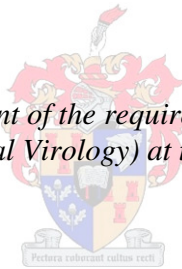# Profiling the approach to the investigation of viral infections in cases of Sudden Unexpected Death in Infancy (SUDI) in the Western Cape Province

by
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Thesis presented in fulfilment of the requirements for the degree Master of Medical Sciences (Medical Virology) at the University of Stellenbosch

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 01 December 2010

Abstract

Sudden Unexpected Death in Infancy (SUDI) refers to any such sudden demise in a child. If the child dies while asleep within the first year of life, and if no conclusive cause of death can be ascertained by means of complete autopsy and investigation into the circumstances surrounding death, including visit of the death scene, such a case is classified as one of Sudden Infant Death Syndrome (SIDS). By South African law, a full medico-legal autopsy is mandated in cases where the cause of death is not evident – including cases of possible SIDS.

There can be little doubt that viral infection can be a cause of death in cases of supposed SUDI. At the Tygerberg medico-legal (forensic) laboratory, the evaluation of lung tissue for the presence of fatal viral lung infections forms part of the institutional protocol for the examination of SUDI cases. Lung samples of these SUDI cases are routinely tested for the presence of Cytomegalovirus (CMV), adenovirus and respiratory syncytial virus (RSV) by means of shell vial cultures. In a retrospective pilot study of 366 SUDI case files from Tygerberg Hospital, Western Cape, from 2004 – 2006, it was evident that in only 13.9% of possible SIDS cases, positive results for one or more of the aforementioned viruses were obtained.

We hypothesise that the current method of virus detection, together with other factors such as the interval between death and post mortem examination, transport time of the specimens to the laboratory etc. might not be optimal to give a realistic picture of death in infancy caused by viral pulmonary infection. As other test modalities exist for the diagnosis of pulmonary viral infections, these methods were compared in terms of positive yield and association with viral pneumonitis, keeping the cost and time needed for each assay in mind.

A total of 82 samples were collected over an 8 month period and routine shell vial cultures were done, followed by real-time Polymerase Chain Reaction (PCR) and immunohistochemical (IHC) staining of the lung sections with consensus pathology opinion. As expected, the real-time PCR method was much more better suited for identifying positive samples than shell vials (35% vs. 3.7% respectively). IHC staining also aided the pathologist in diagnosing viral infections microscopically. We expect the findings to be instrumental in streamlining not only our institutional SIDS

investigation protocol, but also the development of a standardised national SIDS investigation protocol.

Opsomming

"Sudden Unexpected Death in Infancy" (SUDI) verwys na enige skielike sterfte van 'n kind. Indien die kind sterf tydens sy/haar slaap periode en geen oortuigende oorsaak van dood bepaal kan word deur middel van 'n volledige nadoodse ondersoek en ondersoek na die omstandighede tydens die dood, insluitend 'n besoek aan die doodstoneel nie, word so 'n geval as Wiegiedood (SIDS) geklassifiseer. Suid-Afrikaanse wetgewing vereis 'n volledige medies-geregtelike nadoodse ondersoek in gevalle waar die oorsaak van dood onbekend is – insluitend gevalle van moontlike Wiegiedood.

Daar is min twyfel dat virusinfeksie 'n oorsaak van, of bydraende faktor tot dood kan wees in gevalle van moontlike SUDI. By die Tygerberg forensiese laboratorium vorm die evaluasie van long weefsel vir die teenwoordigheid van dodelike virusinfeksies deel van die institusionele protokol vir die ondersoek van SUDI gevalle. Long monsters van hierdie SUDI gevalle ondergaan roetine toetse vir die teenwoordigheid van sitomegaalvirus, respiratoriese sinsitialevirus en adenovirus deur middel van selkulture ("shell vial cultures"). In 'n retrospektiewe steekproef van 366 SUDI gevalle by Tygerberg Hospitaal, Wes-Kaap van 2004 – 2006, is bevind dat in slegs 13.9% van moontlike SUDI gevalle die teenwoordigheid van een of meer van bogenoemde virusse bevestig kon word. Ons hipotese is dat hierdie metode van virus deteksie, tesame met ander faktore soos die tydsinterval tussen dood en nadoodse ondersoek, tyd om monsters na die laboratorium te vervoer ens. moontlik nie optimaal is om 'n realistiese beeld van dood in babas as gevolg van pulmonale virusinfeksie te gee nie. Aangesien ander toets modaliteite bestaan vir die diagnose van pulmonale virusinfeksies, is hierdie metodes vergelyk in terme van positiewe opbrengs en assosiasie met virale pneumonitis, teen 'n agtergrond van die koste en tyd benodig per toets.

'n Totaal van 82 monsters is oor 'n 8 maande periode versamel en roetine selkulture is gedoen, gevolg deur "real-time" Polimerase Ketting Reaksie (PKR), asook immunohistochemiese (IHC) kleuring van long snitte met patologiese verslae. Soos vermoed, is gevind dat die real-time PKR metode baie meer akkuraat is om positiewe monsters te identifiseer as roetine selkulture (35% vs 3.7% onderskeidelik). IHC

kleuring het ook mikroskopiese diagnose van virale infeksies deur die patoloog vergemaklik. Ons verwag dat hierdie bevindinge grootliks kan bydra in die vaartbelyning van ons institusionele SIDS ondersoek protokol, asook in die ontwikkeling van 'n gestandaardiseerde nasionale SIDS ondersoek protokol.

A scientist in his laboratory is not a mere technician: he is also a child confronting

natural phenomena that impress him as though they were fairytales.

Marie Curie (1867 – 1934)

Acknowledgements

List of Abbreviations

ATV	Active Trypsin Versine
Bp	Base pairs
CMV	Cytomegalovirus
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
EM	Electron Microscopy
EQA	External Quality Assessment
FCS	Foetal Calf Serum
FFPE	Formalin-fixed, paraffin-embedded
HF	Human Fibroblast
HIER	Heat-induced Epitope Retrieval
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IFA	Immunofluorescence Assay
IHC	Immunohistochemistry

KSHV	Kaposi's Sarcoma Associated Herpesvirus
LLL	Left Lower Lobe
MDCK	Madin-Darby Canine Kidney
Mt-DNA	Mitochondrial Deoxyribonucleic acid
NAAT	Nucleic Acid Amplification Test
NCBI Information	National Centre for Biotechnology
NHLS	National Health Laboratory Service
NTC	Non-template control
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMTCT Transmission	Prevention of Mother-to-Child
QCMD Diagnostics	Quality Control for Molecular
Real-time PCR	Real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
Rpm	Revolutions per minute

RSV	Respiratory Syncytial Virus
SANAS	South African National Accreditation System
SIDS	Sudden Infant Death Syndrome
SUDI	Sudden Unexpected Death in Infancy
TEM	Transmission Electron Microscopy
VTM	Viral Transport Media
VZV	Varicella Zoster Virus

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Chapter One

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Sudden Unexpected Death in Infancy (SUDI) is the collective term for cases where an infant, younger than one year, dies suddenly and unexpectedly (Weber *et al*., 2008). Similarly, *Sudden Infant Death Syndrome (SIDS)* (also termed "cot death") is defined as the sudden, unexplained death of an infant younger than one year old (Bajanowski *et al*., 2007; Krous *et al.*, 2004), where no other cause of death can be confirmed. SIDS is essentially the unexplained death of a clinically healthy infant, where the cause could not be identified by forensic autopsy and investigation (including an investigation into the scene and circumstances surrounding the death). Therefore, SIDS is a diagnosis of exclusion after elimination of other causes and the validity is dependent upon the accuracy and completeness of the investigations (therefore, by implication a non-diagnosis). In industrialised countries so-called SIDS is the leading cause of death in children between one month and one year of age (Byard & Marshall, 2007).

Autopsies are performed on all SUDI cases and if post-mortem examination and special laboratory investigations still cannot determine a cause of death, the case is regarded as SIDS. It is therefore of utmost importance to note the clear distinction between SUDI and SIDS, even though the terms are used interchangeably and quite often still confused (Figure 1.1). For the purposes of this study the terms SUDI and SIDS will be used as per definition stated above, unless counterintuitive usage of the term is entrenched in the body of research. In these cases, the usage of the term will be explained in the text.
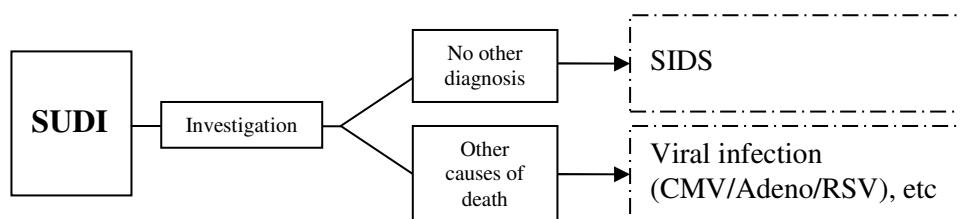
Figure 1.1:   The difference between SUDI and SIDS

Viral infection is considered a possible cause or contributory cause of death in cases of SUDI since an infection that is normally asymptomatic in a healthy adult can cause severe morbidity in an infant. No standardised infant death protocol exists in South Africa, but at the Tygerberg Medico-legal Laboratory in the Western Cape Province all SUDI cases are routinely investigated for the presence of three different viruses, i.e. cytomegalovirus (CMV), adenovirus and respiratory syncytial virus (RSV). The routine diagnostic method for these viruses is the South African National Accreditation Services (SANAS) accredited shell vial culture method. However, a retrospective audit of SUDI cases over a period of three years (2004 – 2006) revealed that in only 13.9% of these cases one of these viruses could be identified with the shell vial method. Since no other cause of death was found in the other 86.1%, these negative cases were classified as SIDS. We therefore hypothesise that more sensitive methods, such as immunohistochemistry (IHC) or real-time Polymerase Chain Reaction (PCR), will be better suited than shell vial cultures to detect viral infections.

1.2 Literature Review

An overview of the history of SIDS (including SUDI) is presented in this section, including possible causes, viruses that might be implicated, as well as different methods of viral detection.

1.2.1 History of SIDS[1]

The history of SIDS can be traced back to Biblical times where infant suffocation was mentioned in 1 Kings 3 in the Old Testament and this was linked by Pope Stephen V, who died in 891 AD, to babies sleeping with their mothers at night (Högberg & Bergström, 2000). This is currently known as bed-sharing or co-sleeping. A Pubmed search (NCBI database) with keywords "SIDS", "SUDI", "cot death" and "unexpected infant death" also revealed articles from as early as 1918 with SIDS as main concern.

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[1] Usage of the term SIDS or cot death in ancient times also includes the entity of SUDI, as formal investigation into these cases is a relatively recent phenomenon

1.2.2 Possible causes of SIDS

1.2.2.1 Genetic Factors

SIDS might have a genetic link. Extensive research had been done to investigate whether the infants who die suddenly and without apparent cause, might be genetically predisposed to this syndrome. A three-hit model (also known as the triple-risk hypothesis) has been proposed specifically for SIDS cases (Opdal & Rognum, 2004) and is presented as Figure 1.2. The model includes:

- The infant is at a vulnerable stage in its development (i.e. younger than 1 year)

- Endogenous factors that can contribute to SIDS are present (e.g. genetic factors)

- The process is triggered by an exogenous factor (e.g. viral infection etc.)
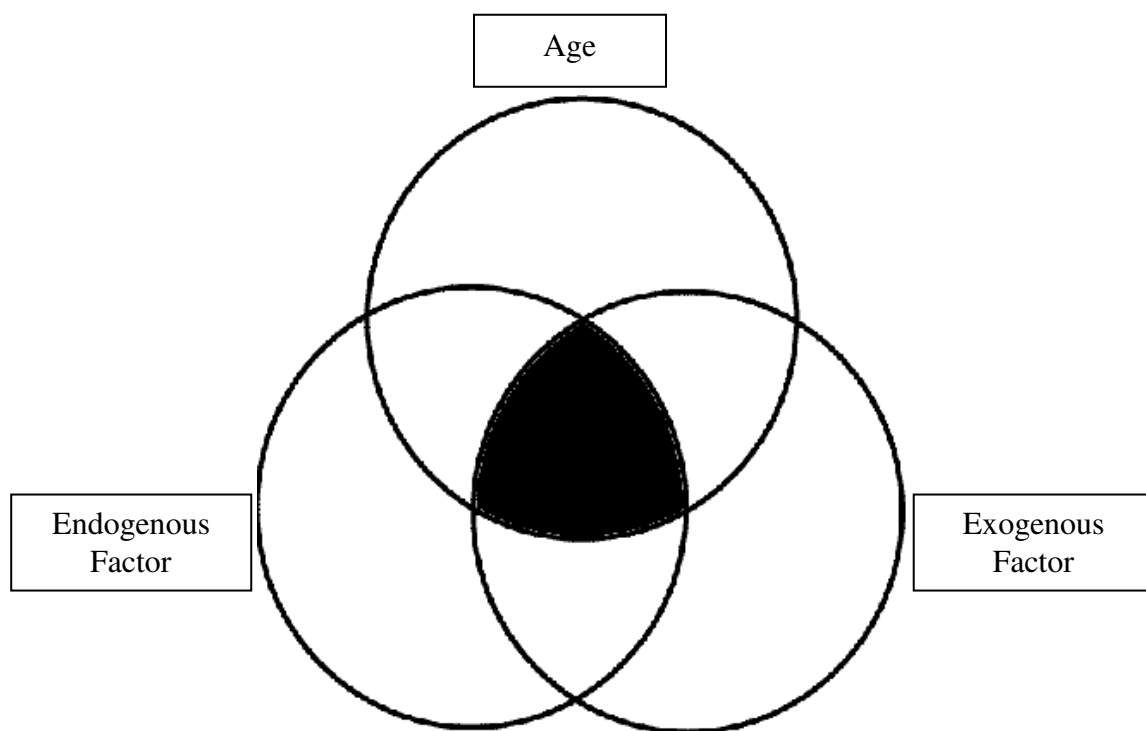
Figure 1.2: **The proposed three-hit or triple risk model for SIDS.** Infants that succumb to SIDS would fall in the central overlapping area.

The genetic link to SIDS has been divided into two groups. It can either be a mutation that leads to the death of the child or a polymorphism that gives the child a disadvantage when confronted with a lethal situation (Opdal & Rognum, 2004), such as a viral infection. In the latter scenario it would mean that the genetic mutation

together with the presence of a lethal situation would lead to the death of the child. Should the child also be younger than one year of age, the three-hit model theory would be applicable.

Genetic alterations in the genome can include fatty acid metabolism, glucose metabolism, long QT syndrome and thrombosis. Gene polymorphisms, including HLD-DR, the serotonin transporter gene, C4, IL-10, mitochondrial-Deoxyribonucleic acid (mt-DNA) and thermal regulation, can also lead to death under certain circumstances (Opdal & Rognum, 2004). Further research in this field is vital to understand the connection between genetic make-up and SIDS.

1.2.2.2   SIDS and Bacteria

It is possible that a bacterial infection might cause the release of toxins that can cause death in an infant, such as those of *Clostridium botulinum* which can lead to respiratory paralysis. Other bacteria have been associated with SIDS cases, such as *Escherichia coli*, *Staphylococcus aureus* and *Clostridium perfringens* (Highet, 2008; Morris, 1999). Toxins excreted by *S.aureus* and *E.coli* can interact with each other and these toxins have been proven to be lethal in gnotobiotic weanling rat models (Morris, 1999).

Whether smoking by one or both parents of an infant contributes to the possibility of SIDS is still a controversial issue. Some scientists believe smoking increases susceptibility to SIDS (Mitchell, 2009; Sayers *et al*., 1995; Scragg *et al*., 1993) and others believe there is no correlation (Farooqi *et al*., 1994). However, the combination of bacterial toxins together with low doses of nicotine proved lethal in chick embryo models (Morris 1999; Sayers *et al.,* 1995) and even though animal models and humans cannot always be readily compared, this is surely cause for concern of whether the combination could also be harmful in human infants.

1.2.2.3 Seasonal Influences

Sudden infant death, across the world, seems to be more prevalent in cooler climates or winter months (Highet, 2008). Even in a country such as Hawaii, where the average temperature between winter and summer months is not significantly different; this has been evident (Mage, 2004). However, a study by Leach *et al*. (1999) showed more

SIDS cases being reported in spring. Since the geographic areas, and thus the climates, of different studies differ, this finding is not seen as unusual.

Sweden has one of the lowest incidences of SIDS worldwide; one death in every 1000 live births in the 1970s – 1980s and even less in the 1990s (Högberg & Bergström, 2000). This country doesn't experience the change in SIDS rates between their winter and summer months. A possible explanation for this can be the national maternity leave programme used in Sweden. This programme allows mothers to stay home and look after their newborn babies for a year after birth (Samuals, 2003). This decrease in SIDS cases might be because mothers can then directly monitor their babies themselves compared to caregivers who have multiple babies and children to look after.

1.2.2.4   Other factors contributing to SIDS

Maternal alcohol abuse during the time of conception and throughout pregnancy is seen as a major risk factor for sudden death in infants (Iyasu *et al.,* 2002). Not only does it lower the unborn infant's ability to fight infections after birth, but it has also been suggested to suppress and alter serotonergic receptor binding in areas of the brain related to cardiorespiratory control. Nicotine, a toxin found in cigarette smoke, also binds to specific receptors in the brain and alters their normal functioning (Duncan *et al*., 2008). All these factors might also play a part in infant death.

The inhalation of cigarette smoke may increase the risk of SIDS. Tobacco smoke contains a number of toxins which impairs the functioning of the pulmonary system which can lead to respiratory infections. Smoke has also been proven to diminish the capability of the killing of bacteria in the lungs of mice. One of these harmful toxins found in smoke is acrolein. This toxic substance causes dyspnoea in experimental animals and decreases the capability of the lungs to act against infections (Astry & Jakab, 1983). According to Samuals (2003), the smoking by one of the infant's parents can increase the risk of SIDS by more than 60%. Multiple risk factors are shared by susceptibility to SIDS and respiratory infections; these include smoking, socio-economic circumstances, gender, birth-weight and prematurity, as well as ethnic origin (Blackwell *et al*., 2004; Samuals, 2003).

Cytokines can also play a role in SIDS. Raza and Blackwell (1999) proposed that certain cytokines associated with infection, such as interleukin 1, interferon and tumour necrosis factor K, can cause somnolence (a severe form of drowsiness) and hypoxia in extreme cases, which can ultimately lead to death. Both these conditions have previously been associated with sudden death in infants (Raza & Blackwell, 1999).

1.2.2.5   SIDS and Viral Infection

Respiratory viruses are important factors in SIDS and are often found in the upper and lower respiratory tract in the autopsies of such cases (Bajanowski *et al*. 2007). A viral infection can trigger a genetic polymorphism that causes death or can interact with bacterial toxins to have a devastating effect (Highet, 2008). Different viral respiratory infections have been implicated as possible triggers for SIDS, with RSV, influenza B, parainfluenza 2, rhinovirus, CMV and adenovirus being the most prevalent (Highet, 2008; Samuals, 2003). Viral infections might not always directly cause mortality in this population group; however, the mere presence could aggravate existing pathology and contribute to death.

The fact that the incidence of SIDS is significantly higher in winter months and cooler climates further strengthens the hypothesis that viruses are possible causative agents, due to their higher prevalence in cooler climates (Highet, 2008; Mage, 2004).

Both Deoxyribonucleic acid (DNA) and Ribonucleic Acid (RNA) viruses have been implicated in the unexpected deaths of infants, including human herpesvirus (HHV)-1 and 2 or herpes simplex svirus (HSV), HHV-3 or varicella zoster virus (VZV), HHV-4 or Epstein-Barr virus (EBV), HHV-5 or CMV, other HHVs (e.g. HHV-6), RSV, enterovirus, influenza and parainfluenza viruses and adenovirus (Álvarez-Lafuente *et al*., 2008; Bajanowski *et al*., 2003; Dettmeyer *et al*., 2008; Fernández-Rodríguez *et al*., 2006 Raza & Blackwell, 1999; Quan *et al*., 2000; Weber, 2010).

Although it is not specifically mentioned to be implicated in SUDI, it can well be suspected that Human Immunodeficiency Virus (HIV) also plays a role in infant death. HIV is a retrovirus that is integrated into the human genome and being HIV positive can increase one's chances of getting secondary viral infections since this virus causes a deficiency in the human immune system. There is, however, no

published research to prove or disprove this. All infants born to HIV infected mothers and who are included in the Prevention of Mother-to-Child Transmission (PMTCT) programme receive HIV prophylaxis and at 6 weeks, an HIV test is performed (South African National AIDS Council, 2010). Should a baby be HIV positive and dies unexpectedly, this leads to an important question of whether this infant should be regarded as a SUDI case, since HIV infection could very likely be the cause of death. The impact of viral infection, including HIV infection, in SUDI therefore warrants further investigation.

The routine protocol for virus detection in SUDI cases at Tygerberg Hospital in the Western Cape Province, South Africa, is the shell vial culture method for CMV, adenovirus and RSV. A positive result on any of these tests will not necessarily conclude that the specific viral infection was the cause of death (Bajanowski *et al.*, 2007), but in such cases the viral infection could possibly be a contributing factor to death. A positive result on shell vials also has to be correlated with the macroscopic autopsy findings and H&E staining before confirming it as official cause of death.

A discussion of the viruses implicated in SUDI with an in-depth discussion of CMV, adenovirus and RSV follows:

1.2.2.5.1 HHVs

HSV, VZV, EBV, CMV are all HHVs belonging to the *Herpesviridae* family in the order *Herpesvirales* which have been mentioned to possibly being involved in SIDS (Forsgren & Klapper, 2009).

These viruses all have latent periods in their lifecycles and it is therefore important to understand the concept of latency as well as the reactivation of a latent virus in comparison to an acute or chronic infection:

- Latency refers to the ability of a virus to remain dormant in the host cell. This is typically referred to as the lysogenic stage. HHVs infect their host and establish and maintain a latent infection, which will be present for the remainder of the individual's life (Forsgren & Klapper, 2009). During this period, only a small number of genes are expressed and even though replication can still occur at low levels, the expression of certain proteins (such

as the immediate-early/α-proteins in HSV) is absent. During this phase, no virions can be detected and no viral antigen is expressed in the infected cell. Since no antigen is present, the immune system of the host cannot recognise infection and therefore cannot respond to the infection (Forsgren & Klapper, 2009).

- Reactivation refers to periods where viral proteins are expressed and where the virus replicates. This is also referred to as the lytic stage. During this time the host can infect other individuals. Since HHVs cause lifelong infection, reactivation can occur frequently after the primary infection. Triggers for reactivation of a latent virus can include a number of stimuli, including stress, sunlight as well as hormone imbalances (Forsgren & Klapper, 2009).

- An acute infection has a rapid onset with a short duration. Since viruses with a latent cycle present a lifelong infection, an acute infection should not be confused with a latent viral infection.

- A chronic infection refers to an infection with a slow onset and long-lasting disease. In this type of infection, infectious particles will be present throughout the duration of infection.

