

# The Contribution of the Placenta to the Diagnosis of Congenital Tuberculosis

by  
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Thesis presented in partial fulfilment of the requirements for the degree  
Master of Pathology in the Faculty of Medicine and Health Sciences at  
Stellenbosch University



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April 2014

## DECLARATION

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**By submitting this thesis electronically, I declare that the entirety of the work contained is my own, original work, that I am the authorship owner therefore and that I have not previously in its entirety or in part submitted it for obtaining any qualification.**

**Signature:**.....

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## ABSTRACT

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The aim of this pilot project was to determine whether mothers with laboratory confirmed or clinically suspected tuberculosis (TB) had evidence of TB in the placenta. A secondary objective was to correlate evidence of placental TB with neonatal outcome. A total of 56 placentas were examined to determine if there were any specific histopathological features predictive of tuberculosis together with Ziehl-Neelsen (ZN) staining. A total of 30 cases were positive for maternal TB and one case was a false positive maternal diagnosis of TB, whilst 25 cases were negative for maternal TB. Biopsies from these 56 placentas were collected for conventional PCR from the paraffin embedded tissue blocks.

The performance of these two diagnostic modalities (histopathology and PCR) was assessed collectively and individually, and compared to the neonatal outcome (presence or absence of active clinical mycobacterial tuberculosis infection) and evidence of maternal pulmonary and extra pulmonary tuberculosis.

The recognition of specific sites of lesions in the placenta (e.g. membranes vs. intervillous space) may lead to an understanding of the pathogenic mechanisms involved in maternal fetal transmission of tuberculosis, and thereby pave the way for further studies in understanding the pathogenesis of congenital TB.

Invaluable knowledge was obtained in the diagnoses of *M.tuberculosis* in the placenta as it was found that micro abscesses and intervillitis were strong indicators of TB infection in the placenta, however, ZN staining still remains the gold standard for diagnosing *M. tuberculosis* infection in the placenta. PCR is found to have limitations, because only *M. tuberculosis* DNA is amplified and does not distinguish live from dead bacteria.

The conclusion reached is that PCR is of limited value in the diagnosis of active *M. tuberculosis* infection in the placenta using FFPE tissue, while certain histological changes may be indicative of such infection; however confirmation of the organism by ZN staining is still essential.

## ABSTRAK

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Die hoof objektief van hierdie projek was om vas te stel of moeders met bevestigde of vermoedelike TB enige indikasie van TB in die plasenta toon. 'n Tweede objektief was om die neonatal uitkoms teenoor die plasentale TB te korreleer. 'n Totale getal van 56 plasentas was geondersoek om vas te stel of daar enige spesifieke histopatologiese indikasies was van tuberkulose tesame met die hulp van die ZN spesiale kleuring. Die totale getal positiewe vir TB was 30 asook 'n fals positiewe geval vir TB en daar was 25 TB negatiewe gevalle. Ses en vyftig biopsy was versamel van paraffien ingebedteerde weefsel vir die gebruik in PKR.

Die uitvoering van hierdie twee diagnostiese modaliteite was geondersoek elk individueel asook tesamentlik om dit te vergelyk teenoor die neonatale uitkoms (m.a.w die verteenwoordigheid of awesigheid van mikobakteriale tuberkulose infeksie) asook die teenwoordigheid van moederlike pulmonere en ekstra-pulmunere tuberkulose.

Die spesifieke ligging van die letsels in die plasenta (bv. membrane vs. intervillus spasie) kan lei tot verbeterde begrip van die patogeniese meganismes betrokke in die moeder fetale oordrag van tuberkulose en dit dui gee 'n area van navorsing in die toekoms aan.

Waardevolle kennis was opgedoen in die diagnose van *M.tuberkulose* in die plasenta, want die letsels van mikro abbesses en intervillitis gee 'n goeie aanduiding van TB infeksie in die plasenta.

Die ZN kleuring bly nog steeds die standaard metode om *M.tuberkulose* in die plasenta te diagnoseer. PKR het baie limiete want dit kan slegs die *M.tuberkulose* DNA vermeningvuldig, maar dit kan nie onderskeid tref tussen lewendige en dooie bakterie nie. The slotsom in hierdie projek is dat PKR 'n beperkte waarde het in die diagnose van aktiewe *M.tuberkulose* in die plasenta, deur die gebruik van formalien gefikseerde paraffien ingebedteerde weefsel nie terwyl sekere histologiese veranderinge 'n aanduiding van sodanige infeksie kan wees maar dat dit deur die spesiale kleruring (ZN) bevestig moet word.

## **DEDICATION**

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To my loving husband, Eldrich Paulsen for his love and support and all the patience and help with all final details of this thesis, without you I would never have completed this thesis. To my mother, and my rest of my family, thank you for all the support and words of wisdom that you have given me.

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## LIST OF ABBRVIATIONS

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<b>%</b>	percent
<b>µl</b>	microliter
<b>µm</b>	micrometre
<b>3'</b>	3-prime end
<b>5'</b>	5-prime end
<b>BCG vaccine</b>	Bacillus Calmette-Guerin vaccine
<b>BLAST</b>	basic local alignment search tool
<b>bp</b>	base pair
<b>CAW</b>	Prof. C A Wright
<b>cDNA</b>	copy deoxyribonucleic acid
<b>CISH</b>	chromogenic in situ hybridisation
<b>CXR</b>	Chest X-ray
<b>ddH<sub>2</sub>O</b>	double distilled water
<b>DIG</b>	Digoxin
<b>DNA</b>	deoxyribonucleic acid
<b>dNTP's</b>	nucleotides
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>FFPE</b>	formalin-fixed paraffin embedded
<b>GA</b>	Gestational Age
<b>G+C</b>	guanine + cytosine
<b>H and E</b>	Haematoxylin and eosin

<b>HIV</b>	Human Immunodeficiency Virus
<b>IDT</b>	integrated DNA technologies
<b>INH</b>	Isoniazid
<b>IPT</b>	INH preventative therapy
<b>ISH</b>	in situ hybridisation
<b><i>M.africanum</i></b>	<i>Mycobacterium.africanum</i>
<b><i>M.bovis</i></b>	<i>Mycobacterium.bovis</i>
<b><i>M.canetti</i></b>	<i>Mycobacterium.canetti</i>
<b><i>M.caprae</i></b>	<i>Mycobacterium.caprae</i>
<b><i>M.microti</i></b>	<i>Mycobacterium.microtti</i>
<b><i>M.pinnipedii</i></b>	<i>Mycobacterium.pinnipedii</i>
<b><i>M.tuberculosis</i></b>	<i>Mycobacterium tuberculosis</i>
<b>MDR</b>	Multiple drug resistant
<b>MgCl<sub>2</sub></b>	Magnesium Chloride
<b>mRNA</b>	messenger ribonucleic acid
<b>MTC</b>	Mycobacterium.tuberculosis complex
<b>MVUP</b>	Maternal vascular underperfusion
<b>NAAT</b>	Nucleic acid amplification testing
<b>ng</b>	nanogram
<b>NHLS</b>	National Health Laboratory Service
<b>NTC</b>	Non template control
<b>°C</b>	degrees Celsius

<b>PAS</b>	para-amino salicylic acid
<b>PCR</b>	polymerase chain reaction
<b>PPE</b>	personal protective equipment
<b>PS</b>	Dr. P Schubert
<b>PTB</b>	Pulmonary Tuberculosis
<b>rpm</b>	revolutions per minute
<b>RT-PCR</b>	reverse transcriptase PCR
<b>rRNA</b>	ribosomal ribonucleic acid
<b>TAE</b>	tris/acetic acid/EDTA buffer
<b>Taq</b>	<u>Thermus aquaticus</u>
<b>TB</b>	Tuberculosis
<b>Tm</b>	melting temperature
<b>Tr</b>	Trans renal primers
<b>TST</b>	Tuberculin skin test
<b>UTI</b>	Urinary tract infection
<b>UV</b>	ultra violet
<b>WHO</b>	World Health Organisation
<b>ZN</b>	Ziehl-Neelsen



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# 1. LITERATURE REVIEW

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## 1.1 Brief History

Tuberculosis (TB) has been a human disease for thousands of years, with evidence dating back to the ancient Egyptian mummies from 3000-2400 BC who showed signs of this disease.(1) This disease has had many names over the centuries with the Greeks calling it phthisis meaning consumption.

Hippocrates identified this disease in 460 BC saying it is “one of the worst diseases of his time” and described it as the coughing up of blood and fever, and observed that every case identified was almost always fatal. In the 17<sup>th</sup> Century Sylvius was the first to identify the tubercles as a characteristic change occurring in the lungs and other areas of the patient’s body.(2) During the European Renaissance, an Italian doctor, Girolamo Fracastoro, recognized the contagious nature of tuberculosis (TB). The English doctor Benjamin Marten in 1720 was the first to suspect that the disease might be caused by “Animalcula or wonderfully minute living creatures” (similar to the ones described by Anton van Leeuwenhoek in 1626). A French military surgeon, Jean-Antoine Villemin, demonstrated the infectious nature of TB. He observed that a rabbit had extensive TB after injection of material from the pulmonary cavity of a patient that died from TB, when he autopsied the animal after 14 days inoculation.(3)

In 1882 Robert Koch revolutionized the medical world when he was the first to demonstrate the causative agent, which he called *Bacillus tuberculosis*, now known as *Mycobacterium tuberculosis*. In 1890 he developed Tuberculin, a concentrated bacteria-free liquid filtrate of *M.tuberculosis*. Although this product was ineffective in treating the disease, it became of great importance in the diagnosis of the disease. This test (Mantoux test) was named after Charles Mantoux, a French physician who built on the work of Robert Koch and Clemens von Pirquet and is still used today.(3)

The 20<sup>th</sup> century produced the (Bacillus Calmette-Guerin) BCG vaccine, developed from attenuated bovine-strain tuberculosis by Albert Calmette and Camille Guérin in 1906. On 18 July 1921 the new vaccine was administered to a 3-day-old infant in France with great success, but it was not until after the World War II that the vaccine came into widespread use in the United States, Great Britain and Germany. In December 1973 the WHO recommended that BCG should be used in high burden settings.(4)

The final and most powerful breakthrough came during the World War II with the discovery of thioacetatezone, the first mycobacteriostatic drug, by Gerhardt Domagk. Jorgen Lehman in Sweden discovered shortly thereafter another mycobacteriostatic drug called para-amino salicylic acid (PAS). Selman Waksman and his colleagues discovered in 1943 streptomycin, an early class of antibiotics which could “crush” the bacterium that had decimated humanity for thousands of years. This antibiotic successfully treated tuberculosis after it was administered to a critically ill patient in 1944 with huge success.

It was soon discovered that drug resistance could be prevented by using two drugs simultaneously, and initially streptomycin was coupled with PAS. Three pharmaceutical companies discovered Isoniazid almost simultaneously in 1952 and in 1963 R-rifampicin was discovered, leading to the modern treatment era.<sup>(5)</sup> The mainstay of the modern treatment therapy also includes PZA (pyrazinamide) which was discovered in 1952 by Kusher.

## 1.2 Tuberculosis today

It is estimated that one third of the world’s population is infected with *Mycobacterium tuberculosis*. In 2012 more than 8.6 million people were infected with TB and more than 1.3 million people died of the disease in that year (including 320 000 deaths among the HIV positive population).<sup>(6, 7)</sup>

The 2012 WHO report estimates the burden of disease in women is 2.9 million new cases of TB and 513,000 new cases in children in 2012.<sup>(7)</sup> Tuberculosis remains one of the top three causes of death in women worldwide.<sup>(7)</sup> Approximately 410,000 women in 2012 died from tuberculosis (160,000 HIV-positive) and an estimated 74,000 HIV-negative children. The figures for the TB and HIV associated mortality in children are not yet available. <sup>(7)</sup>

Between 5-10% of individuals who become infected can subsequently develop clinical disease<sup>(8)</sup>. It is a leading cause of death amongst infectious diseases, accounting for the majority of these avoidable deaths.<sup>(6)</sup> The modern TB epidemic is fuelled by population growth, poverty, immune deficiency (human immunodeficiency virus (HIV) epidemic), drug abuse and the emergence of multidrug-resistant (MDR) TB.<sup>(6)</sup> Approximately 13% of the 8.6 million people who developed TB in 2012 (1.1 million) were also infected with the HIV virus and three quarters of these were in Africa. <sup>(7)</sup> In South Africa

the estimated number of HIV-positive incident TB cases is between 330 000 and 390,000.(7) The increasing burden of HIV on tuberculosis can be demonstrated by figures that show that 58% of HIV-infected individuals in South Africa are also co-infected with TB. (9)

The key public health problem of increasing cases of tuberculosis, can be viewed as an obstacle to delayed achievement of the Fourth Millennium Developmental Goal, namely reducing child mortality by two-thirds between 1990 and 2015 and Goal 5, namely to improve maternal health.

In South Africa, the Western Cape has the highest incidence of tuberculosis, and in 2007, 1005.7 TB cases per 100 000 was notified with a background (10) maternal HIV prevalence of 15 % present.(11) Women carry the greatest TB disease burden during their childbearing years (15-49 years) and more than 80% of all TB deaths occur during these years. (6) In sub Saharan Africa 3 to 4% of HIV-positive mothers die within a year of delivery.(3)

A study done in Durban, Kwazulu Natal in 1996, showed that TB was the third most common cause of maternal death (14.9%), following sepsis and hypertensive disorders of pregnancy(12) and 75% of known cases were HIV-1 positive.

### 1.3 Congenital tuberculosis

Congenital TB results from maternal TB both when the infection involves the genital tract and subsequently the placenta or the bacilli may be introduced hematogenously to the fetus via the umbilical cord, or via infected amniotic fluid that may be ingested or aspirated. (13) Intrapartum aspiration or ingestion of infected amniotic or cervical vaginal fluid is also a source of infection .(13) In the literature from 1952 and 1956, the criteria for diagnosing congenital TB, as proposed by Beitzke (1935) was to confirmed clinical diagnosis of TB within 3 weeks of life. He (Beitzke) proposed that a case should not be accepted as congenital tuberculosis unless (1) the presence of *M. tuberculosis* in the infant was proved, and (2) either (a) a primary tuberculous complex was present in the liver, or (b) the tuberculous lesions were present at birth, or (c) extra-uterine infection could be excluded with certainty.” (14)

Cantwell *et al* (15), 1994, proposed the ‘modern’ diagnostic criteria for congenital TB as a proven tuberculous lesion in the infant plus one or more of the following;

1. Lesions occurring in the first week of life,
2. A primary hepatic complex,
3. Maternal genital tract or placental tuberculosis or
4. Exclusion of postnatal transmission by thorough investigation of contacts.

Although congenital TB occurs rarely (English published literature reports only approximately 400 cases) (16-18) uncertainty exists as to this exact figure. Cases of congenital TB are frequently not reported, or not identified as such. Le Roux *et al* reported the first case of congenital TB in South Africa in 1978. He reported that “..... the early onset and diffuse, evenly spread tubercles throughout both lungs are typical features of congenital TB. The miliary pattern would suggest haematogenous spread to the lungs.” (19)

The risk of TB in pregnancy has increased fourfold due to recent changes in the epidemiology of the disease, which has led to an increased risk of congenital TB. (20)

Infants less than 12 months of age are at highest risk for developing TB after infection (50% approximate risk).(21) This risk is potentially increased in HIV-infected and HIV exposed and uninfected infants due to their immune immaturity, immunocompromised systems, increased exposure to TB from immunodeficient family members and increasing evidence that the BCG vaccine is less efficient in HIV infected infants.(22) In addition the HIV exposed and uninfected infants are immunocompromised temporarily due to exposure to the HIV virus although they are not infected.(22)

High risk factors for transmission of tuberculosis from a mother to the fetus or neonate include:  
maternal miliary or untreated TB

sputum positive smears

disease diagnosed late in pregnancy or post-delivery

lack of prenatal care

primary TB rather than reactivation (3)

Culture-confirmed childhood (<13 years of age) TB surveillance data from March 2003 through February 2009 at Tygerberg Children’s Hospital in the Western Cape province found 72 of 905 cases (8%) were <3 months of age at diagnosis and of these at least 12 (1.3%) had congenital TB(22). Pillay *et al* (23) reported that among 107 mothers with TB in pregnancy 16 babies[15%] developed TB in the first 3 weeks of life, of which 12 were in the first week, which fulfils the modern criteria for congenital TB.

Congenital TB has a very poor prognosis when treatment is delayed. Congenital TB mimics other neonatal illnesses and as it is relentlessly progressive, this treatable disease is unfortunately often

diagnosed too late to effect a cure. (24, 25) The clinical diagnosis of congenital TB is difficult and requires a high index of suspicion. Factors that should alert one to make a clinical diagnosis is a history of maternal TB, a low birth weight infant, hepatosplenomegaly in a neonate, respiratory signs and symptoms, positive gastric washings submitted for TB culture, a positive Tuberculin Skin Test (TST), suggestive chest radiographs and/or positive TB blood culture(26).

Marais et al. have reported in 2004, that in the absence of TB chemoprophylaxis; up to 50 % of TB-exposed untreated infants aged < 1 year will develop tuberculosis within 1-2 years, and up to 30% may develop progressive pulmonary or disseminated (miliary) TB (21).

#### **1.4 Placental histopathology**

The first cases of congenital TB were reported by Schmorl and Birch-Hirschfeld in 1891,(27) however the first reports of placental tuberculosis were in 1904 when Schmorl and Geipl found microscopic lesions in 9/20 placentas from mothers with TB, but all infants remained healthy. The difficulty of identifying placental TB can be illustrated in one case where they examined 2000 sections before demonstrating the organism.(28)

The literature on placental histopathology in TB is rather scarce and the spectrum of lesions associated with TB in the placenta is not well described. The delay in clinical onset of the disease in the neonate often in a mother with clinically undiagnosed TB is one of the explanations for the paucity of reports of placental pathology in congenital TB. When the placentas have been submitted for pathology however, the problem is compounded in that the morphology described in the literature is heterogeneous. The histopathological lesions that have been described associated with documented placental tuberculosis include chorioamnionitis, caseating granulomatous villitis, chronic villitis intervillitis, acute villitis, micro abscesses, perivillous fibrin and microinfarcts. Our knowledge of morphological reactions in the placenta has significantly advanced during the past 15years. Old terms such as placentitis and membranitis have been dropped and replaced by new more specific definitions that are still not universally accepted by all.(29) The mycobacterial organism has been identified on Ziehl Neelsen staining within the amnion, decidua and chorionic villi as well as intervillous space. (30-33) In sites, other than the placenta and meninges, necrotizing granulomatous inflammation is the hallmark of mycobacterial infection. Within the placenta the morphological changes are however less specific. Villitis (often with perivillous extension) is usually a severe, chronic lymphohistiocytic reaction with

many multinucleated histiocytic giant cells without well-formed granulomas. These morphologic changes are not specific for tuberculosis but can also be due to Herpes Simplex virus, Varicella, Toxoplasmosis, Leprosy, Listeria, Chagas' disease, Cytomegalovirus and Blastomycosis. (34, 35)

Increased awareness of the clinical significance of perinatal tuberculosis and therefore heightened awareness by obstetricians and neonatologists as to the potential value of placental pathology have resulted in an increase in cases of congenital TB being diagnosed by histological examination of the placenta (24). At Tygerberg Hospital, placentas from mothers diagnosed with TB within 3 months prior to delivery, or mothers with clinically suspected TB at delivery, are routinely submitted for histological examination. This is done in an attempt to facilitate early diagnosis of congenital TB and to urgently commence a full treatment course rather than one of prophylaxis in the neonate.

### 1.5 The *Mycobacterium tuberculosis* organism

*Mycobacterium tuberculosis* is a gram-positive, rod shaped, bacillus containing a complex cell wall having very high lipid content. (36). The *M.tuberculosis* complex includes *M.tuberculosis*, *M.bovis*, *M.africanum*, *M.canetti*, *M.caprae*, *M.pinnipedii*, *M.microti* which can cause infective disease in humans and animals. (3)

In 1998 the entire genome of *M.tuberculosis* H37Rv was completely sequenced, and revealed that the genome contained 4,411,529 bp and around 4000 genes. It has a very high G+C content of about 66%. In contrast to many other bacteria, many of the genes of *M.tuberculosis* are involved in the synthesis and metabolism of lipids. *M.tuberculosis* contains around 250 enzymes involved in lipid metabolism and much of this metabolic capability enables the mycobacteria to synthesize their very complex lipid-rich cell wall that contributes to their virulence and pathogenicity. (3)

The presence of mycolic acids and other lipids outside the peptidoglycan layer makes mycobacterium an acid fast bacillus. Thus basic fuchsin dye cannot be removed from the cell following treatment by acid alcohol and is the primary principle behind the Ziehl Neelsen (ZN) stain.(36)

Mycobacteria are difficult to demonstrate by the Gram stain technique which is the standard stain for recognising bacterial organisms in histopathological sections, because their capsule is hydrophobic due to long chain fatty acid content. The fatty capsule influences the penetration and resistance to removal of the stain by acid and alcohol, hence the name acid fast bacilli. Heat and phenolic reagents are useful tools in the Ziehl Neelsen (ZN) stain to reduce surface tension and to increase the porosity, so that the dyes may penetrate the capsule.

A positive diagnostic reaction shows magenta coloured slightly curved and beaded bacilli 2-3µm in length indicating acid and alcohol-fastness.(36)

Other stains such as the auramine stain may be used for diagnosis but they destroy the tissue morphology and utilise carcinogenic compounds and require specialised and expensive fluorescent microscopes that usually are not readily available in routine histopathology laboratories.

