Respiratory Pathogens in Cases of Sudden Unexpected Death in Infancy (SUDI) at Tygerberg Forensic Pathology Service Mortuary

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part, submitted it at any university for a degree.

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Signature

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SUMMARY

Background: Sudden infant death syndrome (SIDS) is considered the second most frequent cause of infant mortality worldwide. Research specifically pertaining to SIDS is limited in the South African setting. Identifiable causes for sudden infant death remain challenging despite full medico-legal investigations inclusive of autopsy, scene visit and ancillary studies. Viral infections could contribute to some sudden unexpected death in infancy (SUDI) cases, especially since a multitude of respiratory viruses have been detected from autopsy specimens. The specific contribution of viruses in the events preceding death, including the subsequent involvement of the immature immune response in infants, still warrants deciphering. Infancy is characterised by marked vulnerability to infections due to immaturities of their immune systems that may only resolve as infants grow older when these sudden deaths rarely still occur. In South Africa there is a lack of a standard protocol for investigations into the causes of SIDS, including the lack of standard guidelines as to which specimens should be taken, which viruses should be investigated and which laboratory assays should be utilised.

Objectives: In this prospective descriptive study we aimed to investigate the prevalence of viruses in SUDI and SIDS cases at Tygerberg Forensic Pathology Service (FPS) Mortuary over a one year period. The primary aim was to explore possible respiratory viral infections in SUDI and SIDS cases and to determine the usefulness of molecular techniques to detect viruses from SUDI cases. To determine the significance of viruses, we assessed signs of infection from lung histology. The secondary objectives included collecting demographic data to investigate possible risk factors for SUDI and to look for possible similarities between viruses confirmed in living hospitalised infants at Tygerberg, during the study period compared to viruses detected from SUDI cases.

Methods: Between May 2012 and May 2013 samples were collected from 148 SUDI cases presenting at Tygerberg FPS Mortuary. As part of the mandatory routine investigations into SUDI, shell vial culture (SVC) results were collected from lung and liver tissue specimens and bacterial culture results were collected from left and right lung and heart swabs at autopsy. To investigate the possibility of viruses implicated in some of the infant deaths we used the Seeplex® RV15 Ace detection multiplex polymerase chain reaction (PCR) assay to establish the frequency of 13 ribonucleic acid (RNA) respiratory viruses (influenza A and B, human parainfluenza 1-4, human coronavirus [OC43, 229E/NL63], human rhinovirus A, B and C, respiratory syncytial virus A and B, human enterovirus and human metapneumovirus)

from RNA extracted from tracheal and lower left and right lung lobe swabs. Tissue from the lower left and right lung lobes were also assessed for histology signs of infection.

Results: During our study we confirmed multiple known demographic risk factors for SIDS, such as the age peak around 1-3 months, the male predominance, bed-sharing, sleeping in the prone position, heavy wrapping in warm blankets, prenatal smoke exposure, and socio-economic factors. With the Seeplex® RV15 Ace detection assay between one and three viruses were detected in 59.5% (88/148) of cases. Of the 88 cases that had viruses detected, 75% (66/88) had one virus and 25% (22/88) had co-detections of two to three viruses. The most common viruses detected were HRV in 77% (68/88) of cases, RSV in 18% (16/88) of cases and HCoV in 14% (12/88) of cases. Many of the viruses we detected from our cases are included in the SVC test that forms part of the medico-legal laboratory investigation for all SUDI cases at Tygerberg FPS Mortuary. SVCs were positive in 9.5% (14/148) of all cases only. We showed that the SVC method is potentially missing most of the 13 respiratory viruses we investigated that could contribute to death in some of the SUDI cases.

Conclusion: In some cases that had a *Cause of Death Classification - SIDS*, the PCR viruses detected cannot be ignored, especially when it is supported by histological evidence of infection. We thus propose that the use of PCR could alter a *Cause of Death Classification* from *SIDS* to *Infection* in some of these cases. Further research is needed to determine the significance of detecting viruses from SUDI cases wherein no significant histological evidence of infection was observed. This questions whether PCR may be too sensitive and is detecting past and latent viral infections that do not play any role in the cause of death. The histological picture also requires further characterisation to determine if it accurately predicts infections or lethal events and can truly support virology findings, especially in young infants whose immune systems are still maturing. Without determining the true prevalence of viruses in SUDI cases and the viral-specific immune response, the contribution of virus-specific infections to this syndrome will remain largely undetermined.

OPSOMMING

Agtergrond: Wiegiedood ("SIDS/SUDI") word beskou as die tweede mees algemene oorsaak van sterftes in kinders jonger as een jaar wêreldwyd. Toegewyde *SIDS*-spesifieke navorsing in die Suid-Afrikaanse samelewing is beperk. Dit bly steeds 'n uitdaging om oorsake te probeer identifiseer vir hierdie onverwagte sterftes in kinders (*SUDI*) ten spyte van volledige medies-geregtelike ondersoeke, insluitende die lykskouing, ondersoek van die doodstoneel en aanvullende ondersoeke. Virusinfeksies kan aansienlik bydra tot sommige onverwagte sterftes in kinders, aangesien verskeie respiratoriese virusse alreeds aangetoon is in monsters verkry tydens outopsies. Die spesifieke rol wat virusse speel in die prosesse wat die dood voorafgaan, asook die bydraende rol van 'n onder-ontwikkelde immuunrespons in babas, regverdig verdere ondersoek. Die eerste jaar van lewe word gekenmerk deur verhoogde vatbaarheid vir infeksies weens die ontwikkelende immuunstelsels soos wat babas ouer word, en die voorkoms van *SUDI* neem stelselmatig af met 'n toename in ouderdom. In Suid-Afrika bestaan daar tans geen standaard protokol vir die ondersoek van wiegiedood nie en daar is ook nie standaard riglyne oor die tipe monsters wat geneem moet word, watter virusse ondersoek moet word en watter laboratorium toetse uitgevoer moet word nie.

Doelstellings: In hierdie prospektiewe beskrywende studie is gepoog om die virusse wat in gevalle van wiegiedood of *SUDI* voorkom te ondersoek. Die studie is uitgevoer by die Tygerberg Geregtelike Patologie Dienste lykshuis oor 'n tydperk van een jaar. Molekulêre tegnieke om virusse aan te toon in hierdie gevalle is gebruik om spesifieke virusinfeksies te ondersoek. Die resultate is met histologiese tekens van infeksie in longweefsel gekorreleer. Demografiese data is verder versamel om moontlike risikofaktore vir wiegiedood te ondersoek. Dit is verder vergelyk met virusse wat met dieselfde diagnostiese tegnieke in babas geïdentifiseer is wat tydens die studieperiode in Tygerberg Hospitaal opgeneem was met lugweginfeksies.

Metodes: Monsters van 148 *SUDI* gevalle wat by die Tygerberg lykshuis opgeneem is, is versamel tussen Mei 2012 en Mei 2013. As deel van die roetine ondersoeke in *SUDI* gevalle, was selkultuur resultate verkry van long en lewer weefsel, asook bakteriële kulture van deppers wat van beide longe en hart geneem was tydens die lykskouings. 'n Seeplex® RV15 Ace polimerase kettingreaksie (PKR) toets is gebruik om die teenwoordigheid van virusse te ondersoek wat moontlik by die babasterftes betrokke kon wees. Trageale- en longdeppers wat tydens die lykskouings versamel was, was getoets vir 13 ribonukleïensure (RNS) respiratoriese virusse (influenza A and B, human parainfluenza 1-4, human coronavirus

[OC43, 229E/NL63], human rhinovirus A, B and C, respiratory syncytial virus A and B, human enterovirus and human metapneumovirus).