HSV 1 and 2, also known as HHV-1 and 2, are viruses commonly associated with oro-facial herpes and genital herpes and primary infection thereof is mostly through close contact with someone shedding the virus (HSV-1) or through sexual activity (HSV-2). HSV-2 infection of infants is rare, but not unheard of. Most infants are protected by maternal antibodies against HSV, but should primary infection of infants occur, it can easily be overlooked and symptoms can be thought to be due to 'teething'. Although most infections are mild or asymptomatic, recurring HSV infection may cause *herpes labialis/*fever blisters or recurring genital vesicles. Severe HSV disease is however seen in infants with congenital deficiencies in cell mediated immunity (Forsgren & Klapper, 2009). Since normally HSV infection is easy to misdiagnose in infants, its possible role in SUDI should be investigated.

'Chicken pox' or varicella and 'shingles' or herpes zoster are both well-known diseases caused by VZV infection with chicken pox being from a primary infection

and shingles from reactivation of the virus. VZV is classified as a common childhood disease and in most cases, treatment is not necessary. However, complications can arise and specifically infants are at high risk if the mother is infected with VZV one week prior to or after giving birth, the infant is born prematurely or weighs less than 1 kg at birth with no maternal antibodies. Infants are often protected by maternal antibodies, which leave them vulnerable to future infection in life, but then infection is usually mild and non-life threatening (Breuer, 2009).

EBV is a member of the subfamily *Gammaherpesvirinae* and is associated with many different diseases and tumours. In developing countries, EBV has infected 90% of children by the age of two years. Although most often seroconversion occurs without any symptoms, a primary infection with EBV later in life results in infectious mononucleosis, a disease which is common in adolescents (Haque & Crawford, 2009). EBV has been indentified in different SUDI cases (Álvarez-Lafuente *et al*.2008).

Other HHVs, such as HHV-6, has been suggested as possibly being involved in SUDI (Álvarez-Lafuente *et al*.2008). HHV-6 and 7, also known as roseoloviruses, are members of the subfamily *Betaherpesvirinae.* HHV-6 infections typically cause a rash on the neck and trunk but some cases can present only with a fever, without any other symptoms (Ward & Clark, 2009).

Some of these HHVs (e.g. EBV, HHV-6), have also been detected in SUDI cases with mild myocardial inflammatory infiltrates (Krous *et al*., 2009), leading to the question of what role myocarditis play in SIDS cases.

1.2.2.5.2   Enterovirus

There are a wide variety of enteroviruses, with more than 60 different serotypes and many potential new serotypes. Although these members of the *Picornaviridae* family mostly reside in the gastrointestinal tract it can cause severe illness by spreading to other organs (Minor & Muir, 2009).

Myocarditis has been accepted as a well-known cause of death in cases originally thought to be either unexplained or attributed to SIDS (Dettmeyer *et al*., 2004; Weber *et al*., 2010) and it has been suggested that viral myocarditis is more prevalent in cases of SIDS that is generally accepted (Bajanowski *et al*., 2003). A major threat for

neonates is specifically enteroviral myocarditis, because this can lead to death within two to five days after infection. Coxsackie B virus is the most common enteroviral cause of myocarditis (Minor & Muir, 2009).

The question of how big a part enteroviruses and specifically enteroviral myocarditis play in SIDS remains to be answered.

1.2.2.5.3 Influenza

Influenza is well-known due to the yearly epidemics and occasional pandemics it causes. There are three influenza viruses namely A, B and C and these fall under the genus *Influenzavirus* in the *Orthomyxoviridae* family. Influenza A is primarily viruses of avian species whilst B and C are human viruses (Zambon & Potter, 2009).

Most societies experience a yearly influenza epidemic, generally at times of high humidity, which causes severe respiratory illness. It is this frequent outbreak of the virus that raises questions about its possible involvement in SUDI especially since cases diagnosed as SIDS have been found to have interstitial pneumonia due to influenza infection (Bajanowski *et al*., 2003) raising questions about what was the true cause of death.

1.2.2.5.4 Parainfluenza

The parainfluenza viruses are members of the *Paramyxoviridae* family and the *Paramyxovirinae* subfamily. There are four serotypes of parainfluenza and infection causes both upper and lower respiratory tract infections with complications such as pneumonia and bronchiolitis especially in children. Especially parainfluenza type 3, which has infected nearly 50% of infants in the first year of life, has been proved to cause bronchiolotis in infants and to be the most common cause of the hospitalisation of infants due to respiratory infection (Parrott *et al*., 1962; Psarras *et al*., 2009).

Even though parainfluenza infection is not as frequently included in SUDI research as other viruses, such as CMV and RSV, its involvement should still be considered in theses cases, especially since it can cause severe illness in infants.

1.2.2.5.5   CMV

Globally, CMV is regarded to be one of the most frequent congenital infections. It is also regarded as one of the most successful human parasites and one of the most commonly encountered viral pathogens in newborn infants (Griffiths 2009; Schleiss, 2006). CMV is a double stranded DNA virus, belonging to the *Herpesviridae* family and is also known as HHV-5 (Mans *et al*., 2009) which, like its family members, causes lifelong infection (Cannon *et al*., 2010).

Close contact is the most common mode of transmission for CMV and it can easily be spread from child to child, child to parent, or parent to child. CMV can also be transmitted *in utero* by a mother to her unborn child, leading to congenital CMV in the baby (Luck & Sharland, 2009). It can also be transmitted perinatally (during delivery) or postnatally (via breastfeeding) (Griffiths, 2009; Luck & Sharland, 2009; Schleiss, 2006). It has been estimated that between 40% and 100% of teenagers will already be infected with CMV by the age of 16 (Griffiths, 2009; Cannon *et al*., 2010).

CMV is regarded as a relatively common virus with low morbidity, because it remains asymptomatic in a large proportion of the general population (Luck & Sharland, 2009; Cannon *et al*., 2010). Immunocompromised individuals however, such as organ transplant recipients or patients living with HIV/AIDS, can become severely ill from a CMV infection. Premature infants and neonates with low birth weight are also regarded as being immunocompromised (Luck & Sharland, 2009), because of their underdeveloped immune system, and CMV can cause a variety of complications in these infants, leading to severe morbidity (Hsu *et al*., 2001) and even mortality. CMV is furthermore a risk factor for pregnant women, since this can lead to congenital CMV of the infant. Peri- and postnatal infections of CMV are usually non-life-threatening mild infections, but CMV is commonly detected in perinatally infected infants who develop pneumonitis in the first three months of life. (Griffiths, 2009).

CMV has been mentioned multiple times to possibly being involved in SIDS and forms part of many studies surrounding SUDI.

A variety of methods for the direct detection of CMV are available, including cell culture, EM, IHC, tissue fluorescence and PCR methods. Indirect methods can also be used to detect infection, by measuring an immune response from an individual, rather

than testing for the presence of the virus. This includes serology and enzyme immuno-assays (Griffiths, 2009).

There are however a number of problems with the detection of CMV in infants and to determine whether a positive result is clinically significant. Serological tests might detect maternal antibodies, giving false information regarding the infant's immune status by detecting maternal antibodies instead of the infant's own, and PCR methods might not be very specific in their prediction of disease.

One more concern is the following: CMV is also highly prevalent in general in infants without pathological effect (Álvarez-Lafuente *et al.*2008). This leads to the question whether CMV is then actually involved in SIDS and if so, why many infants who are indeed positive, do not die. A possible explanation might be the triple-risk model discussed earlier, but further research in this instance is therefore warranted.

1.2.2.5.6 Adenovirus

Adenovirus was first discovered in the early 1950s and 51 other species of adenovirus have since been described (Echavarría, 2009; Jones *et al.*, 2007). These non-enveloped, double-stranded DNA viruses, belonging to the *Adenoviridae* family and the *Mastadenovirus* genus, have been classified into 6 species: Human adenovirus A to F, which can cause a whole variety of illnesses (Jones *et al.*, 2007; Echavarría, 2009; Hayashi & Hogg, 2007).

Adenovirus infections are common throughout the year and have a worldwide distribution. It can be transmitted faeco-orally, through water, fomites or by means of aerosols (Echavarría, 2009).

Adenovirus infection, in comparison with a HHV infection, causes an acute infection instead of a lifelong infection. Infection by different species of virus results in different types of disease in humans, including gastroenteritis, pharyngitis, pneumonia, keratoconjunctivitis and acute respiratory disease (Brodzinski & Ruddy, 2009; Hayashi & Hogg, 2007; Jones *et al.*, 2007). Adenovirus is one of the leading causes (along with Rhinovirus, RSV and Influenza) of pneumonia (Figueiredo, 2009; Marcos *et al.*, 2009) and children younger than 5 years of age are more susceptible to

adenovirus infection, which often develops into pneumonia (Hayashi & Hogg, 2007).

A variety of diagnostic methods can be used for the direct and indirect detection (detection of an antibody rather than an antigen) of adenoviruses, including cell cultures, IHC, EM and PCR (direct), as well as serology (indirect) (Echavarría, 2009). Since no latent period is present in this virus's life cycle, similar problems to CMV (and other HHVs) with the detection of the virus is not present and positive diagnostic results can be attributed to primary infection.

1.2.2.5.7   RSV

RSV is a single-stranded RNA virus that belongs to the *Paramyxoviridae* family along with human metapneumo- and parainfluenza viruses and falls under the *Pneumovirus* genus (Figueiredo, 2009; Ogra, 2004; Olson & Varga, 2008). It was discovered in chimpanzees in 1956 and is divided into two major strains, namely RSV A and RSV B (Figueiredo, 2009; Ogra, 2004). These two strains infect approximately 50% of children in their first year of life and by the age of two, most children have been infected already (Vicencio, 2010). Acute respiratory disease in children caused by RSV is the most common cause of hospitalisation and is regarded as the leading cause of morbidity and mortality in children worldwide (Hoffman *et al.*, 2004; Hall, 2009).

RSV has a worldwide distribution and causes annual seasonal outbreaks in different parts of the world. Although it is more prevalent in winter months, countries with warmer or tropical climates can experience the presence of RSV throughout the year (Hall, 2009). Transmission of RSV occurs mainly through fomites and aerosols which enter the eyes or nose, whereafter it spreads to the lower respiratory tract (Hall, 2009). Nosocomial transmission of RSV is quite common, especially since infection generally causes mild infection in adults and symptoms are not severe enough to require an absence from work. Therefore, infected healthcare workers can easily spread RSV to already vulnerable children in clinic and hospital settings (Hall, 2009).

Primary infection with RSV can cause pneumonia and bronchiolitis and is often associated with wheezing (Hall, 2009; Ogra, 2004). Although RSV infection is not normally life-threatening, it can cause severe morbidity and mortality in

immunocompromised patients (Ogra, 2004; Olson & Varga, 2008) and therefore RSV is a huge concern in infants. Research into the effect of RSV in SUDI cases is thus justified.

Since nosocomial transmission can cause large outbreaks in susceptible children, RSV infection should be diagnosed as soon as possible to prevent further spread of the virus. Rapid tests are available for quick diagnosis, but a variety of other laboratory tests also exist to be utilised in RSV diagnosis, such as viral isolation, serology and molecular methods. RSV, like adenovirus, has no latent period and therefore a positive diagnostic test can be interpreted as a primary infection.

1.2.3 SIDS Epidemiology

SIDS and SUDI is a worldwide phenomenon and although lower incidences are seen in some countries, no geographical area is overlooked.There are a number of different factors influencing susceptibility to SIDS. Leach *et al*. (1999) summarised this as follows: prematurity, male gender, low birth weight and age, which was also mentioned previously. The different seasons of the year might also play an important role in SIDS and SUDI. In the same study by Leach *et al*. (1999), most SIDS cases were reported in spring whereas most SUDI cases, with a known cause of death after investigation, were observed in autumn. Other studies have shown that SIDS has a higher prevalence in winter months (Highet, 2008).

Kinney and Thach (2009) reported that black infants in the USA and infants of mixed ancestry in South Africa are at higher risk of SIDS than others. Kitsantas and Gaffney (2010) suggested a possible reason for this being that mothers who are at high risk of post neonatal deaths of their babies often do not have access to high risk neonatal and obstetric services. They also propose that the education of new mothers would be instrumental in the lowering of the amount of SIDS cases in these high risk groups.

1.2.4 Diagnosis in SUDI

A thorough death scene investigation and complete autopsy are required in all cases of SUDI. This should involve different diagnostic approaches, including, but not limited to, microbiology, virology and complete histology of all the organs (Arnestad *et al*., 2002; Bajanowski *et al*., 2007). A positive result in any of these fields does not

necessarily automatically indicate a cause of death, since the finding might not be severe enough to explain death (Highet, 2008) and the decision of when an infection or condition is serious enough to cause death, remains controversial (Arnestad *et al*., 2002; Samuals, 2003).

Common causes of death in forensic investigation of SUDI cases include infections, accidental and non-accidental death, as well as congenital malformations (Arnestad *et al*., 2002; Weber *et al*., 2008). Infections, especially pneumonia, seem to be the most prominent cause of death after full autopsy in the literature (Arnestad *et al*., 2002; Loughrey *et al*., 2005; Prtak *et al*., 2010; Weber *et al*., 2008). If the method of detection, however, is not sensitive enough to detect all true positives, many possible infections could in fact be missed, resulting in cases being wrongly classified as SIDS.

Different diagnostic approaches have been followed in the literature for the investigation of SUDI. Álvarez-Lafuente and colleagues (2008) used a quantitative real-time polymerase chain reaction (real-time PCR) to detect three different HHVs i.e. EBV, CMV and HHV-6 in different types of tissue, including lung, brain, kidney and spleen. Their data showed EBV and HHV-6 to be more prevalent in suspected SIDS cases than in healthy infants (which consisted of a group of violent deaths and one case of sudden death due to natural causes where infection was ruled out). They proposed that SIDS might be caused by the combination of respiratory infections and environmental factors together with underlying causes, such as a genetic predisposition (Álvarez-Lafuente *et al*., 2008).

A different study used PCR in combination with IHC and *in-situ* hybridization to investigate 121 cases of SIDS. Lung tissue from all five lobes as well as a sample from the salivary gland was used and a total of three positive cases, confirmed by one or more of the abovementioned methods, for the presence of CMV were found (Dettmeyer *et al*., 2008).

Another research group used IHC and PCR to investigate two SIDS cases and was able to confirm adenovirus, by means of PCR, in the intrabronchial fluid of a 5-month-old infant. The authors noted that infants around this age are more susceptible to infections due to an underdeveloped or immature defence response against pathogens. This immunological inability to eradicate pathogens can ultimately lead

to sudden death in an infant, whereas the same pathogen would cause only mild illness in older children and adults (Quan *et al*., 2000).

PCR, serology, cell cultures and electron microscopy (EM) were used by a different group to investigate samples from 64 autopsies for the presence of viruses. Samples used include different tissue types (lung, brain, kidney, liver etc.), cerebrospinal fluid (CSF) and bodily fluids (nasal washings, respiratory fluids etc.). These tests were used to confirm the presence of influenza A and B, adenovirus, RSV, VZV, HSV, EBV, HHV-6 and CMV. Positive results were found for adenovirus, influenza A, EBV, CMV, HHV-6 and RSV. The authors noted that traditional microbiological approaches cannot always be readily applied to forensic samples. Tissue autolysis starts occurring immediately after death which compromises cell structure and nucleic acids (Fernández-Rodríguez *et al*., 2006).

These studies all confirm that cultures, PCR and IHC are suitable methods for the detection of specific viruses (Álvarez-Lafuente *et al.,* 2008; Dettmeyer *et al.,* 2008; Fernández-Rodríguez *et al*., 2006). A benefit of PCR compared to the other methods is the ability to utilise small tissue samples, which is often the case in autopsies of SUDI cases.

Histology can also be employed to investigate respiratory infections in autopsy lung tissue. Bajanowski *et al.* (2003) used histology on autopsy lung tissue from 105 SIDS cases, followed by PCR in cases where specific RNA viruses (RSV, influenza A, influenza B and parainfluenza) were confirmed. Adenovirus was the most frequent respiratory infection in these cases (25% compared to 8.5% for Influenza B and 12% for CMV) (Bajanowski *et al*., 2003).

However, isolating viruses from the lung tissue from SIDS cases has proven to be difficult: Sample size often poses the biggest problem (Quan *et al*., 2000). By definition, SIDS infants are younger than 1 year and therefore have very small lungs, resulting in very small samples being available for analyses. Furthermore, samples are usually formalin-fixed after the autopsy, which compromises the quality and integrity of the nucleic acids present in the tissue (Álvarez-Lafuente *et al*., 2008). There is often a time delay of up to seven days between death and autopsy, which could be attributed to different circumstances, including distance from the deceased's home or

place of death to the mortuary, especially in rural areas. Shortages of forensic pathologists or other problems, such as disasters leading to many autopsies and causing backlogs can also result in delays. These time delays can further influence the quality of nucleic acids vital for viral detection.

Other pitfalls specific to post-mortem samples exist, such as low viral loads that can negatively influence both cell culture results and EM findings. It is also unclear whether a viral infection with a low viral load is capable of causing death but when seen in conjunction with other factors, such as secondary infections, malnutrition etc. it might be worthwhile considering the infection as a possible cause of death. It is therefore of utmost importance in forensic pathology to find a reliable diagnostic method that can overcome these problems. Molecular techniques, such as PCR, can improve the the detection and identification of viral infections for as long as the nucleic acid is intact. This is unlike cell culture methods, where post mortem organism overgrowth, which can include organisms such as *α haemolytic streptococci*, *Staphylococcus epidermidis* and *Escherichia coli* (Prtak *et al.*, 2010), can interfere with the detection of viruses, and EM, where autolysis steps in shortly following death and compromises the integrity of cells. Real-time PCR can further add benefit to the diagnosis by quantifying viral load.

Positive PCR results should be carefully interpreted especially in the case of HIV, HHVs (such as CMV) and other infections which might have latent periods. These organisms have periods where there is not an active infection in the body even though the virus is still present, although dormant. These infections are therefore chronic/long lasting. The viral particles then remain dormant until they are reactivated. However, PCR can be used extensively for non-HHVs, such as adenovirus, RSV and parainfluenza since these viruses do not have latent periods (Fernández-Rodríguez *et al*., 2006).

Obtaining fresh samples directly from each autopsy, processing it immediately to preserve nucleic acid integrity and using methods such as real-time PCR might alleviate problems associated with post mortem tissue to some extent.

The methods used in this study will be discussed in section 1.2.5 and its applications in SUDI cases in section 1.2.6.

1.2.5 Methods of viral detection

1.2.5.1 Cell culture Methods

Cell cultures have long been regarded as the gold standard in the detection of respiratory viruses (Ieven, 2007; Leland & Ginocchio, 2007). These methods have the ability to demonstrate the viability of a virus and positive results are mostly linked to active infections. Qualitative molecular techniques and antigen detection assays will only give positive results even if the infection in question is latent (Leland & Ginocchio, 2007).

Shell vial cultures, where a cell monolayer is grown on a coverslip in a small vial into which the test specimen is inoculated, have become a popular derivative of conventional cell culture methods. Shell vials take up less space than normal culture tubes and this method is quicker and cheaper than, but still as sensitive as conventional cell cultures (McAdams & Riley, 2009). There is however evidence that shell vial cultures are not as sensitive as conventional cultures specifically in the case of CMV detection (Erice *et al*., 1992; Mazulli *et al*., 1993; Pedneault *et al*., 1996).

1.2.5.2 Conventional and Real-time PCR

The conventional PCR was first described by Kary Mullis in 1983 (Van Guilder *et al*., 2008) with real-time PCR, a modern molecular technique that is becoming increasingly popular, stemming from the original conventional PCR. Real-time PCR is regarded as an improvement on conventional PCR, producing either qualitative or quantitative results, whilst conventional PCR only provides qualitative information.

PCR is based on the principle of amplifying the target nucleic acid (DNA or RNA) and detecting the product by means of either gel-electrophoresis at the end of the assay (conventional PCR) or a fluorescent dye, which is cleaved off when the probe binds to the target sequence. This omits a signal after being cleaved and can be visualised in real-time (real-time PCR). These methods are useful diagnostic tools for the detections of highly pathogenic pathogens as well as cases where viruses do not proliferate in normal cell cultures, such as hepatitis B and C, and parvovirus B-19. It is also a much faster method than conventional culture methods (Leland & Ginocchio, 2007; Tregoning & Schwarze, 2010).

A major disadvantage of real-time PCR is the high cost of the test (Addendum D) but this can be alleviated by using multiplex PCR reactions i.e. test for the presence of multiple viruses with one PCR reaction. Another disadvantage is the inability of the test to differentiate between an active and latent viral infection (Leland & Ginocchio, 2007) unless a viral load test is performed (by using the real-time PCR quantitatively). This can mean that the test is too sensitive for clinical use and that results may not be clinically relevant (Griffiths, 2009; Machida *et al*., 2000). However, quantitative PCR gives information about the viral load of an infection, and that information can be used to distinguish between active and latent infections.

1.2.5.3   H&E

H&E staining is used routinely to investigate histological changes in tissues and is widely used for medical diagnoses and is routinely used in medico-legal investigations. These stains are used to differentiate between the cell nucleus (stained by haematoxylin) and the cytoplasm (stained by eosin) in tissues.

This method allows significant changes in tissues to be seen (Wittekind, 2003) in a relative inexpensive manner. A disadvantage of this method is its inability to distinguish between different infections and additional tests are required to confirm suspected infections (Bajanowski *et al*., 2007). It is, however, a very valuable test in determining whether a positive infection is indeed significant. If no changes in the lung are present but a virological test is positive, the infection will not be considered as being fatal.

1.2.5.4 IHC

IHC is based on the principle that an antibody, which is bound to a reporter enzyme or fluorescent dye, can be bound to its corresponding antigen whereafter it can be visualised under a light microscope. A diagnosis can then be made according to the distribution of inclusion bodies in tissue and cells, as well as the tissue structure (i.e. whether inflammation and other structural changes are present). (Haines & West, 2005; Linnoila & Petrusz, 1984).

There are two types of antibodies used in IHC, monoclonal and polyclonal antibodies, and IHC can be used on fresh and unfixed tissue, but formalin-fixed, paraffin-

embedded (FFPE) tissue is most commonly used. Antigen retrieval is an important step in the IHC process, since fixation causes the antigenicity of the tissue to be masked by reversible cross-linkages of protein amino acid residues by methylene bridges, rendering the antigen invisible (D'Amico *et al.,* 2008; Shi *et al*., 2001; Sompuram *et al*., 2004). This results in the inability of the specific antibody to bind to the antigen, leading to incorrect results, such as false negatives. The amount of cross-linking in the tissue can be controlled by fixation time, fixative pH and concentration of the fixative (D'Amico *et al*., 2008). Antigen retrieval breaks these cross-linkages and can be performed in a variety of ways, including heat (Heat-induced epitope retrieval or HIER) and enzyme-based methods, radiation by means of a microwave or by boiling the tissue sections (D'Amico *et al*., 2008; Sompuram *et al*., 2004).

IHC is also an expensive method (Addendum E) and even though it can confirm the presence of specific antigen in the tissue, it cannot quantify the infection (Goyal *et al*., 2008). Only small quantities of tissue is needed (Leake, 2000), but if this specific piece of tissue does not contain the antigen in question, a false negative result can occur. Interpretation and reporting of results can often be subjective (Leake, 2000) and care should be taken to differentiate between background staining and true positive results. Factors, such as differences in antigen retrieval times, makes standardising IHC difficult, but this can be overcome by adopting a standard protocol for the processing of all types of tissue before staining (Leake, 2000).