In the past guinea pig inoculation was the preferred method of isolating the *M.tuberculosis* from clinical specimens, such as sputum and CSF. This method of isolating the bacterium has since been phased out due to it not being cost effective, the stringency of safety legislations and the current climate of opinion against using animals for diagnostic work/experiments. Newer culture methods: 1. Kirschner medium with or without antibiotics .(37) 2. Solid media (Lowenstein-Jenson ) containing egg as a basis equalled and surpassed the guinea pig inoculation technique. No consensus was reached at the time to decide which was the superior method. (38)

## 1.6 PCR

PCR is a well-established technique that is used widely as a rapid diagnostic tool for the detection of *M. tuberculosis* DNA. Although this technique is very sensitive and specific it can only show the presence of DNA in the sample and cannot demonstrate live bacilli (39).

A wide variety of PCR methods have been developed in the past few years for detecting *M.tuberculosis* other than the conventional PCR method. These PCR assays may either target DNA or rRNA(40, 41).

- Reverse Transcription PCR (RT-PCR) is a method used to amplify, isolate or identify a known sequence from cellular or tissue RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene.
- Quantitative real time PCR is a method that uses fluorescent dyes such as Syber green, or flourophore containing DNA probes, such as Taqman to measure the amount of amplification product in “real-time” thus there is no measurable end product. The signal produced increases in direct proportion to the amount of PCR product present in the reaction. Recording the amount

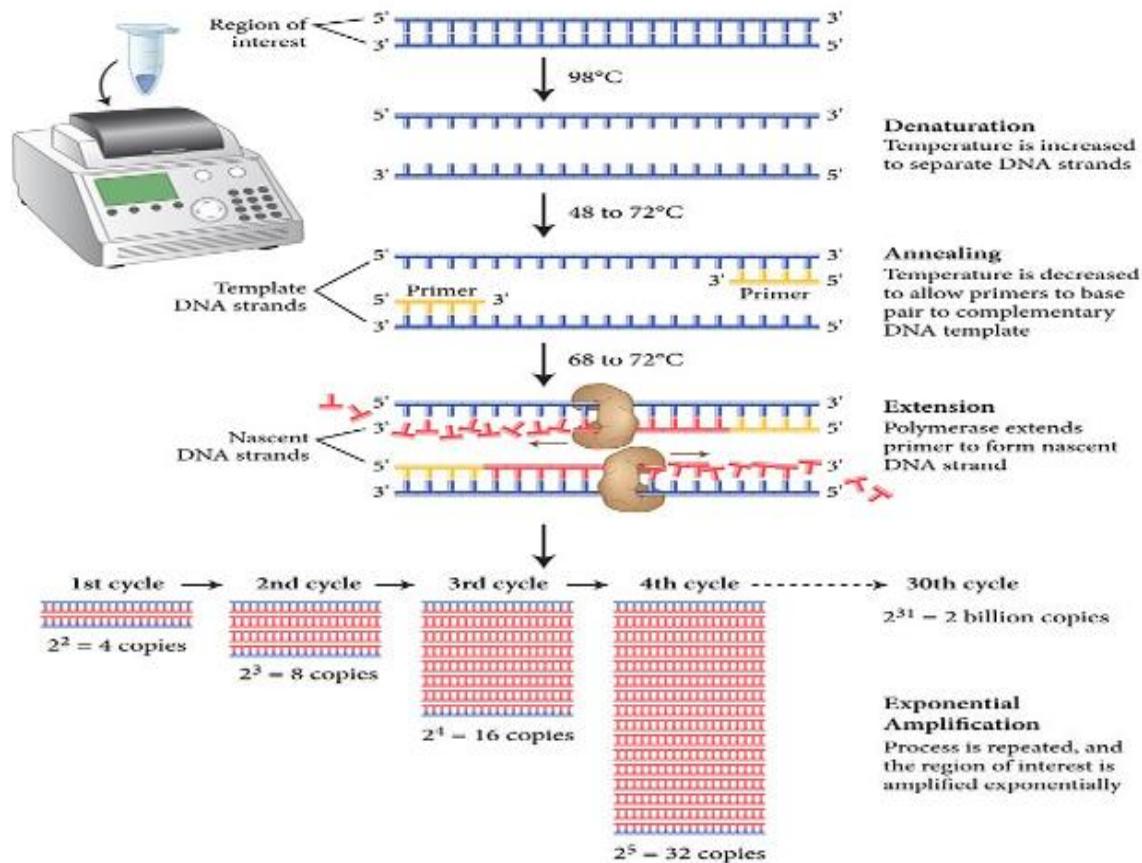


of fluorescence emission at each cycle, it is possible to determine the PCR product amount during the exponential phase and if it correlates to the initial amount of target template added.

- Multiplex PCR is a method that uses multiple primer sets within a single PCR mix. This produces amplicons of varying sizes specific to different DNA sequences. This method is extremely useful in detecting multiple pathogens in a sample. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several reiterations with different primers thereby requiring more reagents and time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis.
- Nested PCR is a method that increases the specificity of DNA amplification by reducing background due to non-specific amplification of DNA. Two sets (instead of one pair) of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The products are then used in a second round of PCR with a set of primers whose binding sites are different from each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

Contamination of PCR is still of major concern in most diagnostic laboratories, but can be managed and reduced with appropriate laboratory design, i.e. Separate rooms for certain stages of the PCR method, strict discipline about sample processing and handling of reagents.

It is a well-known fact that formalin fixed paraffin embedded (FFPE) tissue is often stored for years and presents a huge resource of morphologically well preserved tissues. Many challenges are faced in the application of molecular DNA based techniques to FFPE tissue. It has been reported that FFPE tissue has a tendency to cause nicking of the nucleic acids in the tissue as well as cross-linking of the proteins and it is for this reason that it is so difficult to generate a PCR that produces the desired results. Even though this method of fixation has been used for a hundred years for histology and produces excellent morphology on H and E stained slides, it is not particularly effective when used in molecular methods.[\(42, 43\)](#)



**Fig. 1.1 The principle and exponential amplification of a PCR reaction(44)**

The principle of a PCR reaction (shown in Fig 1.1) demonstrates that DNA can be cloned to millions of copies from one DNA fragment of interest. One of the major difficulties in optimizing PCR from FFPE tissue is the nicking of the DNA into smaller pieces by the action of formalin which can make the primer design difficult.

Chawla demonstrated that a successful PCR could be performed on formalin fixed tissues of a wide variety of tissue types. He came to the conclusion that PCR is a rapid, sensitive and specific test in the early diagnoses of extra pulmonary disease.(45) Gomez-Laguna used real-time and conventional PCR to demonstrate porcine tuberculosis in FFPE; and concluded that RT-PCR is the fastest and most accurate technique to detect *Mycobacterium* complexes in FFPE tissues. (46)

## 1.7 *In situ* Hybridization

*In situ* hybridization, as the name suggests, is a method of localizing and detecting specific DNA, mRNA or cDNA sequences in morphologically preserved tissue sections by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. It is a technique that allows for the precise localization of a specific segment of nucleic acid (mRNA) in a histological section, allowing one to obtain information about gene expression and genetic loci by hybridizing a complementary strand of a nucleotide probe to the sequence of interest. (47)

Detection of RNA and DNA by *in situ* hybridization (ISH) provides a way to detect the presence and expression of various microbial genes in tissue sections with a high specificity. (48) The advantage of using ISH is that the location of the bacilli and spatial variations in gene expression can be seen. (49)

An optimized ISH protocol should serve several goals, with retention of tissue morphology being one of the most important. The tissue must be permeable to the probe to produce a desirable result; while the mRNA target must be retained in the tissue. The probe must be able to effectively penetrate, bind and remain bound during the washing steps aimed at removing non-specifically bound probe. All of these conditions must be met in order to achieve a successful and high quality *in situ* hybridization result. (47)

Hulten *et al* designed a successful *in situ* hybridisation method for the demonstration of *Mycobacterium paratuberculosis* in spheroplasts in FFPE tissues which showed that it may be useful in studying the connection between *M. paratuberculosis*, Crohn's disease and sarcoidosis. (48)

Fenhall *et al.* demonstrated the presence of mRNA from *M. tuberculosis* using ISH techniques, which is thought to be associated with tuberculous granulomas in human lungs. (49).

The diagrammatic picture (figure 1.2) demonstrates the path a slide would follow to successfully demonstrate an ISH protocol. As well as what happens when no detection is visualised. Proper investigations should be followed to solve the problem.



## 1.8 Speciation of *M.tuberculosis* complex

Mapping of Regions of Difference (RD) has become the standard technique for the classification of members of the *M. tuberculosis* complex (MTC) (including *M. africanum*, *M. bovis*, and *M. bovis* BCG vaccine strain, *M. microti*, *M. canettii* and *M.t tuberculosis*) Figure 1.3 shows the evolution history of the MTC by delineating the different RD's in the phylogenetic tree. From this figure it is evident that RD9 is a critical marker to differentiate *M. tuberculosis* and *M. canettii*, from all other members of the *M. tuberculosis* complex. (50) *M. canettii* is very rarely observed in humans and has only been recorded in patients from the horn of Africa.

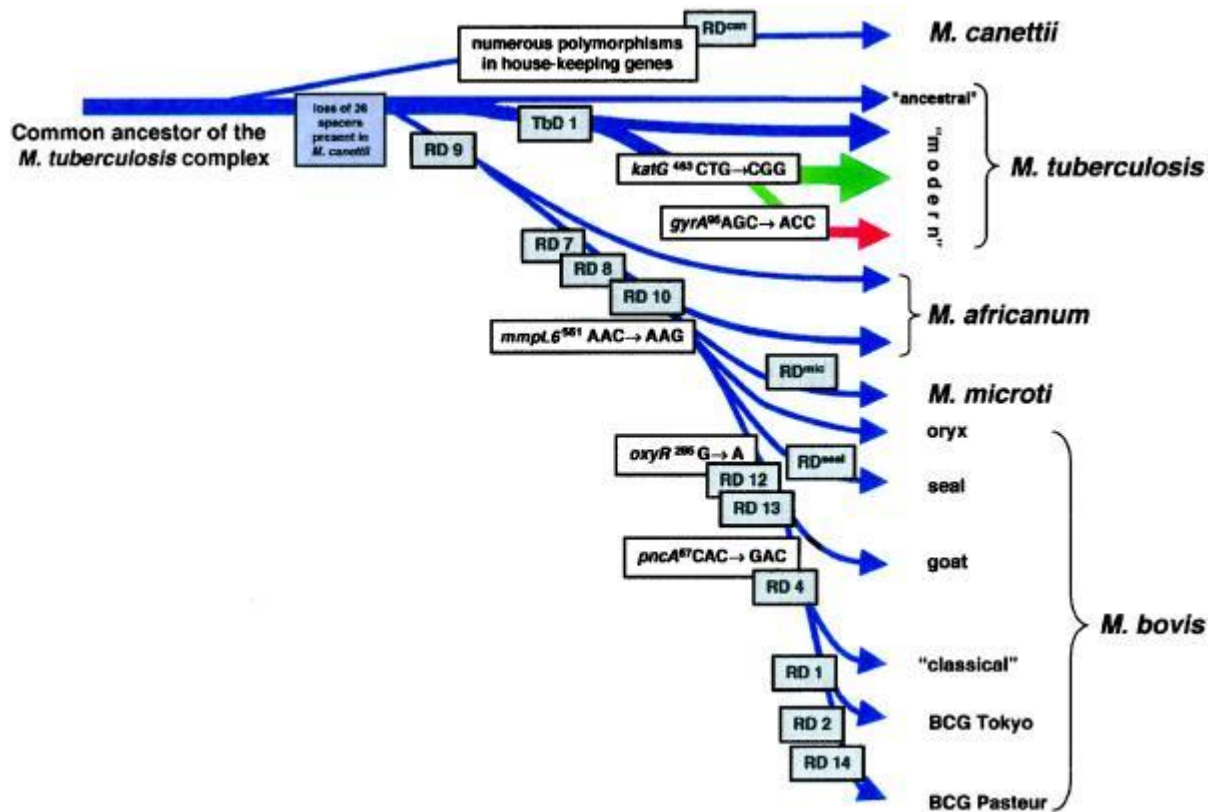


Fig 1.3: An evolutionary tree showing that the Region of Deletion 9 (RD9) is present in *M.tuberculosis* and *M.canettii*, however absent from all other members of the *M.tuberculosis* complex.(51)

## 2. AIM, OBJECTIVES AND HYPOTHESIS

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### 2.1 Aim

The primary aim of this study is to determine whether or not pathology laboratory examination of the placenta from mothers with clinically suspected, untreated or confirmed tuberculosis (on treatment for no longer than three months prior to delivery) can assist in the diagnosis of congenital tuberculosis in their infants. This knowledge would expedite the early diagnosis of congenital tuberculosis and enable appropriate therapy to be initiated.

### 2.2 Methods for achieving the objectives:

#### 2.2.1 Histopathology

Placentas were examined according to standard protocols to determine if specific histopathological features predictive of tuberculosis confirmed by positive Ziehl-Neelsen (ZN) staining could predict placental tuberculosis and congenital infection of the infant.

#### 2.2.2 PCR

DNA was extracted from FFPE tissue sections. PCR was done to identify the presence of *M. tuberculosis* DNA which was visualized using agarose gel electrophoresis.

#### 2.2.3 Outcome of potential neonatal and maternal disease

The performance of the diagnostic modalities was assessed (collectively and individually), and compared to the neonatal clinical outcome (presence or absence of mycobacterial infection) as well as clinical evidence of maternal tuberculosis (pulmonary and extra pulmonary disease). The modalities that were used included: clinical outcomes, radiological imaging studies and laboratory testing (gastric washings from infants and sputum samples from mothers).

## 2.3 Study Hypothesis

Examination of the placenta in cases of suspected or proven maternal tuberculosis using morphology, Ziehl Neelsen (ZN) staining, and PCR will significantly improve and expedite the diagnosis of congenital TB.

## 2.4 Study Design

This was a retrospective laboratory based study, and was conducted using archival material submitted from January 2009 to July 2011 at Tygerberg Hospital, a tertiary, university affiliated institution, which serves as a referral centre for complex TB and HIV deliveries in the Western Cape Province.

The patient population included all patients with suspected or proven TB whose placental tissue had been sent to NHLS Anatomical Pathology, Tygerberg Hospital. This referral centre includes drainage areas with a high burden of tuberculosis, one well-described area with a tuberculosis notification rate of 1000 or more cases per 100 000 population per year.<sup>(7)</sup> Only placentas sent to the laboratory as part of routine clinical practice were eligible for this study. The provincial health authorities were therefore not burdened by any additional study-related admissions.

The suspected or confirmed maternal TB cases were identified from the files of the division of Anatomical Pathology, NHLS Tygerberg Hospital. (See **Appendix D**) Demographic data was collected from the patients' folders. Each mother's TB and HIV-status was documented in their folder (standard of care) and this information together with her CD4 count (if known), and whether or not she was on anti-retroviral therapy (duration) was collected.

Information on the birth weight, sex and gestational age was gathered. (When a positive result for TB was found in the placenta, steps were taken to ensure that appropriate TB treatment was initiated in the newborn).

### **Inclusion criteria**

1. All patients delivering at Tygerberg Hospital from 1 Jan 2009 to 31 July 2011 with clinically suspected and diagnosed TB(HIV-infected and uninfected). Including but not limited to fine needle aspirate (FNA) and/or culture.
2. Either live births or stillbirths.

3. Placentas of patients with no evidence of TB in the mothers or infant's medical records were used as controls.

### **Exclusion criteria**

1. Placentas of patient with unknown TB status

## **2.5 Ethics Approval**

This study was approved by the Committee for Human Research at the University of Stellenbosch. Ethics approval number: (see **Appendix A**)

This study was done in accordance with the relevant ethics guidelines as stipulated by the Health Research Ethics Committee of the Faculty of Medicine and Health Science, University of Stellenbosch. Following data collection, all patients pertaining to the project were treated anonymously to prevent any link between the research material and results of a particular patient. Patient confidentiality was maintained at all times. No patient identifying information (name, hospital file number) was included on the data sheet. Individual patients were identified by a study number on the data capture sheet and the file numbers linked to the study numbers were stored separately.



### 3. MATERIALS AND METHODS

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#### 3.1 Placental Histopathology

##### 3.1.1 Fixation, Tissue processing

Our laboratory receives placentas almost on a daily basis. They all arrive fixed in 10% neutral buffered formalin. A protocol (drawn up between the Departments of Obstetrics and Gynaecology and Anatomical Pathology) is used to select which placentas shall be processed.

Each of the 56 study placentas were fixed in the formalin for at least 24 hours prior to gross examination and processing of the tissue. Gross examination was performed by a training pathologist while the histopathological reporting was performed by a qualified pathologist. Sampling was performed according to standard protocol followed at NHLS, Tygerberg Hospital, Anatomical Pathology Department, which involves taking 1 section which contained 2 pieces of the umbilical cord and free membrane and there were 3-4 sections taken from the placental parenchyma. The tissues were processed and embedded using a Tissue Tek VIP 300 and/or Tissue Tek VIP 500 processor, and a Paraffin tissue embedder (Sakura and Shandon).

##### 3.1.2 Haematoxylin and Eosin stain

The Haematoxylin and eosin (H and E) stain is probably the most widely histological stain used. Its popularity is based on its ability to clearly demonstrate different tissue structures, its widespread applicability in different ways and its comparative simplicity. (36) The Haematoxylin and Eosin stain yielded the following results; the haematoxylin stained the cell nuclei blue-black whereas the eosin stained the cytoplasm and the connective tissue fibres in the cells with varying shades of pink-red. (**Appendix B**).

The placentas were histopathologically reported according to a standard template (**see Appendix C1**). Placental diagnoses were allocated into a series of cluster diagnosis (**see Appendix C2**). Placentas from mothers with a clinical suspicion of TB or known with TB on treatment for less than 3 months qualify for routine histological processing (**Appendix D**).

All placentas were then independently re-evaluated by two pathologists (CAW and PS) and consensus reached.

### 3.1.3 Ziehl Neelsen stain for mycobacteria

After examination of the Haematoxylin and Eosin stained slides the pathologist selected one block for the ZN stain. This was performed in sections cut from the paraffin embedded blocks using standard procedures (see **Appendix E**).

## 3.2 Polymerase Chain Reaction

### 3.2.1 Tissue sectioning

The control and test sections were cut under conditions intended to minimise any sample to sample contamination of the DNA. The control and test section were cut on a microtome (Leica RM 2145) and a new disposable blade was used for each sample cut to prevent any cross contamination between samples being cut. The microtome used to cut the sections was decontaminated first with xylene to remove any waxy particles and tissue sections; thereafter it was decontaminated with absolute alcohol to remove any xylene as this can inhibit PCR reactions. After this, it was decontaminated with a 5% Hypochlorite (Jik) solution to destroy any DNA present on the microtome. Lastly it is sprayed with absolute alcohol solution to remove any residual hydrochloric solution as this may destroy the DNA on the sections. The microtome was allowed to air dry prior to use.

The same procedure was followed for the brush and tweezers used to cut the sections. Dry sections (8-10 in number) of 7µm thickness for each specific pre-selected block were cut and put into sterile DNA & RNAase free Eppendorf tubes. The first few sections of the block were discarded due to their prior exposure of potential DNA contaminant.

### 3.2.2 DNA extraction

The standard protocol as recommended by the manufacturer (Qiagen, Hilden, Germany) was used for the optimization of the DNA extraction. (See **Appendix F**).

The standard protocol was optimised by changing a few steps.

The 1<sup>st</sup> additional step was to heat the tissue sections for 5min at 65° before adding the 1<sup>st</sup> Xylene to the Eppendorf tube as to aid with the wax removal as indicated in step 6 of the protocol suggested by the manufacturer. An extra Xylene wax removal step was introduced in step 6 of the protocol suggested by the manufacturer as only one removal was found to not be sufficient to remove the wax.

The sections were incubated overnight in proteinase K as indicated in step 11 of the protocol suggested by the manufacturer to digest bacterial and tissue proteins.

These steps were necessary as there was residual wax left after the standard DNA extraction method and this acted as a PCR inhibitor.

For the optimisation of the PCR reaction, DNA was extracted from *M. tuberculosis* complex negative and positive FFPE tissue using the commercially available Qiagen FFPE DNA extraction kit. The positive control was taken from the NHLS Histopathology laboratory from a proven case with TB. The negative control was selected to be the testis, which is the least likely tissue type to be infected with *M. tuberculosis*. In addition DNA was extracted from fifty three cases of placental tissue.

### 3.2.3 Primer Design

Correct primer design is essential to ensure that PCR products are specific and sensitive.

Cross-reactivity of primers to other organisms must be avoided so as to ensure amplification of the target region. Primers were designed to specifically amplify RD9 region which is present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the *M. tuberculosis* complex (*M. canettii* is very rarely observed and has not been recorded in this study setting). These primers were subjected to primer design using DNAMAN primer design software. Primer selection criteria included an annealing temperature of 62°C, a G/C content of 40-60% and end on a G/C base at the 3' end. The primers selected were subjected to analysis of hairpin, homo- and hetero-dimer formation using the IDT (integrated DNA technologies, Inc., Coralville, IO, USA)([52](#)) software online. Last a BLAST (NCBI Basic Local Alignment Tool)([53](#)) search was done online to confirm that the sequences were specific to mycobacteria only. PCR was done as previously described ([50](#)) using an annealing temperature of 62°C and the following primers

RD9Fs1 5'-CAA GTT GCC GTT TCG AGC C-3' and RD9FR 5'-GCT ACC CTC GAC CAA GTG TT-3'. (50) The expected product size was 85 base pairs.