Resultate: Ons studie het verskeie bekende demografiese risikofaktore vir *SUDI* bevestig, byvoorbeeld ‘n ouderdomspiek tussen een en drie maande ouderdom, manlike predominansie, deel van ‘n bed met ander persone, slaap posisie op die maag, styf toedraai in warm komberse, blootstelling aan sigaretrook voor geboorte en sosio-ekonomiese faktore. Die Seeplex® RV15 Ace toets het tussen een en drie virusse geïdentifiseer in 59.5% (88/148) van die gevalle. Uit die 88 gevalle waarin virusse opgespoor was, was selgs een virus in 75% (66/88) van gevalle gevind en twee en drie virusse in 25% (22/88). Die mees algemene virusse was HRV in 77% (68/88) van gevalle, RSV in 18% (16/88) van gevalle en HCoV in 14% (12/88) van gevalle. Baie van die virusse wat tydens hierdie studie ondersoek was, was ingesluit in die roetine selkultuur toets wat deel vorm van die standaard medies-geregtelike laboratoriumondersoeke in alle *SUDI* gevalle by die Tygerberg lykshuis, alhoewel die selkulture positief was in slegs 9.5% (14/148) van gevalle. Ons het gevind dat baie respiratoriese virusse potensieel gemisdiagnoseer word wat ‘n rol kon speel in of bydra tot die dood van sommige *SUDI* gevalle.

Gevolgtrekking: In sommige gevalle waarin *SIDS* geklassifiseer is as die oorsaak van dood, kan die virusse wat met PKR toetse opgespoor is nie geïgnoreer word nie, veral waar die bevinding ondersteun word deur histologiese bewyse van infeksie. Ons stel dus voor dat die gebruik van PKR toetse die oorsaak van dood klassifikasie kan verander van *SIDS* na *Infeksie* in sommige van hierdie gevalle. Verdere navorsing is nodig om die waarde van gelyktydige opsporing van virusse in SUDI gevalle te bepaal wanneer daar geen noemenswaardige histologiese bewyse van infeksie gevind word nie. Dit bevraagteken of die PKR toets dalk te sensitief is en gevolglik vorige en latente virusinfeksies identifiseer wat nie noodwendig 'n rol in die oorsaak van dood speel nie. Die diagnostiese en kliniese waarde van die histologiese beeld in terme van die rol van virusinfeksies as bydraende oorsaak van dood moet verder ondersoek word, veral in jong kinders wie se immuunstelsels nog nie volledig ontwikkel is nie. Indien die werklike voorkoms van virusse in *SUDI* gevalle en die virus-spesifieke immuunrespons nie bepaal word nie, sal die rol van virus-spesifieke infeksies in hierdie sindroom grootliks onbekend bly.

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Conference Proceedings

1. la Grange H, Verster J, Louw A, Thompson V, Bezuidenhout J, Dempers J, de Beer C. Assessing the efficacy of posters as educational intervention for pediatric sample collection in forensic pathology practice at the Tygerberg Medico-legal Mortuary.

   - Presented at: South African Association of Health Educationalists (SAAHE) Congress, 27 – 29 June 2013, The Gateway Hotel, Umhlanga Ridge, Durban (oral presentation).
   - Presented at: Faculty of Health Sciences 57th Annual Academic Day in the Centre for Health Professions Education category, August 2013, Tygerberg Campus, Cape Town (poster presentation). **Received reward for one of the best poster presentations.**

2. la Grange H, Dempers J, de Beer C. Investigation of viral respiratory pathogens in cases of sudden unexpected death in infants (SUDI) at the Tygerberg Medico-Legal Mortuary in the Western Cape.

   - Presented at: Faculty of Health Sciences 57th Annual Academic Day in the Pathology category, August 2013, Tygerberg Campus, Cape Town (oral presentation).

Manuscripts in Preparation

1. la Grange H, Verster J, Dempers J, de Beer C. Virology and Immunology in Sudden Unexpected Death in Infancy (SUDI). (Review article).

2. la Grange H, Verster J, Louw A, Thompson V, Bezuidenhout J, Dempers J, de Beer C. Assessing the Efficacy of Posters as Educational Intervention for Pediatric Sample Collection in Forensic Pathology Practice at the Tygerberg Medico-Legal Mortuary. (Research article).

Dedication

This thesis is dedicated to all the South African mothers whose infants have succumbed to SUDI and SIDS; and to the infants who lost their lives.

“What we have done for ourselves alone dies with us; what we have done for others and the world remains and is immortal.”

~Albert Pike

Table of Contents Page

CHAPTER THREE

CHAPTER FOUR

List of Abbreviations

AEC	Airway epithelial cells
AHC	Acute haemorrhagic conjunctivitis
aMPV	Avian metapneumovirus
APC	Antigen presenting cells
ARI	Acute respiratory infections
ATCC	American Type Culture Collection
BALT	Bronchus associated lymphoid tissue
Bp	Base pair
BSL3	Biosafety Level 3
CD	Cluster of differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic acid
FPO	Forensic Pathology Officer
FPS	Forensic Pathology Service
HA	Haemagglutinin
H&E	Haematoxylin and eosin
HAdV	Human adenovirus
HBoV	Human bocavirus
HCoV	Human coronavirus
HF	Human fibroblasts
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
HPIV	Human parainfluenza virus
HRV	Human rhinovirus
HSV	Herpes simplex virus
IC	Internal control
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
IMR	Infant mortality rate
IP	Interstitial pneumonitis
IQR	Interquartile range
LRTI	Lower respiratory tract infection
LRT	Lower respiratory tract
LTQS	Long QT syndrome
MDA-5	Melanoma-differentiation associated gene 5
MERS-CoV	Middle East respiratory syndrome coronavirus
NA	Neuraminidase
NAAT	Nucleic acid amplification test
NHLS	National Health Laboratory Service
NIBSC	National institute of Biological Standards and Control
NK	Natural killer
NTC	Non-template control
ORF	Open reading frame
PAMPS	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PMI	Post-mortem interval
PRR	Pattern recognition receptors
QC	Quality Control
QCMD	Quality Control for Molecular Diagnostics
RIG-1	Retinoic acid-induced gene 1
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
SANAS	South African National Accreditation System
SAQA	South African Qualifications Authority
SARS	Severe acute respiratory syndrome
SD	Standard Deviation
SIDS	Sudden infant death syndrome
SUDI	Sudden unexpected death in infancy
SUCD	Sudden unexpected childhood deaths
SVC	shell vial culture
TAE	Tris-acetate
TB	Tuberculosis

TLR	Toll-like receptors
TNF	Tumour necrosis factor
URT	Upper respiratory tract
URTI	Upper respiratory tract infections
USA	United States of America
UTM-RT	Universal transport media - room temperature
VTM	Viral transport media
VZV	Varicella zoster virus
5-HT	5-hydroxytryptamine

List of Figures Page

List of Tables Page

List of Addenda Page

CHAPTER ONE

INTRODUCTION

The health status of a country can be measured by infant mortality rate (IMR) which is the number of deaths in infants younger than one year of age occurring for every 1 000 live births (Reidpath & Allotey, 2003; Goutas *et al*., 2011). Causes of deaths in infants are a reflection of the economic development of a population, their general living conditions, their social wellbeing, the quality of the living environment and the rate of morbidity in that population (Reidpath & Allotey, 2003). These factors can affect infant death rates depending on variables encompassing socio-demographics, such as race, gender, level of education, annual income per household, as well as geographical and environmental conditions. Autopsy findings have provided fundamental information regarding the nature and causes surrounding deaths in infants. Goutas *et al*. (2011) named the four leading causes of worldwide infant mortality to be congenital abnormalities, Sudden infant death syndrome (SIDS) or otherwise known as “Cot death”, prematurity and low birth weight. Despite a decline in infant mortality rates of about 46% over the last few decades, in many regions SIDS is still considered one of the most frequent causes of infant death during the post neonatal period (Spencer & Logan, 2004; Moon *et al*., 2007; Hauck & Tanabe, 2008; Athanasakis *et al*., 2011; Goutas *et al*., 2011).

During 1974 to 1982, the SIDS incidence in Australia ranged from 1.7 to 2.1 per 1 000 live births (Williams *et al.*, 1984). Early mortality data collected by Malteno *et al.* (1989) from 1983 to 1984 found that approximately 3.8 SIDS cases occurred for every 1 000 live births in Cape Town, almost double that of the 1.7 to 2.1 found in Australia during the 1970s and 1980s (Williams *et al.*, 1984; Molteno *et al*., 1989). The exact incidence of SIDS in South Africa is uncertain (Saayman, 1992).