1.2.5.5 Electron Microscopy

The electron microscope can magnify images up to one million times and enables scientists to see viruses, which are too small to see with a light microscope. Since morphological differences between viruses are visible by means of electron microscopy, this technique plays a very important role in the classification of viruses. A number of new viruses have been described with the aid of EM, including Hendra and Nipah (Henipa) viruses (Goldsmith & Miller, 2009).

No target specific reagent is required to visualise a pathogen by EM. It provides an 'open view' of whatever is present in the test sample (Goldsmith & Miller, 2009; Jeffery & Aarons, 2009) and allows the ultra structure of viruses and other pathogens to be seen. EM can show the presence of all viruses, even those that cannot be

cultured or are highly pathogenic, and it is also a rapid technique, yielding results within a day (Jeffery & Aarons, 2009).

This highly technical process requires a highly trained technician and EM tests are very expensive. Even though all viruses can be detected by EM, a high viral load is required, since EM has a low sensitivity. Approximately 1 million viral particles per millilitre of fluid / tissue culture supernatant are needed for a positive result (Jeffery & Aarons, 2009) and therefore a low positive might be portrayed as being negative. Thus, EM has limited diagnostic applications. Although there is doubt about the future of this method due to its shortcomings (Jeffery & Aarons, 2009) EM currently remains the only direct visualisation method for viruses and other pathogens and EM is of vital importance in the discovery and diagnoses of newly emerging pathogens. These attributes will keep this method of viral detection alive, even if not for diagnostic purposes, then certainly for research applications.

1.2.6   Application of methods in SUDI

1.2.6.1   Cell Culture Methods

Cell culture methods, which include shell vial cultures, are used routinely in cases of suspected SUDI but recently it is being replaced by PCR technology in certain SUDI cases, especially cases of suspected myocarditis (Bajanowski *et al*., 2007; Krous *et al*., 2009; Lozinski *et al*., 1994).

As mentioned earlier, there is evidence that shell vials are not as sensitive for the detection of CMV (Erice *et al*., 1992; Mazulli *et al*., 1993; Pedneault *et al*., 1996) and it should be kept in mind that CMV is a labile virus (Espy & Smith, 1987), a serious cause for concern in post-mortem tissue. The time delay between death and post mortem examination could therefore already influence the results seen on shell vials. Any further delay between post mortem examination and the diagnostic test can also increase degradation of the virus leaving no live virus in the tissue sample, leading to false negative results.

1.2.6.2   Conventional and Real-time PCR

PCR methods are very popular because of its high sensitivity and specificity and mostly because of its rapidness. However, there are a number of problems with these methods in the detection of some viruses in SUDI cases.

As discussed previously, HHVs (and specifically CMV in the case of this study) have latent periods where no active infection is present and although PCR methods are highly sensitive and specific for the detection of CMV, a positive result should be carefully interpreted. Because of the latent period and periodic reactivation of CMV, a positive PCR result might be the result of a primary infection, a latent virus or a reactivated virus (Bhatia *et al.*, 2010). It should, however, be kept in mind that SUDI cases are defined to be less than one year of age. Therefore a positive PCR result will likely be because a primary infection instead of a reactivated virus. Also the effect of CMV on certain infants, specifically those with low birth weight etc., as discussed in 1.2.2.5.5, is more severe than on older individuals. A primary infection might therefore be more cause of concern that would have been in another patient.

The same concern with post-mortem detection of CMV as mentioned in section 1.2.6.1 i.e. degradation of nucleic acid, is a factor and negative results may be apparent in cases where there was actually indeed a CMV infection.

All these concerns highlight the fact that no diagnostic method is flawless and careful interpretation of both positive and negative results are of utmost importance.

1.2.6.3   H&E

H&E stains are routinely used and mandatory in the investigation surrounding death of SUDI cases. Specifically, histology of the respiratory tract is very important because of the significance of respiratory infections in SUDI (Bajanowski *et al.*, 2007). Although H&E stains are very useful in cases of suspected infection, this method on its own as diagnostic method is not very specific as it shows changes due to infection, but different infections cannot always be distinguished.

Certain histological changes are very common in SUDI cases. These include congestion, mild oedema etc. and are non-specific changes (Bajanowski *et al.*, 2007) not necessarily related to death. However, an infection might aggravate these changes

and therefore virological investigations are suggested to correlate with histological changes (Bajanowski *et al.*, 2007; Fernández-Rodríguez *et al.*, 2006).

At the Tygerberg Medico-legal facility the routine protocol is exactly this, to correlate virological investigations with the histology seen on H&E stains.

1.2.6.4 IHC

Even though IHC investigations in SUDI cases are not mandatory, it is recommended in some cases to enhance the diagnoses of certain cases and to distinguish between pathology and non-specific changes in the lungs (Bajanowski *et al.*, 2007).

Even though the method is highly specific due to specific antibodies used, background staining can interfere with detection, leading to inaccurate results. Also, the tissue used for IHC purposes is relatively small due to the size of the infants involved and should the antigen not be present in this sample, negative results can lead to an incorrect interpretation of the case.

1.2.6.5 Electron Microscopy

EM is often used in the investigation of SIDS and SUDI cases and has been used successfully in the detection of viral infections (Fernández-Rodríguez *et al.*, 2006; Weber *et al.*, 2010)

However, EM specifically in the investigation of SUDI cases has a much lower yield than other diagnostic tests (Weber *et al.*, 2010) and the quality of post-mortem tissue is not always suitable for EM purposes ((Fernández-Rodríguez *et al.*, 2006). This is due to the fact that autolysis of cells begins shortly after death, and therefore it is very difficult to distinguish between potential pathogens and cell debris, especially if one considers the magnification that is involved in EM. Also, EM requires a high viral load in order to detect an infection and low viral loads will therefore go by unnoticed, giving a negative result ((Fernández-Rodríguez *et al.*, 2006).

Thus, although a very classic method of detecting viruses, EM might not be entirely suitable for the detection of viruses in SUDI cases.

It is thus clear that not one method is perfect in the diagnosis of a SUDI case. A possible solution could be a combination of different methods, but further research into which methods are best for the detection of specific viruses is therefore vital.

1.3   Aim of this study

Preliminary data from Tygerberg hospital shows a mere 14% positive yield on the current standard testing protocol for viral infections in SUDI cases and investigation into alternative and better suited diagnostic methods seems therefore justified. In this study we will attempt to improve the diagnosis of possible viral infections in SUDI cases. The current medico-legal protocol tests routinely for CMV, adenovirus and RSV with the shell vial culture method and we hypothesise that this method is not best in confirming the presence of specific viruses as more modern techniques, such as real-time PCR and / or IHC. If this hypothesis can be confirmed, it will result in a more better diagnosis of SUDI cases and a decreased number of SIDS cases (where no other diagnosis can be confirmed).

To date, no such research, which compares different methods in SUDI cases, has been conducted in South Africa, where socio-economic and environmental conditions are significantly different from those in developed countries.

Chapter 2

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Chapter Two

2. MATERIALS AND METHODS

2.1 Introduction

The study was conducted as two components. First, a retrospective data audit was conducted to determine the virological yield of cases. This was followed by the prospective analysis of post mortem samples from 82 babies which were obtained from the Tygerberg Mortuary. This chapter outlines three different diagnostic methods that were used to test these samples for the presence of three different respiratory viruses.

2.2 Ethics Approval

The project was approved by the Health Research Ethics Committee of Stellenbosch University on 22 June 2009 and extended for another year on 21 April 2010. Waiver of individual consent for the additional laboratory tests was applied for on the basis that the study material collected, as well as tests performed, do not differ from the protocol currently in use at the Tygerberg Medico-legal Mortuary. Waiver was granted. The ethics reference number for this study is N09/02/046 (Addendum A).

2.3 Retrospective Audit

A retrospective audit was performed by examining the SUDI case files from 2004 – 2006 to determine in how many of these cases positive results for viral infection could be obtained.

2.4 Study Population and Sample Collection

The study population included 82 infants, male and female, less than one year old (with the exception of only one case which was 14 months old but fit all the other criteria) that fit the criteria of SUDI/possible SIDS. Infants that had a known cause of death were excluded. All study infants died unexpectedly in their sleep.

The seasonal distribution of death was classified using the published article of Leach *et al*., (1999) as a guideline:

- Summer: December - February

- Autumn: March - May

- Winter: June - August

- Spring: September - November

Samples were obtained from the post mortem examinations of these cases. The left lower lobe (LLL) of the lung was excised (this is the routine lung lobe used for virological and microbiological tests) and placed into a sterile container, which was transported to the Division of Forensic Pathology, 6th floor, Clinical Building, Tygerberg Campus in a sterile, sealed pathology sample bag. Samples were collected from 29 September until 27 May 2010. The chain of custody was maintained at all times.

The mean post mortem interval (time between death and post mortem examination) was 3.4 ± 2 days, ranging between 0 days (autopsy done on the same day as death) and 8 days.

2.5   Sample Processing

All samples were weighed and this information was sent to the attending pathologist to be recorded on the official post mortem report. The mass of the lung lobes ranged between 7 g and 52 grams (g). The complete results are indicated in Addendum B.

Forceps were flamed for at least 25 seconds over a Bunsen burner prior to use to ensure sterility. Hereafter the sample was placed on a sterile Petri dish (94 mm x 16 mm, triple vent) (Greiner, Austria) for dissection. A sterile surgical blade (Number 23, Fitment No.4) (Paramount Surgimed, Germany) and 4L scalpel handle were used to cut the sample in half and one of the pieces (sample A) was then placed in 10% formalin. Cassettes were marked with the sample number (samples were consecutively numbered in order of collection), the reversed ethics number, as well as "LLL", which indicated the sample was from the lower lobe, left lung. The reversed ethics number was used to ensure samples can be easily identified and has no other significant meaning. A typical example of the number would therefore be "001-N046/02/09 LLL". After a minimum of 24 hours the formalin-fixed samples were trimmed to fit inside the corresponding, standard histology cassettes, which were then processed to prepare formalin-fixed wax blocks for histology purposes.

The other half of the original sample (sample B) was subdivided to be used for the different tests. One piece of approximately 3x1x1 mm was excised from sample B and placed in a 2.0 ml eppendorf tube (Axygen Scientific, USA), containing 2 ml 2.5% gluteraldehyde (Sigma Aldrich, USA). This was stored at 4°C for EM purposes. Gluteraldehyde is the preservative of choice for EM, since it preserves the tissue better than formalin would and it is routinely used and preferred by the Division of Anatomical Pathology's EM technicians.

Another piece of the lung was removed from sample B for routine shell vial cultures. A 5x5x5mm piece of lung was excised (also from sample B) and placed in 2 ml eppendorf tube containing 1.5 ml Viral Transport Medium (VTM) (Highveld Scientific, SA) for transport to Virology Specimen Reception, 8th Floor, Clinical Building, Tygerberg Campus. VTM is used to preserve the virus(es) for shell vial cultures and contains antibiotics to prevent bacterial overgrowth and contamination of the sample.

Two further small pieces of sample B were excised for Real-time PCR purposes. One was placed in VTM until DNA extraction, while the other was placed in RNALater (Sigma Aldrich, USA) for RNA extractions. This was done to ensure the nucleic acid stays intact and does not denature before extraction. Both samples were kept at 4°C until further processing.

The remaining lung tissue was then placed in the original container, covered with between 2 and 15 ml VTM, depending on the size of the remaining tissue, and frozen at -80°C.

2.6   Shell Vial Cultures

2.6.1   Subculture of Cells

All cells were subcultured according to the routine SANAS accredited method. Human Fibroblasts (HF) were used for CMV and combination (combi) cultures containing both HEp2 and Madin-Darby Canine Kidney (MDCK) cells for adenovirus and RSV.

1X Active Trypsin Versine (ATV) (Sigma Aldrich, USA) was prepared by using 90 ml milliQ water (MilliQ™ Water System, Millipore, Ireland) and 10 ml 10XATV.

Culture medium was decanted and discarded from the culture flask and 1XATV (pre-warmed in waterbath to 37ºC) was added to cover the monolayer of cells. The flask was rinsed to remove excess foetal calf serum (FCS) (Sigma Aldrich, USA) to prevent trypsin inactivation caused by FCS. The ATV was decanted and 10 ml fresh, warmed 1XATV was added again. The flasks were then incubated for 5 – 10 minutes at room temperature, whereafter the contents was transferred to centrifuge tubes and centrifuged in a Centaur 2, Sanyo centrifuge for 5 minutes at 1000 revolutions per minute (rpm). The supernatant fluid was discarded and the pellet resuspended in medium by pipetting. Growth medium was added up to 30 ml.

A cell count was done using a Neubauer counting chamber (Figure 2.1) and cells were prepared at a concentration of 15×10^4 cells/ml. These cell cultures were then transferred to shell vials and incubated at 37ºC for 48 hours, until a monolayer of cells was formed.
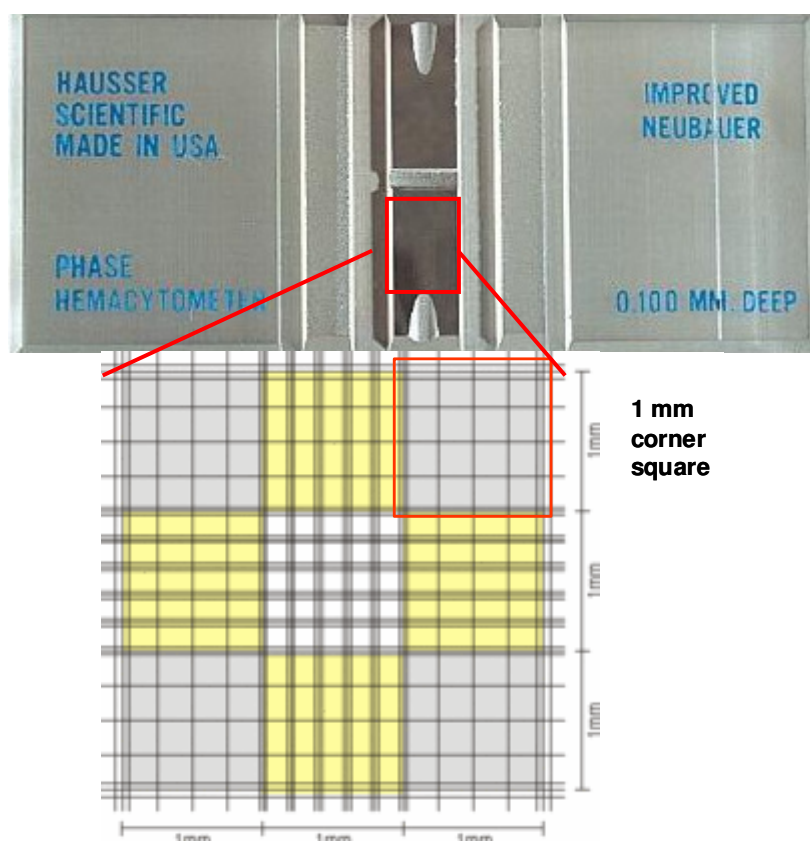
Figure 2.1: **Neubauer Counting Chamber.** All the live cells in any 3 large blocks (containing 16 small blocks) are counted and then the amount of cells/ml can be calculated using the formula below (figures obtained

from Thomas Scientific, USA and Montreal Biotechnologies Inc., Canada)

In order to determine the number of cells per millilitre, cells were diluted in 10 ml media. A small volume of cells (20 microlitre (µl)) and 20µl trypan blue were then mixed and placed onto a Neubauer Counting Chamber. Non-viable cells, e.g. where the cell membrane has been damaged, absorb the trypan blue and distinguish it from viable or live cells, which do not stain.

The amount of cells in three different blocks (Figure 2.1) was then counted and the following formula (Biowhittaker, Biotechnology Products Catalog and Technology Manual, 1993) was used to determine the number of cells/ml (Appendix C).

Total cells/ml = $\underline{\text{total cells in all counted squares x } 10^4 \text{* x } 2\text{**}}$
number of counted squares

*10^4 = volume conversion factor to 1 ml
**2 = specific dilution factor (trypan blue)

2.6.2 Shell vials

Upon receipt, each lung sample was placed in an autoclaved mortar and 2 ml VTM and 0.2 ml antibiotic cocktail were added to the tissue. A small amount of ground glass was used to assist in breaking the tissue and this mixture was then grinded thoroughly with a pestle to create a homogenous suspension.

Another 6 ml of VTM was added to the mixture which was transferred to a labelled 15 ml centrifuge tube and centrifuged at 2000 rpm for 10 minutes at 4ºC in a Beckman GPR centrifuge. After centrifugation the specimen was filtered through a 0.20 µm filter with a 10 ml syringe and inoculated into a labelled shell vial.

CMV and adenovirus shell vials were incubated for 48 hours at 37ºC, which is the normal human body temperature and thus the optimal temperature for normal human pathogens. Combi cultures were incubated for 48 hours at 33ºC since respiratory viruses that are present in the upper respiratory tract, which is cooler than the normal human body temperature, replicate better at this temperature (Message & Johnston, 2004; Papadopoulos *et al*., 2009).

2.6.3 Immunofluorescence Assay (IFA)

IFA staining was done using a LIGHT DIAGNOSTICS™ Respiratory Panel 1 Viral Screening & Identification IFA Kit (Millipore, USA). The protocol in the manufacturer's kit was followed. The cell nucleus in CMV infection is green, while adenovirus infection will display a green cytoplasm. The respiratory virus combi respiratory panel includes screening for parainfluenza 1 – 3, influenza A and B, adenovirus, RSV and human metapneumovirus. All the infected cells will be stained, which means that one or more of the aforementioned viruses is positive. Retesting for individual viruses using the same method must then be done to identify the virus. Shell vials are considered positive if more than 5 cells are stained; if less than 5, but more than 1 cell is positive, the test is repeated.

2.7   Real-time PCR

For this method, nucleic acid extraction must be performed after which the PCR is set up and run on a suitable PCR platform.

2.7.1   DNA Extraction

A QIAamp® DNA Mini Kit (Qiagen, Germany), containing QIAamp Mini Spin Columns, 2 ml Collection Tubes, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE, Buffer ATL and Proteinase K, was used for DNA extractions. Buffer ATL are used with the Proteinase K as tissue lysis buffer, where the Proteinase K causes the denaturing of proteins present in the tissue. Buffer AL is a further lysis buffer and Buffer AW1 and AW are wash buffers used to remove impurities. Buffer AE is the elution buffer, used in the final step of the procedure to elute the DNA. Ethanol and RNase A were also used during the extraction to optimise binding conditions and to ensure that RNA-free genomic DNA is obtained. The DNA extraction procedure is based on the principle that DNA is adsorbed to the silica-gel membrane in the QIAmp Mini spin column during centrifugation; which can then be eluted after purification to yield purified DNA.

After cleaning all work surfaces with freshly prepared biocide to remove potential pathogens and 70% ethanol to remove the the corrosive effect of biocide, DNA extraction was performed using the manufacturer's protocol for DNA purification

from tissues. Tissue lysis was complete in 1 – 3 hours; this was evident when no tissue could be seen in the sample tube. .

The eluted DNA was then stored at -20°C until further use (QIAamp® DNA Mini and Blood Mini Handbook, 2007).

2.7.2   RNA Extraction

A QIAShredder™ Kit (Qiagen, Germany), containing QIAShredder Spin Columns, and an RNeasy® Mini Kit (Qiagen, Germany), containing RNeasy® Mini Spin Columns, Collection Tubes, Buffer RLT, Buffer RW1, Buffer RPE and RNase-free water were used for RNA extractions. Buffer RLT contains guanidine-thiocyanate and is used as denaturing buffer, ensuring pure, intact RNA. Buffer RW1 and RPE are wash buffers used to remove any impurities and contaminants from the RNA. Finally, RNase-free water is used for the elution of the extracted RNA. Ethanol is also used during the procedure to ensure optimal binding conditions. This technology is based on the binding of RNA to the RNeasy® silica membrane in the RNeasy® Mini spin column and combined with the speed of centrifugation, ultimately leading to the extraction of RNase-free RNA.

After cleaning all work surfaces DNA extraction was performed using the manufacturer's protocol for Purification of Total RNA from Animal Tissues, using protocol 3b ("Disruption using a mortar and pestle followed by homogenization using aQIAshredder homogenizer").

The eluted RNA was stored at -80°C until further use (RNeasy® Mini Handbook, 2006).

2.7.3   DNA and RNA Concentrations

Concentrations of DNA and RNA nucleic acids were measured on a Nanodrop Spectrophotometer (Nanodrop Technologies, USA). Nuclease-free water, 1 µl, was used to blank the system whereafter the same volume of either DNA or RNA was added. The appropriate action was taken (i.e. DNA-50 or RNA-40) to measure the nucleic acid concentration and the concentration (nanogram (ng)/µl) was noted. The complete results can be found in Addendum C.

2.7.4 Primer and Probe Design

Although primers and probes in this study were obtained from previously published papers, primers and probes can be also designed by using a variety of online and commercially available programs. The following processes are involved in the designing of primers and probes:

- Collection of gene sequences of target organism. Genebank can be used for this purpose.
- Alignment of sequences with ClustalX version 1.81 or similar programs.
- Manually finding a consensus sequence using Geneious version 4.3 software.
- Choosing primer target sequences in highly conserved region. Probe sequence is then chosen and fluorescent dyes are selected based on the wavelength that must be used e.g. FAM for green channel, VIC for yellow channel etc. Quencher dyes must be compatible with the fluorescent dye, e.g. FAM with TAMRA etc.
- It is also important to ensure that the melting temperatures of forward and reverse primers are not too far apart, otherwise primers will not be able to attach to the target sequence properly.

2.7.5 Real-time PCR Setup

Positive controls for the different assays were obtained from the Quality Control for Molecular Diagnostics (QCMD) programme. This programme is an international External Quality Assessment (EQA) organisation which aids in monitoring the quality of molecular tests in clinical laboratories. All real-time PCR tests were initially run for 45 cycles according to the original protocols. Because low positives can easily be mistaken for negative samples, the cycles were increased to 50 to ensure that low positives could also be detected. The fluorescent dyes used are visualised in different channels. FAM is detected in the green channel at wavelength 510 nanometer (nm), while VIC is detected in the yellow channel at wavelength When a multiplex PCR is used, as in the case of the RSV assay published by Kuypers *et al.* (2004), two different fluorescent dyes are used to ensure that they do not overlap and that both target sequences are detected.

Real-time PCR results were interpreted qualitatively. Both the other methods, shell vial cultures and IHC, only provide qualitative results and therefore the quantification of the real-time PCR results would not contribute to the comparison of the methods.

Although qualitative real-time PCR can be used in the same manner as conventional PCR, real-time PCR was used because this is a modern method with the ability to quantify results which, although not used specifically in this study due to other methods not having the same ability, could be provide valuable information to the pathologist regarding viral loads etc. Therefore real-time PCR was chosen out of a prospective point of view.

2.7.5.1 CMV & Adenovirus PCR Optimisation

Published primer and probe sequences were used for real-time PCR assays. All primers and probes were ordered from Applied Biosystems. Sequences were obtained from the published articles by Preiser *et al*. (2003) and Heim *et al*. (2003) for CMV and adenovirus respectively (Table 2.1). Sequences were BLASTed (NCBI, Pubmed) to ensure they were correct and targeted genes of the target organism.