A second primer set was selected based on a previous study that investigated the presence of Trans renal mycobacterial DNA in urine specimens. These Trans renal protein primers were designed to specifically amplify small fragment of DNA present in urine. We proposed that these primers would be appropriate for the amplification of fragmented DNA extracted from FFPE tissue. These primers were designed to specifically detect *M. tuberculosis* complex (see Figure 3.1) by targeting the insertion segment IS6110 which is only present in members of the *M. tuberculosis* complex. The use of IS6110 as the target element has the potential to increase the sensitivity of the assay since IS6110 is a repeat sequence that may be repeated up to 26times in the *M. tuberculosis* genome. (54) Figure 3.1 shows the principles used for the amplification of short fragments of DNA using the set of primers (F and R) specific to the IS6110 element with ADAPTERS (green) attached at the 5' ends of the respective primers.

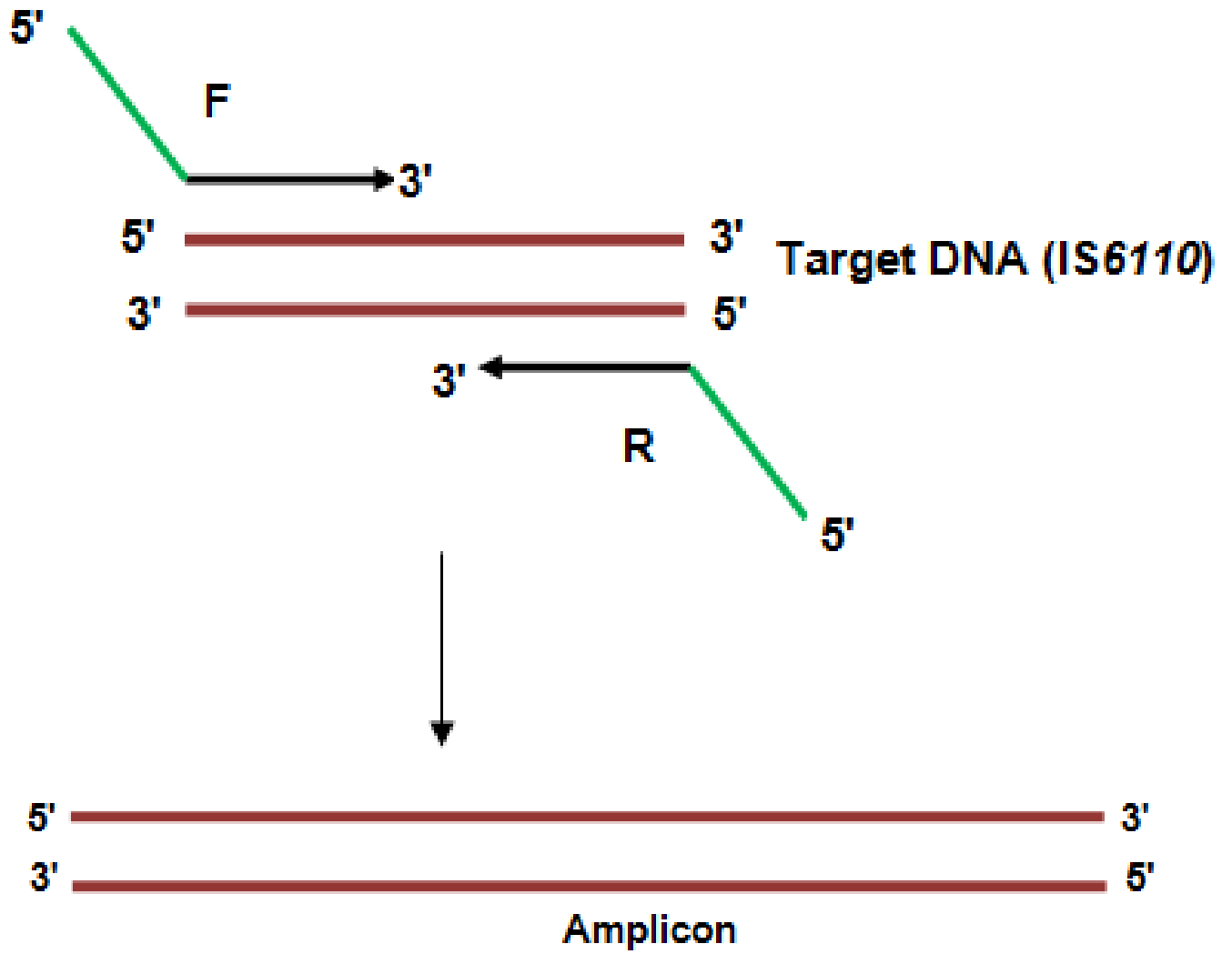


Fig 3.1: PCR Design to detect small DNA fragments

**>IS6110**

```

          tgaac cgccccggca tgtccggaga ctccagttct tggaaaggat
1801 ggggtcatgt caggtggttc atcgaggagg taccgcccg agctgctga gcgggcggtg

                                     Tr-F1 5' tc
1861 cggatggtcg cagagatccg cggtcagcac gattcggagt gggcagcgat cagtgaggtc

gcccgtctac ttggtg 3'
1921 gcccgtctac ttggtggttg ctgcgcggag acggtgcgta agtgggtgcg ccaggcgcag
          3' cctc tgccacgcat tcac 5' Tr-R1

Tr-F2 5' caaagtgt ggctaaccct g 3'
2341 actacggtgt ttacggtgcc cgcaaagtgt ggctaaccct gaaccgtgag ggcacgagg
          3' cactc ccgtagctcc

2401 tggccagatg caccgtcgaa cggctgatga ccaaactcgg cctgtccggg accaccgcg
acc 5' Tr-R2

Tr-F3 5' ggtc ggaagctcct atgac 3'
2821 aaccgtcggg cggagcggtc ggaagctcct atgacaatgc actagccgag acgatcaacg
          3' cg tgatcggctc tgctagt 5'
Tr-R3

```

IS6110 end &lt;

**Figure 3.2: Primer sequence map showing the different locations targeted within the IS6110 insertion element**

The primer sets were designed to target different locations within the *M. tuberculosis* IS6110 element (GenBank: Y14047.1) as shown in figure 3.2. Random ADAPTER sequences (upper case in Table 3.1) were then added to these primers (lower case in Table 3.1) to increase the length of the amplicons.

**Table 3.1: Primer sets designed for Trans renal proteins**

Primer	Function	Target Size (bp)	Product Size (bp)	Sequence
<b>Tr-F1</b> & <b>Tr-R1</b>	Forward Primer Reverse Primer	46	83	5'- ACA TCC TAC ACA CGC TC tcg ccc gtc tac ttg gtg -3' 5'- CAA GCA GAA GAC GGC ATA CG cac tta cgc acc gtc tcc -3'
<b>Tr-F2</b> & <b>Tr-R2</b>	Forward Primer Reverse Primer	41	78	5'- ACA TCC TAC ACA CGC TC caa agt gtg gct aac cct g -3' 5'- CAA GCA GAA GAC GGC ATA CG cca cct cga tgc cct cac -3'
<b>Tr-F3</b> & <b>Tr-R3</b>	Forward Primer Reverse Primer	41	78	5'- ACA TCC TAC ACA CGC TC ggt cgg aag ctc cta tga c -3' 5'- CAA GCA GAA GAC GGC ATA CG tga tcg tct cgg cta gtg c -3'
<b>ext F</b>	External Forward Primer			5'- ACA TCC TAC ACA CGC TC -3'
<b>ext R</b>	External Reverse Primer			5'- CAA GCA GAA GAC GGC ATA CG -3'

All of the above mentioned Trans renal primers (Tr) were evaluated in a previous Masters project. (54) For this project we used the Tr-F3 & Tr-R3 primer set and these primers showed the optimal PCR amplification during the analysis of the control samples (see above).

### 3.2.4 PCR amplification

Each PCR reaction mixture contained: 0.25µl Qiagen's HotStarTaq DNA Polymerase (5 units/µl), 2.5 µl of 10X PCR buffer, 2 µl of 25mM MgCl<sub>2</sub>, 5 µl of 5X Q-solution; 4 µl of 10mM dNTPs (Thermo Scientific, Massachusetts, USA); forward and reverse primer mixes (Tr F3 + Tr R3) were used at a final concentrations of 1 pmol/µl; and 5µl of extracted DNA. The volume of the reaction was adjusted to 25 µl with addition ddH<sub>2</sub>O.

Reagent preparation, addition of DNA and amplification were carried out in separate rooms with restricted access and unidirectional workflow to prevent cross contamination. Each PCR assay included DNA of *M. tuberculosis* strain H37Rv as positive control, as well as a non-Template Control (NTC) to control for contamination.

### 3.2.6 PCR cycle

The PCR cycle started with an initialization step, consisting of heating the reaction to a temperature of 95 °C for 15 minutes. The next step was a [denaturation step](#), consisting of a first regular cycling event and heating the reaction to 94 °C for 30 seconds. This was followed by an annealing step where the reaction temperature was lowered to 62 °C for 30 seconds.

Then an elongation step followed, in which the temperature depended on the DNA polymerase used; [Taq polymerase](#) was used in this experiment at an optimal temperature of 72 °C, which was held for 30 seconds. The denaturation, annealing and elongation steps were repeated for 45 cycles.

Final elongation is the last step of the cycle. This single step is done at a temperature of 72 °C for 10 minutes. PCR amplification was done in the Rotor-Gene Q rotary real-time thermo cycler (Qiagen, Hilden, Germany).

### 3.2.7 Agarose Gel Electrophoresis

PCR products were visualised by agarose gel electrophoresis: Briefly ten micro litres (10 µl) of amplicons mixed with 2µl Blue/Orange 6X Loading Dye (Promega, Wisconsin, USA) was run in a 3% agarose gel (SeaKem LE Agarose, Cambrex Bioscience, Maine, USA) in 1X TAE buffer (containing 40mM Tris, 20mM acetic acid, and 1mM EDTA) at 120V for 1 hour with the PowerPac Basic Power Supply (Bio-Rad, California, USA). The agarose was cast with ethidium bromide (6µl of 10mg/ml per 100ml agarose gel (Promega, Wisconsin, USA).

DNA molecular weight markers were co-electrophoresed to determine the size as the PCR amplicons product: O'GeneRuler 100 bp DNA Ladder Plus (Fermentas Life Sciences, Vilnius, Lithuania). Target bands of 85bp were visualized by ultraviolet (UV) fluorescence using the Alliance 2.7 optic analysis system (UViTec, Cambridge, UK).

The following method was used to make the 3% agarose gel. 3g of agarose was mixed in 100ml 1 x TAE buffer, heated until dissolved, poured into a mould and allowed to cool. 6µl Ethidium Bromide was added to the gel liquid. Caution was exercised when working with this chemical as it is a carcinogen (Handling requires wearing gloves, eye protective goggles, laboratory coat and closed



shoes). This chemical is disposed by an accredited outsourced company and involves being placed in a plastic bin, removed and incinerated.

A new reagent is available now to phase out Ethidium Bromide called Novol Juice, but this reagent is not as sensitive as ethidium bromide. This chemical is not harmful or carcinogenic. The 3% agarose gel is made up as described above. The Novol Juice is mixed with the PCR amplicon, for every 10 $\mu$ l of amplicon add 2 $\mu$ l of Novol Juice. This chemical also acts as a loading dye as well. Add 10 $\mu$ L mixed amplicon mixture into each well of the agarose gel and proceed as previously mentioned. This method was used in the gel electrophoresis of the test samples.

### 3.3 ISH

#### 3.3.1 Primer preparation for Labelling with Digoxin (DIG)

The PCR and subsequent amplification of *IS 6110* were done in the Veriti Thermal Cycler (Applied Biosystems, USA). Reagent preparation, addition of DNA and amplification were carried out in separate rooms with restricted access and unidirectional workflow. Each PCR assay included DNA of *M. tuberculosis* strain H37Rv as positive control, as well as a non-Template Control (NTC) to control for contamination. All precautions to prevent cross-contamination were observed as previously discussed in the PCR section, such as bi directional air flow and working in separate rooms.

A PCR reaction was setup to amplify the primers of *IS6110*

Each reaction mixture of the PCR reaction contained: 0.25µl Qiagen's HotStarTaq DNA Polymerase (5 units/µl), 5 µl of 10X PCR buffer, 2.5 µl of 25mM MgCl<sub>2</sub>, 9 µl of 5X Q-solution; 2 µl of 100mM dNTPs (Thermo Scientific, Massachusetts, USA); 4 µl forward and reverse primer mixes (RD9-Fs1 + RD9-FR) were used in final concentrations of 1 pmol/µl; and 1µl of DNA(*IS6110*) sample was brought to volume (25 µl) with RNase free ddH<sub>2</sub>O.

Reaction mixtures were aliquoted into Eppendorf test tubes and run on a Temperature gradient.

Tube 1 & 2	@ 60°C
Tube 3 & 4	@ 61°C
Tube 5 & 6	@ 62°C
Tube 7 & 8	@63°C
Tube 9	@ 64°C

An agarose gel electrophoresis was done to confirm results: Ten micro litres (10 µl) of amplicons mixed with 2µl Blue/Orange 6X Loading Dye (Promega, Wisconsin, USA) were run at 120V for 1 hour with the PowerPac Basic Power Supply (Bio-Rad, California, USA) on a 1% agarose gel (SeaKem LE Agarose, Cambrex Bioscience, Maine, USA) in 200ml of 1X TAE buffer (containing 40mM Tris, 20mM acetic acid, and 1mM EDTA) and stained with 6µl of 10mg/ml ethidium bromide (Promega, Wisconsin, USA).

DNA molecular weight marker was used (to easily visualize product size as the PCR amplicons form between the DNA ladders): O'GeneRuler 100bp DNA Ladder Plus (Fermentas Life Sciences, Vilnius,

Lithuania). DNA bands were visualized by ultraviolet (UV) fluorescence using the Alliance 2.7 optic analysis system (UViTec, Cambridge, UK).

The following method was used to make the 1% agarose gel: 1g of agarose is mixed in 100ml 1 x SB buffer, heated until dissolved, poured into a mould and allowed to cool. 6µl Ethidium Bromide was added to the gel liquid. Caution was exercised when working with this chemical as it is a carcinogen (Handling requires wearing gloves, eye protective goggles, laboratory coat and closed shoes). This chemical is disposed by an accredited outsourced company and involves being placed in a plastic bin, removed and incinerated.

### 3.3.2 Purification of PCR amplicons (probe)

The PCR amplicons made in the previous section were purified using the Wizard SV gel & PCR clean-up system according to the manufacturer's instructions. (**Appendix G**)

The DNA concentration of the PCR amplicons was determined by spectrometry using the nanodrop 1000 instrument (Thermo Scientific, USA). The IS6110 (RD9) was chosen for the labeling with Digoxin. The primer which was chosen was selected from the nanodrop results (see table 2), which had the most amount of purified DNA present.

### 3.3.3 Labeling of probe with Digoxin (DIG)

The probes were labeled with DIG using the DIG DNA labeling Kit from Roche (Cat No. 11 175 033 910) according to the standard manufacturer's procedure.

The preferred method for quantification of labeled probes is the direct detection method. Labeled probes and the DIG-labeled control DNA (supplied in vial 4) were diluted to 1ng/µl, according to the expected yield of synthesised nucleic acid to start the dilution series below. The control DNA, vial 4 contains approximately 250ng DIG labeled DNA in 50 µl (5µg/ml). A 1:5 dilution of the control DNA as starting material for the dilution chart explained below was prepared.

Apply 1µl spots of tubes 3-10 (table 3.2) from the labeled probe and the labeled control DNA to a strip of nylon membrane (Hydrobond N+) and bake for 120 minutes at 70°C.

Follow the chemiluminescent detection procedure described in the package insert of the substrate CSPD using volumes appropriate to the size of the membrane as explained later

To determine the labeling efficiency a dilution series of the DIG labeled antibody was prepared. (Table 3.2).

**Table 3.2: Dilution series made for membrane spot test**

TUBE	DNA( $\mu$ L)	FROM TUBE NUMBER	DNA DILUTION BUFFER( $\mu$ L)	DILUTION	FINAL CONCENTRATION
1		Dilution of probe and vial 4(supplied in kit)			1 ng/ $\mu$ L
2	2	1	198	1:100	10 pg/ $\mu$ L
3	15	2	35	1:3.3	3 pg/ $\mu$ L
4	5	2	45	1:10	1 pg/ $\mu$ L
5	5	3	45	1:10	0.3 pg/ $\mu$ L
6	5	4	45	1:10	0.1 pg/ $\mu$ L
7	5	5	45	1:10	0.03 pg/ $\mu$ L
8	5	6	45	1:10	0.01 pg/ $\mu$ L
9	0	-	50	-	0

Aliquots of the diluted DIG probe were spotted onto Hybond N+ from a low to high concentrations (tube 9 to tube 3). Thereafter the membrane was covered with Whatman 3MM paper and baked at 70°C for 2 hours to covalently link the DNA to the membrane. The membrane was then stored in a bag at 4°C.

The membrane was rehydrated by washing twice in distilled water for 5 minutes (each) and subsequently washed twice in PBS buffer for 5 minutes (each). These steps were done to ensure that any unincorporated DIG labeled nucleotides were removed from the membrane.

The DIG labeled probe was detected using the IN SITU HYBRIDIZATION DETECTION SYSTEM CORE KIT II (Cat no: DD131-60K) for Digoxin labeled probe/Streptavidin-Alkaline Phosphatase from Biogenix.

This procedure was only started at step 12 of the manual because we were detecting DIG probes on membranes not in FFPE tissue sections. The procedure was followed according to the manufacturer's instructions.

The Power block (15 drops) was added to the membrane and incubated for 20 minutes at room temperature. .

Biotinylated Anti-DIG antibody (15 drops) were placed in the bag and incubated for 20 minutes at room temperature. Thereafter, excess fluid was gently drained from the bag.

The membranes in the bag were than rinsed twice, with 1 x PBS, 0.1% Tween-20 from a wash bottle.

Then the membrane was covered with the PBS-Tween buffer (in the bag) and incubated for 3 minutes. 15 drops of the Streptavidin-enzyme conjugate was added to cover the entire membrane and incubated for 20 minutes at room temperature. Excess fluid was drained and then rinsed from the membrane with 1 x PBS, 0.1% Tween-20 from a wash bottle. Twenty Drops of the activation buffer was added to cover the whole membrane and further incubated for an additional minute at room temperature, before being washed twice in PBS buffer for 2 minutes each time. Finally 5ml of DAB (3, 3'Diaminobenzidine) was added and incubated for 5 minutes.

The manufacturer suggests, in the protocol used above, to use CSPD ready to use substrate to confirm positive labeling of probe results. This method was subsequently followed, as explained below.

The procedure was started at step 2 of the CSPD kit: All of the steps were done at room temperature unless otherwise stated and it is applicable to a membrane of 100cm<sup>2</sup>. The membrane was incubated in Blocking solution for 30 minutes. Antibody solution was added for 30 minutes and was washed twice in washing buffer for 15 minutes. Detection buffer was added next and incubated for 5 minutes. Next it was incubated in CSPD working solution for 5 minutes. The membrane was sealed and the damp membrane incubated for 10 minutes at 37°C. Lastly it was exposed to an x-ray film for 15-25 minutes.

### **3.4 Maternal and Neonatal Follow-up**

In mothers with suspected, but unproven TB (not on treatment); the identification of tuberculous lesions in the placenta will alert physicians to start anti-TB treatment in these patients. Furthermore, placental identification of TB contributes to early identification of high-risk TB-exposed infants, (even if congenital TB cannot be proven by case definition). It also enables clinicians to consider full anti-TB treatment, rather than TB chemoprophylaxis (INH for 6 months) in cases with a high index of suspicion for congenital TB.

It is standard procedure for all infants born to mothers with suspected/ confirmed TB to be followed-up at their local clinic. If the mother is thought to be infectious, their newborn infants routinely receive 6 months of INH chemoprophylaxis. Babies suspected of TB are referred to a level 3 institution for a full TB work-up. Investigations include full clinical examination, radiological studies and gastric washing (which were sent for ZN staining and culture). TB treatment with four drugs (INH, RIF, PZA and Ethionamide) is initiated when the diagnosis of TB is confirmed.

The demographic data of mothers and infants was collated, recorded and analysed as indicated in the results section that follows.

## 4. RESULTS

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### 4.1 Maternal tuberculosis

A total of 56 placentas were collected for the study by means of the request forms that indicated possible or suspected maternal TB. Maternal tuberculosis was confirmed in 30 mothers (53.5%). This was determined on reviewing the patient folders. The diagnosis of confirmed maternal TB was based on laboratory testing results and/or radiological imaging studies. Twenty nine mothers were known with proven TB prior to delivery while 1 mother was still awaiting confirmatory laboratory results. However 30 mothers were on treatment for TB at the time of delivery as 1 mother was falsely diagnosed with TB. Her treatment was stopped shortly after delivery when all the results were reviewed and accessed. (Figure 4.1).

On reviewing the folders, 26 mothers, who initially were suspected of having TB, were negative for tuberculosis on laboratory and radiological investigations, and were included as controls for this study.

