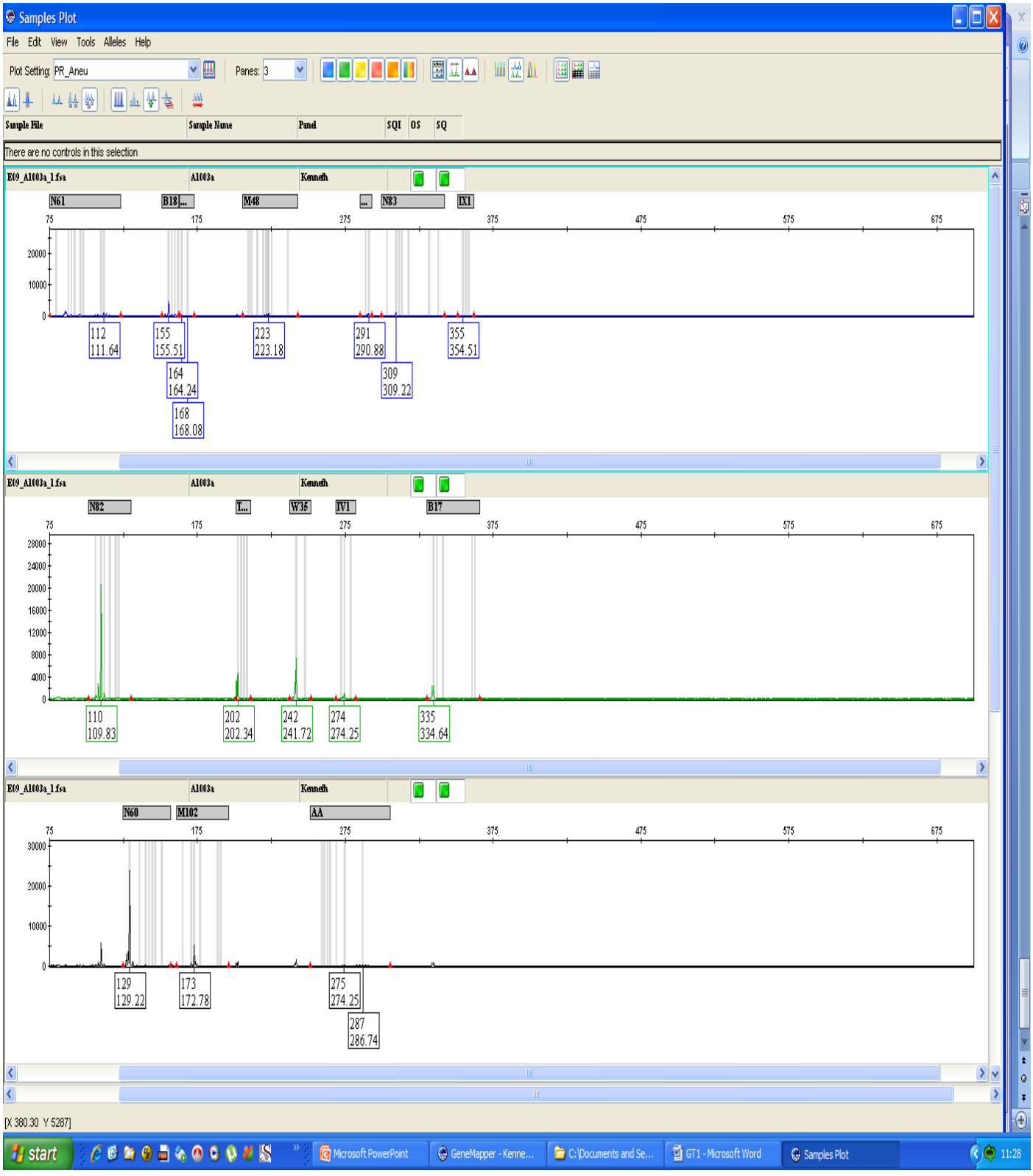
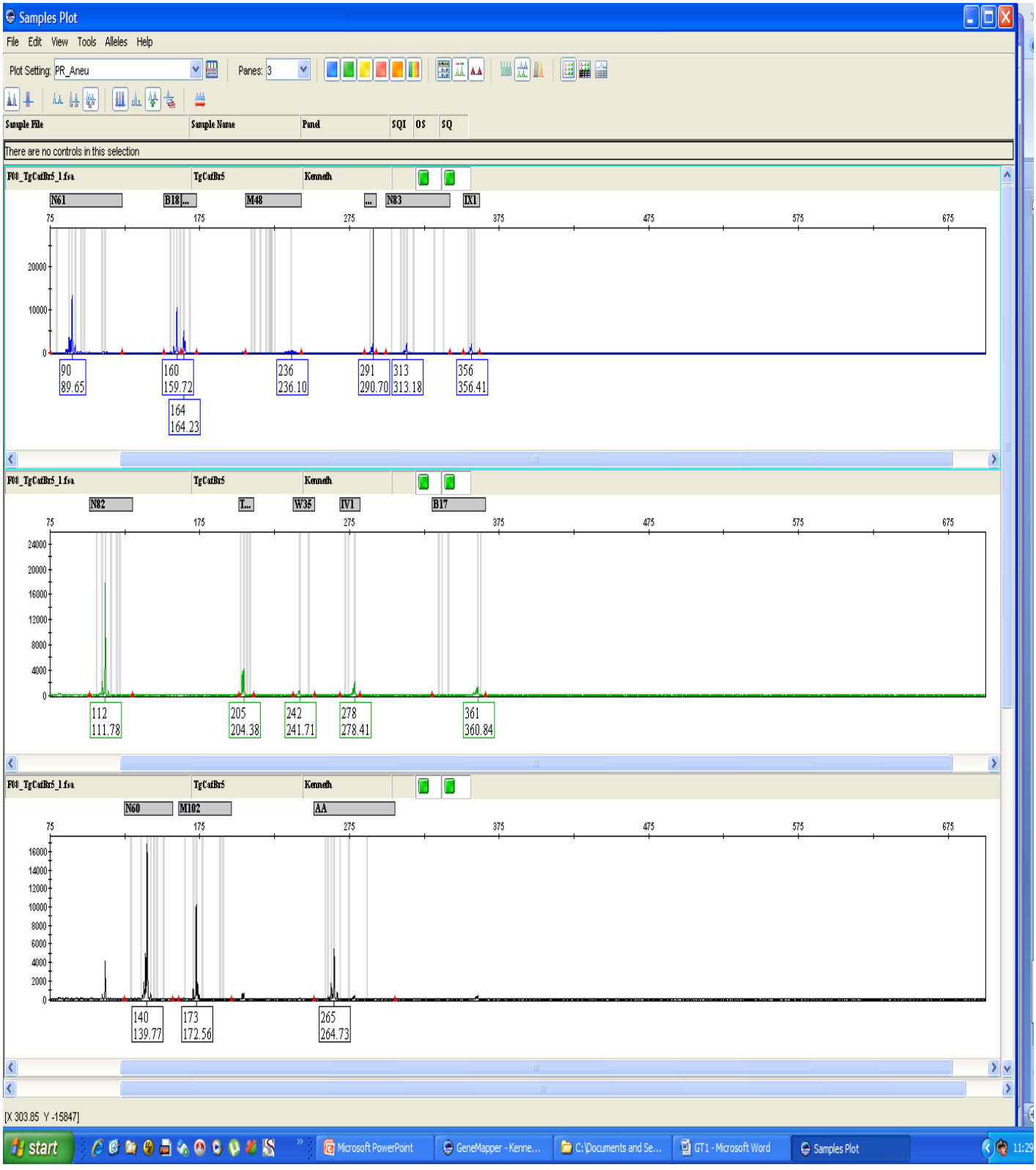