CMV primers and probe were designed to target a conserved region in the UL89 gene which codes for the DNA terminal packaging subunit 1 and amplifies a fragment of approximately 140 base pairs (bp) (Preiser *et al.*, 2003). The adenovirus PCR primers and probe was designed to target highly conserved regions in the hexon gene to detect all 51 types of the human adenovirus (Heim *et al*., 2003). These primers were designed after aligning genomes from five fully sequenced human adenoviruses i.e. type 2, type 5, type 17 and type 40. By aligning the primers with reference adenovirus genomes on Genbank, the amplicon size was determined to also be approximately 140 bp long.

CMV and adenovirus probes were provided in a lyophilised pellet at 50 nmol and primers at 130 nmol. Probes were reconstituted by adding of nuclease-free water (Qiagen, Germany) to both probes to make up a stock solution of 1 millimolar (mM) for each.

Table 2.1: **Primer and probe sequences for the detection of CMV and Adenovirus (Preiser *et al.,* 2003; Heim *et al*., 2003).**

Virus	Sequence (5' - 3')
CMV	
Forward Primer	CGT-TGG-TGT-TGT-AGC-AAG-TGG-C
Reverse Primer	TGT-GCT-CAA-AGA-GGT-CGA-GTT-CC
Probe	VIC-CGC-GAA-GGT-GTG-TGG-CGG-CAG-TAMRA

Adenovirus	
Forward Primer	GCC-ACG-GTG-GGG-TTT-CTA-AAC-TT
Reverse Primer	GCC-CCA-GTG-GTC-TTA-CAT-GCA-CAT-C
Probe	FAM-TGC-ACC-AGA-CCC-GGG-CTC-AGG-TAC-TCC-GA-TAMRA

Aliquots of the stock solution were made and then diluted with nuclease-free water to create a working concentration of 10 µM. The primer stock solutions were prepared in the same manner for all primers and 10 µM working concentrations were prepared. This was done to prevent multiple freeze-thaw cycles of the working solution, as this can reduce the fluorescent signal strength and damage the primers/probe. A final concentration of 10 µM was used to ensure that volumes are not too small to pipette accurately.

Preiser *et al*. (2003) published optimal concentrations of the primers and probes in each real-time PCR reaction for CMV, which was 300 nanomolar (nM) for forward- and reverse primers, as well as the probe. The same concentrations were first tested using positive controls and later used in this study.

Heim *et al*. (2003) reported using final concentrations of 0.5 millimolar (mM) (each primer) and 0.4 mM (probe) for adenovirus. These concentrations seemed extremely high and likely to be a printing error. To avoid non-specific binding of the primers and the wasting of expensive reagents, the concentrations were adjusted to 500nM for each primer and 400 nM for the probe before the first real-time PCR run. The adjusted values were tested using positive controls and confirmed to be correct.

A real-time PCR mastermix was prepared for each virus (Table 2.2 for CMV and 2.3 for adenovirus) using a Sensimix™ Probe Kit buffer (Bioline, Germany) and nuclease-free water. The purpose of the mastermix was to eliminate pipetting and other systematic errors by working with one large volume instead of multiple smaller volumes. A fixed volume of 20 µl of the mastermix could then be transferred to 0.2 ml Thin Wall PCR® tubes (QSP, California, USA) and 5 µl of known positive nucleic acid (positive control) or nuclease-free water (non-template control) was added to each reaction in a separate room to eliminate contamination of primers/probes and buffer with nucleic acid. Two non-template controls were included in each run where

nuclease-free water was used instead of nucleic acid. Tubes were placed in the Rotor-Gene 6000 (Corbett life science, Australia) real-time PCR platform and conditions were set for the specific viruses. These conditions are described in Tables 2.4 (CMV) and 2.5 (adenovirus).

Table 2.2:   Mastermix setup for CMV real-time PCR

Reagent	Concentration (µM)	Concentration (µM)	µl per 1 reaction
	Working solution	Final	
2 x uMM Buffer	2 x	1 x	12.50
FP (forward primer)	10	300	0.75
RP (reverse primer)	10	300	0.75
P (probe)	10	300	0.75
water			5.25
Template			5.00
total volume µl			25.00

Table 2.3:   Mastermix setup for adenovirus real-time PCR

Reagent	Concentration (µM)	Concentration (µM)	µl per 1 reaction
	Working solution	Final	
2 x uMM Buffer	2 x	1 x	12.50
FP (forward primer)	10	500	1.25
RP (reverse primer)	10	500	1.25
P (probe)	10	400	1.00
water			4.00
Template			5.00
total volume µl			25.00

Table 2.4:   Cycling conditions for CMV in the Rotor-Gene 8000.

Cycles	Time	Temperature (ºC)
	2 min	50
	10 min	95
50x	10 sec	95
	45 sec	60

Table 2.5:   Cycling conditions for adenovirus in the Rotor-Gene 8000.

Cycles	Time	Temperature (ºC)
	5 min	35

	10 min	95
50x	3 sec	95
	10 sec	55

After optimisation with positive controls, all samples were tested in duplicate and where inconclusive results were found, a third real-time PCR assay was performed.

2.7.5.2 RSV PCR Optimisation

The RSV real-time PCR was initially done according to the method published by Kuypers *et al*. (2004). The sequences can be found in Table 2.6. However, this assay did not work correctly and the method according to Watzinger *et al*., (2004) was rather used.

The RSV primers and probes in the Kuypers *et al*. (2004) method were designed to target the RSV polymerase gene from both RSV A and RSV B, amplifying a 94 bp fragment whilst the Watzinger *et al*. (2004) method was designed to target specifically the N gene of the virus for the amplification of a 149 bp fragment (Watzinger *et al*., 2004).

Both forward and reverse primers (for Kuypers *et al*., (2004) method) were manufactured by Applied Biosystems and supplied in a lyophilised pellet form. These were reconstituted and a working solution of 10 µM was prepared. Two probes were used for this assay by Kuypers *et al*., (2004) for the two different subtypes, one FAM-labelled probe for RSV type A and another VIC-labelled probe for RSV type B. Different fluorescent dyes are used to differentiate between the two virus types.

The final concentration of the primers and probes in the reaction were also obtained from Kuypers *et al*. (2004) and 250 nM for both primers and 100 nM for both probes were used. A mastermix was prepared (Table 2.7), using a Sensimix™ Probe One-Step Kit (Bioline, Germany) and nuclease-free water (Qiagen, Germany). A final volume of 40 µl in each PCR tube contained 30 µl mastermix and 10 µl positive control or nuclease-free water, for the non-template control (NTC).

Table 2.6:   Published primer and probe sequences for the detection of RSV.

Virus / Publisher	Sequence (5' - 3')
RSV / Kuypers *et al*., 2004	
Forward Primer	AAT-ACA-GCC-AAA-TCT-AAC-CAA-CTT-TAC-A
Reverse Primer	GCC-AAG-GAA-GCA-TGC-AAT-AAA
Probe A	FAM-TGC-TAT-TGT-GCA-CTA-AAG-MGBNFQ
Probe B	VIC-CAC-TAT-TCC-TTA-CTA-AAG-ATG-TC-MGBNFQ
RSV / Watzinger *et al*., 2004	
Forward Primer	GGC-AGT-AGA-GTT-GAA-GG
Reverse Primer	ACA-ACT-TGT-TCC-ATT-TCT-GC
Probe	FAM-ACT-TGC-CCT-GCA-CCA-TAG-GCA-TTC-ATA-AAC-AAT-TAMRA

Table 2.7:   Mastermix setup for RSV Real-time PCR (Kuypers *et al*., 2004).

Reagent	Concentration (µM)	Concentration (µM)	µl per 1 reaction
	Working solution	**Final**	
2 x uM Buffer	2 x	1 x	20.00
FP forward primer	10	250	1.00
RP reverse primer	10	250	1.00
P probe A	10	100	0.40
P probe B	10	100	0.40
template			10.00
water			7.20
total volume µl			40.00

The PCR tubes were placed in the Rotor-Gene 6000 real-time PCR machine and conditions were set for the specific viruses (Table 2.8.)

Table 2.8:   Cycling conditions for RSV real-time PCR (Kuypers *et al.*, 2004)

Author	Cycles	Time	Temperature (ºC)
Kuypers *et al*., 2004		5 min	35
		10 min	95
	50x	3 sec	95
		10 sec	55

However, this assay did not work correctly (Figure 2.3) and negative results were obtained when the assay was tested with positive controls. Concentrations were therefore adjusted by means of a checkerboard titration with concentrations 300, 600 and 900 nM for primers and 200, 400 and 500 nM for the probes. The same reaction was also run on the ABI-7900 real-time PCR machine (Applied Biosystems, Germany) in an attempt to identify the problem. After multiple runs, it was concluded that the probes were damaged.
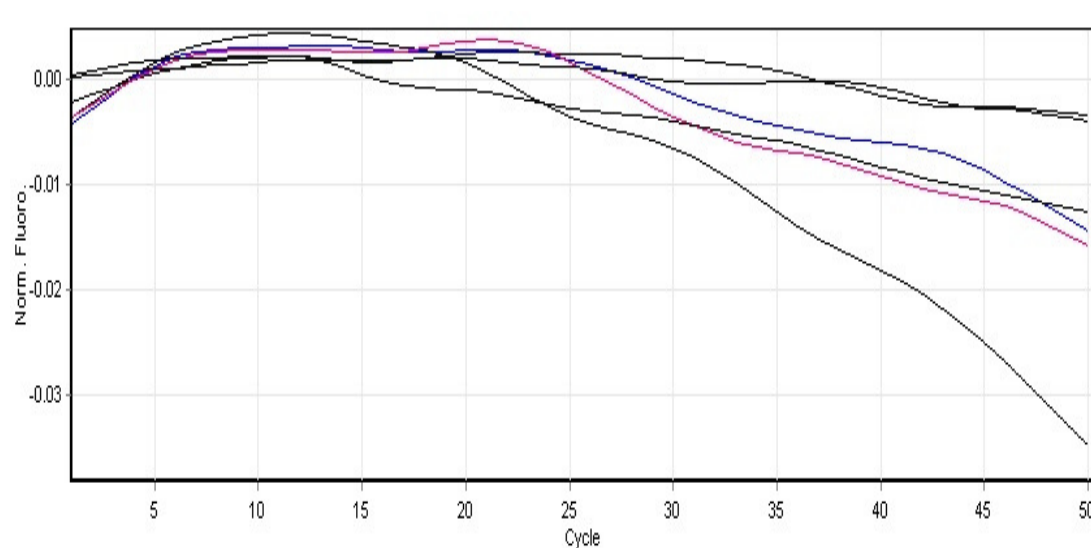
Figure 2.2: **RSV real-time PCR – Kuypers *et al*. (2004).** This real-time PCR was run according to the method of Kuypers *et al*. (2004) with positive controls, but did not work properly.

Another set of primers and a probe were obtained from Integrated DNA Technologies from the published sequence by Watzinger *et al*. (2004) (Table 2.6). These were reconstituted to a working concentration of 10 µM in the case of the primers and 40 µM for the probe. After confirming that concentrations used in the published article was correct by performing PCR runs with positive control for this purpose, the final concentrations of the forward primer, reverse primer and probe in each reaction were 900 nM, 300 nM and 200 nM respectively. A mastermix was set up (Table 2.9) and 19 µl of mastermix and 6 µl of known positive template were mixed in a PCR tube. Two non-template controls were added in each run where nuclease-free water was used instead of nucleic acid. This was placed on the Rotor-Gene 6000 with the cycling conditions in Table 2.10.

Table 2.9:   Mastermix setup for RSV Real-time PCR (Watzinger *et al*., 2004).

Reagent	Concentration μM	Concentration μM	μl per 1 rxn
	Stock	Final	
2 x uMM Buffer	2 x	1 x	12.50
FP forward primer	10	900	2.25
RP reverse primer	10	300	0.75
P probe	40	200	0.13
template			6.00
water			3.38
total volume μl			25.00

Table 2.10:   Cycling conditions for RSV real-time PCR (Watzinger *et al.*, 2004)

Author	Cycles	Time	Temperature (ºC)
Watzinger *et al.*, 2004		5 min	35
		10 min	95
	50x	3 sec	95
		10 sec	55

After optimisation, all samples were tested in duplicate and where inconclusive results were found, a third real-time PCR assay was performed.

2.8   Histology

2.8.1   Processing of tissue for histological examination

Tissue was fixed in formalin for at least 24 hours. Thereafter, it was trimmed into smaller pieces and put into histological cassettes as described under sections 2.5 (Sample Processing). The preparation of the wax blocks was done in a Tissue Tek VIP 5 Tissue Processor, which uses a graded series of ethanol, starting at 70% and ending with 100%. This was used to dehydrate the tissue, i.e. remove all water and fixative from the tissue. The dehydration fluid was replaced with xylene (Merck, Germany) by soaking the section in two changes of concentrated xylene, whereafter it was impregnated with paraffin wax (Paraplast Plus) (Merck, Germany) and embedded into a wax block manually.

Slides were prepared with the corresponding sample numbers and the wax blocks were cut into 3-5 μ sections on a MICROM Rotary microtome (Thermo Scientific,

Germany). These sections were floated out on a waterbath at 45ºC to prevent fold and cracks in the sections. Each section was mounted on positively charged Superfrost Plus slides (Thermo Scientific, Germany). The slides were then placed at 65°C for at least 20 minutes to ensure binding of the section to the slide and to melt all excess wax.

2.8.2   H&E Staining

H&E staining was done manually. Formalin pigment on these slides was not removed, since removal of the pigment is not routinely done. Slides were used also for routine medico-legal purposes and the standard protocol was therefore adhered to.

Slides were taken from the incubator and immediately immersed in xylene for 5 minutes. This was done to remove paraffin wax to ensure that the slide contents are mixable with water, since the dyes used are water-soluble. This process was repeated three times. It was then placed in decreasing concentrations of ethanol, starting with three changes 100% ethanol (Sigma Aldrich, USA) for 5 minutes each, followed by 96% and then 70% ethanol, each for 5 minutes. The slides were then placed in normal tap water at room temperature for 5 minutes to wash off all remaining ethanol, whereafter it was submerged in Mayers-Haematoxylin (Merck, Germany) for 5 minutes. This was rinsed with running tap water for 5 minutes and placed in 2.5% Eosin (Merck, Germany) for 1 minute, whereafter it was washed with running tap water again. The slides were subjected to rising concentrations of ethanol, starting with 5 minutes in 70% followed by 5 minutes in 96% and ending with 5 minutes in three changes of 100% ethanol. The final step was to transfer the slides to xylene, this was done in three changes for 5 minutes each to ensure proper washing and prevent carry-over of the ethanol. Coverslides were mounted on the slides with a resinous mounting media (DPX) (Merck, Germany). The slides were left for a few hours to dry at room temperature.

2.8.3   Optimisation

Optimisation and testing were done on the Leica BOND-MAX™ automated system (Leica Microsystems), using a Bond™ Polymer Refine Detection System. Positive controls were used for optimisation and included in each test batch of ten slides (nine tests and one control). A CMV positive control was obtained from Mrs Rochelle van

Wijk, Southern Cross Biotechnologies. Adenovirus and RSV controls were donated by Mr Ronald Houston, Anatomical Pathology Manager of Childlab, a Division of Nationwide Children's Hospital, USA.

Overfixation of some samples occurred due to prolonged storage in formalin. This resulted in the factory settings for antigen retrieval and staining not being used. Processes were therefore adjusted whereafter it was optimised. Formalin pigment, which is commonly found in post mortem tissue and is associated with overfixation due to prolonged periods in formalin, had to be removed on all slides. This was done using a picric acid (Merck, Germany) treatment and is described in section 2.8.4. All IHC slides, even those without formalin pigment, were subjected to this treatment prior to staining to ensure uniformity of the IHC process.

Heat-induced Epitope Retrieval (HIER) was used for antigen retrieval. The following protocols were used in the optimisation: ER-1 20 min; ER-2 20 min; ER-2 30 min and finally ER-2 40 min at 97ºC using Ethylenediaminetetraacetic Acid (EDTA)/citrate buffer. Antibodies were tested at a 1:100 and 1:50 dilution and were diluted in Bond Primary Antibody Diluent. .

The final protocol used for all three antibodies was a primary antibody dilution of 1:50 with an ER-2 40 min at 97ºC antigen retrieval step and an antibody incubation time of 50 minutes.

2.8.4   Treatment for removal of formalin pigment

Picric acid and absolute ethanol (Sigma Aldrich, USA) were used to remove formalin pigment, which was present in the tissue.

Slides were placed on a hot plate (60ºC) for 2 minutes to melt all excess wax and then further de-waxed by placing it in xylene for 5 minutes. They were then transferred to two changes of xylene, each for 5 minutes. In order to hydrate the tissue, slides were placed in three changes of 100% ethanol and then transferred to saturated picric acid to remove the pigment caused by formalin fixation. After 40 minutes, the slides were rinsed in 90% ethanol for 5 minutes, followed by another 5 minutes in 70% ethanol for hydration of the tissue. Finally, running tap water was used to wash the slides for 15 minutes before staining.

2.8.5 IHC Staining

After treating the slides to remove the formalin pigment, it was dried thoroughly with paper towel. Labels were printed using the BOND-MAX™ software and affixed to each slide. Every slide had therefore two identification numbers; the original identification number as described under section 2.5. (Sample Processing) and the BOND-MAX™ number used by the system to identify the different slides.

After labelling, slides were placed on a slide tray and five drops of Bond Wash Buffer was added to every slide. A covertile was placed on each slide. The covertile works through capillary action, spreading fluid evenly across the slide.

The full slide tray, as well as already diluted antibodies (described in Table 2.11), was then placed in the BOND-MAX™ system. The BOND-MAX™ protocol was followed for IHC staining with a prolonged antigen retrieval step of 40 minutes at 97ºC. The protocol was set on a computer containing the BOND-MAX™ software and the process was initiated.

Table 2.11: Antibodies used for IHC staining.

Antibody	Supplier	Optimised Retrieval	Retrieval Time & Temperature	Antibody dilution
CMV	Leica Microsystems	HIER ER-2[*]	40 min @ 97ºC	1:50
RSV	Leica Microsystems	HIER ER-2	40 min @ 97ºC	1:50
Adenovirus	Leica Microsystems	HIER ER-2	40 min @ 97ºC	1:50

*Bond Epitope Retrieval Solution 2 (EDTA based, pH 9)

Slides were taken from the BOND-MAX™ system, placed in three changes of 100% ethanol and transferred to three changes of xylene. Finally slides were placed in a Tissue-Tek® SCA (Sakwa) automated coverslipper and coverslips were attached to each slide, using xylene as adhesive.

2.8.6 Grading of H&E stains

All IHC and H&E stains were investigated by two blinded individuals, the primary researcher and a forensic pathologist.

Interstitial pneumonitis on H&E stains were graded as follows (for purposes of this study and in SUDI cases in general, grade 2 or 3 interstitial pneumonitis is seen to be severe enough to cause death) (Krous *et al*., 2003):

0. No interstitial pneumonitis present
1. Interstitial pneumonitis is hardly present, focal with no overall alteration of the alveolar septa
2. Interstitial pneumonitis is relatively diffuse with mild thickening of alveolar septa
3. Interstitial pneumonitis associated with interstitial oedema, diffuse involvement.
4. Diffuse infiltration associated with significantly widened alveolar septa.

2.9   Transmission Electron Microscopy

Three positive samples for each one of the viruses (CMV, adenovirus and RSV) and one negative sample (acting as negative control) were used for EM. Samples were considered positive if one or more of the aforementioned assays produced positive results.

2.9.1   Sample Preparation

Processing was done automatically in a Leica EMTP Processor and the protocol for processing by this manufacturer was followed (Leica Microsystems, Germany).

The processed tissue was then trimmed to an acceptable size to view under a light microscope after toluidine blue staining: these sections are slightly larger than the sections used for viewing under the electron microscope. A Resin Block Trimmer (Leica Microsystems, Germany) was used for this purpose. Slides were cut into 2 µm blocks using a newly made glass knife, made with a LKG 7800 Knifemaker, in a Reichert Ultra Microtome (Reichert, Austria). Cut sections were placed on a clean microscope slide and stained with toluidine blue.

2.9.2   Toluidine Blue Staining

A toluidine blue stain was done on sections of the different tissue to aid in the detection of possible viruses. The area in the tissue where infection is suspected should be used for the further processing of the sections used for EM since EM is directed at such a small target. Failure to use infected tissue will lead to false negative results and therefore the toluidine blue staining is used to detect areas of possible infection.

Slides were placed in sodium methoxid, prepared by dissolving 5 g Sodium in 100 ml methanol, for 1 minute, followed by two rinses in absolute ethanol (Sigma Aldrich, USA) to remove excess resin. Running water was used to rinse the slides for 1 minute, whereafter the sections were covered in toluidine blue and heated with a Bunsen burner for one minute to ensure proper penetration of the dye in the tissue. Slides were again rinsed with running water for one minute whereafter they were dried with filter paper and kept on a hot place (60º) until no water drops were visible. Coverslips were added to each slide, using DPX mounting media.

The toluidine blue stained slides were then visualised under a dissection microscope (American Optimal Corporation).

2.9.3   EM

After toluidine blue staining and visualisation of the slides under a light microscope, the processed tissue was trimmed down further, including areas of suspected infection, and sections were cut using a new glass knife. The sections were then placed on a copper mesh grip for viewing by means of a JEM-1011 electron microscope (JEOL, Japan).

2.10 Statistical Analysis

The statistical analysis for this study was done by Mr Justin Harvey from the Centre for Statistical Analysis, University of Stellenbosch.

Descriptive statistics were done using STATISTICA 9 and Kappa Statistics were performed using SAS version 9.1.

2.10.1   Descriptive Statistics

Frequency tables and histograms of the following variables were drawn: ethnicity, gender, presence of bedsharing, maternal smoking or alcohol use during pregnancy, prematurity, prone sleeping position, history of illness prior to death, post mortem interval, as well as histology, IHC, real-time PCR and shell vial results for each different virus (CMV, adenovirus and RSV). Histograms and frequency tables of either grade 1 or grade 2/3 interstitial pneumonitis were drawn as well. Results will be discussed in Chapter 3 and 4.

When a diagnosis of a grade 2/3 interstitial pneumonitis in the absence of any other positive findings could be made, this was strongly considered as being the most likely cause of death. Positivity on any of the three methods (shell vials, real-time PCR or IHC) was considered an overall positive for the presence of the virus. This positivity (referred to as 'mixed positivity') and the presence of grade 2/3 interstitial pneumonitis were cross tabulated in a 2-way summary table. The statistics thereof were calculated using the Pearson Chi-square and Fisher exact two tailed tests. Should a value of "0" exist the Pearson Chi-square test should not be used as this test is not considered to be accurate in the statistical analysis of "0"-values. A p-value of less than 0.05 is considered significant.

2.10.2   Kappa Statistics

The amount of agreement between tests was calculated using Kappa statistics. Results of every test (shell vials, real-time PCR, IHC, as well as histology) were compared with that of every other test for each virus. When there is no significant difference between the tests, the Kappa Coefficient will be high (1 = absolute agreement, 0 = no agreement). A Pr>S value (p-value) is considered significant (i.e. there is a significant difference between the two tests) if it is smaller than 0.05.