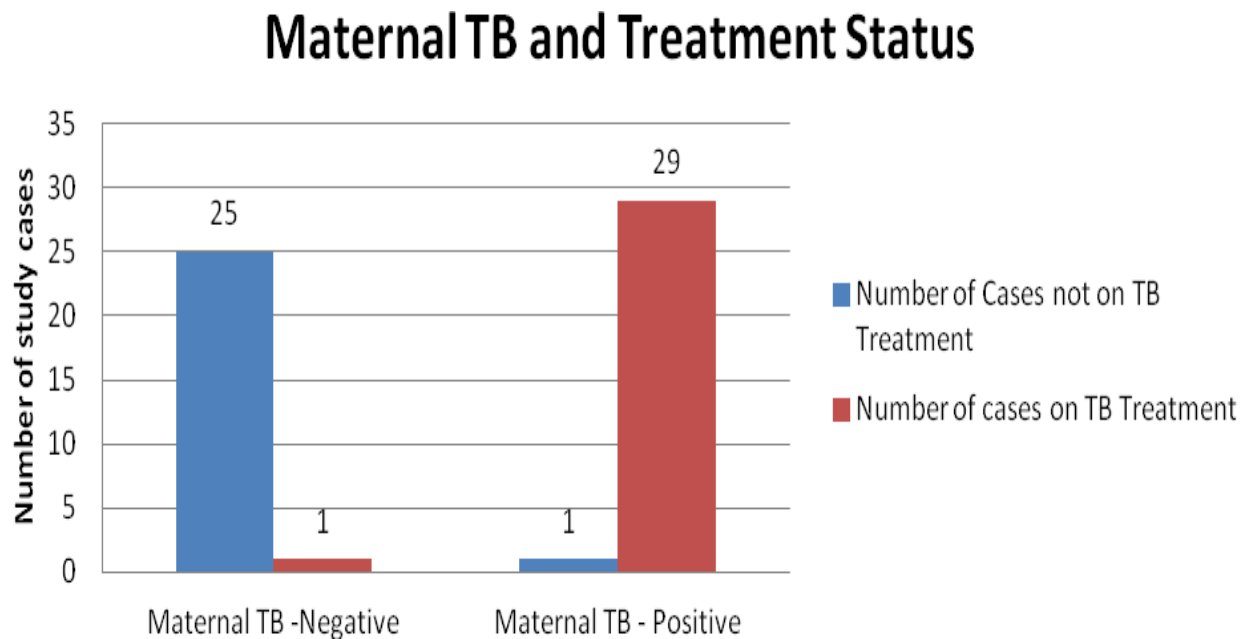


Figure 4.1 Status of maternal TB and treatment of study cases

#### 4.1.1 Treatment status of TB positive mothers

Four of the 30 mothers (13%) were on anti-tuberculous treatment for longer than 2 months at the time of delivery (Figure 4.2). Twenty-four mothers (80%) were on treatment for less than 2 months with 4 of these mothers receiving treatment for less than 1 week. Two mothers (6%) received treatment for an unknown duration. The mothers who received treatment for a short duration could be ascribed to late presentation and the difficulty of diagnosing TB in pregnancy. One patient was on anti-tuberculous therapy due to clinical suspicion of disease, but subsequently laboratory and radiological investigations were negative in the mother (false positive treatment case).

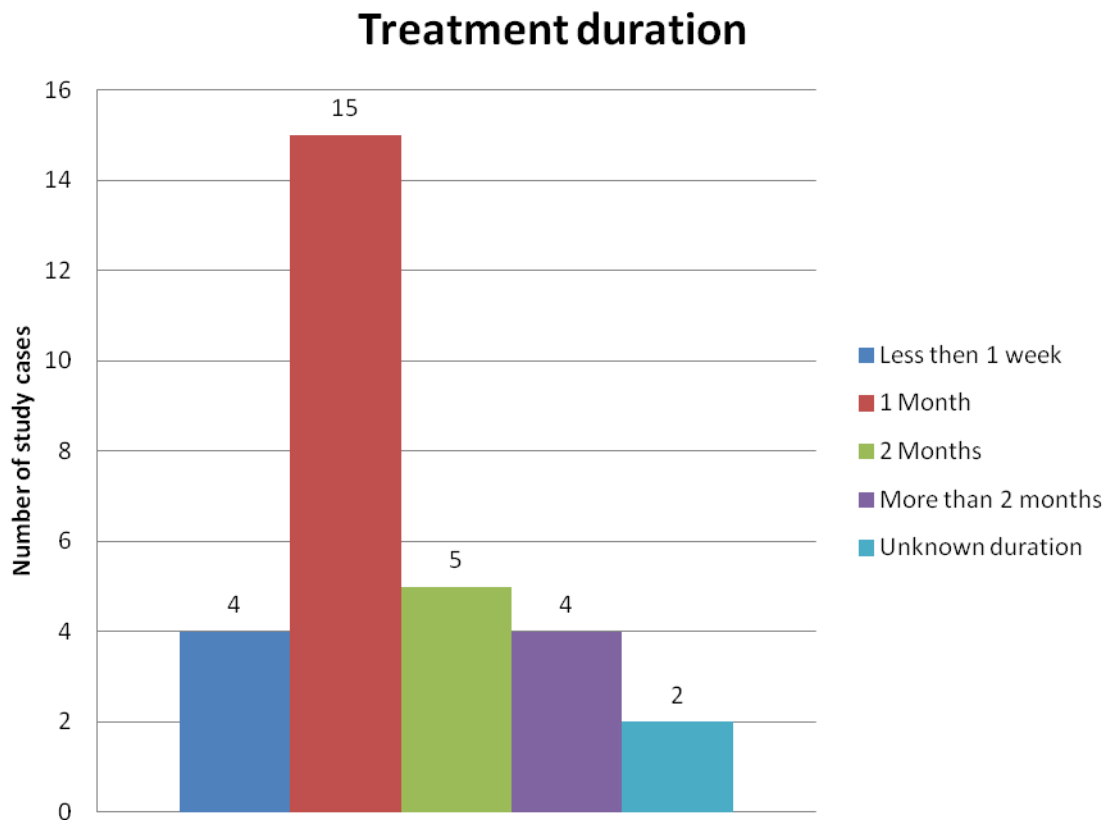


Figure 4.2. Treatment duration status of TB positive mothers in study.



#### 4.1.2 Different types of TB in TB positive mothers

Twenty of 30 mothers (66%) were diagnosed on presentation as pulmonary TB with 5 of these presenting as pleural effusions. Eleven of 30 mothers (36%) were diagnosed with extra pulmonary disease; 2 had TB meningitis, 2 had miliary TB, 2 had TB lymphadenitis, 1 had disseminated TB, 2 were categorized into other TB infections (1 had positive TB blood culture, 1 had placental TB) and 1 are uncategorized (Figure 4.3).

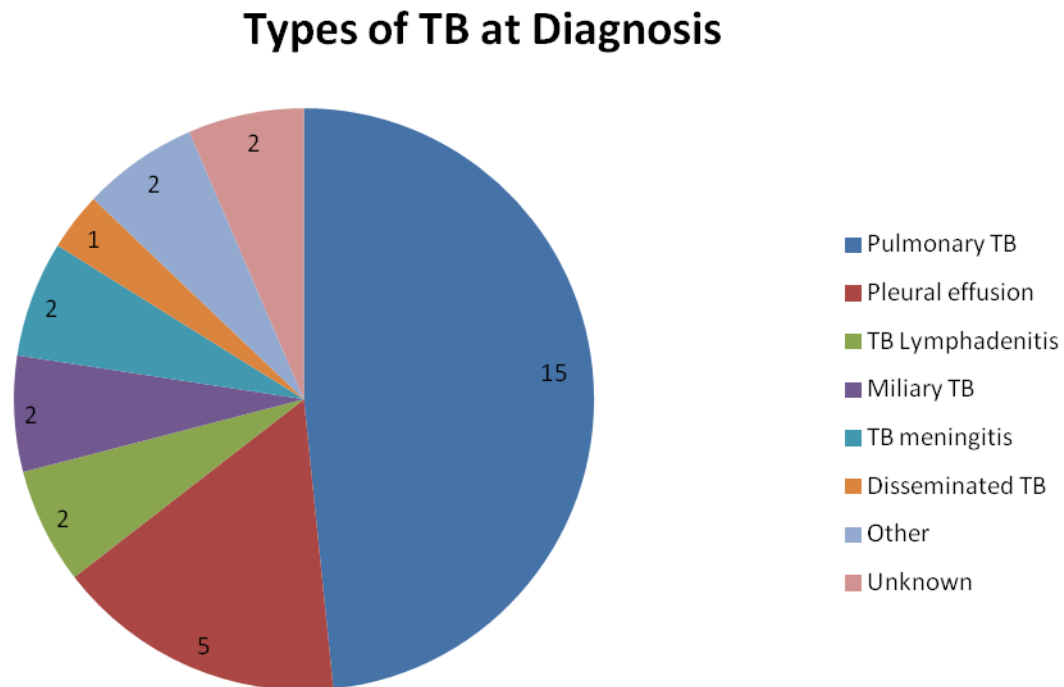


Figure 4.3. Types of TB diagnosed in TB positive mothers.

#### 4.1.3 HIV status of study patients

Of the 56 patients in the study, 26 (46%) were HIV positive and 17(30%) were HIV and TB positive (Figure 4.4). In the mothers with TB, co-infection of HIV and TB was higher at 56% than mothers who were HIV negative and had maternal tuberculosis (42%). This is consistent with the current literature (22). The HIV status of 6 TB negative mothers was unknown, due to missing hospital folders

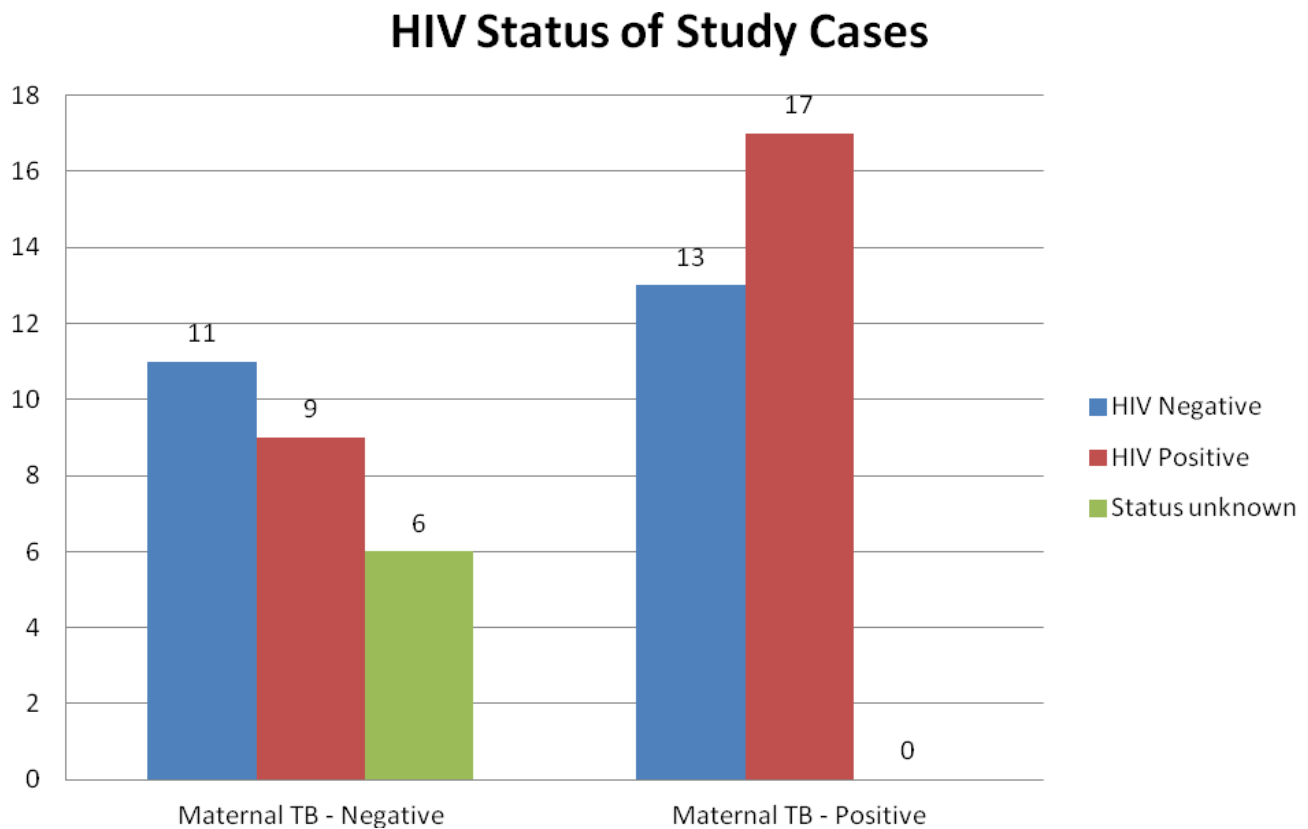


Figure 4.4. HIV status of study cases

## **4.2 Neonatal tuberculosis**

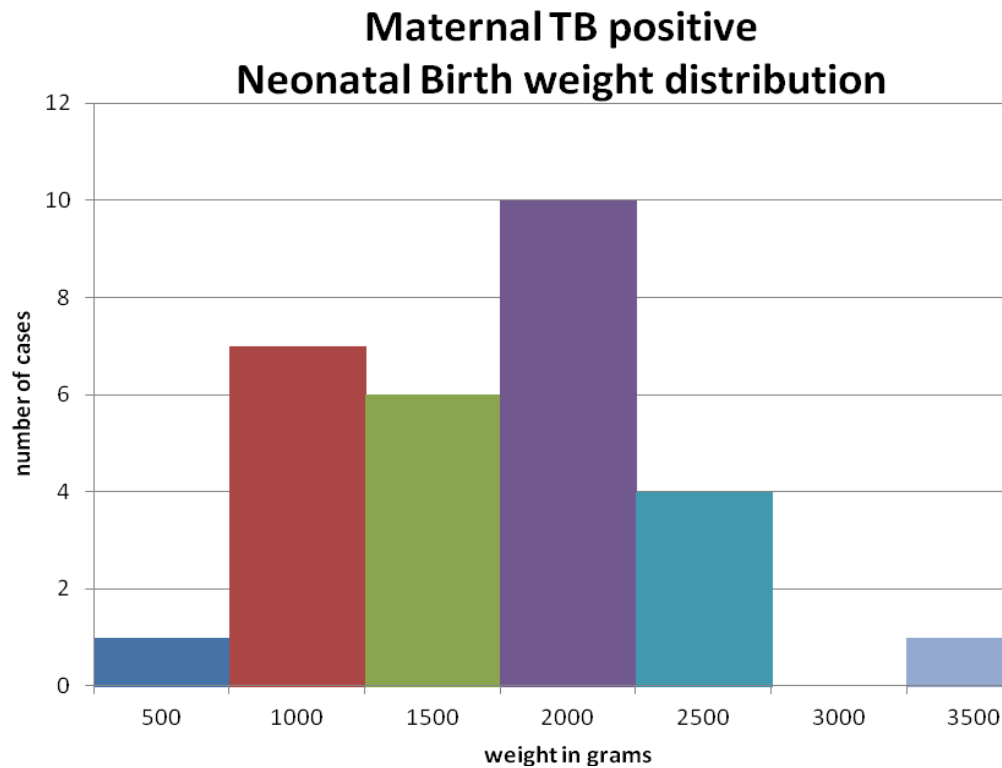
### **4.2.1 Outcome**

Of the 56 study infants, data was available on 43 (77%). Of the missing 13 neonates, 8 were stillbirths with the remaining 5 having either unavailable or unrecorded data in clinical files. From the 30 TB positive mothers only 29 infants had data available for inclusion in the statistical analysis. From the 29 infants, 5 (17%) turned out to have congenital TB (diagnosed according to current diagnostic criteria)(Cantwell et al 1994). The low number of confirmation may reflect the rarity of congenital TB, the limitations of gastric washings and the fact that INH therapy prevents progression of tuberculosis in high risk neonates.

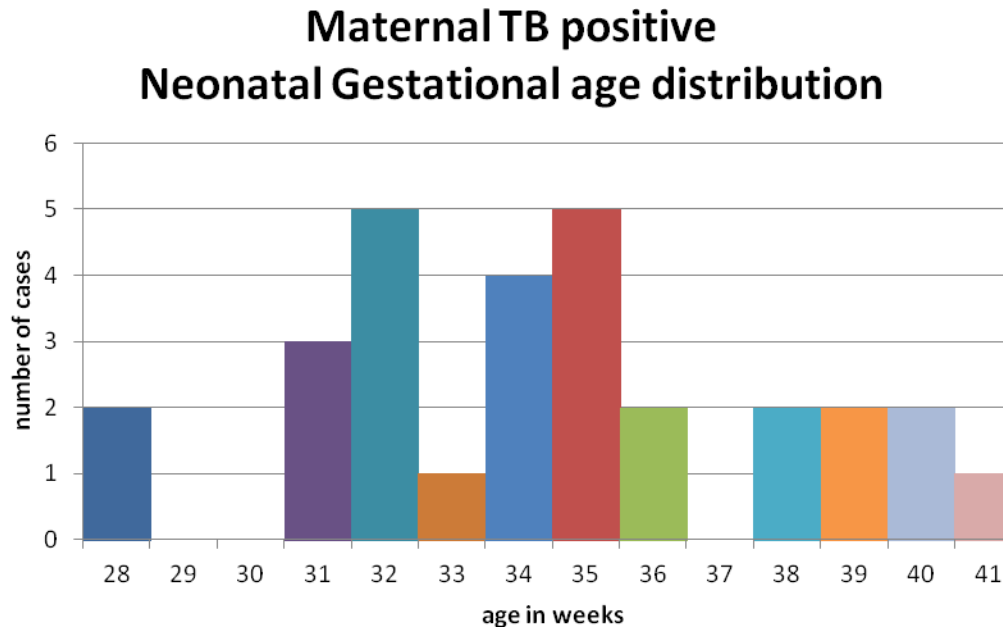
### 4.2.2 Gestational age and birth weights

A median birth weight of 2030 grams (IQR 1440-2360) and a median gestational age of 34 weeks (IQR 32-36) were observed, in this group illustrating a high proportion of low birth weight and premature infants in the cohort. One mother had diabetes in the birth weight range of 3500-4000. Table 4.1 and Figure 4.4 and 4.5 indicate the birth weights and gestation age of the infants included in this study to all the mothers with maternal TB.

<b>Table 4.1: Maternal TB positive Birth weight and gestational age analysis (N=29)</b>							
<b>Variable</b>	<b>Mean</b>	<b>Median</b>	<b>Min</b>	<b>Max</b>	<b>Lower - Quartile</b>	<b>Upper - Quartile</b>	<b>Std.Dev.</b>
<b>Birth weight</b>	<b>1983</b>	<b>2030</b>	<b>800</b>	<b>3780</b>	<b>1440</b>	<b>2360</b>	<b>640.2763</b>
<b>GA</b>	<b>34</b>	<b>34</b>	<b>28</b>	<b>41</b>	<b>32</b>	<b>36</b>	<b>3.4291</b>



**Figure 4.5: Histogram of infant birthweights distrubution**



**Figure 4.6: Histogram of infant gestational age distribution**

In the control (TB negative mothers) group, a total of 14 infants were included in the statistical analysis. Then median birth weight was 1985g (IQR 1336-2970) and the median gestational age was 35 weeks (IQR 31-40). Table 4.2 provides the data.

<b>Table 4.2 Maternal TB Negative(control) Birth weight and gestational age analysis (N=14)</b>							
<b>Variable</b>	<b>Mean</b>	<b>Median</b>	<b>Min</b>	<b>Max</b>	<b>Lower - Quartile</b>	<b>Upper - Quartile</b>	<b>Std.Dev.</b>
<b>Birth weight</b>	<b>2140</b>	<b>1985</b>	<b>810</b>	<b>3785</b>	<b>1336.667</b>	<b>2970</b>	<b>968.562</b>
<b>GA</b>	<b>34</b>	<b>35</b>	<b>25</b>	<b>40</b>	<b>31</b>	<b>38</b>	<b>4.8431</b>

#### 4.2.3 Radiology

Radiological studies (e.g. Chest Roentgenogram) can help to identify neonates with congenital TB. Out of the 5 congenital TB positive neonates, 4 (80%) had positive radiological features consistent with congenital tuberculosis.

#### 4.2.4 Laboratory tests

Of the 5 infants treated for congenital TB, 4 (80%) infants had positive radiological features suggestive of congenital tuberculosis. Three (60%) infants had positive gastric washings for tuberculosis, and this figure is similar, though lower, than that quoted in the literature (80% sensitivity). This discrepancy is most likely due to our low numbers of infants with congenital TB.

Additionally, most of the mothers were on TB treatment at the time of delivery and this could have played a role in lowering the transmission rates. The risk of transmission of congenital TB is lowered with the length of duration of maternal TB treatment.[\(23\)](#)

From the 5 infants that had congenital TB, only 3 mothers had positive placental diagnosis of TB. Of these 3 infants, 1 had positive gastric washings and 2 had radiological features of tuberculosis with negative gastric washings. The other 2 infants, whose mothers had no placental diagnosis of tuberculosis, both had positive gastric washings and radiological features of TB.

### 4.2.5 Treatment

Out of the 29 maternal TB infants, 22 (76%) received INH chemoprophylaxis, 5 (17%) had full TB treatment and 1 (3%) received no treatment at all. 1 infant was from a mother that was falsely presumed to have maternal TB and hence her therapy was stopped and the infant received no therapy at all. The infant was well on clinical follow-up. (Figure 4.7)

From the 14 infants born to TB negative mothers, 3 (21%) had received INH chemoprophylaxis. This resulted in a total of 25 infants receiving INH chemoprophylaxis in our study.