SIDS was first defined in 1969 at the Second International Conference on Causes of Sudden Death in Infants as "the sudden death of any infant or young child, which is unexpected by history, and in which a thorough post-mortem examination fails to demonstrate an adequate cause for death” (Willinger *et al*., 1991; Beckwith, 2003). The definition was revised in 1989 as “the sudden death of an infant under one year of age, which remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history” (Willinger *et al*., 1991; Beckwith, 2003). The 1989 definition included an accompanying statement; “cases failing to meet the standards of this definition, including those without a post-mortem investigation, should not be diagnosed as SIDS” (Willinger *et al*., 1991; Beckwith, 2003). Prior to any medico-legal

investigation, all sudden and unexpected infant deaths are categorised as sudden and unexpected death in infancy (SUDI), as is the case at the Western Cape Forensic Pathology Service (FPS) Mortuary at Tygerberg. Pulmonary infections are the most common diagnoses for a defined cause of death in SUDI cases in South Africa and many other centres in the world (Nicod, 2005; Weber *et al*., 2008a; Côté, 2010; du Toit-Prinsloo *et al.*, 2013).

All SUDI cases admitted to FPS Mortuaries in South Africa for examination and autopsy are investigated according to the auspices of the country's Inquests Act (58 of 1959). Despite a relatively well developed FPS, there exists no standardised *national* guidelines for the examination of these cases; neither at the seven large academic FPS Mortuaries, nor at a multitude of small, rural peripheral mortuaries (du Toit-Prinsloo *et al*., 2013). At the Tygerberg FPS Mortuary, all SUDI cases are investigated according to a standard *facility* protocol which includes autopsy, case note review, and laboratory investigations, including virology and microbiology, in an effort to identify possible causes of death. Toxicology and other tests are not performed routinely, but as indicated by case. Routine samples collected at autopsy at this facility for microbiology culture include sterile swabs from the left lower lung lobe, the right lower lung lobe and the heart. Virology samples include tissue from the lower left lung lobe, tissue from the liver and blood drawn from the base of the heart.

From a virological perspective, every SUDI case is subjected to a routine viral screen by the National Health Laboratory Service (NHLS) using a centrifugation enhanced Shell Vial Culture (SVC) technique. Lung and liver tissue from these cases are screened for human adenovirus (HAdV), cytomegalovirus (CMV), respiratory syncytial virus (RSV), influenza A and B, human parainfluenza (HPIV) 1, 2 and 3 and Human metapneumovirus (HMPV) as the standard procedure to determine natural infectious deaths. The role and contribution of viruses in the sequence of events that may lead to SUDI, including the systemic response to such infections, are not well defined; even so, SVC screening for only nine viruses may be inadequate. Culture techniques, although still considered the gold standard for isolation of respiratory viruses, lack the degree of sensitivity of nucleic acid amplification tests (NAATs) (Lee *et al.*, 2010).

Viruses are common in infants and can lead to acute respiratory infections (ARI) and fatalities (O'Callaghan-Gordo *et al*., 2011; Ghani *et al*., 2012; Kwofie *et al.*, 2012). Numerous RNA and deoxyribonucleic acid (DNA) respiratory viruses have been isolated from SUDI cases and new respiratory viruses are continuously being discovered (Bajanowski *et al*., 2003; Dettmeyer *et al.*, 2004; Fernández-Rodríguez *et al*., 2006; Álvarez-Lafuente *et al*., 2008; Prtak *et al*., 2010). This justifies the need for continual updated screening methods to ensure that viral respiratory infections contributing to such deaths are not missed. Exploring

alternative detection methods that will improve the accuracy of diagnosis and include a broader range of viral pathogens can provide crucial information in the search for answers. Questions that still remain include which viruses should routinely be investigated in SUDI cases and which techniques and types of specimens should be used. Routine viral screening focuses on RNA viruses at Tygerberg and because seven RNA viruses are included in the screen, and only two DNA viruses, we focused on the RNA viruses. Extensive international research has been conducted on various aspects of SUDI and the medico-legal investigations involved, but there is a limited amount of locally published literature available in South Africa (du Toit-Prinsloo *et al*., 2011; Douglas, 2012).

The primary aim of our study was to investigate respiratory pathogens and likely infections in SUDI cases at the Tygerberg FPS Mortuary in South Africa. Using the Seeplex® RV15 Ace detection assay (a multiplex polymerase chain reaction [PCR] assay), 13 respiratory ribonucleic acid (RNA) viruses were investigated as possible causes for viral pneumonia in SUDI cases. These viruses included influenza A and B, HPIV 1-4, human coronavirus (HCoV) OC43/HKU1 and 229E/NL63, human rhinovirus (HRV) A, B and C, RSV virus A and B, human enterovirus and HMPV. The clinical significance of pathogens detected was interpreted against the histological manifestations of infection in the lungs.

The background and literature review to follow will explore some of the important factors encompassing SUDI and SIDS investigations including respiratory pathogens and immunology that may play an important role in some of these cases.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER CONTENTS

2.1 Early History of SUDI and SIDS

The occurrence of SIDS, where no cause of death can be ascertained, spans centuries. The first historical case alluding to SIDS appears in the Book of Kings in the Bible, during the ruling of Solomon, where it was presumed that a woman had lost her infant due to overlaying (bed-sharing). Until the late twentieth century, the practice of bed-sharing between infants and adults, where the infants allegedly suffocate in their sleep, were previously thought to be the leading cause of infant mortality of unknown cause (Byard & Krous, 2003).

2.2 Definition of SUDI and SIDS

In more recent years a definite distinction has been made between the terms SIDS and SUDI (Krous, 2010). Prior to any medico-legal investigation the term SUDI is used, which is a heterogeneous category of death for all sudden and unexpected infant deaths. The purpose of the medico-legal investigation is to provide sufficient evidence to explain the death of the infant. Upon completion of the autopsy, the term *Borderline SIDS* is used when pathology changes are detected in SUDI cases that are presumed non-lethal or non-specific and insufficient to result in death. If a SUDI case remains unexplained after a thorough medico-legal investigation that includes reviewing the medical history of the deceased infant, the duration of gestation, delivery and postnatal development, the death scene investigation, the psychosocial history of the family and a complete autopsy, it is classified as *SIDS* (Bajanowski *et al*., 2007) (Figure 2.1).

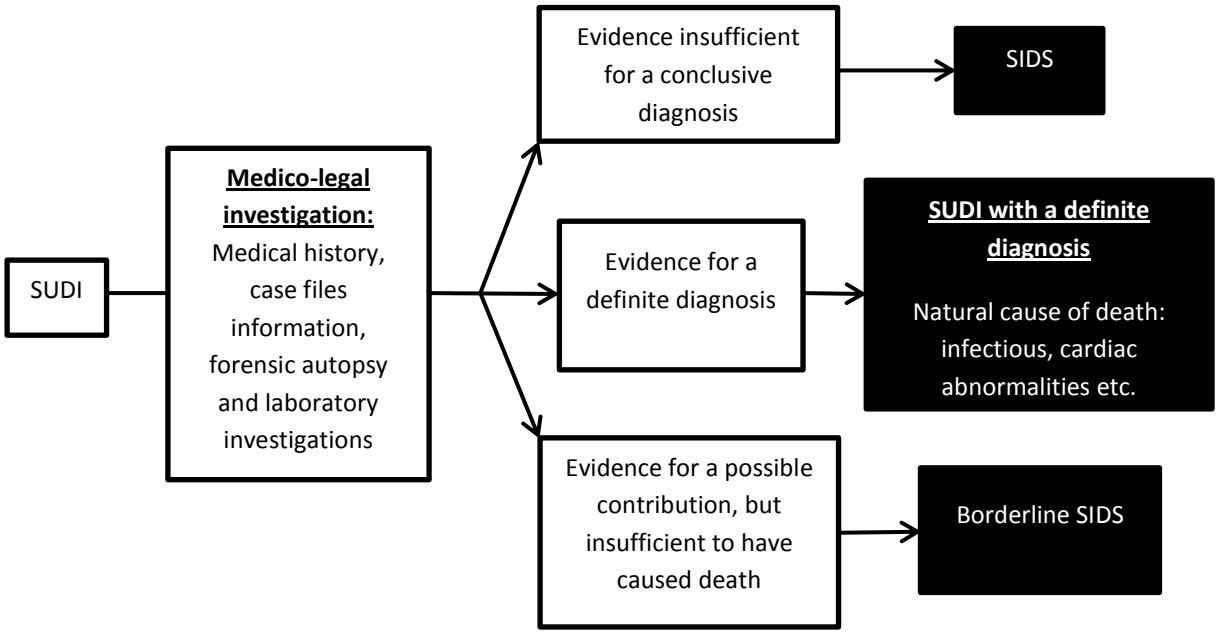


Figure 2.1: Categories for SUDI and SIDS

Thus, SIDS is a subcategory of SUDI that is diagnosed when the evidence fails to uncover anything out of the ordinary and after all the processes encompassing the medico-legal investigation have exhausted other causes of death. This indicates that the accuracy of the diagnosis strongly relies on the thoroughness of the medico-legal investigations (Beckwith, 2003; Bajanowski *et al.*, 2007; Krous, 2010).