According to the statistical test there should be a difference in values of at least 10 between the positivity of the two tests being compared for it to be an accurate presentation of the results.

Chapter Three

3.   RESULTS

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Chapter Three

3. RESULTS

3.1 Introduction

A total of 82 samples were tested with three different methods and the results will be discussed in this chapter.

3.2 Retrospective Audit

The retrospective audit done on all SUDI cases from 2004 – 2006 revealed that only 14% (n = 51) of cases were positive for the presence of CMV, adenovirus and/or RSV out of a potential 366 cases. These positives included 46 positive CMV cases, six positive for adenovirus and only one for RSV with two being dual infections (CMV and adenovirus).

No information regarding demographic distribution of the cases in the retrospective audit was available to the researcher due to ethical constraints and this information could therefore not be included in the study.

3.3 Demographics – Prospective Study

The ethnic and gender distribution of the SUDI cases are presented in Figure 3.1.
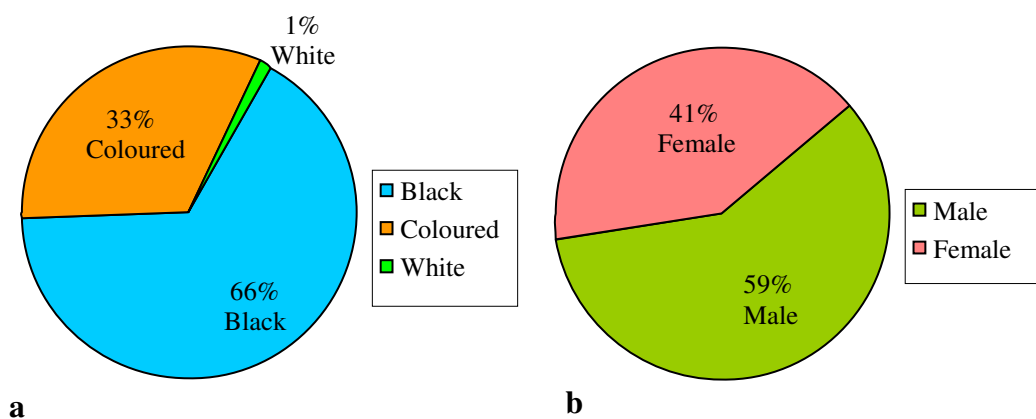
Figure 3.1(a) and (b): Ethnic and gender composition. As indicated by (a), two thirds of the total number of infants was black. The ratio between male and female, as indicated by (b) was more equal, although there was a slight male predominance.

The mean age of the infants was 11.6 ± 10.5 weeks (2.9 months) with ages ranging from

When the seasonal distribution of the study population is considered it is important to keep in mind that sample collection of this study started in September and ended in May, thus excluding winter. This was done because of time constraints of the study. Ideally such a study should run over more than one calendar year, to include all different seasons as well as seasonal infections (i.e. different strains of influenza) and also for comparing of the same seasons in different years. However, the seasonal distribution of this study included 24% (n = 20) cases of SUDI in spring, 30% (n = 25) cases in summer and 45% (n = 27) in autumn.

3.4   Shell vial cultures

3.4.1   CMV

Results from shell vial cultures identified three positive results for CMV (Figure 3.2), while the rest of the cultures were all negative. One shell vial culture was contaminated by bacteria and was discarded.

a   b
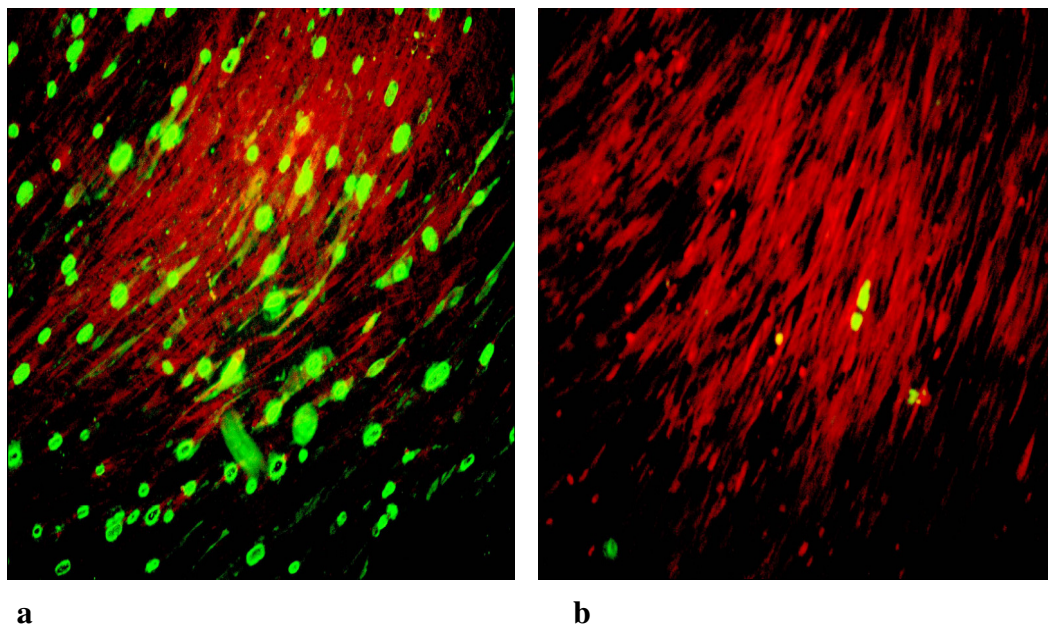
Figure 3.2(a) and (b): CMV shell vial cultures. Shell vial (a) shows several fluorescent nuclei, whereas (b) seems mostly negative, but with 4 fluorescent nuclei, therefore (b) will be repeated to confirm the result (Photographs provided by Ms Marna Blomerus, NHLS).

3.4.2 Adenovirus

None of the shell vials were positive for the presence of adenovirus (Figure 3.3). One of the shell vials (the same as the case in CMV) was contaminated with bacteria and was thus discarded.
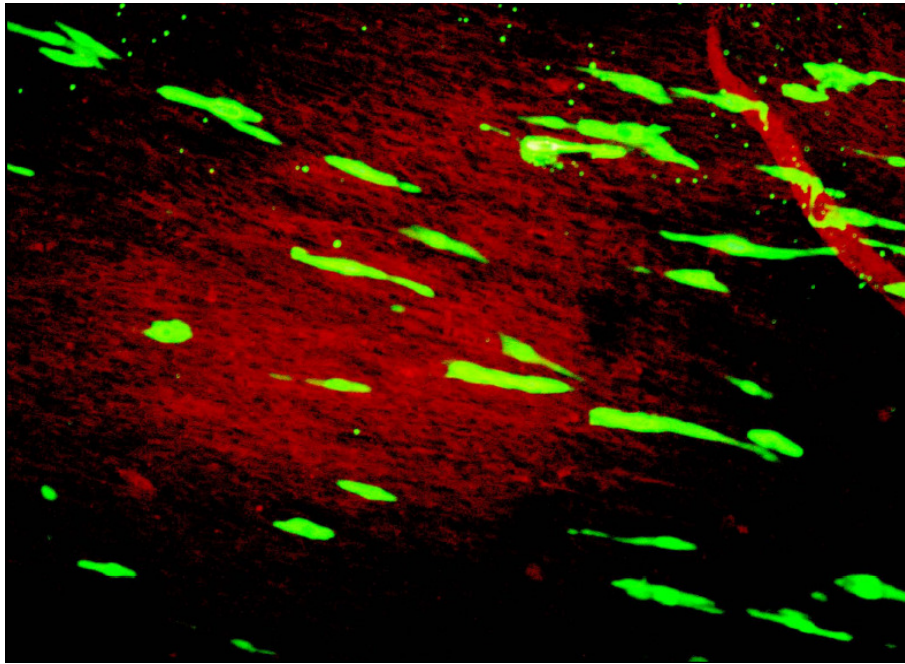
Figure 3.3: **Positive adenovirus on shell vials.** An example of a positive adenovirus shell vial (Photographs provided by Ms Marna Blomerus, NHLS).

3.4.3 RSV

One combi shell vial result was positive for the presence of a respiratory virus, but the viral load was too low to determine which specific virus it was.
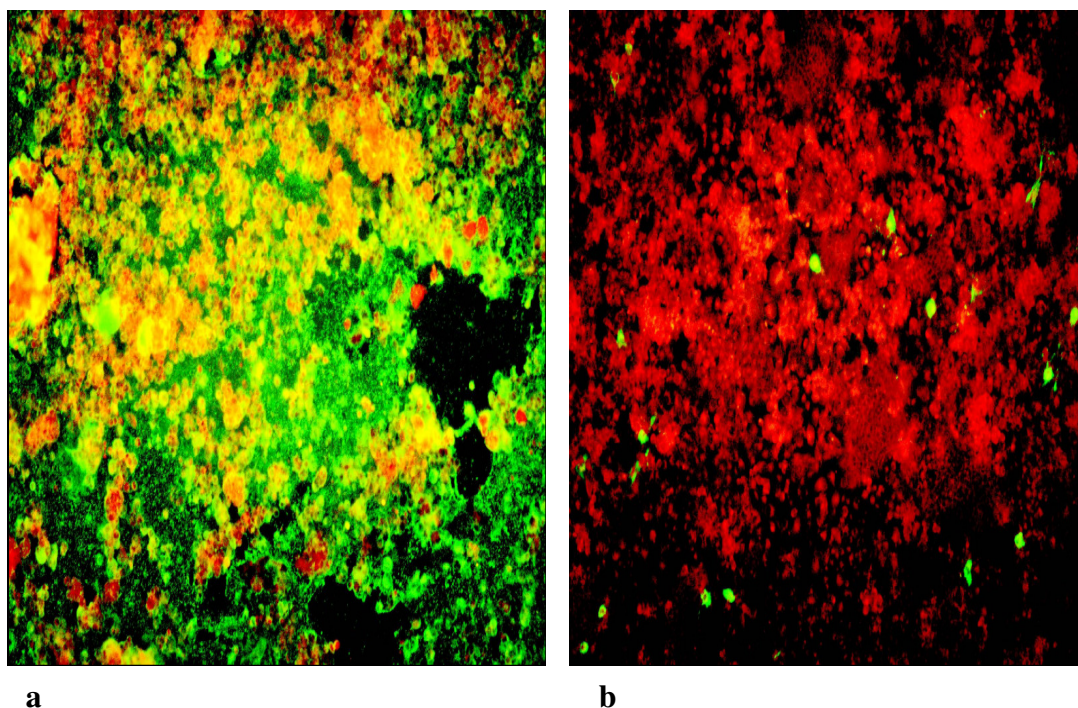
Figure 3.4(a) and (b): **Positive respiratory virus on combi shell vials.** (a) indicates a high positive and (b) a lower positive (Photographs provided by Ms Marna Blomerus, NHLS).

3.5 Real-time PCR

The results of the real-time PCR tests are discussed in this section.

3.5.1 DNA and RNA Concentrations

DNA concentrations were between 7.6 ng/µl and 247.6 ng/µl with a mean value of 61.1 ± 49 ng/µl. RNA concentrations were between 8.4 ng/µl and 545.1 ng/µl with a mean value of 97.3 ± 92 ng/µl. The complete results can be found in Addendum C. Examples of random DNA and RNA concentrations are shown in Figures 3.5 and 3.6.
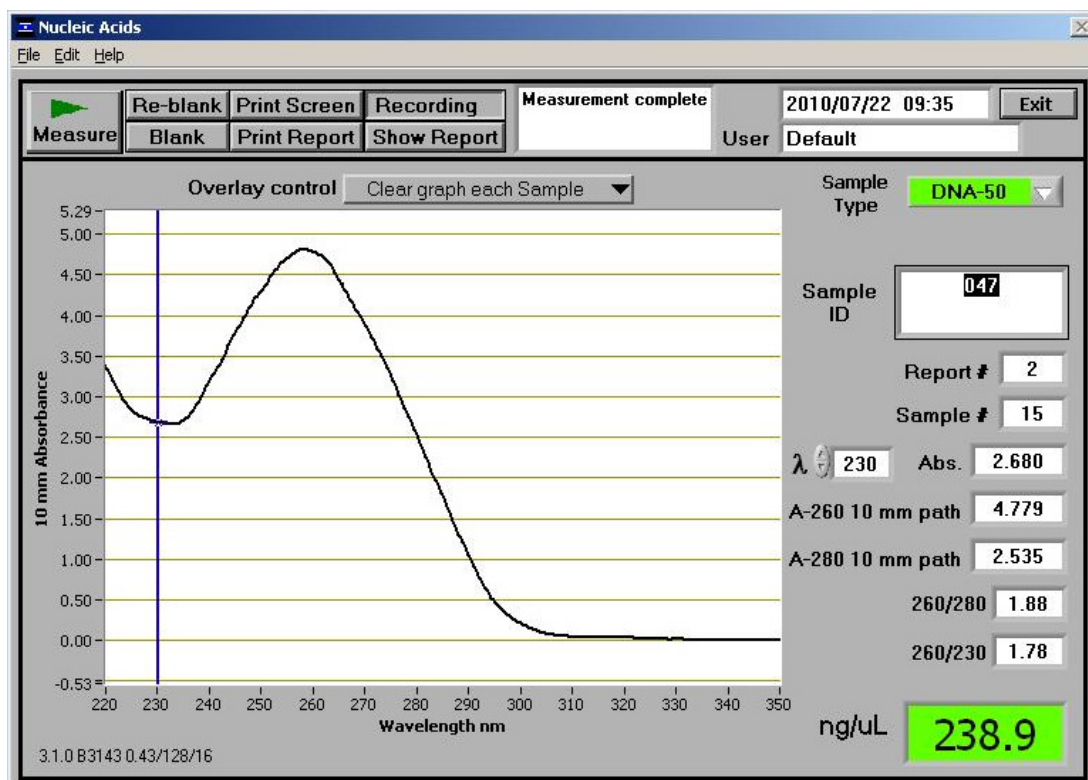
Figure 3.5: **DNA concentration.** Sample N046/02/09-047.
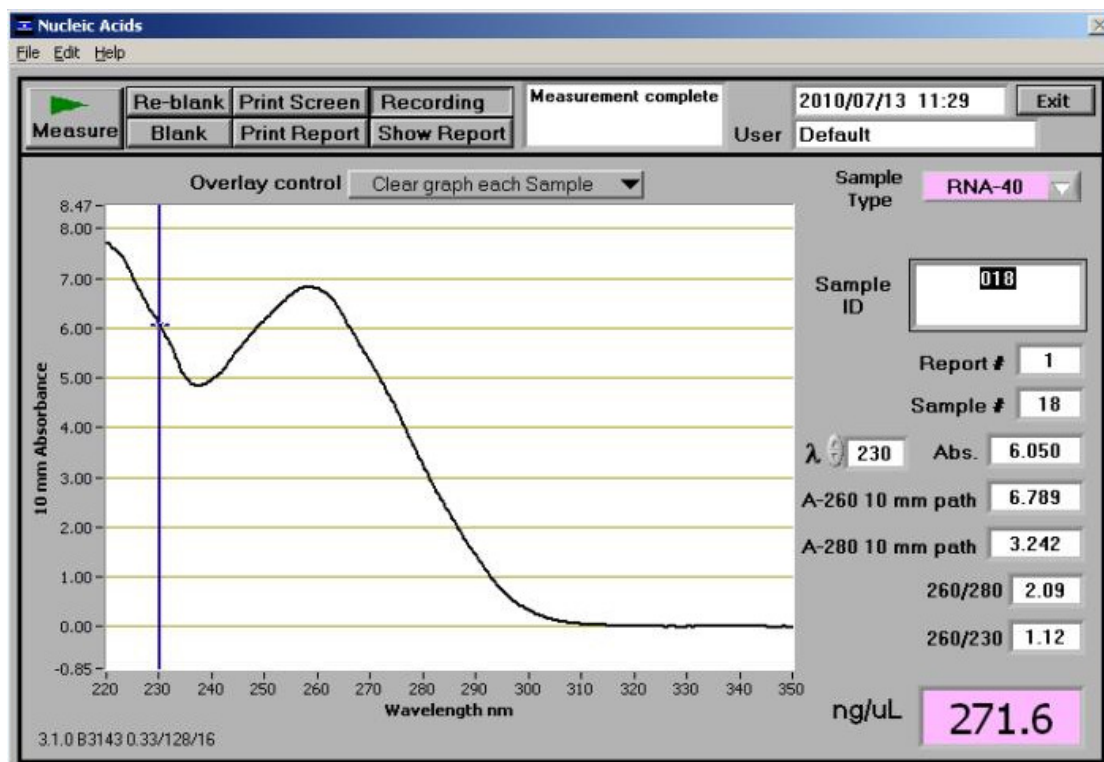
Figure 3.6: **RNA concentration.** Sample N046/02/09-018.

3.5.2   CMV

A total of 29 samples tested positive for CMV on real-time PCR, which was 35% of the total number of samples. Figure 3.7 is an example of the real-time PCR graph containing multiple positive results. A threshold was added just above the NTCs to simplify the process of determining which samples were negative compared to low positives. Ct-values of positive controls were consistently between cycles 23.30 and 32.39. Sample Ct-values were between cycles 18.54 and 39.27. The colours in which samples and controls are visualised were chosen at random.
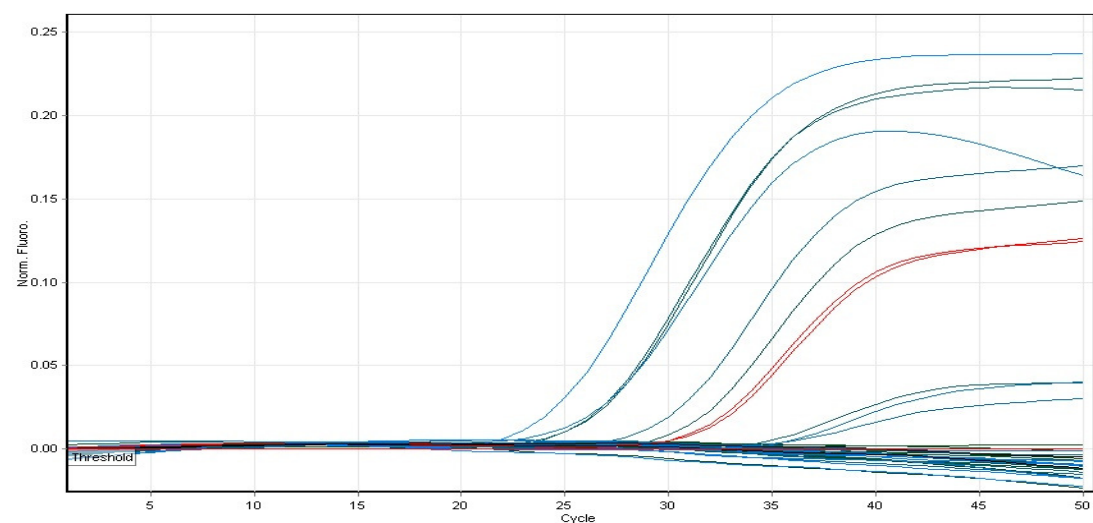
a
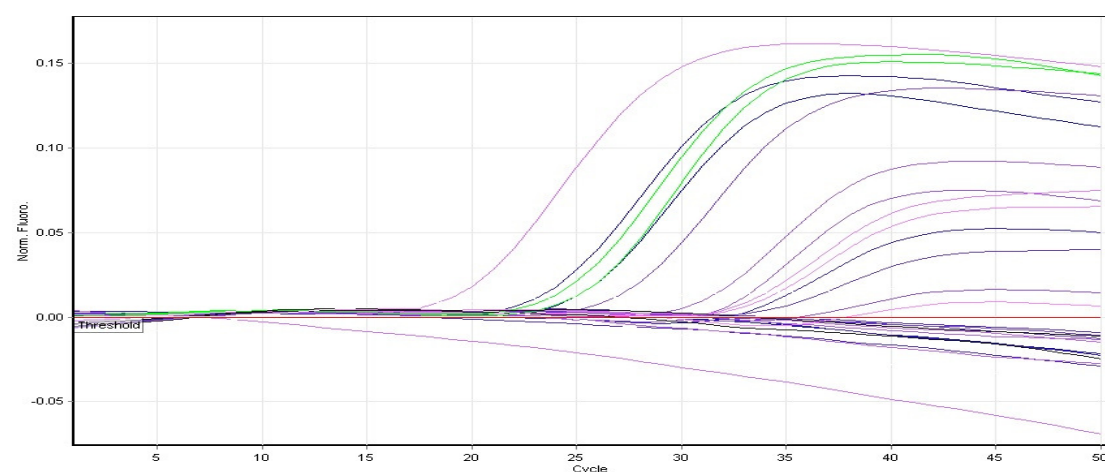
b

Figure 3.7(a) and (b): Multiple real-time PCR positive results for CMV. Where the curve exceeds the threshold and has a typical logarithmic curve. Straight or slightly curved lines toward the x-axis are

considered negative. The positive control is visualised in red (a) and green (b).

3.5.3 Adenovirus

Two samples (014- & 015-N046/02/09) tested positive on real-time PCR for adenovirus. Ct-values were between cycles 26.88 and 34.51 for positive controls and at cycles 33.80 and 43.20 for the two positive samples. Figure 3.8 shows the adenovirus real-time PCR run for three different concentrations of positive controls.
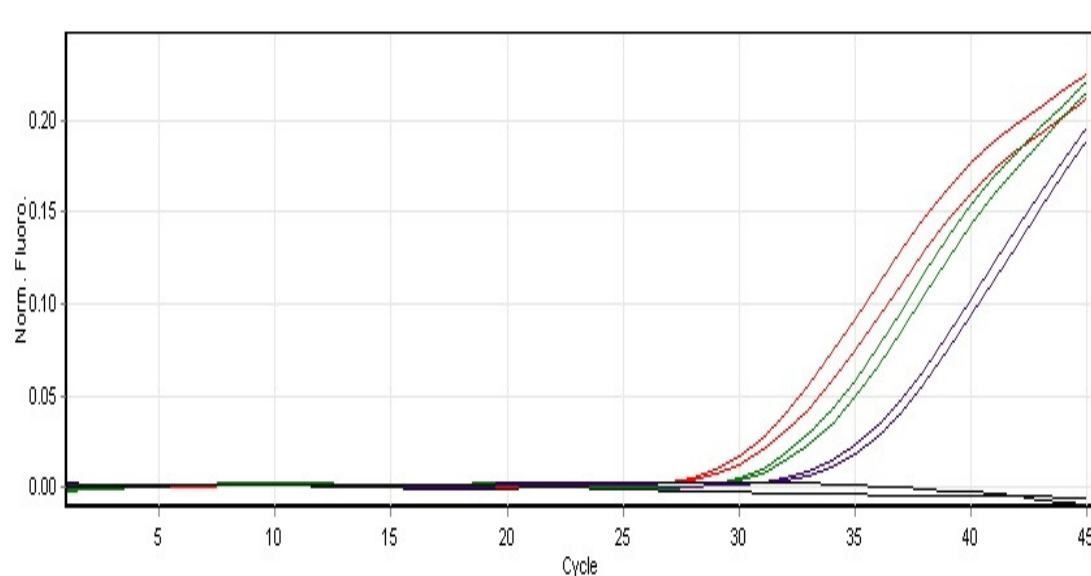
Figure 3.8: **Adenovirus real-time PCR – Positive controls.** A test run was done with three concentrations of positive controls to ensure validity of the PCR. Concentrations were 1:1, 1:10 and 1:100.