There were 7 positive placental TB cases, 5 of these infants received full TB treatment and 2 had received INH chemoprophylaxis.

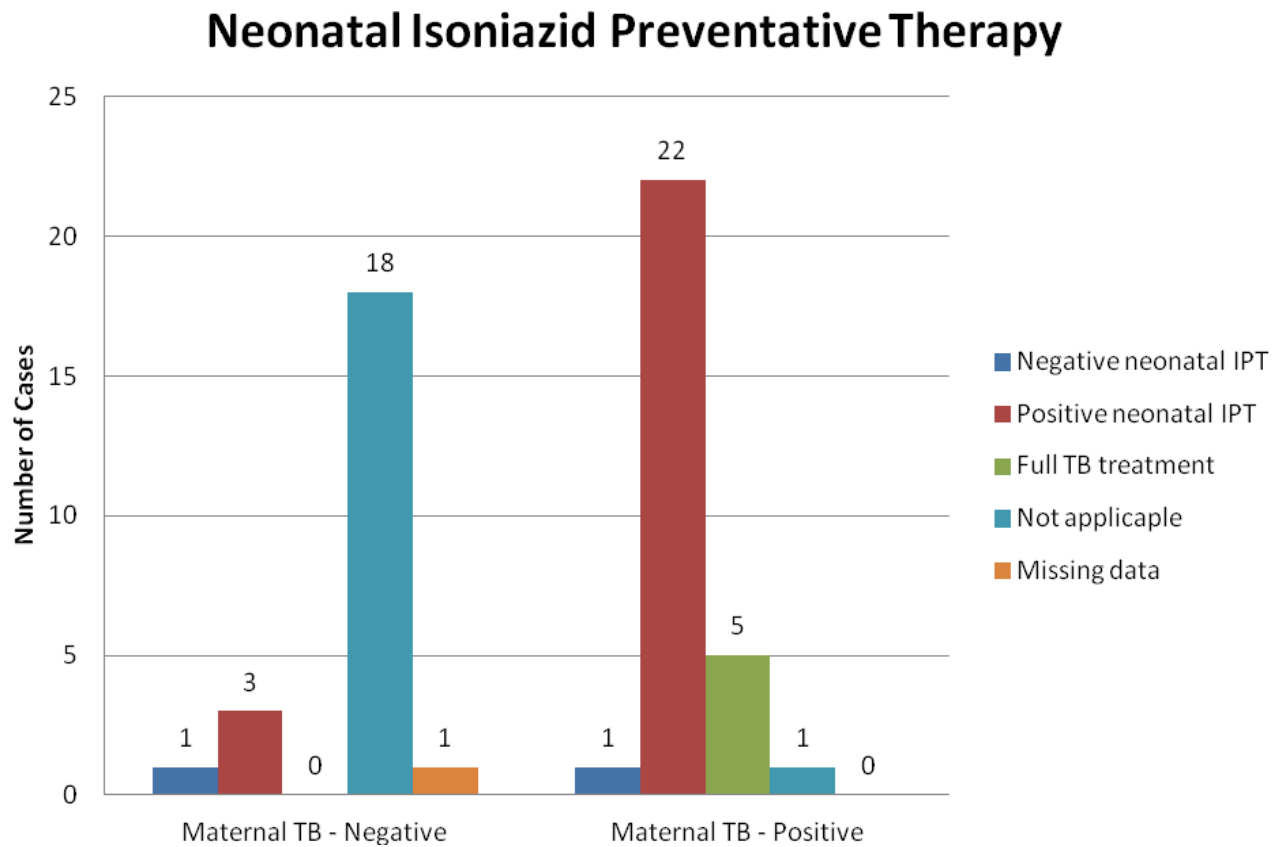


Figure 4.7. Histogram illustrating the number of infants receiving Isoniazid chemoprophylaxis.

### **4.3 Placental tuberculosis**

Of the 56 mothers that were clinically suspected of having tuberculosis, 30 (53.6%) were diagnosed with TB. Twenty nine mothers were diagnosed pre-delivery while 1 mother was diagnosed post delivery. Of the 30 TB positive mothers, 7 (23%) had placental TB. All 7 cases had morphological features that were consistent with tuberculosis, although they did display the heterogeneous pattern described in the literature.

As a quality assurance measure, all placentas from the control group were blindly reviewed by the study pathologists and no case of tuberculosis was diagnosed in this control group.



### 4.4 Placental histological Features

After histological examination, the 2 most statistically significant features seen in placentas with TB were acute intervillitis and intervillous micro abscesses. In the 7 placenta with TB, 6 contained acute intervillitis ( $p=0.00033$ ) and 2 contained intervillous micro abscesses ( $p=0.01364$ ). The micro abscesses were not seen in any of the control group placentas. ZN stains were positive 7 of the 7 placental cases.

Statistically non-significant histological findings included: (1) Chorioamnionitis which was present in 13 (23%) of the 56 placentas and in none of the 7 TB placentas ( $p=0.18233$ ). (2) Acute villitis was present in 5(9%) of the 56 placentas and in only 1 placenta with TB ( $p=0.5007$ ). (3) Chronic villitis was present in 15 (27%) of the 56 placentas and in 3 (43%) of the 7 TB placentas ( $p=0.36$ ). (4) Placental pathological abruption was present in 10(18%) of the 56 placentas and in 1(14%) of the 7 TB placentas ( $p=1.00$ ). (5) Fetal thrombotic vasculopathy was present in 9(16%) of the 56 placentas and in 1 (14%) if the 7 TB placentas ( $p=1.00$ ). (6) Maternal vascular underperfusion (MVUP) was present in 23(41%) of the 56 placentas and in 4 (57%) of the 7 TB placentas ( $p=0.4261$ ).

Acute chorioamnionitis and MVUP are frequent diagnoses in the placentas in our population.

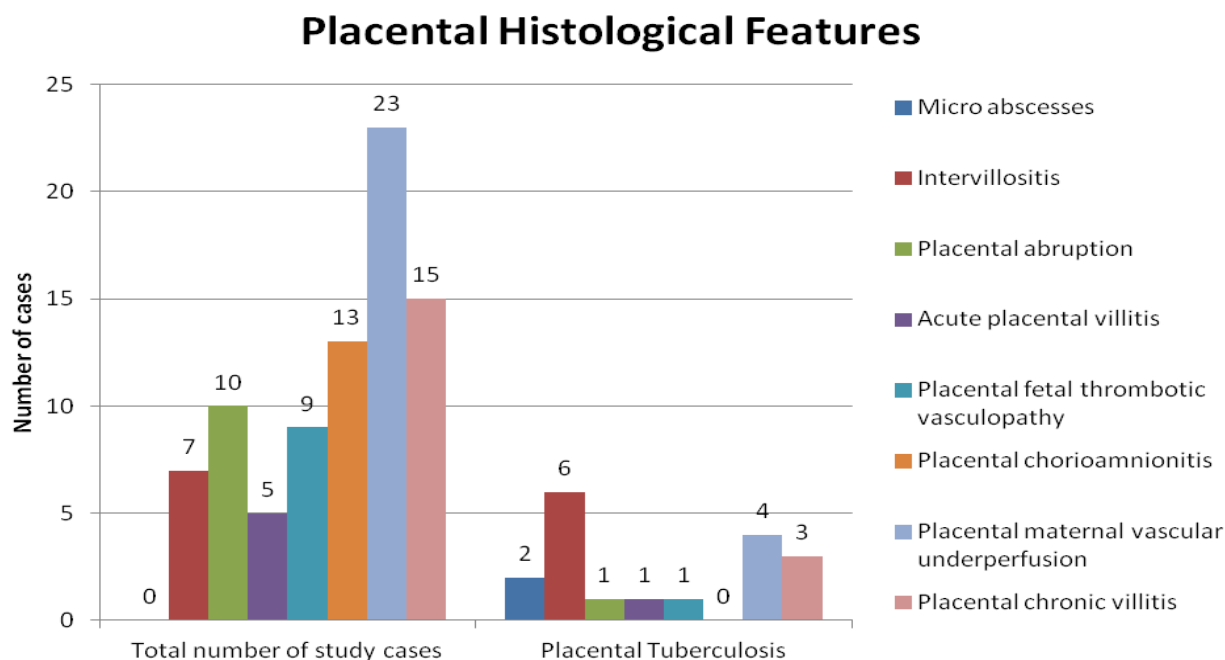


Figure 4.8. Histogram illustrating the placental histological features present in the study.

## **Presentation of the Positive Placental TB cases found in this study**

### **CASE 1**

#### Clinical history

25 weeks GA, Mother HIV-positive on HAART, has a TB contact

Maternal TB confirmed on culture (sputum)

#### Placental histopathology

Chronic villitis, lymphohistiocytic, with villous lysis

Acute intervillitis

ZN positive

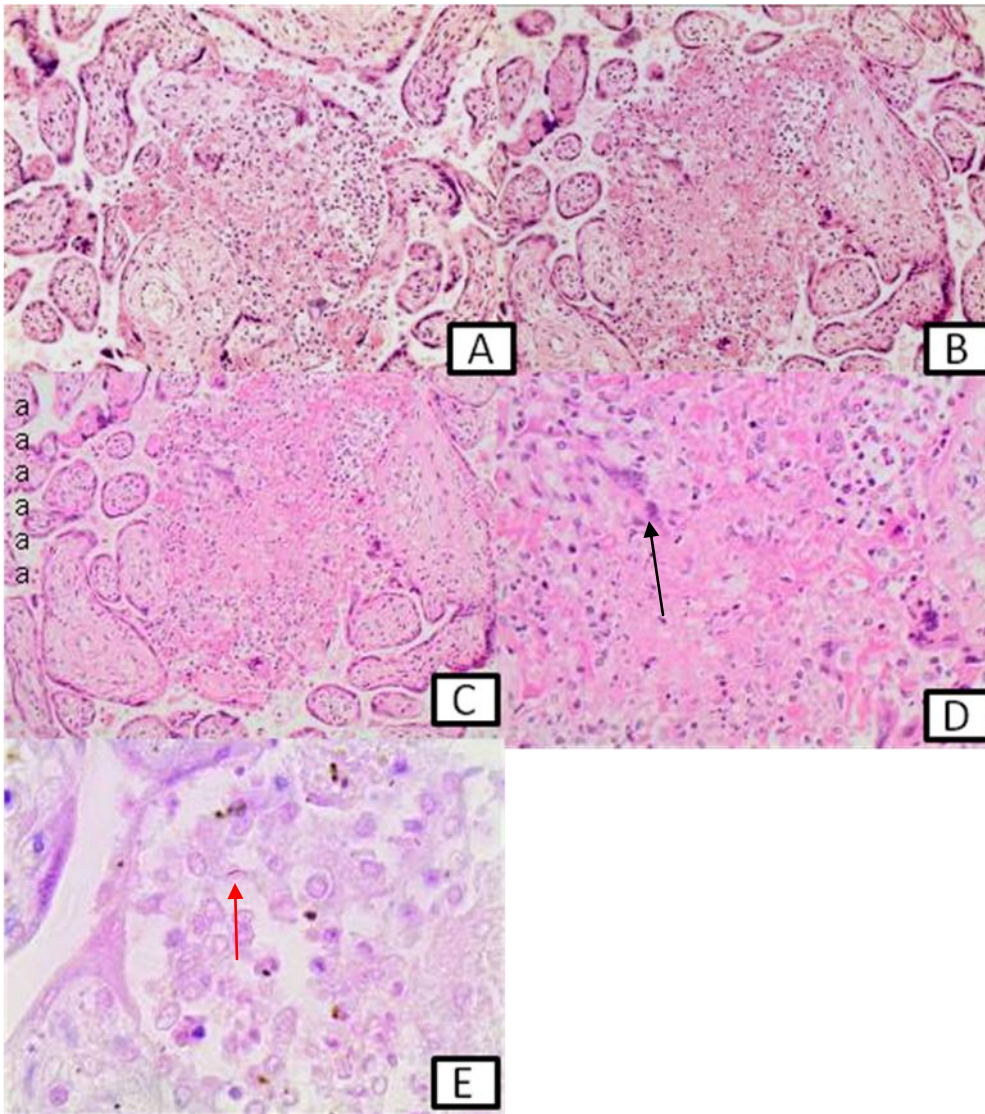
#### MVUP

Placenta < 10<sup>th</sup> centile

Increased perivillous fibrin

Accelerated maturation

Thrombi in the stem villi and chorionic plate vessels



**Figure 4.9.1 Case 1**

Histologically confirmed mycobacterial disease

Figures (a-d) show acute intervillositis associated with fibrin. Entrapped syncytiotrophoblast giant cells mimic Langhans giant cells (black arrow) but classical granulomas are absent. In addition chronic villitis associated with marked villous lysis, predominantly lymphohistiocytic is present.

Figure (e) shows a positive ZN acid and alcohol fast bacilli (red arrow) confirming mycobacterial infection

## **CASE 12**

### Clinical History

G4 P2 M1, 31 years old

HIV-positive, CD4 count 50

Mother diagnosed with miliary TB three days prior to delivery, on treatment

Maternal TB confirmed on culture (pleural fluid)

### Placental histopathology

Chronic villitis with plasma cells

Acute intervillitis, abscess formation

ZN positive

Meconium laden macrophages

Multifocal avascular villi

Karyorrhexis and calcification of villi

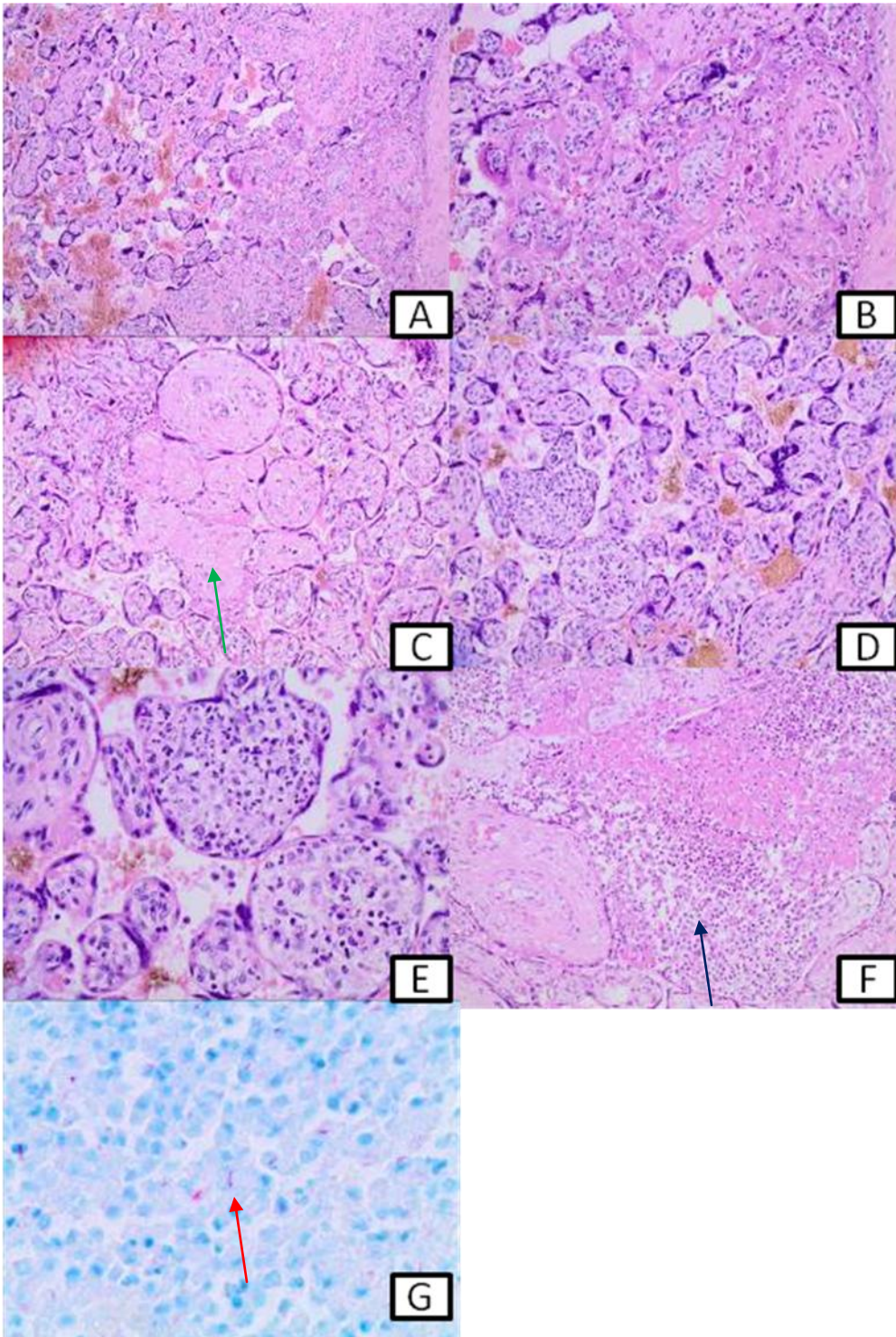
MVUP

- increased syncytial knots

- micro-infarcts

- increased perivillous fibrin

Cytomegalovirus positive on immunohistochemistry



**Figure 4.9.2 Case 12**

Histologically confirmed mycobacterial disease.

Figures (a-b) show maternal vascular underperfusion, while Figures (c-e) demonstrate foci of avascular villi (green arrow). Figures (e-f) show acute intervillitis (blue arrow) associated with chronic villitis with plasma cells. Figure (g) shows a positive ZN confirming mycobacterial infection (red arrow).

## **CASE 20**

### Clinical History

G3 P2, 34 weeks GA

Premature labour, PROM for 3 weeks, clinically suspected chorioamnionitis

Resistant UTI defaulting treatment

Normal vaginal delivery after induction of labour

Placenta reviewed after neonate symptomatic

Multiple +++ sections

Maternal TB confirmed on culture (sputum) after maternal CXR showed miliary TB

### Placental histopathology

Focal acute intervillitis and focal chronic villitis with decidual micro abscesses

ZN positive

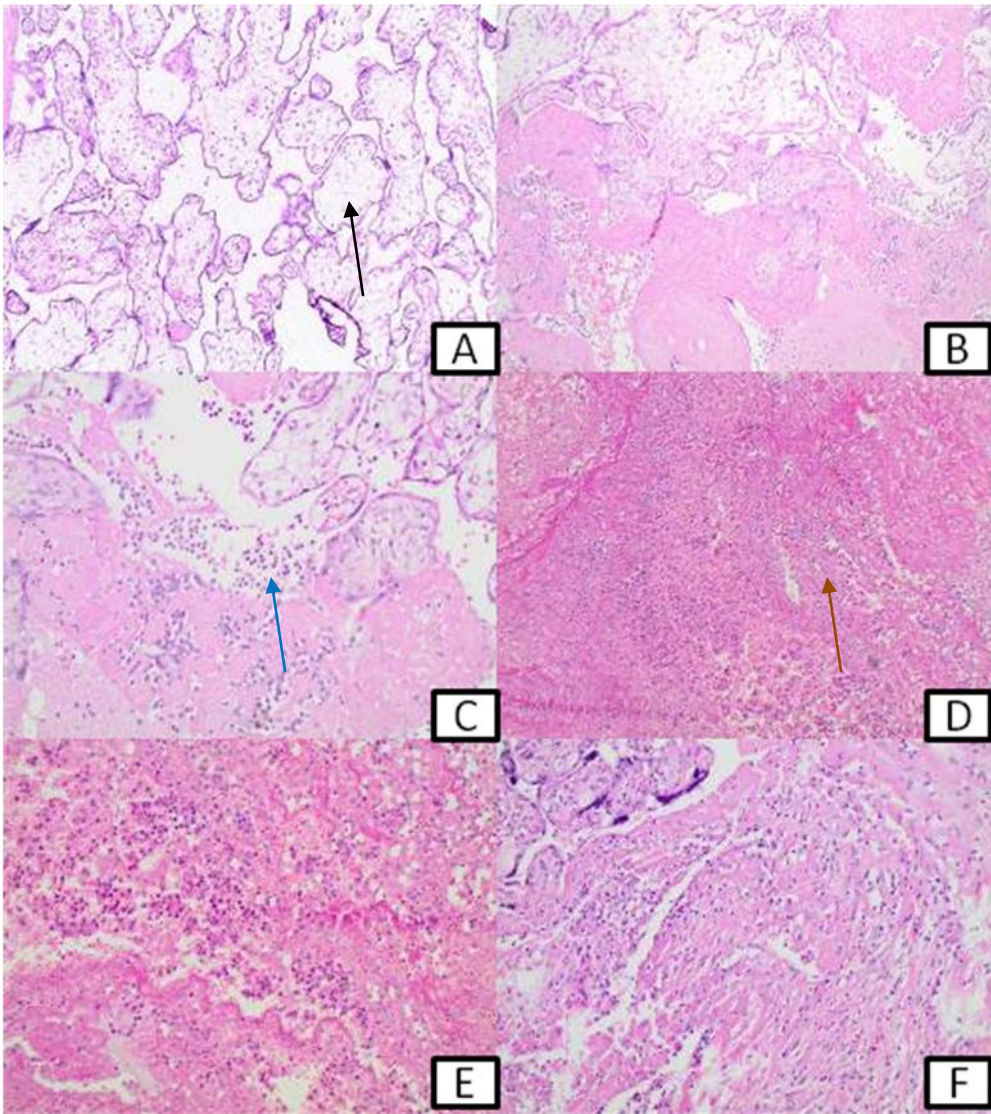
NO CHORIOAMNIONITIS

Retroplacental haemorrhage (? Due to induction labour)

Villous edema

Increased syncytial knots

Increased perivillous fibrin



**Figure 4.9.3 Case 20**

Histologically confirmed mycobacterial disease.