The following categories were derived for SIDS cases meeting the requirements for the definition of SIDS including specific criteria (Krous *et al*., 2004):

Category IA SIDS:

- Age between 21 days and nine months
- Normal clinical history and term pregnancy
- No history of SIDS in the home and family environment
- Death scene investigation does not reveal possible explanations for the mode of death
- The autopsy does not reveal fatal pathological findings, but may reveal minor inflammatory infiltrates in the respiratory tract and although intrathoracic petechial haemorrhage is considered a supportive finding it is not considered to be obligatory or diagnostic in SIDS cases
- There must be no indications of abuse and neglect including unexplained or unintentional trauma or injuries and signs of negligence
- Substantial thymic stress should be absent and screening for microbiology, toxicology, vitreous chemistry, metabolic screening and radiology results should be negative

Category IB SIDS:

This category meets the criteria of Category IA, but excludes the death scene investigation and/or excludes one or more of the screening processes mentioned in Category IA.

Category II SIDS:

This category encompasses infant deaths that meet criteria of Categories IA and IB, but excludes one or more of the below mentioned:

- Age range between 0-21 days and/or up to one year
- History of SIDS in the home and family environment but excluding possible infanticide or genetic disorders

- Neonatal or perinatal conditions such as prematurity that had resolved prior to death
- Possible mechanical asphyxia or suffocation
- Abnormal growth and development and inflammatory disorders insufficient to contribute to death

Category Unclassified Sudden Infant Death:

Infant deaths in his Category fail to meet the criteria for Categories IA, IB and II, but which excludes definite diagnoses of natural or unnatural causes of death. This Category also includes cases where no autopsies were performed.

For the purpose of this thesis, SUDI will include all sudden and unexpected deaths, while SIDS will refer to cases where no diagnosis could be made after a complete and thorough medico-legal investigation and autopsy were performed, as per facility protocol. When literature is cited, the terms SUDI and SIDS will be used according to author definition and preference.

2.3 Medico-legal investigation into SUDI and SIDS

After many years of research the attempts to identify and confirm the cause of death for some of these cases are still unsuccessful, leaving SIDS a medical mystery in desperate need of elucidation (Koehler, 2010). Therefore medico-legal investigations into the causes of these deaths are warranted.

Although investigations into causes of infant deaths at forensic mortuaries conform to regional protocols, some of which are aligned with international protocols (most notably those at selected academic centres), a national standardised SUDI protocol for South Africa has not been formulated or adopted. Most of the current regional protocols include a review of the clinical history and the circumstances surrounding the event, as well as an autopsy, but rarely investigation of the death scene. Unfortunately, the degree of detail with which SUDI cases are investigated and the quality of the information collected, especially in developing countries, is largely dependent on the availability of resources, equipment and trained personnel at institutes (Koehler, 2010; du Toit-Prinsloo *et al.*, 2011). Therefore, investigations into the causes of SUDI and SIDS, including the limited information available pertaining to South Africa may currently, at best, be lacking.

Upon completion of medico-legal investigations, those deaths classified as natural causes include unexpected congenital abnormalities or malfunctions of bodily organs, and any

infections that were not diagnosed, recorded, or regarded as significant, by a treating physician.

Deaths defined as unnatural (Regulations Regarding the Rendering of Forensic Pathology Service, National Health Act [No. 61 of 2003] [N.R.929]) include amongst other factors physical injuries, trauma, abuse, homicide and any evidence of suffocation or aspiration of foreign material or gastric contents.

If unnatural causes of death can be ruled out after autopsy and death scene investigation, valuable community and forensic resources can be saved and the potential miscarriage of justice can be avoided. Furthermore, if contagious or pathogenic organisms, that could cause or contribute to natural death, can be confirmed after the autopsy with appropriate specimen collection and laboratory investigations, it could have significant implications for the improvement of public health (Dempers *et al.*, 2011).

A retrospective pilot study that involved reviewing SUDI case files from 2000 to 2004 at the Tygerberg FPS Mortuary in the Western Cape and Pretoria FPS Mortuary in Gauteng revealed prominent discrepancies between the standard SUDI investigation protocols for these two large academic facilities. At Tygerberg a full macroscopic autopsy was performed for almost all the SUDI cases and a detailed histological analysis was conducted in about 81% of the cases. At Pretoria a full macroscopic autopsy was performed in just over 60% of the cases and histology was done in only about 29% of the cases. Additionally, samples from about 60% of cases were screened for bacteria and viruses at Tygerberg, whereas Pretoria did not screen for bacteria and only performed viral screening in less than 3% of the cases (du Toit-Prinsloo *et al*., 2013). In a follow-up five-year multicentre study by du Toit-Prinsloo *et al*. (2013), Tygerberg conducted the most virology and microbiology investigations (57% of cases) compared to Pretoria's virology (31%) and microbiology (29%) investigations. Almost no virology and microbiology investigations were conducted at facilities in Durban, Bloemfontein and Johannesburg. Thus, Tygerberg appears to currently be the academic institute in South Africa where SUDI cases are most extensively investigated. Despite the thorough investigations at Tygerberg, another important component in SUDI investigations includes an examination of the death scene. The death scene investigation may, in some instances, reveal the cause of death and could also be crucial in the identification of hazardous environments for modification for future siblings of the same households (Dempers *et al.*, 2011). Such examinations were not performed during the four year study period at either institute (du Toit-Prinsloo *et al.*, 2011) and during the multicentre study, death scene investigations were only performed by pathologists in 0.5% of cases. The discrepancies between institutional protocols for macroscopic examinations and extent of

ancillary investigations such as virology and microbiology, may affect the final diagnostic categories for infant death classification (du Toit-Prinsloo *et al.*, 2011; du Toit-Prinsloo *et al*., 2013).

Multidisciplinary approaches are required where standardised protocols are considered crucial to ensure consistency of data collected (Mitchell *et al.*, 2000; Howatson, 2006; Prtak *et al.*, 2010). Therefore, a single ancillary piece of evidence cannot be relied upon in isolation in SUDI investigations. Despite the lack of universal protocols there has been a definite trend internationally towards employing standard protocols to improve the quality of SUDI investigations and diagnoses, because SIDS had been used in the past as a "conventional diagnostic dustbin" (Mitchell *et al.,* 2000). This implies that the diagnosis of SIDS has been used inaccurately for a diverse range of natural and unnatural infant deaths, mainly due to the complexity of identifying and distinguishing between various diagnoses. Continued efforts are needed to define and improve national infant death investigations to streamline accurate assessment and inter-institutional comparison of SUDI data. This would not only contribute to identifying and confirming risk factors, but also to increase the understanding and insight into the pathophysiological mechanisms involved in these infant deaths (Weber *et al.*, 2008a).

A case study by Dempers *et al.* (2011) highlighted the importance of a complete autopsy to diagnose tuberculosis (TB) in an infant, which lead to the identification of TB in a close family member. This case also emphasised the difficulty of recognising TB in infants, because they do not present with the same clinical symptoms as adults. If a thorough autopsy had not been performed and histology analysis including a specific bacteriology stain (Ziehl-Neelsen stain) that revealed acid fast bacilli, the cause of death might have remained undetermined and TB would not have been diagnosed in another family member during that period. If a complete autopsy is not conducted with the aid of ancillary investigations, infections contributing to or responsible for death in infants may easily go unnoticed. The need for an autopsy further becomes necessary when the immature state of the immune system in infants is considered. Due to immune system still developing, clinical symptoms of infections could possibly be so minor that they are not recognised by parents and doctors (Dempers *et al.*, 2011).