Reference strain TgCatBr5



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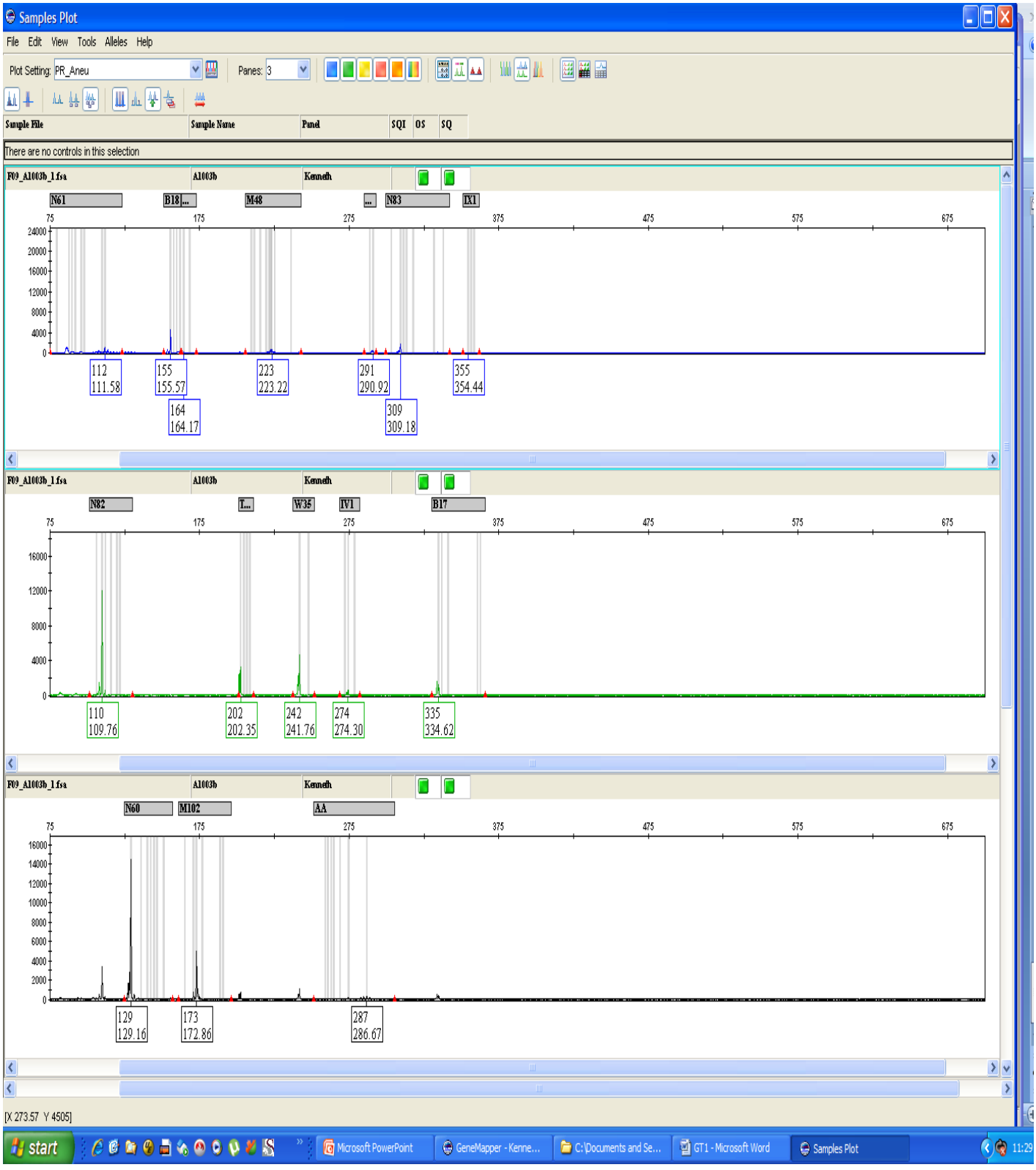

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