Figures (a) shows villous edema of villi (black arrow) (b-c) show acute intervillitis (blue arrow).

Figures (d-e) show decidual micro abscesses (brown arrow). Figure (f) shows chronic basal villitis

## **CASE 42**

### Clinical History

Caesarean section at 31 weeks

HIV-positive, CD 4 count 123, Mother clinically ill

? PTB with Acute Respiratory Distress Syndrome / ? miliary TB

Maternal TB confirmed on culture (sputum, blood)

Mother died

### Placental histopathology

Intervillositis, micro abscess formation, Acute suppurative villitis

ZN positive

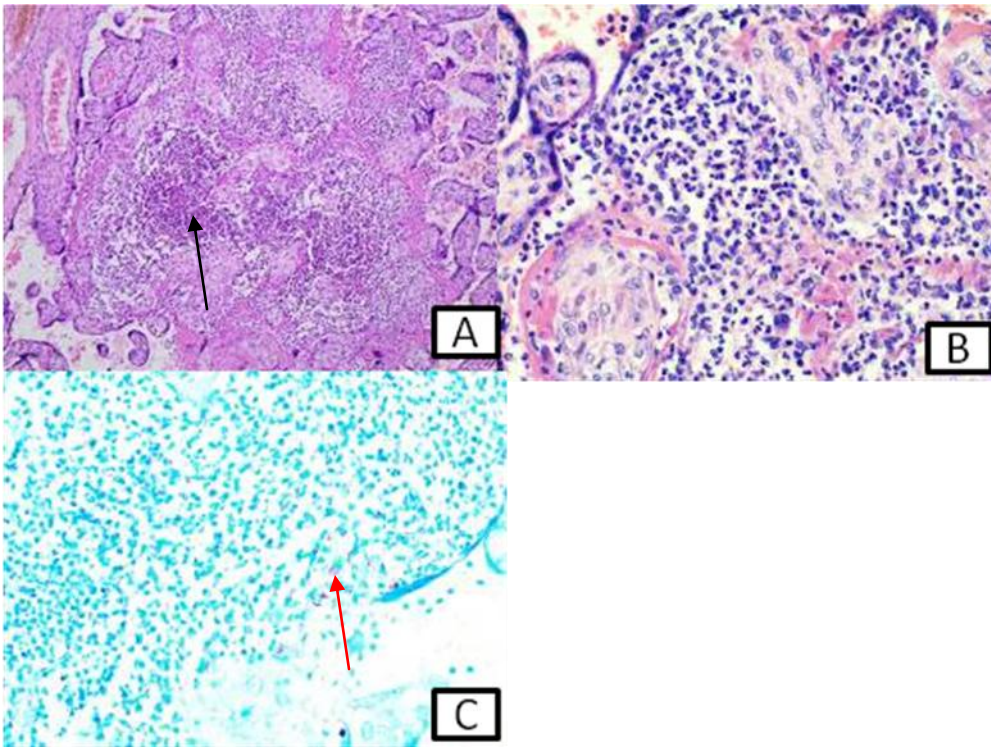
MVUP

- accelerated maturation

- distal villous hypoplasia

- increased syncytial knots





**Figure 4.9.4 Case 42**

Histologically confirmed mycobacterial disease.

Figure (a) shows acute intervillitis with microabscess (black arrow) formation (b) show acute suppurative villitis and Figure (c) shows a positive ZN stain (red arrow).

### CASE 43

#### Clinical History

G3 P2, , GA 30 weeks, HIV-positive

Spontaneous preterm labour

Mother symptomatic / TB, confirmed on culture MDR (sputum)

#### Placental histopathology

Chronic villitis, lymphocytic and Intervillositis

ZN positive

Meconium laden macrophages

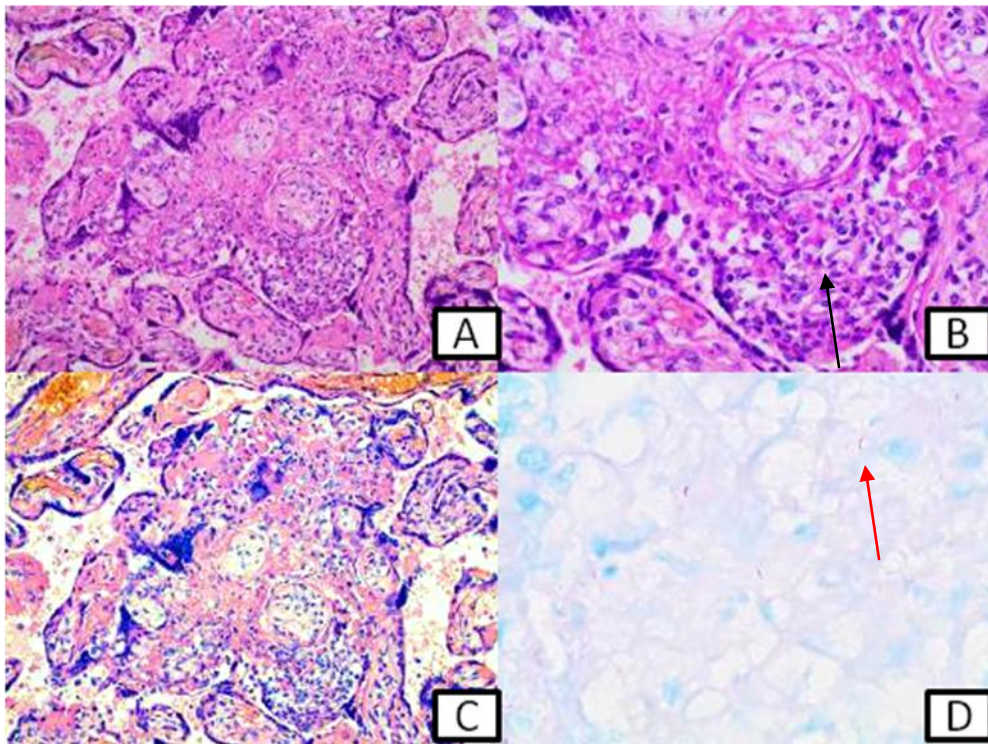
MVUP

accelerated maturation

increase in syncytial knots

micro-infarction

distal villous hypoplasia



**Figure 4.9.5 Case 43**

Histologically confirmed mycobacterial disease.

Figures (a-c) show chronic villitis (black arrow) and Figure (d) shows a positive ZN stain (red arrow).

## **CASE 44**

### Clinical History

G3 P3, 26 years old, GA 33 weeks

HIV-positive, CD4 count 23

Spontaneous preterm labour

Previous TB, now reactivation with left pleural effusion

Maternal TB confirmed on culture (sputum, MDR)

### Placental histopathology

Microabscess formation and Intervillositis

ZN positive

Meconium laden macrophages, diffuse villous edema

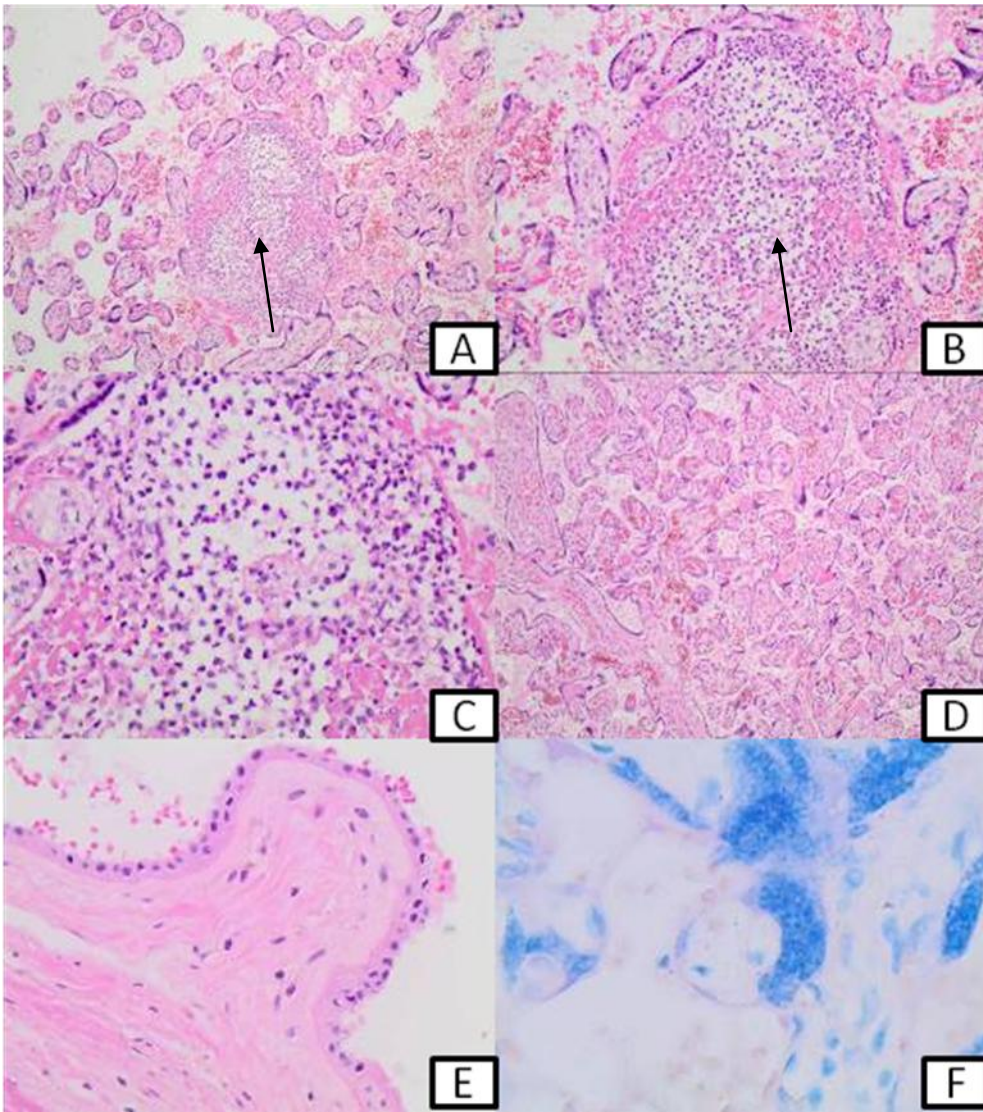
MVUP

placenta < 10<sup>th</sup> centile

accelerated maturation

increase in syncytial knots

increased perivillous fibrin



**Figure 4.9.6 Case 44**

Histologically confirmed mycobacterial disease.

Figures (a-c) show acute intervillitis and microabscess formation (black arrow). Figure (d) demonstrates maternal vascular underperfusion with increased syncytial knots and Figure (e) meconium laden macrophages in the amnion while Figure (f) shows a positive ZN stain

## **CASE 28**

### Clinical History

HIV– positive

Maternal TB confirmed on culture, on TB treatment for 1 week prior to delivery

### Placental histopathology

Acute intervillitis and chronic granulomatous villitis,

Decidual microabscess formation

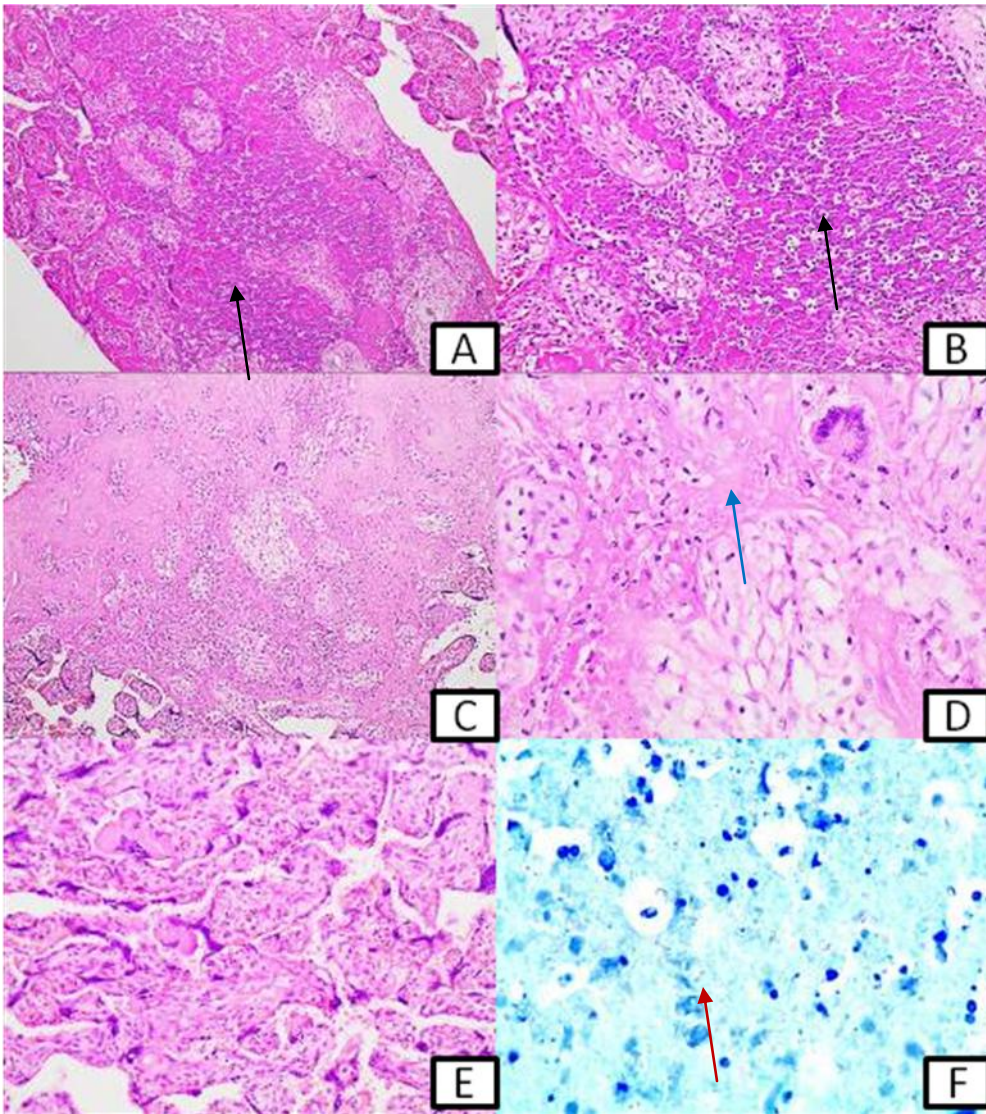
ZN positive, numerous organisms

MVUP

placenta < 10<sup>th</sup> centile

increased perivillous fibrin

recent and old infarction



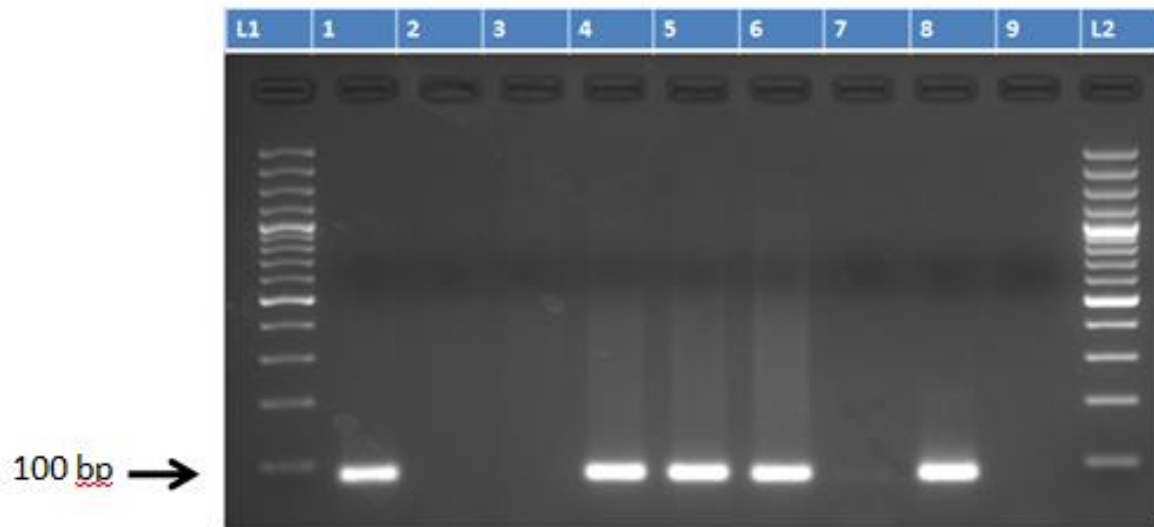
**Figure 4.9.7 Case 28**

Histologically confirmed mycobacterial disease.

Figures (a-b) show acute intervillitis and microabscess formation (black arrow). Figure (c-d) demonstrates granulomatous villitis (blue arrow). Figure (e) shows maternal vascular underperfusion with increased syncytial knots while Figure (f) shows a positive ZN stain (red arrow).

## 4.5 PCR

In order to identify the presence of *M. tuberculosis* in FFPE placental tissue, extracted DNA was initially amplified using primers complementary to the RD9 region. The figure seen below (figure 4.10) demonstrated that the amplification achieved from the controls selected for the optimisation process.



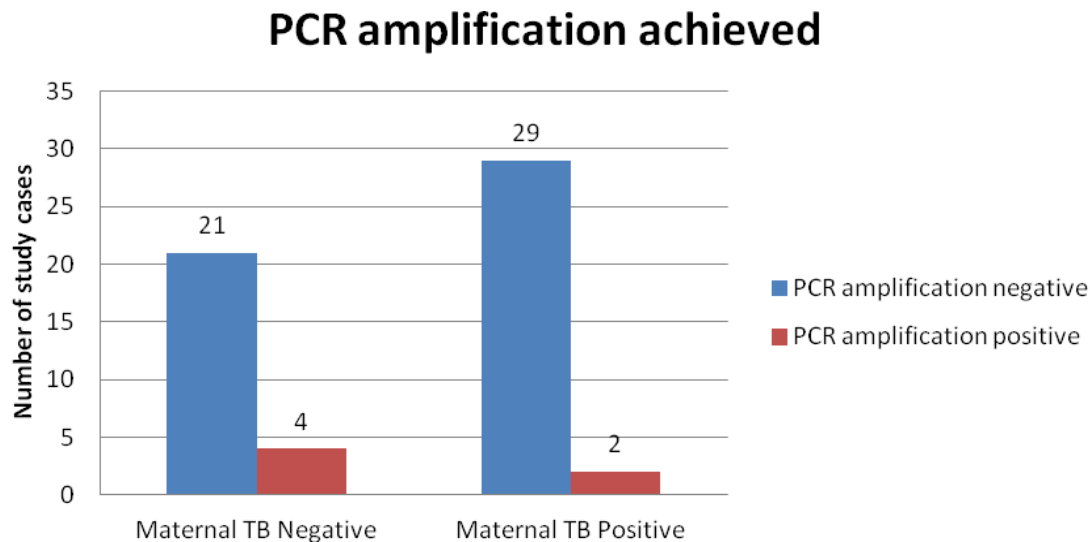
**Figure 4.10 Primer optimisation gel electrophoresis using known TB positive disease as positive control and testis as negative control.**

L1: Ladder 1; 1: Negative control 1; 2: Negative control 2; 3: Negative control 3; 4: Positive control 1; 5: Positive control 2; 6: Positive control 3; 7: NTC; 8: H37Rv; 9: NTC; L2: ladder 2

This primer optimisation PCR experiment was repeated numerous times to confirm that no DNA contamination was present in the negative control. The repeat test also showed that positive amplification was achieved in the negative control and these were thought to be due to primer dimer amplification.

To improve the detection of fragmented DNA a second PCR amplification strategy was followed which targeted DNA fragments of >40 bp. This PCR strategy amplified a region of the IS6110 transposon element thereby enabling the identification of members of the *M. tuberculosis* complex.

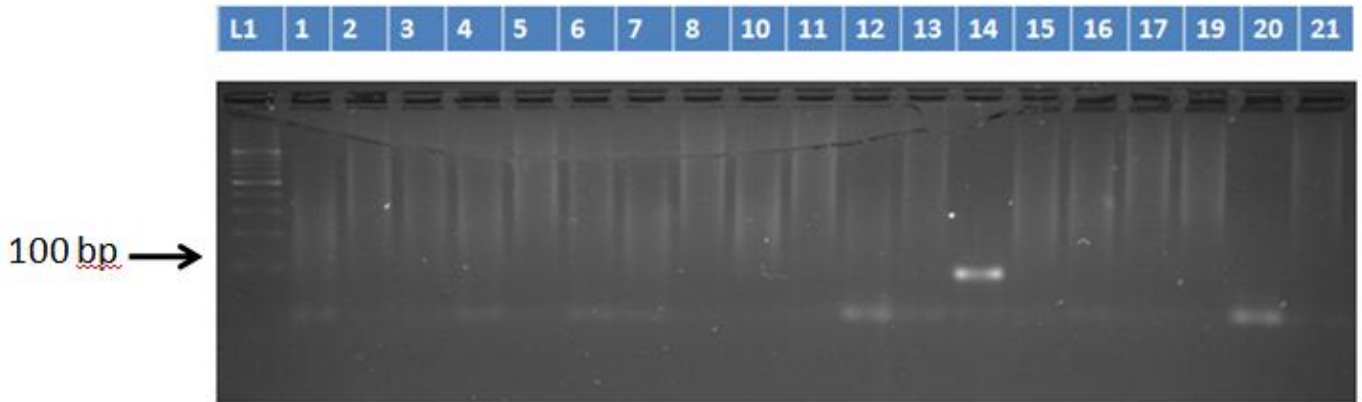
The following figure illustrates the positive PCR results in this study and is categorised into positive results found in TB positive mothers and TB negative mothers. Positive PCR results were found in 6.45% of TB positive mothers and a positive PCR result was found in 16% of TB negative mothers.