2.4 Suggested guidelines in the literature for SUDI and SIDS autopsies

Proposed guidelines for investigations into the causes of death include that SUDI autopsies should be performed according to a methodical protocol aided by distinct supplementary investigations and conducted by pathologists that are familiar with paediatric pathology. Ancillary investigations could include skeletal radiology, histology of the major organs,

microbiology and biochemical and metabolic analysis, but to include all of these tests may not always be practical in a resource-poor setting, such as South Africa.

It has been suggested that microbiological sampling during an autopsy should include heart blood collection into sterile syringes, followed by bronchial swab collection once the trachea and bronchi have been cut open from the thorax. Prior to lung sample collection the surfaces should be wiped with ethanol or seared to limit the likelihood of acquiring post-mortem contaminants. Swabs should then be taken from the lungs after a sterile incision is made into the lung surface. Bacteriological sampling can further include lung fluid, tissue samples and colonic swabs (Howatson, 2006; Prtak *et al.*, 2010).

Virological sampling could include post-nasal swabs, nasopharyngeal aspirates, lung tissue and serum samples (Fernández-Rodríguez *et al.*, 2006; Howatson, 2006).

An additional examination modality was instituted in SUDI investigations at the Forensic Mortuary in Salt River in South Africa where a digital radiography system (LODOX Statscan®) was set up in 2007. They screened 192 SUDI cases using this system (Douglas, 2012). Lung disease was the most prevalent imaging pathological finding and this correlated well with their subsequent autopsy findings for those cases. In cases of natural infant death when no autopsies are performed, radiology may aid the diagnosis in combination with the medical history of the case, if the death was determined to be of an infectious nature. Radiology during post-mortems are generally used to reveal fractures and visualise foreign objects, such as bullets, but is surprisingly not widely used in South Africa for SUDI investigation, possibly due to limited funding and resources. There are also no guidelines regarding the documentation of radiological findings in SUDI cases (du Toit-Prinsloo *et al.*, 2011; Douglas, 2012). Thus SUDI investigations and determination of natural causes of death due to infections strongly rely on ancillary investigations, such as virological and microbiological culture and histological examination of body organs (Fernández-Rodríguez *et al*., 2006; Douglas, 2012).

In a study conducted over a 10 year period (1996-2005) 546 SUDI cases from London, England were investigated to determine the role of specific autopsy investigations in concluding the final cause of death. After autopsy examinations the cause of death remained undetermined in 63% of the cases. The most common explained cause of death in the remaining cases was due to infections. Histology was the primary component towards determining the final diagnosis in 46% of the explained SUDI cases, other components included macroscopic examinations in 30% of the cases, microbiology investigations in 19% of the cases and in 5% of the cases the clinical history primarily determined the cause of

death (Weber *et al.*, 2008a). Thus histological examination remains one of the most valuable supplementary autopsy investigations to identify a possible cause of death in SUDI (Weber *et al.*, 2008a; Weber *et al.*, 2012). Ansari *et al*. (2003) conducted a study in Botswana in the late 1990s where they emphasised the value of autopsy investigations, even in children dying from known infectious causes; thus non-SUDI cases. An investigative tool such as the autopsy can assist with a definite diagnosis. To emphasise the value of the autopsy, even outside the scope of the forensic pathology examinations for SIDS, a thorough anatomical autopsy can also allow the correlation between autopsy results and clinical information so that ante-mortem diagnosis and patient management can be improved (Ansari, *et al*., 2003).

Another aspect that could affect the compilation of a feasible SUDI investigation protocol is the dilemma of the lack of agreement as to what is considered sufficient microbiological and virological investigations during SUDI autopsies (Weber & Sebire, 2010). The existing autopsy protocols also lack guidelines for interpretation of positive pathology results. In addition, variations occur when different pathologists interpret the findings as either significant to explain death, or insignificant; and therefore a final diagnosis of SIDS. In the case of microbiological findings the difficulty arises during the interpretation of positive results where it is required to distinguish between significant findings, supposedly insignificant positive findings and post-mortem contaminants. Weber & Sebire (2010) further suggested that methods are required to investigate the systemic responses to the pathogens isolated instead of simply relying on the isolation of pathogens. This would give a better idea of whether these pathogens were involved in the disease progression and whether pathogen detection correlated with pathological findings of infection or whether it was mere contaminants.

Desmons *et al*. (2013) reported on a 22-month-old male SUDI case diagnosed with Varicella zoster virus (VZV) a few days prior to death. The infection was seen as being mild and symptoms were not considered alarming. The autopsy revealed skin lesions, but the organs were macroscopically normal and no cause or mechanism of death could be determined. Only after molecular analysis on frozen tissue samples, CMV and VZV were detected with the highest viral loads in the lungs, implicating the co-infection in the death. The CMV and VZV results correlated with histology signs of extensive necrotising inflammation. Their case study highlights the importance of a thorough histology and molecular virology investigation for determining a definite cause of death in a SUDI case. If such a diagnostic approach is not undertaken, the contribution of viral infections could be underestimated and underreported in SUDI cases (Desmons *et al*., 2013).

2.5 Viral pathogens in SUDI and SIDS cases

The effect of viral infections contributing to, or causing SUDI, is not well defined and although often implicated in these cases, only a hand-full of possible viruses have received attention. Therefore, the role of infections still requires further investigation and should not be overlooked. Infectious organisms have been isolated in up to 49% of SUDI cases in the United Kingdom (Prtak *et al.*, 2010). Weber *et al*. (2008a) found that a definite cause of death could be determined in 37% of SUDI cases in London, England after autopsies were performed. Of these, 58% were due to infections, of which 22% were caused by pneumonia. In a multicentre study in South Africa, about 77% of SUDI cases died from natural disease processes (pneumonia) with 26% dying from bronchopneumonia (du Toit-Prinsloo *et al.,* 2013). Blood-Siegfried (2000) pointed out that at least 70% of infants that have died of SIDS, where no cause of death could be found after investigations, had records of mild viral infections prior to their demise. Parents had also reported a visit to a healthcare provider, as well as symptoms of rhinitis, pharyngitis and a cough in their infants. This strongly suggests that infections play a role in a subset of SUDI cases. Once a severe or lethal viral or bacterial disease has been diagnosed in a SUDI case, the case cannot be categorised as SIDS, but rather as an infection and therefore a natural death (Blackwell & Weir, 1999).

Over the past three decades, there has been consensus in the literature about the importance of viral infections in the epidemiology of SUDI and SIDS, especially when it occurs in combination with other risk factors. Several viruses have been detected in autopsy samples from cases of SUDI and SIDS (Table 2.1), but the significance of such findings has remained undetermined in the context of SUDI (Williams *et al.*, 1984; Zink *et al.*, 1987; Bajanowski *et al.*, 2003; Dettmeyer *et al.*, 2004; Álvarez-Lafuente *et al.*, 2008).

Williams *et al.* (1984) detected viruses in 26% of SIDS cases with a greater proportion in cases older than three months. They also detected viruses in 18% of their control cases that died due to accidents, which raised the question whether viral detection was associated with the events leading to SIDS or only an incidental finding. Patrick *et al*. (1989) identified a similar viral retrieval rate from cot death cases and controls. The absence of infection in some of their cot death cases created doubt regarding the importance of viral findings and they suggested that the standardisation of sample collection and viral retrieval processes could possibly yield more significant results. Patrick *et al*. (1989) found viruses in 19% (44/237) of cot death cases in Glasgow in 1979-1986, and broadly stated that viruses could be expected in about one-fifth of cot deaths. Cot death cases from their study that were older than 16 weeks ($p<0.05$) and with a post-mortem interval (PMI) of less than 24 hours ($p<0.01$) yielded significantly more positive viral results when using cell culture, immunofluorescence

and immune-enzyme assays. In 12 cases the same virus was detected from more than one sampling site and dual isolates were found in three cases (Patrick *et al*., 1989).