3.5.4 RSV

No positive results were found for RSV with the real-time PCR method. The Ct-values of the positive controls of the runs were between cycle 27.00 and cycle 38.16. Figure 3.9 shows results obtained from the Watzinger *et al*. (2004) real-time PCR method
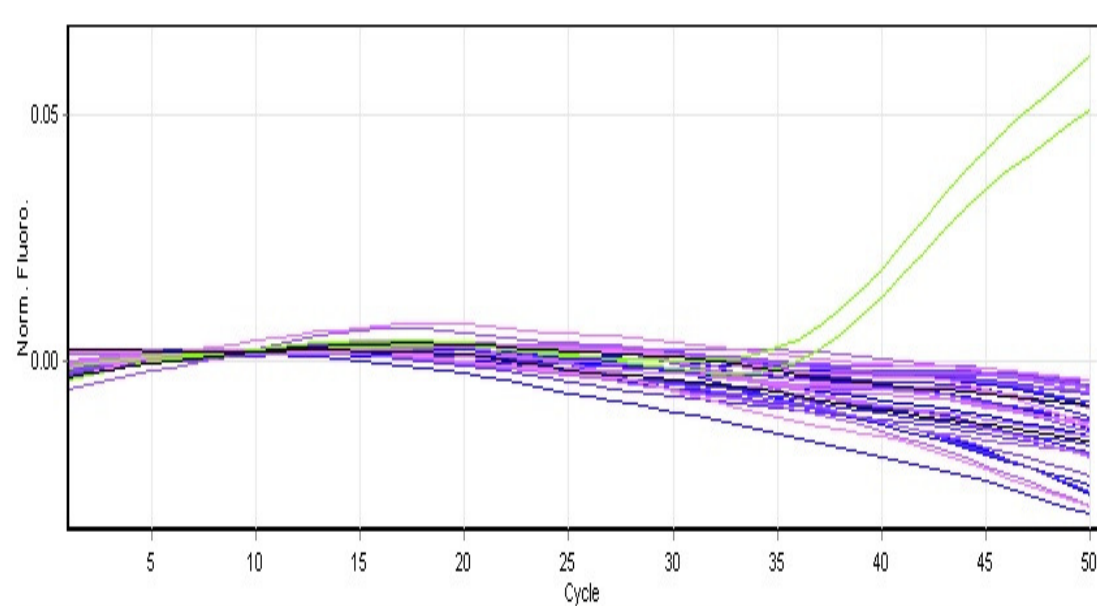
Figure 3.9: **RSV real-time PCR – Watzinger *et al*. (2004).** All samples tested negative. The positive controls can be seen in green.

3.6   Histology and IHC

3.6.1   H&E

Interstitial pneumonitis, severe enough to be considered a cause of death (i.e. grade 2 – 3) was present in 15% (n=12) of the cases. Other noteworthy histological changes can be found in Table 3.1.

Table 3.1:   **Distribution of histological changes in the LLL.**

Histological Condition	Percentage	n
Congestion	68	56
Oedema	37	30
Collapse	60	49
Alveolar Debris	15	12
Interstitial Alveolar Haemorrhage	18	15
Sloughing of lining epithelium	4	3
Formalin pigment	18	15
PM Organism overgrowth	11	9

3.6.2   CMV

Two samples were positive on IHC when examined for CMV. Inclusion bodies were evident in the histology and IHC of these cases (An example of a positive IHC stain on CMV tissue is shown in Figure 3.10 (a)).

3.6.3   Adenovirus

No samples were positive for adenovirus on IHC. The positive control used in IHC for adenovirus is shown in Figure 3.10 (b).

3.6.4   RSV

A total of four samples were positive for the presence of RSV on IHC. Figure 3.10 (c) shows the positive control for RSV on IHC.
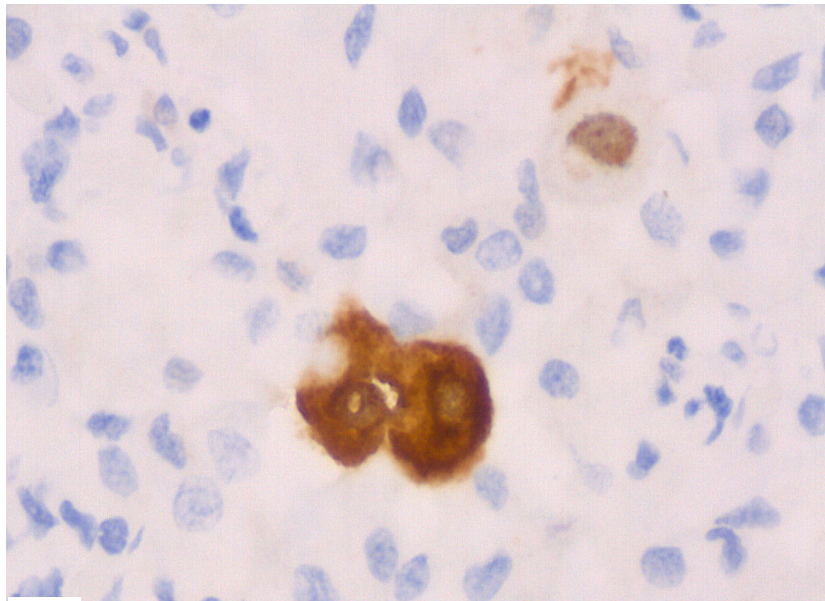
a
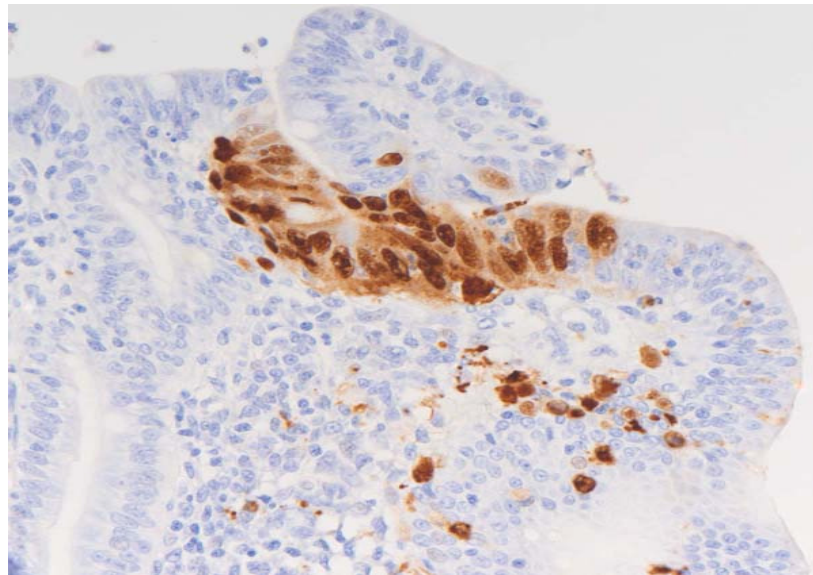
b
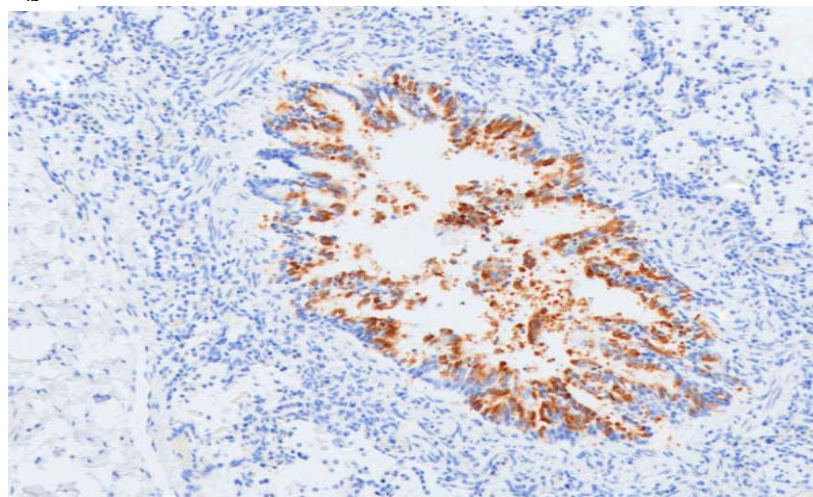
c

Figure 3.10(a), (b) and (c): IHC staining of positive tissues. An example of a positive CMV IHC stain can be seen in (a). The positive controls for adenovirus and RSV are shown in (b) and (c) respectively. (Figures (b) and (c) courtesy of Mr Ronald Houston, Childlab, Nationwide Children's Hospital, OH, USA)

3.7 Comparison of the three methods

Three samples tested positive for CMV with the shell vial method compared to 29 positives on real-time PCR and 2 positives on IHC. Two samples were positive for the presence of adenovirus by means of real-time PCR testing and four samples revealed the presence of RSV by means of IHC. These results, plus the presence of a grade 2/3

interstitial pneumonitis and the medical history (i.e. whether the infant was sick or displayed symptoms of illness prior to death), are summarised in Table 3.2.

Table 3.2: **Grade 2/3 Interstitial Pneumonitis and results of viral detection by different methods**

Sample Number	Gr2/3 Int Pneumonitis	CMV	Adeno	RSV	Sick prior to death
001-N046/02/09	No				**Yes**
002-N046/02/09	**Yes**				**Yes**
003-N046/02/09	No	SV**/PCR***			No
004-N046/02/09	No	PCR			No
005-N046/02/09	No	PCR			No
006-N046/02/09	No	PCR			No
007-N046/02/09	No	*BC	*BC	*BC	**Yes**
008-N046/02/09	No	SV/PCR			No
009-N046/02/09	No				No
010-N046/02/09	**Yes**	SV/PCR			No
011-N046/02/09	No				**Yes**
012-N046/02/09	No				No
013-N046/02/09	No				No
014-N046/02/09	No		PCR		No
015-N046/02/09	No		PCR		**Yes**
016-N046/02/09	No				No
017-N046/02/09	No				No
018-N046/02/09	No				No
019-N046/02/09	No				No

Sample Number	Gr2/3 Int Pneumonitis	CMV	Adeno	RSV	Sick Prior to Death
020-N046/02/09	No				No
021-N046/02/09	No	PCR			**Yes**
022-N046/02/09	No	PCR			No
023-N046/02/09	No				**Yes**
024-N046/02/09	No				No
025-N046/02/09	No	PCR			**Yes**
026-N046/02/09	No				No
027-N046/02/09	No				No
028-N046/02/09	No				No
029-N046/02/09	No				No
030-N046/02/09	**Yes**	PCR			**Yes**
031-N046/02/09	**Yes**				**Yes**
032-N046/02/09	No				**Yes**
033-N046/02/09	No				No
034-N046/02/09	No				No
035-N046/02/09	No				No
036-N046/02/09	**Yes**				No
037-N046/02/09	No				**Yes**
038-N046/02/09	No				No
039-N046/02/09	No				No
040-N046/02/09	No	PCR			No
041-N046/02/09	No				No
042-N046/02/09	No				No
043-N046/02/09	**Yes**	PCR			**Yes**
044-N046/02/09	No				No

Sample Number	Gr2/3 Int Pneumonitis	CMV	Adeno	RSV	Sick Prior to Death
045-N046/02/09	No	PCR			No
046-N046/02/09	No				**Yes**
047-N046/02/09	No				No
048-N046/02/09	**Yes**				No
049-N046/02/09	No	PCR			No
050-N046/02/09	No				**Yes**
051-N046/02/09	No				No
052-N046/02/09	No				**Yes**
053-N046/02/09	**Yes**	PCR		IHC	**Yes**
054-N046/02/09	No			IHC	No
055-N046/02/09	No	PCR			**Yes**
056-N046/02/09	No				**Yes**
057-N046/02/09	No				**Yes**
058-N046/02/09	**Yes**			IHC	No
059-N046/02/09	No				No
060-N046/02/09	No				No
061-N046/02/09	**Yes**	PCR			No
062-N046/02/09	No	PCR			No
063-N046/02/09	No	PCR			**Yes**
064-N046/02/09	No	PCR			**Yes**
065-N046/02/09	No				No
066-N046/02/09	No				**Yes**
067-N046/02/09	No				**Yes**
068-N046/02/09	No	PCR			No
069-N046/02/09	No				No

Sample Number	Gr2/3 Int Pneumonitis	CMV	Adeno	RSV	Sick Prior to Death
070-N046/02/09	**Yes**	PCR			No
071-N046/02/09	No	PCR			No
072-N046/02/09	No				No
073-N046/02/09	No	PCR		IHC	No
074-N046/02/09	No				No
075-N046/02/09	No				No
076-N046/02/09	No				No
077-N046/02/09	No				No
078-N046/02/09	No	PCR			No
079-N046/02/09	**Yes**	PCR			No
080-N046/02/09	No	PCR			No
081-N046/02/09	No	PCR			No
082-N046/02/09	No	PCR			No

* - Bacterial Contamination
**SV – Shell Vials
***PCR – Real-time PCR

3.8   Transmission Electron Microscopy

TEM was performed only on randomly selected positive samples, since this is a very expensive method, costing approximately R950 per sample. Sample number 003-N046/02/09 was used for CMV, 014-N046/02/09 for adenovirus (Figure 3.11) and 050-N046/02/09 for RSV. Although the latter sample was confirmed negative on real-time PCR, it was the only RSV sample to test positive once (once in three tests).
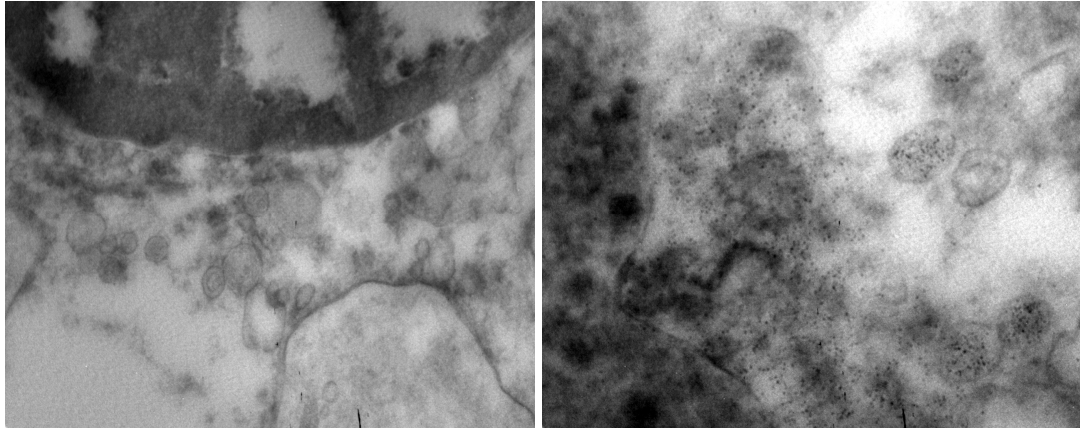
Figure 3.11: **TEM Micrographs for Adenovirus and CMV.** Due to autolysis of the tissue, individual viruses could not be recognised.

3.9 Statistical Analysis

3.9.1 Descriptive Statistics

3.9.1.1 CMV

A total of 7 cases had grade 2 or 3 interstitial pneumonitis and were positive for CMV. A Pearson chi-squared test resulted in a p-value of 0.06605 and the p-value of a Fisher exact, two tailed test was 0.09938. An interaction plot of each the 2-way summary tables were drawn (Figure 3.12). If the two lines intersect, an association is expected.
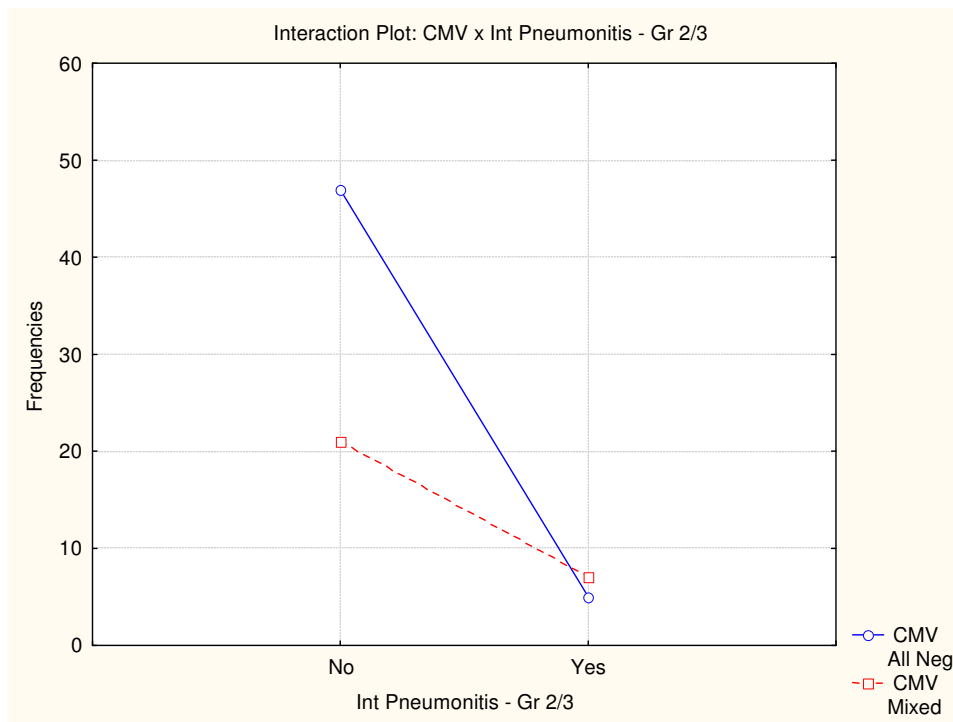
Figure 3.12:   Interaction Plot: CMV x Interstitial Pneumonitis Grade 2/3

3.9.1.2   Adenovirus and RSV

None of the cases had both interstitial pneumonitis and a confirmed adenovirus infection and only two cases were positive for RSV and had interstitial pneumonitis. No statistical significance could be found in either the Pearson Chi-squared or the Fisher exact, two-tailed tests. These results are evident in the interaction plots (Figure 3.13 and 3.14).
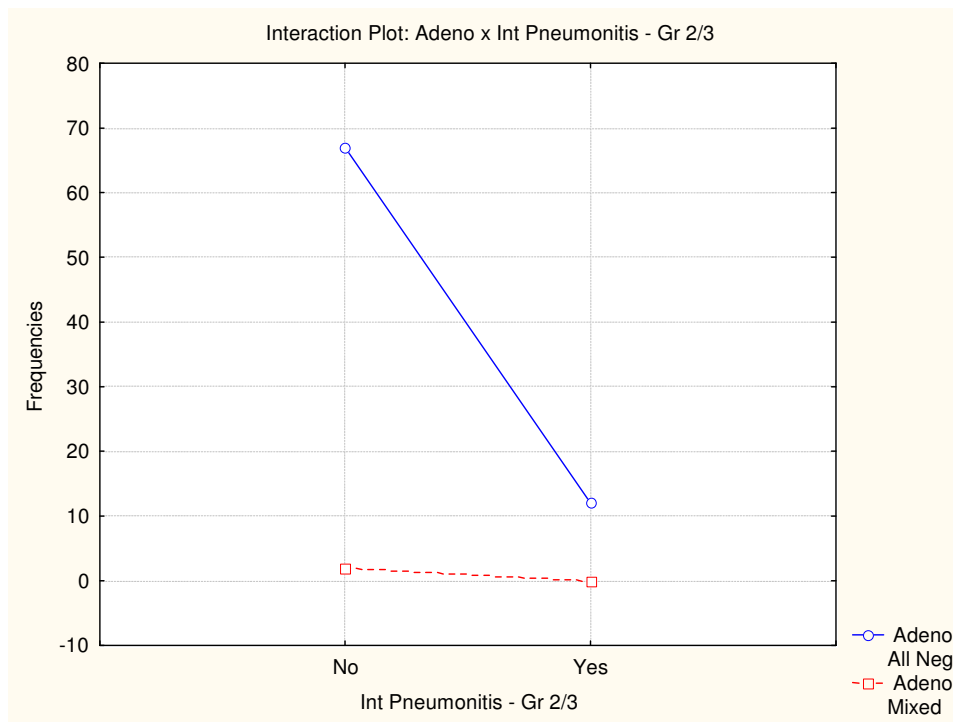
Figure 3.13:   Interaction Plot: Adenovirus x Interstitial Pneumonitis Grade 2/3
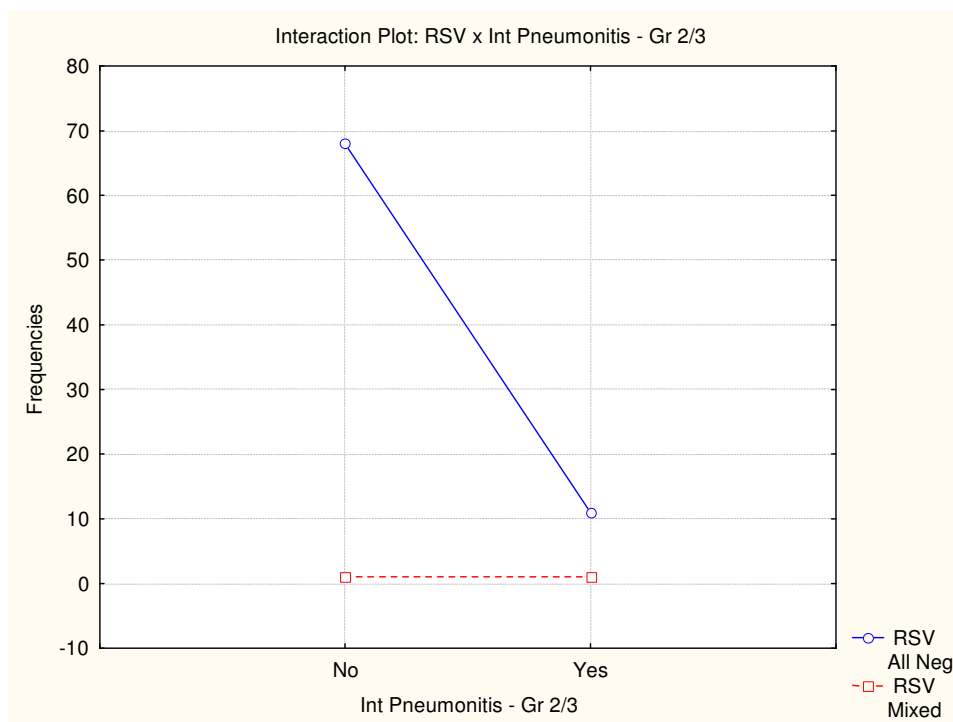
Figure 3.14:   Interaction Plot: RSV x Interstitial Pneumonitis Grade 2/3

3.9.2 Kappa Statistics

The amount of deviation between methods for adenovirus and RSV was too small to perform a statistical analysis. There were however significant differences between the methods for CMV (Table 3.5). Agreement between Histology, IHC and shell vials were good, but a highly significant was seen against real-time PCR.