**Figure 4.11: Histogram of PCR amplification achieved**

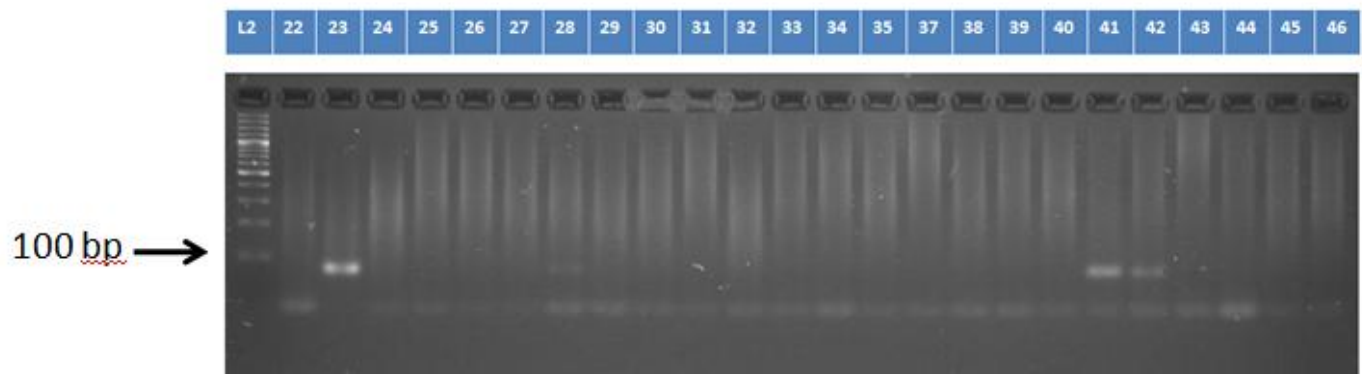
Positive amplification was observed in six patient specimens (samples 14, 23, 28, 41, 42 and 47) by the presence of an amplification product of 85 bp, using the second primers as described above. In 2 of these cases positive placental TB was found and none of the neonates developed congenital TB, this might be due to the neonates that received preventative therapy (INH for 6 months).





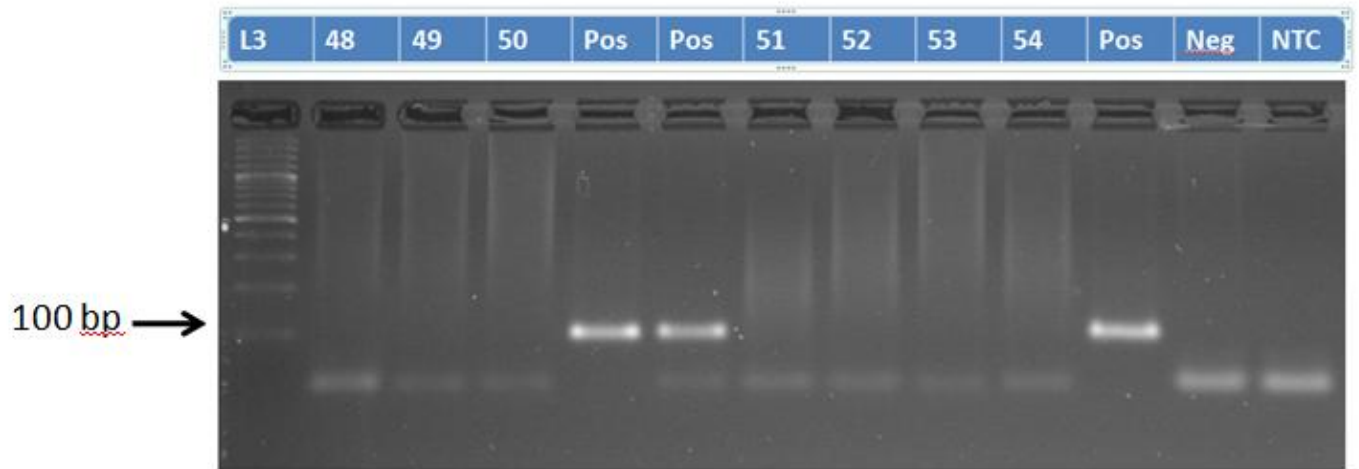
**Figure 4.12: Gel A showing PCR amplification of sample 14.**

L1: ladder 1; 1: sample 1; 2: sample 2; 3: sample 3; 4: sample 4; 5: sample 5; 6: sample 6; 7: sample 7; 10: sample 10; 11: sample 11; 12: sample 12; 13: sample 13; 14: sample 14; 15: sample 15; 16: sample 16; 17: sample 17; 19: sample 19; 20: sample 20; 21: sample 21



**Figure 4.13: Gel B showing PCR amplification of samples 23, 41 and 42.**

L2: ladder 2; 22: sample 22; 23: sample 23; 24: sample 24; 25: sample 25; 26: sample 26; 27: sample 27; 28: sample 28; 29: sample 29; 30: sample 30; 31: sample 31; 32: sample 32; 33: sample 33; 34: sample 34; 35: sample 35; 37: sample 37; 38: sample 38; 39: sample 39; 40: sample 40; 41: sample 41; 42: sample 42; 43: sample 43; 44: sample 44; 45: sample 45; 46: sample 46



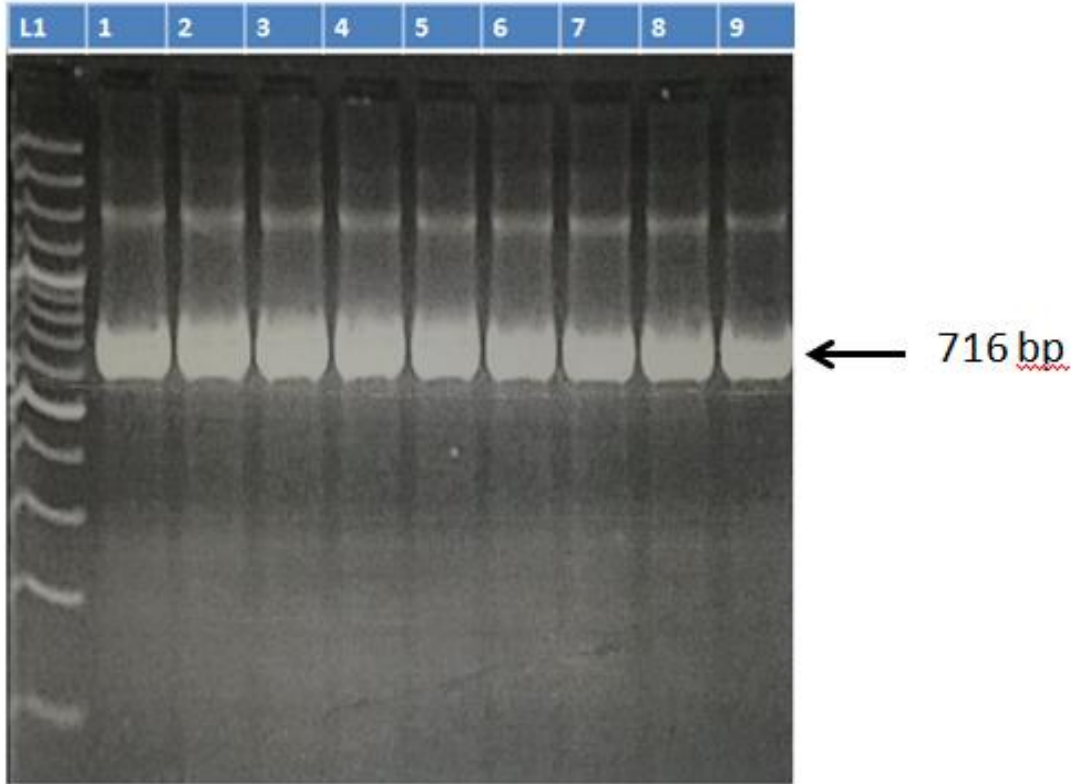
**Figure 4.14: Gel C showing PCR amplification of positive control samples**

L3: ladder 3; 48: sample 48; 49: sample 49; 50: sample 50; pos: positive control sample1; pos: positive control sample2; 51: sample 51; 52: sample 52; 530: sample 53; 54: sample 54; Pos: H37Rv; Neg: Negative control sample; NTC: non template control to check for contamination.

## 4.6 ISH

### 4.6.1 Amplification of *IS 6110*

*IS6110* was amplified and the products run on the temperature gradient and subsequent gel electrophoresis in a 3% agarose gel.

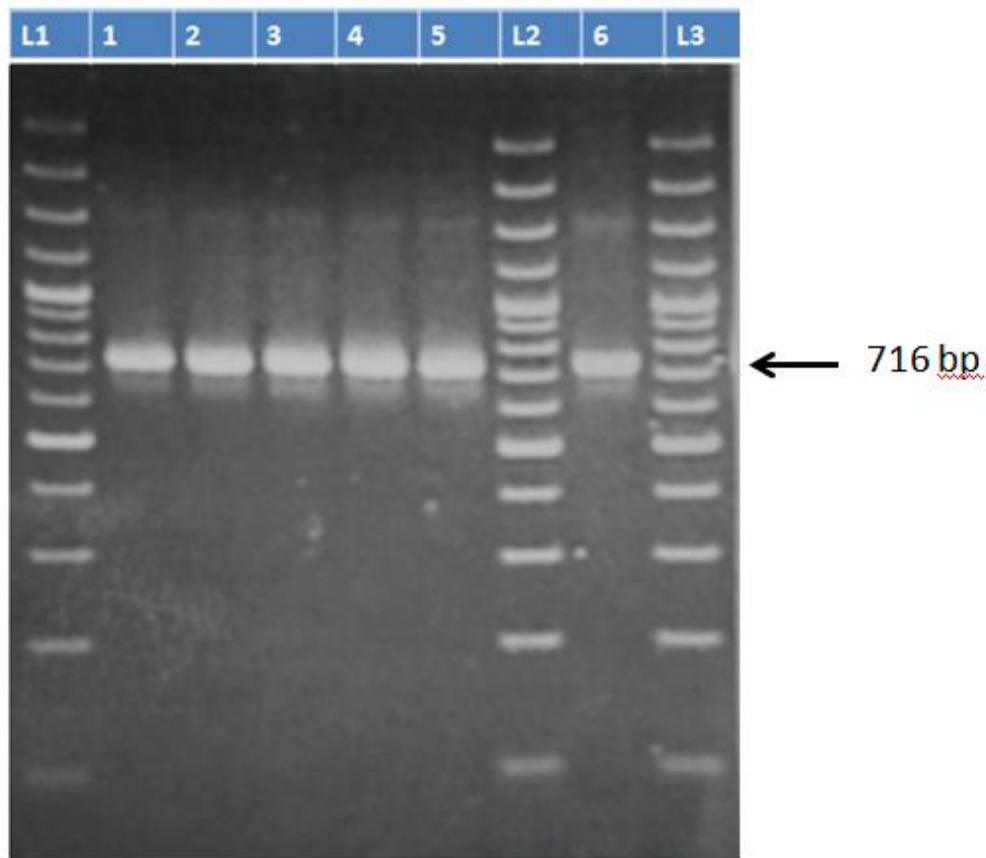


**Figure 4.15 Temperature gradient PCR amplification**

L1: Ladder 1; 1: PCR done at 60°C; 2: PCR done at 60°C; 3: PCR done at 61°C; 4: PCR done at 61°C; 5: PCR done at 62°C; 6: PCR done at 62°C; 7: PCR done at 63°C; 8: PCR done at 63°C; 9: PCR done at 64°C.

The temperature gradient revealed positive amplification by the presence of an amplification product of 713 bp.

#### 4.6.2 Purification of amplified PCR (IS6110) amplicons



**Figure 4.16 Gel electrophoresis of purified PCR products.**

L1: Ladder 1; 1: Sample 1 & 2 mixture from above results; 2: Sample 3 & 4 mixture from above results; 3: Sample 5 & 6 mixture from above results; 4: Sample 6 & 7 mixture from above results; 5: Sample 7 & 8 mixture from above results; L2: Ladder 2; 6: Sample 9 from above results; L3: Ladder 3

The above results showed that successful amplification and purification of IS6110 was achieved. These purified DNA was quantified using the nanodrop machine in order to identify the vial that had the most amount of DNA present in it. (see Appendix H)

IS6110 vial 5 was chosen as the probe to be labelled with Digoxin as it was identified as the one with the most amount of DNA present in it.

### 4.6.3 Labeling of probes with DIG

No results were seen on the membrane when the product membrane was hybridised with IN SITU HYBRIDIZATION DETECTION SYSTEM CORE KIT II(Cat no: DD131-60K) for Digoxin labeled probe/Streptavidin-Alkaline Phosphatase supplied by Biogenix, and subsequently stained with DAB. This kit from Biogenix was specific for the use with FFPE kit and not membranes. A positive reaction would have been seen with a brown precipitate forming on the membrane. The procedure was repeated with a different kit (CSPD) as recommended by the manufacturer used for the labeling of the probes (Roche) as explained above, to check for successful labeling of the probe with Digoxin. The membrane needs to be exposed to UV light to confirm positive labeling.



**Figure 4.17 Membrane stained with DAB**

o positive product was visualised on the X-ray film when the product membrane was stained with the CSPD, ready to use kit from Roche (cat no; 11 755 633 001) and the DIG wash and block buffer set from Roche (cat no 11585762001). Several X-rays was taken to optimise the UV light exposure in order to visualize if a positive product was present on the membrane.



**Figure 4.18 X-ray film of DIG labelled probes**

## 5. DISCUSSION

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The diagnosis of congenital tuberculosis is known to be fraught with difficulty as the clinical signs can be non-specific and sometimes mimic other diseases. It is said that most infected neonates initially appear well and the disease becomes apparent only in the late neonatal or early infant period .(31) Clinical signs may suggest a chronic intrauterine infection that may mimic acute infection, while diagnosis may be extremely difficult and complex and often is missed. In our study affected infants are significantly lighter, as shown in the results section, than controls and often are delivered prematurely. In addition the neonates frequently show intrauterine growth retardation and there may be an increase in perinatal death. Infants with congenital TB often present with reduced appetite, vomiting and weight loss. Upper respiratory, fever and an enlarged spleen and liver have been reported.(55, 56) Neonatal mortality of up to 30% has been reported in infants born to untreated mothers with active TB disease(57) and the overall mortality from congenital tuberculosis is 22 to 50%. (3, 31)

A familial history of TB (including but not limited to the mother) or a mother who is symptomatic is a strong pointer to the diagnosis of congenital and/or perinatal TB. An additional factor that causes one to suspect congenital or perinatal tuberculosis is known maternal HIV infection which should lead to further investigations being undertaken in the neonates. Such investigation could include chest radiograph and microbiologic testing of gastric aspirate, endotracheal aspirates, CSF, liver, skin and lymph node biopsies, broncho alveolar lavages and ear swabs. In the few published cases on congenital or perinatal tuberculosis examination and reporting on the placenta is conspicuously absent. (13, 18, 23, 55, 57)

This may be due to delay in recognising the clinical onset of the disease, and the placenta is therefore not submitted for histopathology. Secondly the paucity of reports may be due to the placental lesions often being focal or not histologically recognised.

In most laboratories routine sampling of the placenta is limited to 4-5 blocks, and while this may be adequate to identify most pathological lesions it may not be appropriate for tuberculosis. Second early lack of a granulomatous response which is usually regarded as the hallmark of mycobacterial infection is almost always absent in placental tuberculosis. This was noted in our study where granulomatous inflammation was present in only one of the seven cases in which tuberculosis was identified on histopathology. It has been proposed that women with chronic tuberculosis have low fertility and therefore most cases of perinatal TB are associated with acute rapidly progressive or a new infection in the mother similar to primary tuberculosis infection in children. The response therefore is one of an

acute neutrophilic or histiocytic inflammatory focus in the placenta rather than the formation of typical granulomas.(35) This focus can be either an acute villitis that causes villous lysis with spillage into the intervillous space or cause primarily an intervillousitis with or without intervillous microabscess formation and secondary involvement of villi. This was the major findings that we also encountered in this study. Decidual abscesses can also be seen. This pattern involves the early or innate inflammatory response. The differential diagnosis includes bacterial infections such as *Listeria*, *Staphylococcus* and *E. coli*. (35) In our study the two histological features which were statistically significant despite the small numbers were intervillousitis ( $p=0,0005$ ) and micro abscess formation ( $p=0.01$ ). The Fishers two tailed test was used due to the small numbers. This is consistent with histopathological findings in the more recent literature that are seen in mycobacterial infection.(30) Finding acute villitis, intervillousitis and micro abscesses formation should always prompt the pathologist to perform a ZN stain to exclude the possibility of TB in an African setting or any other high risk area for TB.

As the histopathology literature of tuberculous infection of the placenta is sparse and the pathology non-specific, it is not possible for pathologists to definitively confirm infection with *M.tuberculosis* without bacteriological confirmation. This can be done by using a number of specialised stains; the one most frequently used being the ZN stain. This has a wide reported sensitivity, from 20-70%, depending on the skill and patience of the laboratory and the pathologist, and the number of bacilli present. The ZN stain still remains the gold standard in histopathology to diagnose *M.tuberculosis* in a histological section, despite its limitations. In all seven cases reported as positive in our study the diagnosis was confirmed on a positive ZN stain.

Pregnancy often delays the diagnoses of TB in the mother as symptoms that point to tuberculosis may be masked by being attributed to gestational-associated fatigue and malaise and often there is reluctance by the clinician to undertake imaging studies. The outcome of starting early treatment for active disease outweighs the risk any possible future drug toxicity to the mother or infant.

The recognition of tuberculosis in the placentas of women with clinically suspected but as yet unproven infection has the benefit of alerting physicians to quickly start TB treatment in both the mothers and their infants. This is especially relevant when they are co-infected with HIV. Furthermore, placental identification of TB contributes to identifying high-risk TB-exposed infants early, and provides clinicians with an opportunity to start antituberculous therapy early and so prevent progressive



infection. Early diagnosis is of the utmost importance due to the high risk of developing active disease in these infants.

It is recognised that proven placental tuberculosis does not necessarily mean that the baby has congenital TB. We have seen in our study that only 3 of the cases with placental TB on histology and/or PCR developed symptoms and/or signs of neonatal tuberculosis. This is likely to be as a consequence of the prophylaxis which the neonates were placed on after identifying maternal tuberculosis as a risk factor.

The more recent techniques of PCR have revolutionised the laboratory diagnosis of TB. PCR is a highly sensitive test with proven high specificity. It has a fast turn-around time with a result being available within 24-48 hrs, potentially having better patient outcome and faster treatment options. However there are many factors that influence a successful PCR result. One of the major concerns is that formalin fixation of the tissue may cause significant damage to the DNA structure; causing cross linking of the DNA and breakage of the proteins. Another concern is that of contamination. It is of empirical importance that strict procedures are followed when cutting the sections to limit the potential of contamination. It has also been noted that PCR can trace *M.tuberculosis* DNA in a successfully treated patient, as the technique does not distinguish between live and dead bacteria but only identifies its presence in the particular section being analysed. It is precisely for this reason that we propose that the cases confirmed to be positive by PCR in the study have had the DNA of *M.tuberculosis* identified but not necessarily the bacilli. All of the positive PCR results (16%) found in the study from TB negative patients were also HIV negative. By way of explanation can be argued that the patients might have had latent TB, or having been previously treated or exposed to the bacterium, as it is known that most of our hospital population is infected by TB without acquiring disease.

A healthy individual with a healthy immune system can overcome the disease, but because the *M.tuberculosis* bacilli are protected by a waxy protein layer, they can lie dormant for many years. It is only when the immune system is weakened or compromised (e.g. By HIV, poor nutrition, advancing age, corticosteroid therapy and renal failure) that the risk of disease increases exponentially. Only 5-10% of the population who are HIV negative are likely to become ill with TB. HIV and *M.tuberculosis* co-infected individuals are much more likely to develop active TB. This co-infection is a lethal combination as each speeds up the others progression.(58)

A second consideration is that a maternal placental PCR positive result from smear/culture negative mothers does not necessarily mean an absence of genital tuberculosis and potential *in-utero* placental infection or transmission to the infant.

The low number of positive cases diagnosed by PCR relative to histopathology may be due to inhibitors e.g. the paraffin wax embedding; even though due care was taken to make sure all paraffin wax was removed from the sample. Paraffin wax is known to inhibit DNA extraction and subsequent PCR amplification. When protein digestion is insufficient DNA extraction is inhibited and thus the amplification of PCR products is compromised. All these factors may have contributed to the low positive *M.tuberculosis* results found by the PCR method. It is well known that formalin fixation influences results negatively.

PCR should be of greater value in the diagnosis of *M.tuberculosis* on the placenta when fresh tissue is utilised.

Alternatively both RNA and DNA in situ hybridization provide ways to detect the presence and expression of various microbes in tissue sections with a high specificity.(48). Optimized ISH protocols should serve several goals, thus the retention of tissue morphology is highly important. The tissue must be permeable to the probe to produce a desirable result so that the mRNA target must be retained in the tissue. The chosen probe must be able to effectively penetrate and bind and lastly there must be minimal non-specific background staining. All of these conditions must be met for a successful and high quality *in situ* hybridization experiment and results.