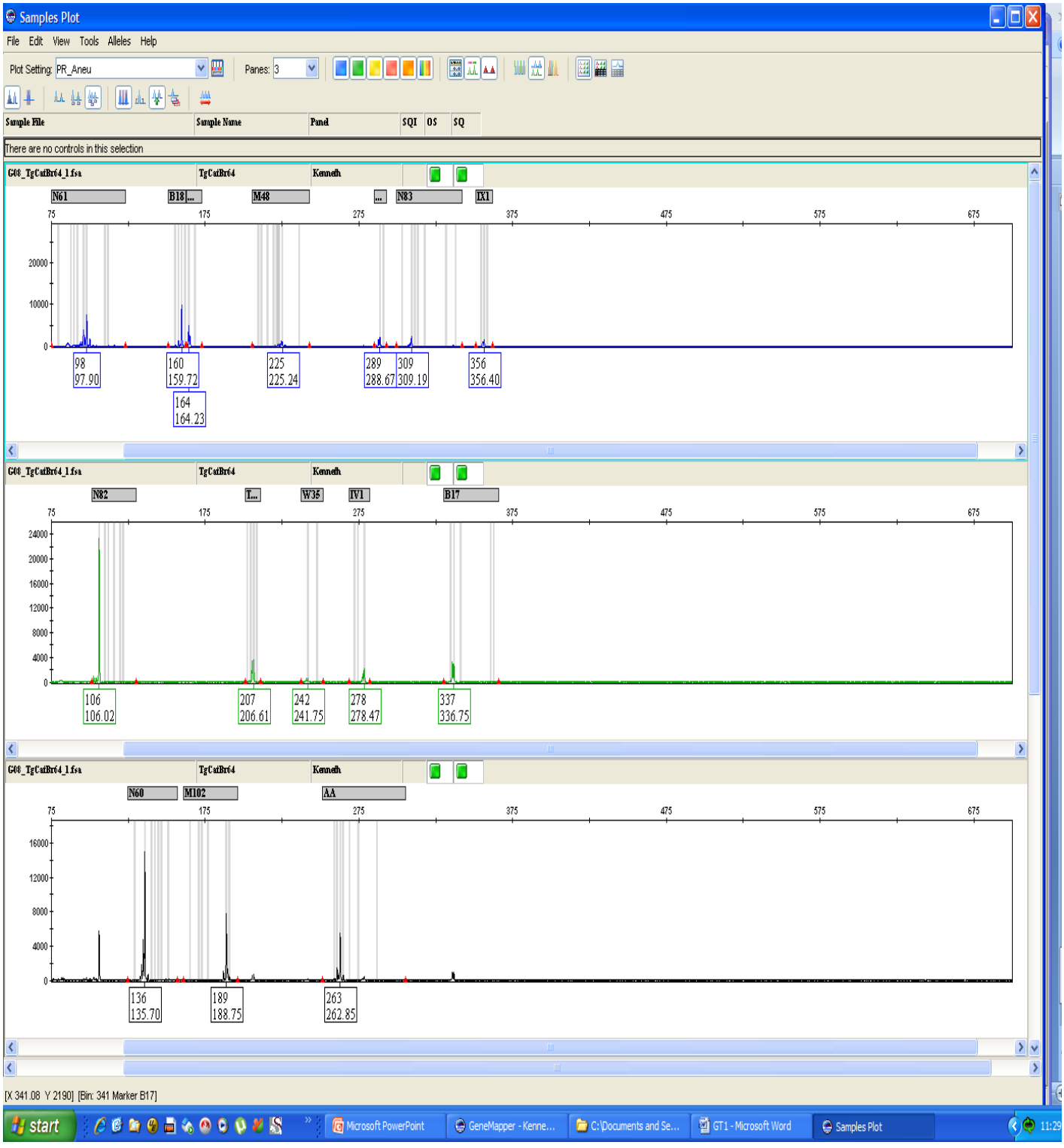

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