Table 3.3:   Kappa Coefficient and P-values for method comparison – CMV

CMV Method Comparison	Kappa Coefficient	Pr > S (p-value)	Comment
IHC & Histology	0.6611	0.3173	No significant difference between methods.
qPCR & Histology	0.0438	< 0.0001	Significant difference between methods
Shell vials & Histology	-0.0191	0.3173	No significant difference between methods.
qPCR & IHC	0.0874	< 0.0001	Significant difference between methods
Shell vials & IHC	-0.0305	0.6547	No significant difference between methods.
Shell vials & qPCR	0.129	< 0.0001	Significant difference between methods

3.10 Time- and Cost Analysis

Formal quotes from the National Health Laboratory Services (NHLS) for the three methods used (shell vial cultures, real-time PCR and IHC) are attached (Addendums D and E).

The minimum time required for one test (including preparation time, incubation time and running time on the instrument), as well as the cost for each method, is indicated (including IHC processing) in Table 3.6. Although these times are for a single test, most samples are batched to save costs and time. The maximum time from when a sample is received until a result is available is also indicated.

An important factor which was not considered by this study, is the possibility of using multiplex real-time PCRs. This method will decrease the cost for the detection of viruses by means of real-time PCR considerably as more than one virus can be detected by one PCR. A single PCR might have a higher cost, due to the complexity of developing this type of PCR, but it will still be less expensive than performing

different PCRs for each different virus. More research into the development of multiplex PCRs for detection of multiple viruses in SUDI cases is thus definitely justified.

Table 3.4: **Time and cost analysis for diagnostic methods**

Method	Minimum Time Needed	Maximum Time Needed	Cost per sample (NHLS)
Shell vial culture	48 hours*	48 hours*	~R200 – R400
Real-time PCR	8 hours*	48 hours*	~R550
IHC (excl. processing)	24 hours**	48 hours**	~R380
IHC (incl. processing)	24 hours**	48 hours**	~R560

* Personal communication – Ms Mathilda Claassen, Chief Medical Technologist, NHLS

** Personal communication – Ms Charlene Williams, Immunohistochemistry laboratory manager, Pathcare

Chapter Four

4.   DISCUSSION AND CONCLUSION

4.1   Discussion

   4.1.1 Introduction

   4.1.2 Retrospective Audit

   4.1.3 Demographic Data

   4.1.4 Laboratory Assays

   4.1.5 Comparison of Laboratory Assays

   4.1.6 Histology – H&E

   4.1.7 Time and Cost Analysis

   4.1.8 Future Research

4.2   Conclusion

Chapter Four

4. DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Introduction

The objective of this study was to compare three different laboratory assays, i.e. shell vial cultures, real-time PCR and IHC, for the detection of three different viruses in lung tissue of SUDI cases. These viruses, CMV, adenovirus and RSV, are known to cause respiratory disease and pneumonitis in children and especially infants. It is possible that viral pneumonitis accounts for many deaths of infants younger than one year old, but that the current routine method, shell vial cultures, may not be sensitive enough to confirm positive infections (Erice *et al*., 1992; Mazulli *et al*., 1993; Pedneault *et al*., 1996). Therefore, many deaths are attributed to SIDS rather than having a known cause of death, even though there *is* a cause of death, but the method of detection is just not able to confirm it. Eighty-two (82) lung samples were collected from SUDI cases and were tested in parallel. The major findings will be discussed in this chapter.

4.1.2 Retrospective Audit

A difference between the retrospective audit and the prospective study for the amount of SUDI cases positive for adenovirus on shell vial cultures is evident. If one considers that the time delay between autopsy and laboratory tests were significantly lower during the prospective study, because SUDI samples were collected directly from the mortuary and taken to the laboratory for immediate testing instead of being couriered, this is quite an unexpected finding.

However, it must be taken into account that the retrospective study reviewed cases of three full years, including all seasons whereas the prospective study only reviewed cases seen in a nine month period. Thus, in the prospective study, no cases were collected during winter. It is unfortunately not possible to directly compare the number of cases per season with each other, as the date of death of babies included in the retrospective study is not available and therefore no conclusion about the season in which death occurred is available.

4.1.3   Demographic Data

The majority of SUDI cases (58%) in this study were male. This finding corresponds with the literature, indicating that male gender shows a slight predomination in SIDS cohorts (Kinney & Thach, 2009; Raza & Blackwell, 1999; Thach, 2008; Unger *et al.,* 2003). The reason for this phenomenon is unclear and further research into the reasons for the gender distribution is required.

Ethnic distribution included predominantly black infants with 66% and infants with mixed ancestry (coloured) making up 33% of the total. Only one infant (1%) was white. Certain ethnic groups are more prone to SIDS (Blackwell *et al*., 2004) and black infants and infants of mixed ancestry (coloured) are singled out by Kinney & Thach (2009) to be at higher risk than other infants. It is however postulated that this tendency is not the only reason responsible for the ethnic distribution in this particular study, but also the geographical area covered by the Tygerberg mortuary as areas such as Khayelitsha, Mfuleni and Delft, where a similar ethnic distribution can be found, are included in this region. These findings are however also similar to a different study performed in South Africa, which compared the demographic profiles of SUDI cases in Pretoria and Tygerberg (Du Toit-Prinsloo *et al*., 2010).

The mean age of the infants was 2.9±2.6 months and median age was 2 months. This is in accordance with the literature that SIDS deaths reach a peak at three months (Blackwell *et al*., 2004; Kinney & Thach, 2009). A possible explanation for the age distribution of SUDI cases could include that maternity leave is only two to three months after which mothers have to leave their babies at daycare centres etc. where there are sometimes multiple babies/children and individual attention is not given to infants. A more likely explanation is the maturation of the central nervous system (CNS) as well as different immune responses of the infant. Whatever possible reasons exist for the peak of SIDS cases between two and four months, it is clear that this is a multifactorial phenomenon.

Published literature shows that lower socio-economic circumstances places infants at higher risk for SIDS (Blackwell & Weir, 1999; Blackwell *et al*., 2004; Dwyer & Ponsonby, 2009; Kinney & Thach, 2009). There are many possible explanations for poor socio-economic circumstances being a risk factor; this includes malnutrition

which could play a role in acquiring infections, also exposure to multiple pathogens and even domestic violence, which is common in such settings. All SUDI cases were collected from the Tygerberg area with the largest number of cases (n = 24) obtained from Khayelitsha, a predominantly black township. Kraaifontein and Delft followed with ten cases each and Mfuleni contributed eight cases to the study. According to the 5 Year Integrated Development Plan published by the City of Cape Town in 2006, these areas are regarded as having lower socio-economic status.

In 65% of the cases (n = 53), bedsharing with up to three people was reported. Twenty-four percent (24%, n = 20) of infants were either put to sleep, or were found, on their stomach. Sleeping in the prone position, as well as bedsharing, has been recognised as a possible risk factor for SIDS (Bajanowski *et al.*, 2007; Dwyer & Ponsonby, 2009; Högberg & Bergström, 2000; Kinney & Thach, 2009; Mitchell, 2009). The Back-to-Sleep campaign, which was launched in 1994, therefore encouraged mothers to place their babies on their backs to sleep. Although the dangers of bedsharing and prone sleeping position are widely known, it is possible that many of the parents of the SUDI cases in this study are not aware of these dangers. As previously mentioned, the majority of SUDI cases in this study are from low socio-economic areas (5 Year Integrated Development Plan, City of Cape Town, 2006). New mothers might not be aware that it may be detrimental to place young infants in the prone position, or why they should not allow the infant to co-sleep with them. Another problem in these areas is overcrowding, where there is often only one bed available in a home and bedsharing is inevitable (Randall *et al*., 2009).

Even though all mothers were interviewed before the autopsy of their babies and asked whether they knew the dangers of cigarette smoke and alcohol use during pregnancy and all confirmed that they did, alcohol use and cigarette smoke during time of conception and pregnancy were reported in 24% (n = 20) and 29% (n = 24) of the cases respectively. One mother admitted to using other types of drugs, including marijuana and methamphetamine (street name: tik) during pregnancy. Maternal smoking is reported to be the most preventable risk factor for SUDI (Matturri *et al*., 2006) and therefore education of new mothers are of vital importance to prevent SUDI.

At least 28% (n = 23) of the cases were reported to be ill at least 24 hours prior to death. Illness included vomiting, rhinorrhoea, coughing, pyrexia and diarrhoea. Although these symptoms are probably not enough to independently cause death, the underlying cause (i.e. an infection) might be a contributing factor to death. Especially rhinorrhoea and coughing might be an indication of a respiratory infection, and proper screening for respiratory viruses is therefore very important. It is also important to keep in mind the three-hit model/triple risk hypothesis, which suggests that an infection might be the trigger of a genetic predisposition to SIDS (Opdal & Rognum, 2004).

4.1.4   Laboratory Assays

Bajanowski *et al.* (2007) recommended that two samples (one peripheral and one central) of each of the five lung lobes should be taken for histology in all SIDS cases. They further recommended that two samples be taken of the lung parenchyma and one of the lower trachea for IHC, PCR and other purposes, such as genetic testing (Bajanowski *et al.*, 2007). The current protocol at the Tygerberg mortuary is to take swabs from the left lower lung lobe for bacterial cultures and to send a piece of that same tissue, as well as a piece of liver, for virological testing. The lack of a national protocol for the investigation of SUDI cases in South Africa (Du Toit-Prinsloo *et al.*, 2010) leaves institutions to determine their own protocols in the handling of such cases.

Shell vial cultures are still regarded as the gold standard for respiratory virus detection in some settings (Lee *et al.*, 2010). This method was initially developed to speed up the detection of CMV, which can take up to 30 days on conventional cell cultures before the virus can be detected, but is now used for a variety of viruses including adenovirus, RSV and other respiratory viruses (Leland & Ginocchio, 2007). Shell vial cultures are however known to be less sensitive for the detection of CMV infections than conventional cell culture and other methods of detection (Erice *et al.*, 1992; Mazulli *et al.*, 1993; Pedneault *et al.*, 1996). This was also observed in this study when only three samples were found to be positive for CMV. No samples were positive for either adenovirus or RSV, which could mean that either all samples were truly negative for the presence of these viruses or that shell vial cultures are not sensitive and specific enough for the detection of these two viruses. The lack of a gold

standard for the investigation of viral infections specifically in SUDI cases makes it very difficult to interpret results such as these because there is no information about the sensitivity and specificity of the assay. Thus the agreement between methods is very important to make an educated guess as to which method is better suited for diagnostic purposes.

Considering the shell vial results for all three viruses, an infection could only be confirmed in three cases. Of these three positive viral infections, only one had an interstitial pneumonitis diagnosed by a forensic pathologist as seen on H&E stains, severe enough (i.e. grade 2 or 3) to cause death. Thus, only one case was successful in determining a possible cause of death. Virus infections, specifically CMV, adenovirus and RSV, have been mentioned numerous times to be prevalent in SUDI cases (Bajanowski *et al.*, 2007; Highet, 2008; Samuals, 2003; Weber *et al.*, 2010) and therefore a this low positivity rate raises questions about possible positive infections not being detected. The fact that post-mortem tissue is involved should be considered in the explanation of this low positivity due to the fact that degradation of tissue as a result of delays between death and the laboratory assay might hinder viral detection. There is however not currently a suitable solution for this problem and therefore the correlation of any positive results with H&E, which gives a picture of the pathology in the lung at the time of death, is of vital importance.

Even though shell vials have a very low positive yield which one would expect to be higher, this is the most inexpensive method currently available. In a resource limited country this is an important factor which carries a lot of weight in the decision of which method is best.

Real-time PCR is widely considered to be the gold standard for the detection of infectious pathogens (Álvarez-Lafuente *et al.*, 2008). It is quicker than conventional methods (Leland & Ginocchio, 2007) and has a higher sensitivity (Jeffery & Aarons, 2009), but is unfortunately a very expensive method. In our study, real-time PCR revealed far more positive CMV infections than any of the other methods and has, potentially, a much faster turnaround time than both shell vial cultures and IHC. The implication of a positive HHV, in this case CMV, should however be very carefully considered. As previously discussed, PCR methods can detect the presence of the virus, even though the infection is not clinically relevant. This could be due to either

the detection of a latent virus (i.e. asymptomatic period with little or no replication and no viral antigen expressed) or that of a reactivated virus. Even though reactivation in infants is not very likely since their young age would lead one to believe that infection is due to primary infection, this factor cannot be proved and thus cannot be discarded.

A total of 29 positive CMV cases were confirmed compared to two positive cases on shell vials and two on IHC. The latter methods did not indicate the same samples as being positive for CMV, but real-time PCR confirmed positives with both shell vials and IHC.

The difference between the different methods (considering positive yield) is statistically highly significant when real-time PCR is compared with either shell vial cultures or IHC (Kappa Statistical Test: Pr>S = 0.0001) and shows that there is not good agreement between real-time PCR and both the other methods of viral detection (Table 3.5). This highly significant p-value indicates that there is a great difference between the detection yields of the different methods, therefore illustrating that one method is detecting positives (true and/or false positives) the other is not. Again, it must be kept in mind that these positive results are not necessarily indicative of clinical disease, only of the presence of the virus in the body.

There are two possible reasons for the differences between the methods of viral detection. One could be that the specificity of the CMV real-time PCR assay is low and thus false positives are detected, leading to a very high number of seemingly positive CMV infections. However, this is not a likely explanation: the primers and probe for the CMV real-time PCR were obtained from a published article by Preiser *et al*. (2003) who specifically tested the assay against other HHV's to ensure that it has a high specificity, which was indeed the case. The fact that a specific probe is used in real-time PCR also increases the assay's specificity (Preiser *et al*., 2003). These facts therefore show that the CMV real-time PCR did not detect false positives and that a low specificity can therefore not be to blamed for the high number of positive CMV cases compared to the other two methods.

Another reason for the big difference in positive yields between methods is probably that the PCR method is able to detect not only active infections, but also latent

infections (Griffiths, 2009), an asymptomatic period where little or no replication of the virus occurs. Latent infections are not necessarily clinically relevant (Griffiths, 2009) and whether a latent CMV infection can actually contribute to death even though it did not cause death directly, is an unanswered question which requires further research (Dettmeyer *et al.*, 2008). It has been reported that PCR methods are often too sensitive for clinical use in the detection of CMV (Machida *et al.*, 2000). Our study confirmed that to identity a direct cause of death, real-time PCR used qualitatively is not optimal for the purpose of diagnosing CMV infections since there cannot be distinguished between active and latent infections and thus clinically relevant and non-relevant infeections. Therefore the clinical significance of a positive result is unknown. Conventional PCR would have the same problem, and therefore a possible solution would be to use real-time PCR quantitatively.

At the Forensic Pathology Division of Tygerberg Hospital, all results are correlated with the findings of the microscopic evaluation of H&E stains and positive virological results may be discarded if the H&E does not also indicate changes in the lung that suggest an infection. Using a method that detects latent infections will therefore not necessarily contribute to the overall diagnosis, because there will be no virological changes in the lung that can be detected on histology, without an active viral infection.

Seven of the 29 positive CMV cases showed, as diagnosed on histology slides, an interstitial pneumonitis of either grade 2 or 3, a pneumonitis severe enough to cause death. Although this is not a statistically significant value (Pearson Chi-square test: $p = 0.06605$), there could indeed be an association between the two factors (Figure 3.12), which should be confirmed in larger studies with more cases. However, it must also be kept in mind that, although more cases as seen with the other methods, still only a small percentage (24%, n = 7) of positive CMV infections (as tested by real-time PCR) had pneumonitis severe enough to cause death. This leads to the question of how specific this method is for the prediction of pneumonitis. If it was indeed as specific for the prediction of pneumonitis as initially thought, a much higher agreement between positivity and grade 2/3 interstitial pneumonitis would have been seen. Thus, the method might be very sensitive in detection of CMV but it might not

be very specific in the prediction of whether the CMV detected is relevant and therefore indicate non-relevant infection as being relevant.

Yet again, the possibility of association between CMV infection and severe interstitial pneumonitis, as well as the specificity of real-time PCR in the prediction of pneumonitis should be further researched to draw any significant conclusions. The ideal method in the detection of CMV in SUDI cases would be one with a high sensitivity and specificity, but also a high positive and negative predictive value.

Using real-time PCR quantitatively might be a good indicator of whether CMV can be held responsible for viral pneumonitis or whether there might be other causes to consider and therefore this method might solve some of the abovementioned problems.

Two samples were positive for adenovirus using qualitative real-time PCR but neither of these cases had a grade 2/3 interstitial pneumonitis. If the tests had been done for routine diagnostic purposes, the results would have been disregarded and not considered in the final diagnosis of death. This low positive yield of adenovirus in SUDI cases was also found in a previous study which used IHC and PCR for viral detection (Dettmeyer *et al*., 2004). This poses the question of whether adenovirus is not a virus commonly found in SUDI cases in a South African setting or whether none of the methods are ideal in the detection of adenovirus. It must also be kept in mind that no samples were collected during winter and this could explain why so few positives were detected, especially if it is compared with the retrospective audit where six cases of adenovirus were found in a three-year period. Since adenovirus is more prevalent during winter months (Echavarrìa, 2009) this is a plausible explanation.

No RSV could be confirmed with real-time PCR. The sensitivity of the primers and probe used might be in question, but since it forms part of a multiplex PCR for the detection of multiple viruses designed for use in a clinical setting (Watzinger *et al*., 2004), this is not a likely explanation. The fact that so few samples were positive for the presence of RSV by means of real-time PCR might indicate that RSV, similar to adenovirus, is not a virus commonly found in SUDI cases in a South African setting. The reason for this is unclear and this finding needs to be ratified by larger studies which consists of more cases, is multi-centred (and thus covers not only an area of the

Western Cape, but also all other provinces in South Africa) and that runs over several years (to include not only different calendar seasons, but different "flu seasons" as well).

IHC can be used as an aid in routine histological examination of H&E stains (Dettmeyer *et al*., 2008) and that was confirmed in this study where IHC detected slightly more CMV positives than routine H&E stains. Two samples were positive for the presence of CMV when tested with IHC and macrophages were clear in one and CMV inclusion bodies were clear in both cases. Both macrophages and inclusion bodies are typically found in CMV infections. One of the two cases also had a grade 2/3 interstitial pneumonitis. Therefore IHC, when testing for CMV, had similar results to shell vial cultures: contributing a possible cause of death due to CMV infection, to one case. IHC did not reveal the presence of adenovirus in any of the samples. Four samples were positive for the presence of RSV thus making IHC the only method that detected this virus in SUDI cases in this study.. However, the positive yield of this specific test was only 4.9% (n = 4), revealing that even though there is a difference between the detection rates, there is good agreement between IHC and the other methods used for the detection of RSV (Table 3.5). Half of the positive RSV cases (n = 2) also had a grade 2/3 interstitial pneumonitis indicating that a cause of death could be clarified in only two cases when using IHC as RSV detection method.

Consequently IHC is not ideal for the detection of viral infections in SUDI cases, detecting six infections out of all 82 cases. Only three of the 6 infections also had a grade 2/3 interstitial pneumonitis and the infection could possibly be considered the cause of death. The shortcomings of this particular study should however be kept in mind, including problems with overfixation and formalin pigment which is not likely to, but may have contributed to poor positive yields.

Even though IHC seemed to be the best method for the detection of RSV in SUDI cases since no other method detected the virus at all, there were only 4 positive RSV infections, which still revealed good agreement between the different methods (Table 3.5).

When considering the results of all three methods for the detection of adenovirus and RSV, it seems that these viruses are not commonly seen in the Tygerberg area in

SUDI cases. These viruses have been included in the routine protocol for years and no research of this type has been performed to prove or disprove the prevalence of the viruses in the Tygerberg area until now. This finding also correspondeds with the retrospective audit (Section 3.2) where six cases were positive for adenovirus and only one for RSV, over a period of three years. This indicates that the positive yield for adenovirus and RSV is low consistently over a period of time. All three methods used in this study are either gold standards for other detection purposes or known for its sensitivity and thus agreement between them, that the viruses are rare in this setting, should be seriously considered. The detection of adenovirus in two cases did not contribute at all to a possible diagnosis of death and only two RSV were recognised for its possible involvement in death. However, it must be kept in mind that this study has several limitations, including a low number of study subjects and that sample collection did not include winter, a month in which both adenovirus and RSV are highly prevalent ((Echavarrìa, 2009; Hall, 2009). Thus, the findings observed in this study should be confirmed, as previously mentioned, by studies with more subjects and over a period of several years.

When considering only H&E stains it is clear that, as has been discussed before, this method cannot be used on its own to determine what type of infection is present. It can only describe changes due to infection. The results of the H&E stains showed a high prevalence of collapse and congestion in the lungs. Other changes in the lungs were not significant in that it did not appear often or in most cases. Since congestion and collapse were highly prevalent, these findings were correlated with the medical histories as well as whether cases with congestion and/or collapse were positive for any viruses. The data revealed that infants who were sick prior to death had 27% (n = 16) collapse and 21% (n = 14) congestion. Viral infection together with congestion and/or collapse was less prevalent, with 29% and 23% respectively. Many of the structural changes in the lungs which were mentioned are frequently seen in SUDI cases and are not necessarily related to infection or a cause of death. The reasons for the changes occurring remains unclear.

When correlating the medical histories of the babies with the results of the different methods, if was found that in five cases of severe interstitial pneumonitis being present, the infants displayed symptoms of illness prior to death. Eight positive CMV

cases and one each of adenovirus and RSV were sick prior to death. These symptoms included vomiting, rhinorrhoea, coughing, pyrexia and diarrhoea. If these numbers are considered against the background of how many positive infections was found, it is not particularly surprising and only serves to underline the questions already asked: what role does infection play in SUDI and what causes the difference in sick infants that do not die of infection as opposed to SUDI cases.

4.1.5   Comparison of Laboratory Assays

All this information leads to very important questions. Is the method currently used, which includes histology being correlated with shell vial results, the ideal method for the detection of respiratory viruses in SUDI cases? If not, which different method/s should be used and is it economically justified? Furthermore, to what extent do viruses play a role in the causation of interstitial pneumonitis and are we in fact including the wrong viruses by excluding so many other potential viruses in the routine protocol?

When the positive yield of the three different laboratory tests is considered, the only test with a significant difference compared to the other methods is real-time PCR when testing for CMV. However, this technique reveals active as well as latent infections which might not necessarily be clinically relevant. Also, the problem of primary versus reactivated CMV infection still remains. Qualitative real-time PCR is not the recommended method for the detection of CMV in SUDI cases. Different methods, such as a viral load on real-time PCR or a PP65 antigen test (which detects the presence of the PP65 antigen which is present during the replication of the CMV virus and is thus an indication of an active infection), can be used to detect active infections will thus give more information regarding a specific case and whether a positive result in fact does have a clinical implication. A latent virus will result in a very low viral load in a real-time PCR test and this could be a good indication of whether infection contributed to death or not. There is no specific cut-off viral load value to determine whether a virus was the cause of death or not, but when it is considered in conjunction with histology, clinical information and pathologist opinion, viral load can be a very useful tool in the diagnosis of death.

Ideally, a method should exist that can solve all abovementioned problems. This would be a method that has a high positive predictive value (i.e. a positive result that is truly positive among all positive results) and a high negative predictive value (ie.e a negative result being truly negative among all detected negative results). This would then indicate whether positive viral infections have indeed played a role in death or not. However, on the basis of the methods examined in this study, this is not possible and therefore a combination of methods, such as which is currently used, might be the best option to try and detect as many true positive infections which played a role in causing death. Further research into this matter is thus justified.