The possibility of pursuing these methods was attempted and positive amplification was achieved by amplification of *IS 6110*. Successful purification of these amplicons was achieved as shown in figure 4.22. The probes was labelled with DIG and no positive results was seen when the product membrane was stained with IN SITU HYBRIDIZATION DETECTION SYSTEM CORE KIT II for Digoxin labeled probe/Streptavidin-Alkaline Phosphatase by Biogenix. This kit from Biogenix was specific for the use with FFPE tissue and not membranes. It was decided to use the CSPD ready to use kit as recommended by the manufacturer used to label the probes with DIG, instead of the kit available from Biogenix. No result was encountered when the product membrane was stained with the CSPD, ready to use kit from Roche and the DIG wash and block buffer set from Roche on the X-ray film. Although due care was taken to optimize the exposure of UV light to the X-ray film. It is important that appropriate probes are chosen for the correct assay.

The labeling of the primers with DIG was unsuccessful in this study setting. Limited funding constrained any further optimization and use of different kits available for the application of ISH protocols, as already 3 different kits specific for this methodology had produced unfavorable results. This methodology is a very interesting field that may be explored in further projects and/or by other students. It was decided to exclude the ISH protocol from the study.

## **CONCLUSION**

Invaluable knowledge was obtained in the diagnoses of *M.tuberculosis* in the placenta as it was found that micro abscesses and intervillitis show a strong suggestion of TB infection in the placenta and this must be confirmed with identification of the organism using ZN staining.

PCR has its limits, in that only *M.tuberculosis* DNA can be amplified, but it cannot distinguish latent from active disease and is further inhibited by formalin fixation that causes fragmentation of the *M.tuberculosis* DNA and breakage of the proteins. The conclusion that we reached, is that PCR is of limited value in the diagnosis of *M.tuberculosis* in the placenta using FFPE tissue.

Due to the low occurrence of congenital TB, the value of placental examination for the diagnosis of tuberculosis in relation to congenital TB in the infant could not be accurately determined in this study setting.

We recommend that future project(s) could explore the usage of using fresh placental tissues(s) and newer NAAT such as the Xpert<sup>®</sup> MTB/RIF to diagnose TB.

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## **Appendix A: Ethics approval**



UNIVERSITEIT•STELLENBOSCH•UNIVERSITY  
jou kennisvenoot • your knowledge partner

20 August 2010

**MAILED**

Miss U Rabie  
Anatomical Pathology  
Pathology  
TBH, 10th Floor/ West Wing  
Tygerberg Hospital

Dear Miss Rabie

**The contribution of the placenta to the diagnosis of congenital tuberculosis.**

**ETHICS REFERENCE NO: N10/05/156**

**RE : APPROVAL**

A panel of the Health Research Ethics Committee reviewed this project on 27 May 2010; the above project was approved on condition that further information is submitted.

This information was supplied and the project was finally approved on 19 August 2010 for a period of one year from this date. This project is therefore now registered and you can proceed with the work.

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division: [www.sun.ac.za/rds](http://www.sun.ac.za/rds)) 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 and subjected to an external audit. Translations of the consent document in the languages 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).

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](mailto:healthres@pgwc.gov.za) Tel: +27 21 483 9907) and Dr Hélène Visser at City Health ([Helene.Visser@capetown.gov.za](mailto: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.

Approval Date: 19 August 2010

Expiry Date: 19 August 2011

20 August 2010 14:48

Page 1 of 2



Fakulteit Gesondheidswetenskappe - Faculty of Health Sciences



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## **Appendix B: Haematoxylin and Eosin Stain**

1. Cut 3-5 $\mu$ m sections and mount on a slide using a water bath set at 45-
2. Incubate the slides in an oven set at 65°C for 20 minutes.
3. Dewax sections in Xylene for 5 minutes.
4. Dehydrate sections through graded alcohols to water for 1 min each, thus 100%, 96%, 80% and 60% alcohol each.
5. Stain with Mayers Haematoxylin for 10 minutes to stain the nuclei.
6. Wash in tap water.
7. Blue in Scotts tap water solution for 2 minutes.
8. Wash in tap water.
9. Put sections in 100% alcohol for 1min to prepare for alcoholic Phloxine Eosin.
10. Stain with alcoholic Phloxine Eosin solution for 2 minutes.
11. Dehydrate sections through graded alcohols as in step 4 but this time start at the lowest concentration ending in 100% alcohol.
12. Clear in Xylene.
13. Mount sections with Sakura Tissue TEK automated cover slipper.

### **Expected Results:**

**Nuclei:** Blue-black

**Cytoplasm** and most connective tissue fibers stain in varying shades and, intensities of pink, orange and red.



**Appendix C: Placenta Reporting Template**

Data Set (Checklist)  
Female Genital Tract

**Placenta - Singleton**

Department of Anatomical Pathology  
University of Stellenbosch & NHLS  
November 2010

**MACROSCOPIC:**

Weight: \_\_\_\_\_ g (N= \_\_\_\_\_ g)  
 Dimensions: \_\_\_\_\_ x \_\_\_\_\_ x \_\_\_\_\_ mm  
**MEMBRANES:**  
 Insertion: normal / placenta circumvallate/circummarginate – percentage \_\_\_\_\_ %  
 Meconium: Absent / Present  
**CORD:**  
 Length: \_\_\_\_\_ mm Vessels: 2 / 3 Diameter (average): \_\_\_\_\_ mm  
 Insertion: parenchymal / marginal / velamentous / furcate insertion  
 Obstructive lesions: absent / present : type – knot / torsion / stricture / hypercoiling. No of coils \_\_\_\_\_  
**FOETAL SURFACE OF PLACENTA:**  
 Congested / Thrombosed chorionic vessels: absent / present – artery / Vein / Both / Indeterminate  
**MATERNAL SURFACE OF PLACENTA:**  
 Complete: yes / no  
 Indentations: absent / present - \_\_\_\_\_ %  
 Retro placental blood clot: absent / present - \_\_\_\_\_ %  
**PARENCHYMA**  
 Infarction: recent: absent / marginal / central - \_\_\_\_\_ %  
 old: absent / marginal / central - \_\_\_\_\_ %  
 Other \_\_\_\_\_

Name: \_\_\_\_\_  
 STA \_\_\_\_\_

Coil Cord Index (CCI):  
 No of coils/length in cm  
 ≤0.1: hypocoiling  
 ≥0.3 hypercoiling

**MICROSCOPIC:**

**CORD:**  
 Vasculitis: absent / present – no of vessels: \_\_\_\_\_  
 Funisitis: absent / present – perivascular / necrotizing  
 Thrombosis: absent / present – artery / vein / both  
 Meconium induced vascular necrosis: absent / present – site: cord / chorionic plate  
**MEMBRANES & CHORIONIC PLATE:**  
 Chorion amnionitis: \$absent / present : \* Inflammatory type: Acute / chronic / Eosinophilic  
 \* Location: subchorionic / chorion / chorioamnion  
 \* Intensity: nonconfluent / confluent  
 \* Other: necrotizing / microabscesses / amnion necrosis  
 Meconium-laden macrophages: absent / present - amnion / chorion / deciduas / amnion necrosis  
 Vasculitis vessels chorionic plate: absent / present - artery / vein / both / indeterminate - focal / multifocal  
 Thrombosis chorionic plate/stem vessels: absent / present – focal / multifocal  
 Amnion nodosum: absent / present  
 Decidual vasculopathy: absent / present – muscular hypertrophy / fibrinoid necrosis / atherosclerosis  
 Retromembranous haemorrhage: absent / present - recent / old / hemosiderin

**PARENCHYMA:**

Maturation: appropriate / accelerated / delayed / variable / arrest  
 Distal villous hypoplasia: absent / present  
 Increased syncytial knots: absent / present - regional / diffuse  
 Perivillous fibrin: normal / increased - patchy / geographic / maternal floor infarction / MPFD disease  
 Infarcts: absent / microinfarcts / recent / old / both - regional / diffuse  
 Extravillous fibrinoid / trophoblastic islands: normal / increased  
 Villitis: absent / present - acute / chronic / chronic-nonspecific  
 Extent: patchy / multifocal / diffuse / diffuse with fetal obliterative vasculopathy  
 Intervillitis: absent / present - acute / histiocytic  
 Retroplacental bleeding: absent / present - with overlying infarction / with haemorrhage into parenchyma  
 Intravillous hemorrhage, recent: absent / present  
 Avascular villi: absent / present - regional / diffuse  
 Villous-stromal karyorrhexis ± calcification: absent / present - regional / diffuse  
 Edema villi: absent / present - patchy / diffuse  
 Increased nucleated RBC in fetal vessels: absent / present - mild / moderate / massive  
 Chorangiomas: absent / present - focal / diffuse  
 Other:

**DIAGNOSIS:**  
**PLACENTA:**

**Appendix C2:**

**Department of Anatomical Pathology**  
**Placenta Cluster Diagnosis Expanded Version:**

**A) Utero-placental Insufficiency (UPI)/Maternal vascular underperfusion:**

Criteria:

- Small placenta (below the 10<sup>th</sup> centile for gestational age)
- Extensive infarction – macroscopically: >20% at term, less if premature or other placental insult
- Extensive infarction – microscopically.
- Accelerated maturation
- Distal villous hypoplasia
- Decidual vasculopathy (muscular hypertrophy, fibrinoid necrosis & acute atherosclerosis)
- Increased syncytial knots
- Increase in perivillous fibrin
- Increase in extravillous trophoblast

**Diagnosis:** Minimum of 3 criteria (preferably 4) – UPI

Need a small placenta (below 10<sup>th</sup> centile) with 2 (preferably 3) more features.

If >1 but <3 or if ≥3 but placenta within normal ranges → placental compromise.

If only 1 – isolated finding (e.g. decidual vasculopathy) – other causes)

**B) Placental Insufficiency:**

- Fetal Trombotic vasculopathy / fetal vascular thrombosis
  - Umbilical thrombosis
  - Chorionic plate / stem villous thrombosis
  - Avascular villi (regional in clusters)
  - Villous-stromal karyorrhexis (regional)
- Maternal floor infarction
  - Villi of the entire maternal floor are embedded in fibrin, to a thickness of at least 3mm, evident on at least one slide.
- Massive perivillous fibrin deposition disease
  - Entrapment of at least 50% of the villi as seen on at least 1 slide.
- Diffuse villous oedema
- Maturation arrest
- Massive histiocytic intervillitis

Each entity is a diagnosis on it's own.

**C) Placental Abruptio:**

Criteria need 2 major:

- Clinical diagnosis of abruptio placenta
- Macroscopic abruptio: >15% adherent retroplacental haematoma or indentation
- Microscopic abruptio – need at least 2 minor:
  - Retroplacental &/or retromembranous &/or marginal sinus haemorrhage
  - Dissection into the deciduas and placental parenchyma
  - Overlying infarction
  - Intravillous haemorrhage

#### D) Chorioamnionic Hemosiderosis (chronic/venous abruption)

- Iron deposits in the membranes, chorionic plate or decidua.

#### E) Subacute Fetal Hypoxia with compensatory response:

- Focal villous oedema
- Chorangiomas (focal or diffuse)
- Increase in fetal nucleated red blood cells

#### F) Ascending Infection - chorioamnionitis:

- Criteria: clusters of at least 5 neutrophils in the subchorionic layer.
- Response types:
  - Maternal: subchorion, chorion and chorioamnion ± amnion necrosis ± subchorionic microabscess formation ± demonstrable organism.
  - Fetal: Chorionic plate vasculitis, umbilical cord vasculitis and funisitis.
- Can have an associated: minor component of intervillitis and chorionic plate, stem villous or umbilical cord vascular thrombosis.
- Intensity:
  - Confluent - continuous inflammatory infiltrate over an entire chorionic plate on a slide.
  - Non-confluent – noncontinuous inflammatory infiltrate.

#### G) Haematogenous infection:

- Types:
  - Acute Villitis – neutrophils in the villi (major pattern) ± overflow into the intervillous space. Prominent numbers of neutrophils in the villous vessels.
  - Chronic villitis – lymphocytes, histiocytes &/or plasma cells in the villi ± villous lysis ± overflow into the intervillous space. Characterized by focal areas of inflammation with mononuclear cells and areas of fibrinoid necrosis in chorionic villi.
  - Acute intervillitis – neutrophils in the intervillous space ± secondary overflow into the villi.
  - Intervillous micro abscesses are defined: when the acute intervillous inflammatory aggregate (intervillitis) starts to push apart neighbouring villi.

#### H) Immunological damage:

- Types:
  - a. Chronic villitis of unknown aetiology
  - b. Massive histiocytic intervillitis

#### I) Within normal limits / no pathological diagnosis

#### J) Small placenta, nos

## **Appendix D: Guidelines for requesting placental histopathology**

### **INDICATIONS FOR PLACENTAL HISTOLOGY**

Placental histology must be requested in all the following cases of singleton and multiple pregnancies.

1. All unexplained stillbirths 24+ weeks or  $\geq 0.500$  kg.

This excludes cases of abruptio placentae and cord prolapse. In cases of uncertainty, the placenta must be kept with the body for Prof de Jong, Drs Mike Urban, Heidi Bezuidenhout or Emma Krizinski opinion.

2. Indications of asphyxia in a viable baby. This group consists of all neonates who required resuscitation, unless clearly due to abruptio placentae or cord prolapse.

3. Second or higher order midtrimester loss.

4. Idiopathic preterm labour (gestational age < 34 weeks or birth weight < 1800g).

5. Suspected clinical chorioamnionitis.

6. Cases of severe intrauterine growth restriction ***without*** antenatal work-up (Doppler and ultrasound).

7. Multiple pregnancies:

All applicable indications that would be relevant in singleton pregnancies.

All multiple pregnancies with uncertain chorionicity at time of birth.

8. Congenital abnormalities without prior diagnosis (unless otherwise requested by Prof G de Jong, Prof L Geerts, Dr's M Urban, H Bezuidenhout or E Krizinski).

9. Cases of severe pre-eclampsia if requested by Special Care Unit.

### **HOW TO ARRANGE FOR PLACENTAL HISTOLOGY**

Submit placenta in 10% buffered formalin in plastic container sufficiently large so not to distort placenta.

Provide essential clinical information if available and/or relevant:

Gestational age

Live baby or stillbirth

Retroviral, syphilis serology

Recurrent loss

Maternal disease

## **Appendix E: Zhiel Neelsen stain for acid fast bacilli**

1. Cut 3-5 $\mu$ m sections and mount on a slide using a waterbath set at 45-55°C. Pick up the slide on positive charged slides or slides coated with Poly-L-Lysine.
2. Incubate the slides in an oven set at 65°C for 20 minutes.
3. Dewax sections in Xylene for 5 minutes.
4. Dehydrate sections through graded alcohols to water for 1 min each, thus 100%, 96%, 80% and 60% alcohol each.
5. Wash sections in distilled water.
6. Flood the slide with Carbol Fuchsin and heat until steaming. Do not let the solution boil. Allow the sections to cool for 5 minutes and repeat the flaming process.
7. Wash the section in tap water.
8. Decolourise in 1% acid alcohol for 30 seconds.
9. Rinse in tap water.
10. Decolourise in 10% sulphuric acid for 3 minutes.
11. Rinse in tap water.
12. Wash the sections in running tap water for 5 minutes.
13. Counterstain the sections with 0.25% methylene blue for 10-20 minutes until the sections appear blue.
14. Rinse in tap water.
15. Dehydrate sections through graded alcohols as in step 4 but this time start at the lowest concentration ending in 100% alcohol.
16. Clear in Xylene.
17. Mount sections with Sakura Tissue TEK automated cover slipper.

### **Expected Results:**

**Acid fast bacilli:** Red

**Tissue elements:** Shades of Blue

**Appendix F: QIAGEN® QIAamp® DNA FFPE Tissue Handbook. For purification of genomic DNA from formalin-fixed, paraffin-embedded tissue**  
Protocol:

Important points before starting:

- Perform all centrifugation steps at room temperature (15-25°C)

Things to do before starting:

- Equilibrate all buffers to room temperature (15-25°C)
- Set a thermomixer or heated orbital incubator to 56°C for use in step 11.
- If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation.
- Ensure that Buffer AW1 and AW2 have been prepared according to instructions.

PROCEDURE:

1. Using a scalpel, trim excess paraffin wax off the sample block.
2. Cut up to 8 sections 5-10µm thick. Discard the first 2-3 sections. (Remember to follow strict conditions to avoid cross contamination between samples.)
3. Place the sections in a 1.5 or 2ml microcentrifuge tube and add 1ml Xylene to the sample. Close the lid and vortex vigorously for 10 seconds.
4. Centrifuge at full speed for 2 min.
5. Remove the supernatant by pipetting; do not remove any of the pellets.
6. Add 1ml ethanol (96-100%) to the pellet, and mix by vortexing. The ethanol extracts any residual xylene from the sample.
7. Centrifuge at full speed for 2 minutes.
8. Remove the supernatant by pipetting. Do not remove any of the pellet. Carefully remove any residual ethanol using a fine pipet tip.

9. Open the tube and incubate at room temperature (15-25°C) or up to 37°C. Incubate for 10 minutes or until all residual ethanol has evaporated from the sample.
10. Resuspend the pellet in 180µl Buffer ATL. Add 20µl proteinase K, and mix by vortexing.
11. Incubate at 56°C for 1h (or until the sample has been completely lysed).
12. Incubate at 90°C for 1h. The incubation at this temperature in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 90°C
13. Briefly centrifuge the 1.5ml tube to remove drops from the inside of the lid.
14. Add 200µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200µl ethanol (96-100%), and mix again thoroughly by vortexing. It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the QIAamp procedure.
15. Briefly centrifuge the tube to remove drops from the inside of the lid.
16. Carefully transfer the entire lysate to the QIAamp MinElute column (in a 2ml collection tube) without wetting the rim, close the lid and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp MinElute column in a clean 2ml collection tube, and discard the collection tube containing the flow-through.
17. Carefully open the QIAamp MinElute column and add 500µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp MinElute column in a clean 2ml collection tube, and discard the collection tube containing the flow-through.
18. Carefully open the QIAamp MinElute column and add 500µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp MinElute column in a clean 2ml collection tube, and discard the collection

tube containing the flow-through. Contact between the QIAamp MinElute column and the flow-through should be avoided at all times.

19. Centrifuge at full speed (20 000 x g; 14 000 rpm) for 3 minutes to dry the membrane completely. This step is very necessary, since ethanol carryover into the elute may interfere with some downstream applications.
20. Place the QIAamp MinElute column in a clean 1.5ml microcentrifuge tube, and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 20-100µl Buffer ATE to the centre of the membrane. (For the purpose of our Experiment 50µl Buffer ATE was added.)
21. Close the lid and incubate at room temperature for 5 minute. Centrifuge at full speed (20 000 x g; 14 000 rpm) for 1 minute. Incubating the elute in Buffer ATE for 5 minutes before centrifugation generally increases DNA yield.



## **Appendix G: Wizard SV gel and PCR Purification system**

### **Processing PCR amplification products:**

1. Amplify target of choice using standard amplifications conditions.
2. Add an equal volume of Membrane Binding Solution to the PCR amplification.
3. Proceed with the DNA Purification by centrifuge method.
4. Place one SV Minicolumn in a collection tube for each PCR amplification.
5. Transfer the prepared PCR product to the SV Minicolumn assembly and incubate for 1 minute at room temperature.
6. Centrifuge the SV minicolumn assembly in a microcentrifuge at 16 000 x g (14 000 rpm) for 1 minutes. Remove the SV minicolumn from the spin column assembly and discard the liquid in the collection tube. Return the SV minicolumn to the collection tube.
7. Wash the column by adding 700µl of membrane wash solution, previously diluted with 95% ethanol, to the minicolumn. Centrifuge the SV minicolumn assembly for 1 minute at 16 000 x g (14 000 rpm). Empty the collection tube as before and place the SV minicolumn back in the collection tube. Repeat the wash with 500µl of membrane wash solution and centrifuge for 5 minutes.
8. Remove the SV minicolumn assembly from the centrifuge, being careful not the wet the bottom of the column with the flowthrough. Empty the collection tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol.
9. Carefully transfer the SV minicolumn to a clean 1.5ml microcentrifuge tube. Apply 50µl of Nuclease-Free Water directly to the centre of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 16 000 x g (14 000 rpm).
10. Discard the SV minicolumn and store the microcentrifuge tube containing the eluted DNA at 4° or -20°C

**Appendix H: Quantification of purified DNA**

DNA quantified of purified IS6110 PCR amplicons using Nanodrop 1000(Thermo Scientific, USA)

Module: Nucleic Acid

Path: 10mm

Software: 3.7.1

Firmware: USB2000 2.41.3 ND2

**Table 4.15: Quantification of DNA from purified PCR products**

Sample ID	User ID	Date	Time	ng\ul	A260	A280	260\280	260\230
CLEAN	DEFAULT	14-03-13	07:35	0	0	0	NaN	NaN
UR IS6110-1	DEFAULT	14-03-13	07:36	260.6	5.212	2.726	1.91	2.22
UR IS6110-2	DEFAULT	14-03-13	07:37	259.5	5.19	2.759	1.88	2.15
UR IS 6110-3	DEFAULT	14-03-13	07:38	255.73	5.115	2.671	1.91	2.2
UR IS 6110-4	DEFAULT	14-03-13	07:39	232.31	4.646	2.429	1.91	2.21
UR IS 6110-5	DEFAULT	14-03-13	07:40	237.15	4.743	2.463	1.93	2.14