Table 2.1: Viruses detected by immunological and molecular techniques from autopsy samples of sudden infant death cases

Viruses isolated / Identified	References
Enterovirus	Urquhart & Grist, 1972; Uren *et al*., 1980; Williams *et al.*, 1984; Grangeot-Keros *et al.*, 1996; Dettmeyer *et al.*, 2004; Weber *et al.*, 2010a
Coxsackie virus	Balduzzi & Greendyke, 1966; Patrick *et al*., 1989
Echovirus	Balduzzi & Greendyke, 1966; Patrick *et al*., 1989
Polio virus	Patrick *et al*., 1989
Adenovirus	Balduzzi & Greendyke, 1966; Urquhart & Grist, 1972; Uren *et al*., 1980; Williams *et al.*, 1984; Zink *et al.*, 1987; Patrick *et al*., 1989; An *et al*., 1993; Dettmeyer *et al*., 2004; Weber *et al.*, 2010a
Epstein-Barr virus	Dettmeyer *et al.*, 2004; Álvarez-Lafuente *et al.*, 2008
Parvovirus B19	Dettmeyer *et al.*, 2004
Human herpes simplex virus	Uren *et al*., 1980; Dettmeyer *et al.*, 2004; Álvarez-Lafuente *et al.*, 2008; Weber *et al.*, 2010a
Cytomegalovirus	Uren *et al*., 1980; Zink *et al.*, 1987; Patrick *et al*., 1989; Álvarez-Lafuente *et al.*, 2008; Weber *et al.*, 2010a; Desmons *et al*., 2013
Rhinovirus	Urquhart & Grist, 1972; Uren *et al*., 1980; Williams *et al.*, 1984 Zink *et al.*, 1987; Patrick *et al*., 1989
Influenza virus	Uren *et al*., 1980; Williams *et al.*, 1984; Zink *et al.*, 1987; Patrick *et al*., 1989; Bajanowski *et al.*, 2003; Weber *et al.*, 2010a
Respiratory syncytial virus	Uren *et al.,* 1980; Williams *et al.*, 1984; Patrick *et al*., 1989; An *et al*., 1993; Weber *et al.*, 2010a
Parainfluenza virus	Uren *et al.,* 1980; Zink *et al.*, 1987; Patrick *et al*., 1989; An *et al*., 1993
Rotavirus	Yolken & Murphy, 1982; Patrick *et al*., 1989
Varicella Zoster Virus	Desmons *et al*., 2013

Some of the viruses isolated from SUDI cases (Table 2.1) were found in specimens from the myocardium, bowel, recto-sigmoid loop, suprarenal fat, liver tissue and post-nasal swabs (Patrick *et al*., 1989; Dettmeyer *et al*., 2004; Weber *et al*., 2010a). At the Tygerberg FPS Mortuary myocardial, post-nasal swabs, bowel, recto-sigmoid loop and suprarenal fat specimens are not considered for routine viral screening in SUDI cases, and SVC also does not screen for HRV, Epstein-Barr virus (EBV), parvovirus B19, herpes simplex virus (HSV), human enterovirus, coxsackie virus, echovirus or rotavirus.

One factor challenging the significance of positive viral identification from SUDI cases is the frequency of communal viruses in both SUDI and infants who died as a result of unnatural causes (Williams *et al.*, 1984). Several of these viruses were also found to be similar to those circulating in living infants in the general population, some of which often cause self-limiting infections (Urquhart & Grist, 1972).

If an infant has an underlying vulnerability, such as immunosuppression, then an infection with one or more of the viruses in Table 2.1 could well contribute to the infant's demise, especially if the infection goes unnoticed by parents, care-givers and physicians. Furthermore, the viral load required to contribute to, or result in, death is still undefined (Krous *et al.*, 2009). Neither a single virus, nor combinations of specific viruses that have been detected from SUDI cases, have consistently been associated with such cases (Nelson *et al*., 1975; Samuels, 2003; Weber *et al.*, 2010a). Nelson *et al*. (1975) concluded that if there was an association between respiratory viral infections and SIDS it seems to be a complex or even indirect association. Therefore, viral pathogens alone are considered a risk factor and may act as a trigger, but have not been recognised as a direct cause of SIDS (Álvarez-Lafuente *et al.*, 2008; Weber *et al.*, 2010a).

Dettmeyer *et al*. (2004) found human enterovirus in 14 cases and the DNA viruses at much lower prevalence with parvovirus B19 detected in seven cases, EBV in three cases and HAdV detected in two cases. Álvarez-Lafuente *et al*. (2008) investigated DNA viruses only and found human herpes virus (HHV)-6 in seven cases, EBV in six cases and CMV in five cases. Weber *et al*. (2010a) detected RNA viruses such as human enterovirus in five cases, RSV in four cases and influenza in one case. They also detected DNA viruses such as HSV in three cases, CMV in three cases and HAdV in one case. Gaaloul *et al*. (2012) only investigated human enterovirus in SIDS cases and found a prevalence of 12.5%. Both DNA and RNA viruses have been detected from SUDI and SIDS cases in the literature but the specific contribution of a group of RNA or DNA viruses to SUDI has not been establish because studies differed in the types of viruses and the amount of viruses investigated.

2.6 Respiratory viral pathogenesis

For a virus to survive and infect humans it needs to gain entry into the host by binding to host receptors on cellular membranes. When respiratory viruses are inhaled they generally gain entry through the nasopharyngeal epithelium, mucous membranes and airway epithelial cells (AEC) of the respiratory tract. They also encounter macrophages and dendritic cells (DC) which form part of the innate immune system, expressing pattern recognition receptors (PRR). Successful entry is followed by viral replication inside the nasopharyngeal epithelium,

mucous membranes and AECs that are completely dependent on the host's cellular machinery in the surrounding tissues. This induces the characteristic release of interferon (IFN)-α and IFN-γ by AECs and other innate immune cells (Schwarze & Mackenzie, 2013). The innate immune system is the body's first response to infections and is continually surveying the external and internal environment for foreign antigens in order to rapidly mount a non-specific response (See & Wark, 2008). The PRRs on cells of the immune system detect viruses through recognition of pathogen-associated molecular patterns (PAMPS). The PRRs include Toll-like receptors (TLR) such as TLR-4 that binds to the fusion protein of RSV, intracellular adhesion molecule 1 (ICAM-1) which is a PRR for HRVs, retinoic acid-induced gene 1 (RIG-1), and melanoma-differentiation associated gene 5 (MDA-5) (Rossmann, 1994; See & Wark, 2008; Schwarze & Mackenzie, 2013). After receptor binding viral entry occurs through fusion of the envelope with the membrane of the cell. For capsulated viruses, entry into the cell cytoplasm is not well characterised yet (Rossmann, 1994). Upon detection of viruses by PRRs the DCs and macrophages of the innate immune response release interferons that impair viral replication (See & Wark, 2008). The production of interferons stimulates programmed cell death of infected AECs thus limiting further replication and subsequent infection of other cells (Schwarze & Mackenzie, 2013) and also activates natural killer (NK) cells (Schwarze & Mackenzie 2012). In asthmatics the epithelial cells in the respiratory tract release less interferon which limits programmed cell death in the infected cells and thus enhance viral replication. When AECs recognise viral pathogens a host of proinflammatory cytokines, chemokines and growth factors are released. During viral lung inflammation the AECs produce pro-inflammatory cytokines which include Tumour necrosis factors (TNF) and ILs that leads to the further recruitment of macrophages, DCs and neutrophils (Schwarze & Mackenzie 2012). NK cells are important effector cells forming part of the innate immune response to control viral infections. These cells lyse host cells infected with viruses and if extensive NK cells are activated it can contribute to virus-associated pathology (Jost & Altfeld 2013).

The release of interferon and the ability of DCs and macrophages to act as antigen presenting cells (APC) serve as a link between the innate and the adaptive immune system by activating and recruiting naïve T cells (See & Wark, 2008). DCs can activate both effector and memory T cells to respond to viral infections and re-infections respectively (Schwarze & Mackenzie 2012). NK cells can also interact with DCs and produce cytokines and chemokines to regulate adaptive immune responses (Jost & Altfeld 2013). Although the adaptive immune system is not a rapid response it is very specific and effective. Cytotoxic $CD8^+$ T cells are recruited to areas of acute inflammation during viral infection and secrete interferon but they can also function to dampen an exaggerated innate immune response to

decrease tissue damage (See & Wark, 2008). $CD4^+$ T cells secrete cytokines during a viral infection and help to activate the production of antibodies by B cells that could neutralise foreign microorganisms or toxins (Gordon *et al*. 1999; Koziel 2005). Immunological adaptive memory is acquired after recovery from infection that allows the host to respond faster upon re-exposure to the same pathogen or a closely related one (Hikono *et al.* 2006)

2.7 Introduction of respiratory pathogens investigated in this study

The following sections will briefly introduce and characterise the thirteen respiratory viruses and subtypes that will be investigated during this study, namely influenza A and B, HPIV 1-4, HCoV (OC43, 229E/NL63), HRV A, B and C, RSV A and B, human enterovirus and HMPV.