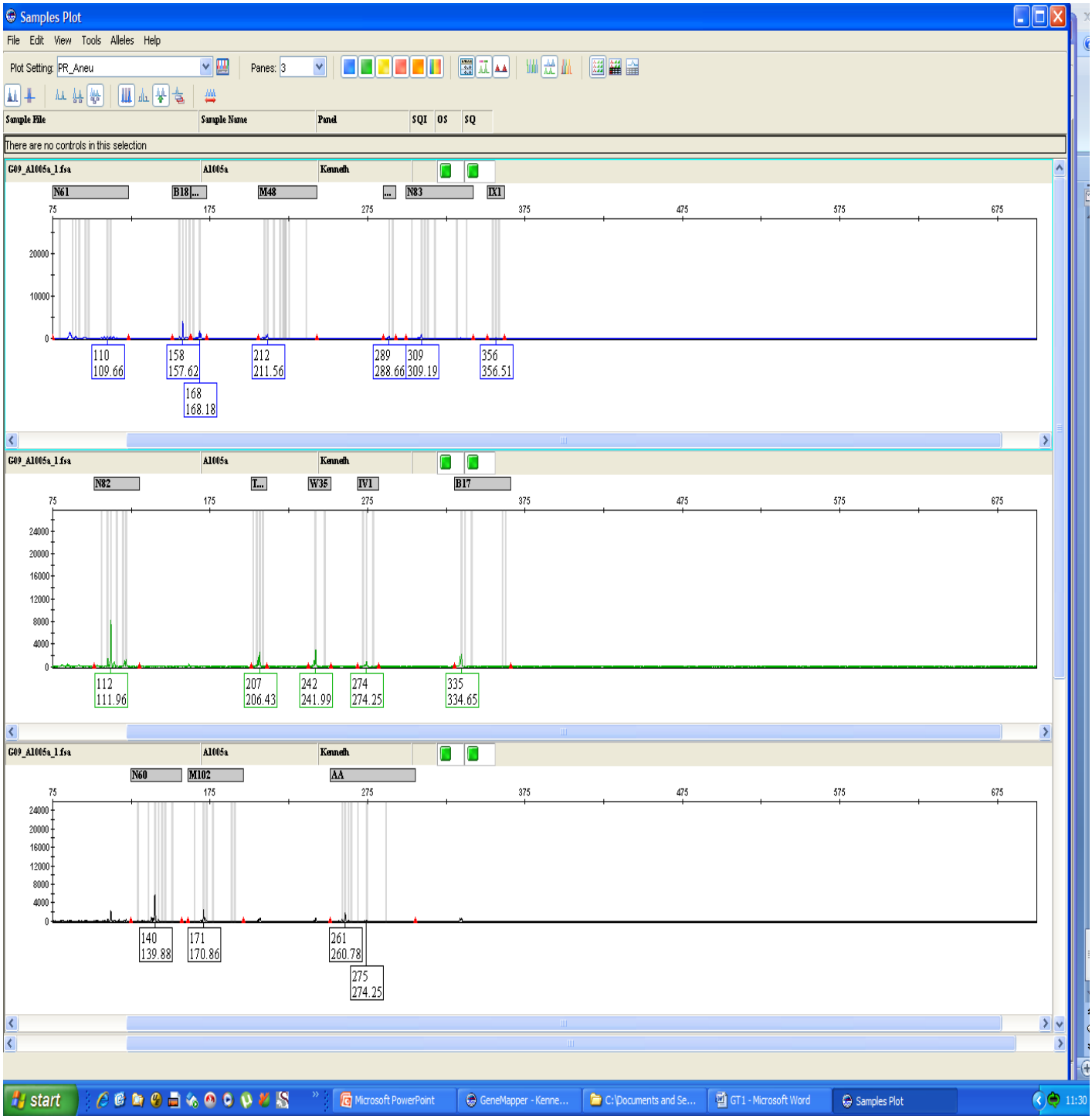

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