4.1.6   Histology – H&E

H&E staining is routine in all SUDI autopsies. Not only are the lungs examined, but also all other organs which might reveal a possible cause of death, including the brain, gastrointestinal tract and liver. Many different architectural changes are visible in the H&E stains of lungs, which can include congestion, collapse, oedema, interstitial pneumonitis, pneumonia and other.

The histology of all SUDI specimens is thus very important and is used to interpret any results of additional tests that had been done on tissue, i.e. virology and microbiology tests. Should the findings of the additional test not correspond with what is found in the histology of the case, the additional results will be discarded and not considered in the final diagnosis of death. Architectural changes indicative of infection are often seen on histology, and further investigations such as virology and bacteriology might be able to prove the aetiology (Bajanowski *et al.*, 2007). Thus these tests, such as shell vial cultures etc. aid in the diagnosis of the case. An example is interstitial pneumonitis, which can be caused by viral infection but also by other factors. Therefore further tests are needed to confirm whether a virus was indeed the cause of the interstitial pneumonitis. Should a laboratory test reveal the presence of an infection, but this is not evident in the histology, it is concluded that the infection did not cause death and is merely a co-infection. This presence of viral infections, without them causing death, has been noted before in SUDI cases (Weber *et al.*, 2010). In this study, a grade 2/3 interstitial pneumonitis was found in twelve (14.6%) of the cases and eight of the 12 cases (66.7%) were positive for the presence of a viral infection on one or more methods. This indicates that the specific viral infection might have

indeed been responsible for the pneumonitis, which caused death in these cases. Furthermore it indicates that in four cases, the interstitial pneumonitis was not caused by a viral infection, thus showing how important it is to know whether a virus is present or not.

An H&E stain on its own and without the backing of other tests is not ideal in the detection of respiratory virus infections specifically, only for the changes due to viral infection. Only one stain was positive for CMV on histology and CMV inclusion bodies were clear in this case. None of the other stains revealed the presence of any of the other viruses. Histology is however a valuable tool in determining whether an infection was contributing towards death because architectural changes in the tissue can indicate whether the effects of infection were life-threatening or not.

4.1.7   Time and Cost Analysis

Although the turnaround time of a specific test is not of vital importance in SUDI since the results are not required for clinical management of a patient, it is still important that pathologists receive results as soon as possible to avoid an accumulation of case reports and results.

Although the processing of samples for shell vial cultures is relatively quick and easy, the incubation time of this method increases the overall turnaround time until a result is available to 48 hours. Although real-time PCR has the potential to show results within 8 hours, many results are only available after 48 hours (Table 3.6), because samples in a diagnostic setting are mostly tested in batches to save cost and time. IHC processing and staining for a typical SUDI sample also takes up to 48 hours until completion. The maximum time needed for any of the three methods is thus 48 hours.

Skilled laboratory staff is needed for all three methods, whether it is to subculture and interpret shell vial culture results microscopically, to set-up and run a real-time PCR and be able to interpret results, or to process samples for IHC (a process which is mostly done on automated systems) and interpret the results.

Table 3.6 summarises the cost of all three methods and formal quotations for the tests from the NHLS can be found in Addendums D and E.

The total cost for viral detection in one SUDI case according to the current protocol adds up to R584.54. This includes the pricing of two cultures and four shell vials (different cultures are used for CMV and adenovirus/RSV, plus two viruses are tested for simultaneously per shell vial).

The main disadvantage of using real-time PCR for diagnostic purposes is the high cost of the method. One test costs R547.68 and therefore the combined cost of testing for three viruses in SUDI cases, will be approximately R1600. However, an important factor that should be considered in the case of real-time PCR is the prospect of developing multiplex real-time PCRs. Although multiplex PCRs are complex to develop and the cost of a single run might increase slightly, the overall cost of the detection of multiple viruses will decrease considerably.

IHC staining costs R374.34 per sample, but if the processing of each sample is taken into account (i.e. processing tissue sample to wax block, cutting of slides etc.), the cost of one test is R561.51, making IHC the most expensive method of all the tests used in this study.

There is thus a significant difference in the costs of the different methods. This factor should be taken into account when considering which method is ideal to use in the detection of viruses in SUDI cases, especially in a resource-limited setting. Also, the applicability of the different methods and the cost thereof should be carefully considered as it will be futile to spend resources on methods that are not proven to be significantly better in the detection of viral infections in these cases.

The conclusion reached by this study would be to investigate the possibilities of quantitative multiplex PCRs for the detection of different respiratory viruses and then compare it with information currently available. This method could then include more viruses that are currently included in the routine protocol and more information regarding the viral load of positive infections will be available. Also, more research on the incidence of some of the viruses mentioned is required so that these viruses can stay included or be removed from the routine protocol.

4.1.8   Future research

Although it is known that CMV can cause interstitial pneumonitis (Smith *et al*., 1977), the association between CMV infection and severe interstitial pneumonitis, leading to death, should be further explored. Specific questions that need to be addressed include at which level of infection and at what specific viral load value is CMV considered to be able to cause interstitial pneumonitis severe enough to cause death; and why is CMV asymptomatic in some individuals but cause a severe pneumonitis in others. If there is a significant association between the amount of CMV infections that lead to severe interstitial pneumonitis and thus death, it is vital to detect this early in life to avoid infections severe enough to cause death. It is also important that an accurate method of detecting CMV infections is used in SUDI cases to obtain a clear result of whether the virus was the cause of death or not. It is evident that many problems exist with the detection of CMV regardless of which diagnostic method is used, and thus this is one of the most important issues that require attention and further research. A possible solution could be to make use of more than one method and use this combination to get a more realistic and trustworthy picture of what role CMV has played in death.

Another factor that might be useful is determining the prevalence of CMV in non-SUDI related infant deaths. If CMV is shown to be highly prevalent in these cases, it might indicate that CMV is not related to death. Yet, the triple-risk model (Opdal & Rognum, 2004) should always be kept in mind.

The literature mentions many other viruses, including HSV, VZV, EBV, HHV-6, influenza and parainfluenza viruses (Álvarez-Lafuente *et al*, 2008; Bajanowski *et al*, 2003; Raza & Blackwell, 1999) which do not form part of the current routine diagnostic investigation of SUDI cases in South Africa. Research on the incidence of these viruses in SUDI cases could prove very valuable to adjust the routine diagnosis for viral infections. The consequence of HIV should also be investigated to determine what influence this virus, specifically in South Africa, has on SUDI cases.

The possibility and development of a multiplex real-time PCR that include all viruses that are implicated in SUDI can prove to be very valuable since it will not only decrease the cost, but it will also save time, since different tests would not be needed.

Another important factor in future SUDI research is the need for a method or combination of methods that is specific not only for the viruses it tests for, but also for the prediction of whether a positive result is in fact meaningful.

It is important to remember that even though the presence of a viral agent might not be enough to cause severe illness and death, the mere presence could be the trigger that offsets the three-hit model/triple risk hypothesis (Opdal & Rognum, 2004). Therefore something that might not seem important, such as a mild viral infection which can even be completely asymptomatic in most individuals, can in fact contribute to death even if it didn't cause death directly. This definitely warrants further research into the effect the presence of a virus can have in a baby that is already predisposed to SUDI, especially if the three-hit model (Opdal & Rognum, 2004) is considered.

4.2 Conclusion

In cases of SUDI, no cause of death can be identified by only reviewing the history of the case or performing an autopsy. Any laboratory tests done are therefore of vital importance to aid in identifying potential causes of death, which will decrease the amount of cases that are classified as having no cause of death and thus falling into the SUDI category. A nationalised protocol of the investigations performed in SUDI cases is also vital, to ensure proper investigations and comparable results of SUDI cases across South Africa.

The lack of a gold standard for the detection of viral infections in SUDI cases makes it difficult to determine whether a different method is better suited for this diagnostic purpose. Specificity and sensitivity of the different methods cannot be determined without a gold standard because there is no method with known statistical measures for the new methods to be compared to (regarding true and false positive and negative results). Therefore only agreement between methods could be assessed without being able to determine whether one method is statistically better than another. All that is possible is making an educated guess, based on the results as well as previous research, to decide which method is better suited for this specific purpose.

If the results of all the tests are considered, it is clear that more research into the prevalence of the different viruses in SUDI cases of the Western Province, Tygerberg

are. Also, no single method seems to be ideal for the detection of any of the current viruses included in the routine protocol and therefore an investigation into which method or combination of methods is best, should be investigated.

Using this method in SUDI cases cannot be compared to the usage in other routine applications since the circumstances regarding the test differ greatly. One difference between the methods is the type of sample used: post-mortem lung tissue, which might already have begun degrading or might be overgrown by post mortem flora, is used in SUDI cases. Other samples, such as urine, stool, nasal and throat swabs and tracheal aspirates are obtained directly from a live patient to be used in other applications. Even though a direct comparison cannot be made, data mining of all shell vial results, in the same laboratory, over the same period as this study revealed that only 4.7% of cases were positive when tested for CMV, 3.3% showed an adenovirus infection and 5.7% of all combi shell vials were positive for RSV. These results correspond with the low positive yields found in this study, but leads to questions about the validity of the shell vial culture method.

The study further concludes that IHC might not be a good option for the routine detection of viruses either. Although this method revealed more RSV infections than any of the other two methods, the positive yield is still too low to justify the high cost of the method.

Real-time PCR might be a good option for the routine detection of respiratory viruses, but it is important to distinguish between active and latent viral infections. By using quantitative real-time PCR, not only the presence of a specific virus can be confirmed, but also the viral load. This approach should alleviate the problem of detecting latent viruses, such as HHVs. Latent viruses will cause a very low viral load, whereas active viruses will have a high viral load. This will also aid the pathologist in making a decision on whether a viral infection was severe enough to cause death. Unfortunately, real-time PCR is a very expensive method and thus investigation into the development of multiplex PCRs is required. Not only will it decrease the cost of the detection of different viruses, but more viruses can be included in the assay.

Currently, the routine diagnosis of viral infections in SUDI cases includes testing for CMV, adenovirus and RSV. As previously mentioned, these viruses are mentioned in

the literature to be prevalent in SUDI cases and therefore have been included in the routine protocol at Tygerberg mortuary. No research has been undertaken until now to determine whether these viruses are indeed prevalent in SUDI cases of this specific area. If the results of this study, as well as other published studies are taken into account, it seems adenovirus and RSV are not commonly found in SUDI cases, especially in a South African setting. These should however be confirmed by larger studies as this study was relatively small.

The major limitation of this study was the relatively small sample size of only 82 subjects. A larger study should be able to show clearer statistical differences between variables, such as CMV positivity and grade 2/3 interstitial pneumonia. Also, larger studies, over several years, might indicate whether adenovirus and RSV are indeed scarce in this particular setting, as well as what influence the different seasons have on viral prevalence. Another limitation was that only the left lower lung lobes of SUDI cases were used since this is the lobe routinely used for viral and bacterial detection. Obtaining and combining small samples from each lung lobe and using that for pathogen detection might give a better overall view of the flora found in the lungs. The lack of a gold standard in the routine testing for viral presence in SUDI cases at Tygerberg mortuary poses another limitation, because the sensitivity and specificity of the different methods used could not be calculated.

There are a few recommendations sprouting from this study: The first is to develop multiplex PCRs for the detection of viral infections in SUDI cases. More viruses can then be included and the overall cost of the assay will decrease. The second suggestion is to determine to what extent CMV is present in non-SUDI related deaths. This will answer important questions about whether CMV might be as involved in SUDI as suspected. Another related suggestion is further research on the relationship between CMV infection and severe interstitial pneumonitis and to what degree CMV is responsible for the presence of pneumonitis.

It is hoped that this study will contribute to the streamlining of investigation into the viral causes of death in SUDI, our institutional SIDS investigation protocol, as well as the standardisation of a national SIDS investigation protocol.

Chapter Five

5. REFERENCES

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List of Addendums

Addendum A: Ethics Approval (incl. waiver of consent) and extension.

Addendum B: Weight of left lower lung lobe of SUDI cases

Addendum C: DNA & RNA Concentrations

Addendum D: NHLS Quotations

Addendum E: NHLS Quotations

Addendum A: Ethics Approval (incl. waiver of consent) and extension

21 April 2009

MAILED

Ms MC Burger
Department of Pathology
Stellenbosch University
PO Box 19063
Tygerberg
7505

Dear Ms Burger

"Profiling the approach to the investigation of viral infections as a cause of death in sudden infant death cases-the Tygerberg Medico-legal Laboratory experience."

ETHICS REFERENCE NO: N09/02/046

RE : PROVISIONAL APPROVAL

It is my pleasure to inform you that the abovementioned project has been provisionally approved on 21 April 2009 for a period of one year from this date.   Waiver of individual consent has been granted. You may start with the project, but this approval will however be submitted at the next meeting of the Health Research Ethics Committee for ratification, after which we will contact you again.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary to make their final decision.

Please quote the abovementioned project number in all future correspondence.

Please note that a progress report (obtainable on the website of our Division) 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.

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).

21 April 2009 11:55

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Yours faithfully

MRS ELVIRA ROHLAND
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9677 / E-mail: elr@sun.ac.za
Fax: 021 931 3352

21 April 2009 11:55

Fakulteit Gesondheidswetenskappe · Faculty of Health Sciences

07 May 2010   **MAILED**

Ms MC Burger
Department of Pathology
Medical Virology
Tygerberg
7505

Dear Ms Burger

"Profiling the approach to the investigation of viral infections as a cause of death in sudden infant death cases-the Tygerberg Medico-legal Laboratory experience."

ETHICS REFERENCE NO: N09/02/046

RE : PROGRESS REPORT

At a meeting of the Health Research Ethics Committee that was held on 21 April 2010, the progress report for the abovementioned project has been approved and the study has been granted an extension for a period of one year from this date.

Please remember to submit progress reports in good time for annual renewal in the standard HREC format.

Approval Date: 21 April 2010   Expiry Date: 21 April 2011

Yours faithfully

MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
Fax: 021 931 3352

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Addendum B: Weight of left lower lung lobe of SUDI cases.

Study Number	LLL Weight (g)
N046/02/09-001	20.30
N046/02/09-002	21.31
N046/02/09-003	27.15
N046/02/09-004	15.82
N046/02/09-005	20.01
N046/02/09-006	11.04
N046/02/09-007	9.24
N046/02/09-008	19.89
N046/02/09-009	30.26
N046/02/09-010	31.09
N046/02/09-011	14.09
N046/02/09-012	15.50
N046/02/09-013	19.79
N046/02/09-014	33.52
N046/02/09-015	26.95
N046/02/09-016	21.63
N046/02/09-017	25.14
N046/02/09-018	12.51
N046/02/09-019	23.16
N046/02/09-020	9.45
N046/02/09-021	29.23
N046/02/09-022	7.63
N046/02/09-023	28.72
N046/02/09-024	23.69
N046/02/09-025	24.46
N046/02/09-026	30.33
N046/02/09-027	18.20
N046/02/09-028	15.74
N046/02/09-029	43.88
N046/02/09-030	33.54
N046/02/09-031	38.91
N046/02/09-032	16.98
N046/02/09-033	36.12
N046/02/09-034	15.93
N046/02/09-035	35.13
N046/02/09-036	26.48
N046/02/09-037	19.97
N046/02/09-038	24.71
N046/02/09-039	12.63
N046/02/09-040	13.36

Study Number	LLL Weight (g)
N046/02/09-041	15.06
N046/02/09-042	13.88
N046/02/09-043	31.6
N046/02/09-044	26.80
N046/02/09-045	10.66
N046/02/09-046	51.79
N046/02/09-047	22.53
N046/02/09-048	9.75
N046/02/09-049	23.16
N046/02/09-050	27.70
N046/02/09-051	19.46
N046/02/09-052	30.75
N046/02/09-053	14.15
N046/02/09-054	17.02
N046/02/09-055	22.34
N046/02/09-056	29.59
N046/02/09-057	29.05
N046/02/09-058	16.53
N046/02/09-059	10.10
N046/02/09-060	13.64
N046/02/09-061	19.37
N046/02/09-062	14.88
N046/02/09-063	24.49
N046/02/09-064	46.59
N046/02/09-065	19.90
N046/02/09-066	26.64
N046/02/09-067	27.27
N046/02/09-068	29.23
N046/02/09-069	25.84
N046/02/09-070	13.55
N046/02/09-071	N/A*
N046/02/09-072	25.38
N046/02/09-073	22.37
N046/02/09-074	36.40
N046/02/09-075	11.88
N046/02/09-076	12.98
N046/02/09-077	36.05
N046/02/09-078	32.48
N046/02/09-079	24.39
N046/02/09-080	27.21
N046/02/09-081	14.24
N046/02/09-082	12.82

* - Due to only a sample of the lung received from the mortuary instead of the entire LLL, the weight of the lobe could not be established.

Addendum C: **DNA & RNA Concentrations**

Sample Number	DNA concentration (ng/µl)	RNA concentration (ng/µl)
N046/02/09-001	85.7	545.1
N046/02/09-002	34.5	45.3
N046/02/09-003	53.1	128.1
N046/02/09-004	41.8	534.6
N046/02/09-005	88.1	156.6
N046/02/09-006	122.1	397.8
N046/02/09-007	92.7	97.3
N046/02/09-008	55.7	55.9
N046/02/09-009	42.3	30.6
N046/02/09-010	41.7	92.6
N046/02/09-011	43.9	86.4
N046/02/09-012	36.1	69.2
N046/02/09-013	116.1	118.2
N046/02/09-014	49.5	122.2
N046/02/09-015	37.2	86.1
N046/02/09-016	152.0	104.2
N046/02/09-017	42.8	31.6
N046/02/09-018	165.6	271.6
N046/02/09-019	25.2	72.9
N046/02/09-020	40.0	58.9
N046/02/09-021	53.3	160.8
N046/02/09-022	88.7	41.4
N046/02/09-023	55.1	68.1
N046/02/09-024	39.6	24.6
N046/02/09-025	33.3	66.9
N046/02/09-026	31.1	70.4
N046/02/09-027	55.4	94.4
N046/02/09-028	21.6	59.9
N046/02/09-029	13.7	97.9
N046/02/09-030	34.8	44.4
N046/02/09-031	6.4	46.2
N046/02/09-032	110.1	43.2
N046/02/09-033	7.6	53.4
N046/02/09-034	20.9	56.8
N046/02/09-035	11.3	40.4
N046/02/09-036	15.6	30.7
N046/02/09-037	14.1	112.9
N046/02/09-038	39.4	167.0
N046/02/09-039	35.8	63.1
N046/02/09-040	21.4	160.7
N046/02/09-041	22.2	40.1
N046/02/09-042	74.0	75.1
N046/02/09-043	51.3	208.7
N046/02/09-044	49.2	49.2
N046/02/09-045	82.1	77.4

Sample Number	DNA concentration (ng/µl)	RNA concentration (ng/µl)
N046/02/09-046	33.9	99.0
N046/02/09-047	238.9	62.5
N046/02/09-048	60.4	159.1
N046/02/09-049	83.4	77.2
N046/02/09-050	31.8	41.7
N046/02/09-051	17.0	48.1
N046/02/09-052	52.1	31.7
N046/02/09-053	41.6	94.0
N046/02/09-054	132.0	76.7
N046/02/09-055	38.2	64.3
N046/02/09-056	27.2	47.9
N046/02/09-057	121.1	119.6
N046/02/09-058	39.8	142.3
N046/02/09-059	36.9	48.4
N046/02/09-060	68.8	57.6
N046/02/09-061	247.6	129.8
N046/02/09-062	84.4	73.2
N046/02/09-063	43.5	176.6
N046/02/09-064	66.6	8.4
N046/02/09-065	44.8	29.8
N046/02/09-066	128.6	19.6
N046/02/09-067	30.3	38.5
N046/02/09-068	174.2	76.0
N046/02/09-069	41.0	133.8
N046/02/09-070	40.3	11.8
N046/02/09-071	20.9	91.0
N046/02/09-072	119.1	90.6
N046/02/09-073	153.0	172.4
N046/02/09-074	28.8	93.7
N046/02/09-075	35.2	166.8
N046/02/09-076	34.3	75.2
N046/02/09-077	157.7	91.1
N046/02/09-078	42.6	10.9
N046/02/09-079	20.2	72.5
N046/02/09-080	54.1	75.4
N046/02/09-081	29.2	16.6
N046/02/09-082	35.4	99.1

Addendum D: NHLS Quotations - Shell vial cultures & Real-time PCR

NATIONAL HEALTH LABORATORY SERVICE

9ᵗʰ Floor, West side
Tygerberg Hospital
Tel 021 938 4102
Fax 021 938 4457

E-mail: nanette.spencer@nhls.ac.za

To: Marilize Burger

Date: 30 June 2010
From: Nanette Spencer

Re: Quotation for Sudden Unexpected Death in Infants (SUDI) in the Western Cape Province

Dear Me Burger

I thank you for your enquiry regarding the analysis of laboratory specimens and have the pleasure in enclosing the following quotation.

TARIFF	TEST	FEES
Shell Vial Culture:		
5506 3897	Cell 1 Culture	R116.91
5510 3975	Shell Vial Culture Respiratory	R 87.68
5559 3975	Shell Vial Culture Adenovirus	R 87.68
5509 3975	Shell Vial Culture Cytomegalo	R 87.68
Viral PCR		
5563 3974	Cytomegalovirus PCR	R547.68
5564 3974	Virus PCR	R547.68

Billing is as follows:
Respiratory virus: 2 x culture + 2 x shell vial
Adenovirus: 2 x culture + 2 x shell vial
Cytomegalovirus: 1 x culture + 1 x shell vial

PRICES INCLUDE:
1. VAT
2. Distribution of results via phone, fax or post.
3. All prices are based on State Tariffs
4. Price increases are effective from 1ˢᵗ April each year

Please do not hesitate to contact me should you require any further assistance.

Yours sincerely

Mrs. NY Spencer
Business Manager
NHLS Tygerberg Hospital

Addendum E: NHLS Quotations - Processing and staining of IHC

NATIONAL HEALTH LABORATORY SERVICE

9ᵗʰ Floor, West side
Tygerberg Hospital
Tel 021 938 4102
Fax 021 938 4457

E-mail: nanette.spencer@nhls.ac.za

NATIONAL HEALTH
LABORATORY SERVICE

To:	Marlize Burger
Date:	22 October 2010
From:	Nanette Spencer
Re:	Quotation for CMV Immunohistochemistry on Lung tissue

Dear Me Burger

I thank you for your enquiry regarding the analysis of laboratory specimens and have the pleasure in enclosing the following quotation. Please note that this quote replaces the quote dated 18ᵗʰ July 2010

TEST	TARIFF	FEES
Immunohistochemistry Stain CMV (per test)	1565	R 374.34
Histology 1 Block (Including, processing, H&E stain and reporting)	1395	R 187.17

PRICES INCLUDE:
1. VAT
2. Distribution of results via phone, fax or post.
3. All prices are based on State Tariffs
4. Price increases are effective from 1ˢᵗ April each year

Please do not hesitate to contact me should you require any further assistance.

Yours sincerely

Mrs. NY Spencer
Business Manager
NHLS Tygerberg Hospital