2.7.1 Human coronavirus (HCoV)

The HCoVs are from the family Coronaviridae and the order Nidovirales. They are single-stranded positive RNA viruses with their nucleic acids ranging from 27 000-33 000 base pairs (bps) in size. The HCoVs share common genes that encode for structural proteins that include the envelope, the membrane and the spike proteins. The HCoVs are divided into two groups; group one containing HCoV-229E and HCoV-NL63, and group two containing HCoV-OC43 and HCoV-HKU1. Mutations in HCoV strains seem to occur frequently and if two viruses are present in the same cell genetic recombination can occur. The common symptoms observed in patients infected with HCoVs include coughing, fever, rhinorrhoea, bronchiolitis and croup (Kahn & McIntosh, 2005; Kuypers *et al.*, 2007; Abdul-Rasool & Fielding, 2010).

The first HCoV types, HCoV-OC43 and HCoV-229E, were already described in the 1960s (Kahn & McIntosh, 2005; Abdul-Rasool & Fielding, 2010). In 2003 a HCoV strain that caused severe acute respiratory syndrome (SARS) was identified. This SARS HCoV virus was distributed worldwide causing severe respiratory disease with a 10% mortality rate. As quickly as the outbreak occurred and the virus appeared, it disappeared again. No others had been detected in human populations since then. Several HCoV strains cause severe disease in animals, but only mild symptoms in humans, however, as was the case with the SARS epidemic, some animal coronaviruses (CoV) may be potentially life-threatening when introduced into the human population. The HCoV-NL63 viral strain was discovered in 2004 in The Netherlands when it was isolated from a seven-month-old girl who was suffering from symptoms that included coryza, conjunctivitis, fever and bronchiolitis. The following year HCoV-HKU1 was isolated from a man that was 71 years old who showed symptoms of respiratory disease, which included a fever and coughing (Kahn & McIntosh, 2005; Kuypers *et al.*, 2007; Abdul-Rasool & Fielding, 2010). Recently the Middle East respiratory syndrome

coronavirus (MERS-CoV) was detected from a man with a fatal outcome who had shown signs of acute pneumonia and renal failure in Saudi Arabia. Both authors claimed that the clinical features seemed similar to that of the SARS-CoV outbreak (Zaki *et al*., 2012; Breban *et al*., 2013). MERS-CoV cases are being closely monitored and may have the potential to cause a human pandemic as was the case with SARS-CoV. The animal host for MERS-CoV is still unknown (Breban *et al*., 2013).

Since the discovery and subsequent culture and characterisation of HCoVs, they have been found in a substantial proportion of children presenting with symptoms of a common cold. The HCoV strains can infect any age group, but the majority of these infections occur during childhood. Apart from SARS-CoV, the pathogenicity of HCoVs are generally low, causing upper respiratory tract infections (URTI), occasional pneumonia and can also contribute to asthma exacerbation in childhood. The only HCoV virus that has been thoroughly studied is SARS-coronavirus and this may be due in part to the notion that HCoVs only result in mild infections of the respiratory tract and has thus far not been considered as serious threats (Kahn & McIntosh, 2005; Kuypers *et al.*, 2007; Abdul-Rasool & Fielding, 2010).

2.7.2 Human enterovirus

The human enteroviruses are non-enveloped icosahedral viruses from the picornaviridae family with positive single-stranded RNA and a genome size of about 7 500 bps. They cause illnesses that range from mild respiratory infections, such as the common cold, hand-, foot- and mouth disease, conjunctivitis, carditis, general neonatal infections, meningitis, encephalitis and paralysis (Hyypiä *et al.*, 1997; Han *et al*., 2010).

Originally, the human enteroviruses were grouped into polioviruses, coxsackie A and B viruses and echoviruses, but because genetic similarities between echoviruses and coxsackie viruses became apparent, the human enteroviruses have been grouped into five species, which include the poliovirus and human enterovirus groups A-D (Oberste *et al.*, 2004).

The first isolation of human enterovirus 68 occurred in 1962 in California, Unites States of America (USA), where it was found in four children showing signs of respiratory disease. From that time onwards this virus was rarely isolated, but when it was isolated, it was always from respiratory specimens of patients that suffered from respiratory disease, including bronchiolitis and pneumonia. Unlike human enterovirus 68, most of the other human enterovirus strains are typically isolated from faecal specimens from patients with meningitis (Oberste *et al.*, 2004). In 1971 human enterovirus 70 was isolated during a pandemic of acute haemorrhagic conjunctivitis (AHC). Human enterovirus 71 was isolated in 1969 from

the brain of a patient who had suffered from fatal encephalitis. Outbreaks of hand-, foot- and mouth disease were also associated with human enterovirus 71 in Sarawak, Malaysia, in 1997. This strain led to serious myocardial dysfunction, paralysis and death in some of the paediatric patients. These deaths had a major impact on public health and resulted in the Government of Malaysia declaring hand-, foot- and mouth disease one of the notifiable diseases in 2007 (Yusof *et al.*, 2011).

2.7.3 Human metapneumovirus (HMPV)

HMPV belongs to the paramyxoviridae family and is an enveloped virus characterised by negative-sense single-stranded RNA with a genome size of about 13 000 bps and is divided into two main genetic lineages, A and B. The genome of HMPV encodes for several proteins that include the attachment, nucleoprotein, fusion protein, matrix protein, phosphoprotein, small hydrophobic and the large polymerase proteins. Of these, the fusion protein is involved in immune protection and neutralising monoclonal antibodies against the fusion protein have been observed, whereas the attachment and small hydrophobic proteins seem to be only weakly immunogenic (de Graaf *et al*., 2008; Lo Pesti *et al*., 2011).

HMPV was isolated from respiratory specimens of young children in The Netherlands during 2001 and has since become recognised as a noteworthy cause of lower respiratory tract infection (LRTI), prevalent amongst infants. HMPV infection is not restricted to the paediatric populations and has been observed in the elderly and immunocompromised patients as well (Van den Hoogen *et al*., 2001; Williams *et al*., 2004; Lo Presti *et al*., 2011). Avian metapneumovirus (aMPV) was discovered during the 1970s when it was implicated in respiratory illnesses in birds. It was classified into subgroups A and B found in South Africa in 1978, C in the United States of America (USA) and D in France. The aMPV subtype C that was isolated in 1996, was genetically related to HMPV. It was thus suggested that HMPV may have originated from birds and entered the human population through zoonosis (de Graaf *et al*., 2008; Lo Presti *et al*., 2011).

Currently, HMPV is divided into subtypes A1, A2a, A2b, B1 and B2. There is no HMPV vaccine available yet, but research is on-going towards developing one. Cold-adapted vaccine strains of aMPV, which do not grow at normal body temperature, have been successful in preventing infections in poultry. Replication of HMPV in cell lines which normally support the growth of various respiratory viruses, such as human larynx carcinoma cell line (i.e. Hep-2), only weakly supports HMPV replication and this may be why this virus has only recently been identified. The recent identification was achieved through PCR tests

that employed random primers to increase the likelihood of detecting novel viral species (Van den Hoogen *et al*., 2001; Crowe, 2004; Lo Presti *et al*., 2011; *Li et al*., 2012).

The symptoms of HMPV infection include fever, coughing and rhinorrhoea accompanied by vomiting. Infection with HMPV was related to clinical features that included bronchiolitis, croup, pneumonia, acute otitis media and asthma exacerbation. Recurrent infections with HMPV have also been observed in infants. In these infections the strains that were isolated during the first and subsequent episodes of infection were genetically distinct from each other, which may suggest some genetic variability (Van den Hoogen *et al*., 2001; Williams *et al*., 2004; Pesti *et al*., 2011).

2.7.4 Human rhinovirus (HRV)

The HRVs belong to the family picornaviridae and were first isolated in the 1950s. They are non-enveloped, single-stranded positive sense RNA viruses with a genome size of about 7 200 bps. Over a hundred serotypes have been described and are mainly divided into HRV-A and HRV-B after sequence analysis. More recently two other possible groups emerged, HRV-C and HRV-D. The high number of serotypes described can be accounted for by the error-prone polymerase that can result in mutations in the nucleotides of the HRV genome, driving viral adaptation and evolution (Tapparel *et al*., 2007; Cordey *et al*., 2010; Smuts *et al*., 2011).