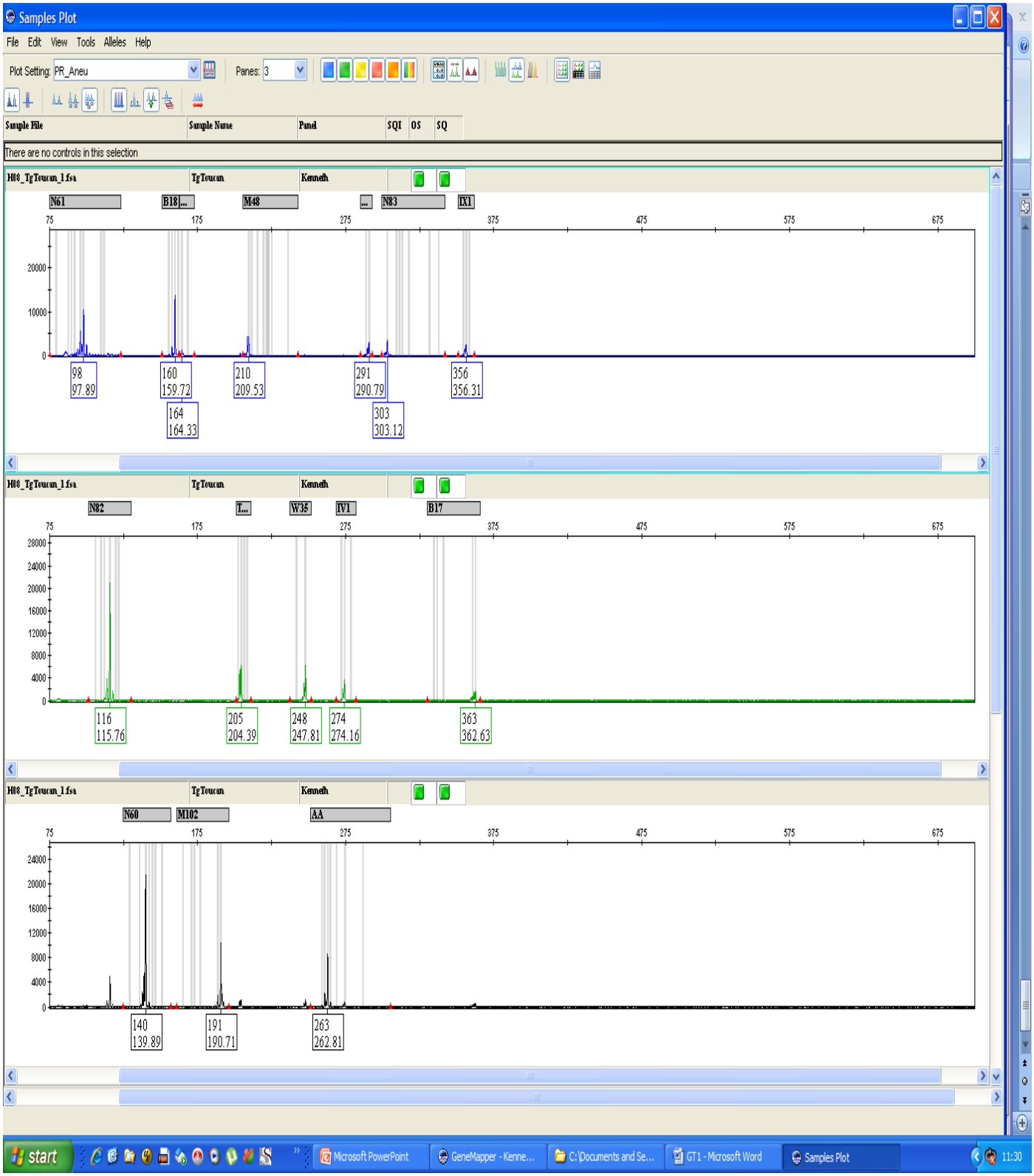

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