HRV is found in low concentrations in saliva and was shown to be poorly transmitted during coughing and sneezing, which involves mainly oral secretions. For this reason, the main route of identified transmission was suggested to occur via contaminated nasal secretions and eyes. The virus can then be acquired through self-inoculation or from contaminated hands to the nose or eyes of susceptible persons. The virus initially enters through the nose and establishes infection in the ciliated epithelial cells of the upper respiratory tract (URT). In cases where the infection is self-limiting, after viral acquisition, the concentration of HRV increases in nasal concentrations, reaching a peak at about 48 hours, after which it starts to decrease. Yet, viral shedding can still occur for another three weeks after initial establishment of infection (Hendley *et al*., 1973; Gwaltney, 1995).

HRVs are the frequent mild pathogens of the URT. These viruses are responsible for the common cold and other infections that include otitis media and sinusitis, but have also been associated with asthma exacerbations and severe LRTI in immunocompromised patients (Papadopoulos, 2004; Tapparel *et al*., 2007; Cordey *et al*., 2010). In hospital-admitted paediatric patients (<18 years) in California in 2007, 49% were infected with HRV genotypes A and C (Louie *et al*., 2009). Louie *et al*. (2009) reported the common symptoms to include

coughing in 71% of the patients, wheezing in 52% of the patients and fever in 48% of the patients. Although HRV was previously thought to be responsible for mild infections, the findings from Louie *et al*. (2009) suggested that HRV could also be associated with severe LRTI.

2.7.5 Influenza A and B

The human influenza viruses belong to the orthomyxoviridae family and can be further divided into three genera, namely influenza A, discovered during 1933, influenza B, discovered in 1940, and influenza C, discovered in 1950. The influenza viruses are enveloped, single-stranded negative-sense RNA viruses with influenza having a genome size of about 13 588 bps. The genome of influenza viruses codes for three membrane proteins that include the haemagglutinin (HA), neuraminidase (NA), the ion channel protein and a matrix protein (Chan *et al*., 2006; Gao *et al*., 2012). Several subtypes exist for influenza A. These are named according to the HA and NA proteins combined with H1, H2 and H3 and N1 and N2 found to circulating in human populations within the last hundred years. Three known large influenza pandemics have occurred; the first in 1918, as a result of the H1N1 subtype, the second during 1957 as a result of the H2N2 subtype, and the third in 1968 as a result of the H3N2 subtype (Zambon, 2001).

Influenza A is a significant human pathogen responsible for annual epidemics during winter months and can also cause infections in birds, pigs and horses. Influenza B infections are mostly limited to humans where it causes significant disease, but epidemics rarely occur compared to influenza A. Influenza C is a less significant human pathogen and also generally restricted to humans, although groups B and C have both been isolated from mammalian species on occasions. Influenza infections can be asymptomatic or in more serious cases, characterised by a high fever, chills, coughing, headaches and a sore throat (Hilleman, 2002; Zambon, 2001; Petric *et al*., 2006).

The RNA-dependent RNA polymerase of influenza A has a high replication error rate, which supports the rapid evolution of this virus. This also means that complete immunity to influenza virus infection cannot be achieved. Point mutations, especially in the HA surface glycoprotein, enable the virus to surpass the hosts immune defence of persons that had previously been exposed to influenza, which can result in annual re-infection. The ribonucleoprotein core of influenza A and B is divided into eight segments, whereas influenza C only has seven segments. The segmented ribonucleoprotein core permits re-assortment of genetic material and recombination amongst different influenza subtypes, giving rise to a substantial reservoir of novel influenza strains over time (Zambon, 2001; Gao *et al*., 2012). In

contrast to the majority of respiratory viruses, a vaccine exists for influenza, which serves as a preventative tool against severe infection. Even so, the influenza vaccine needs to be modified annually according to the circulating strains (Schwarze & Mackenzie, 2013).

2.7.6 Human parainfluenza virus (HPIV) 1-4

The HPIVs belong to the paramyxoviridae family and are divided into HPIV 1 (described in 1956 together with HPIV 3), HPIV 2 (described in 1956), HPIV 3 and HPIV 4-A and HPIV 4-B (described during 1960-1964). They are small viruses ranging from 150-250 nm, characterised by an envelope, negative-sense single-stranded RNA molecule, and a genome size that is approximately 15 000 bps. The genome encodes for structural proteins that include the large nucleocapsid protein, the phosphoprotein, the nucleocapsid protein, the surface glycoproteins Haemagglutinin-neuraminidase (HN), the fusion protein and the membrane protein (Henrickson, 2003). Illnesses observed after HPIV isolation in children include mild colds, croup, bronchitis, bronchiolitis and less frequently, pneumonia. URTI include otitis media, pharyngitis and conjunctivitis (Gardner, 1969; Downham *et al*., 1974; Henrickson, 2003).

The humoral immune response to infection with HPIVs include antibody production against the HN and fusion proteins of the virus, and to date there are no effective vaccines available (Henrickson, 2003).

2.7.7 Respiratory syncytial virus (RSV) A and B

RSV was first recognised as a cause of coryza in chimpanzees in 1965 and was subsequently detected in children suffering from respiratory illness (Simoes, 1999). Isolated RNA shows that the virions are single-stranded with 90% of the RNA having a negative polarity, and the genome size was determined to be close to 14 705.88 bps. RSVs are enveloped RNA viruses organised into the genus pneumovirus and the family paramyxovirus (Huang & Wertz, 1982; Domachowske & Rosenberg, 1999).

Two of the transmembrane surface antigen determinants for RSV are the fusion and attachment glycoproteins. Despite the detection of neutralising antibodies against these glycoproteins during RSV infection, cellular immune response is required in order to effectively clear the virus and allow recovery from infection. Annual RSV infections with the same viral strains often occur within the first few years of life, suggesting that immunity may be incomplete during early life and only establishes after several infections where the immune response progressively strengthens with each re-infection. Even though multiple re-infections are thought to occur throughout early life, severe infection is often only restricted to

the first two re-infections in childhood (Collins *et al*., 1995; Domachowske & Rosenberg, 1999; Simoes, 1999).

The most significant cause of LRTI in early childhood is RSV infection. The acquisition of RSV occurs form the nasopharynx and the eyes, followed by an incubation period of about five days, after which substantial viral replication occurs in the nasopharynx. RSV infection is characterised by rhinitis, coughing, fever and roughly a third of childhood cases develop acute otitis media. LRTI predominantly results in bronchiolitis and pneumonia, but may also develop into croup (Collins *et al*., 1995; Domachowske & Rosenberg, 1999; Simoes, 1999).

Co-infection of some of the respiratory viruses outlined above is common, especially for HMPV and RSV (IJpma *et al.,* 2004). Many respiratory viruses show definite seasonal variation, including RSV (Ghani *et al*., 2012; Kwofie *et al*., 2012), HMPV, influenza (O'Callaghan-Gordo *et al*., 2011) and HPIV viruses, with peaks during winter and rainy months (IJpma *et al.,* 2004; Smuts *et al.,* 2011). HRV infections usually peak in autumn and spring (Smuts *et al*., 2011). Zink *et al*. (1987) and Grangeot-Keros *et al*. (1996) have discussed correlations between the increase of infections in colder climates and the incidence of SUDI and SIDS. Nelson *et al*. (1975) found a significant association between SIDS cases and infants hospitalised as a result of influenza in a study in Chicago from January 1970 to June 1976.

Viral infections may act in combination with other factors, including the host's immune response to infection, the initiation of bacterial toxin production, as well as co-infection with other microorganisms, thereby contributing to SUDI. Viruses can also enhance cytokine activity, leading to the unregulated expression of inflammatory mediators. Inflammatory changes have regularly been observed in the URT and lower respiratory tract (LRT) in SUDI cases and clinical symptoms have often been reported in the days preceding death. The cytokine interleukin (IL)-6 has been detected in similar concentrations in cerebrospinal fluid (CSF) from SIDS cases, as well as those who died from serious diseases, such as meningitis and septicaemia. The combination of a viral infection and the subsequent activation of bacterial toxin production can increase pro-inflammatory reactions. To date no method exists for determining the degree of inflammation required for lethality. Despite this, severely elevated pro-inflammatory levels have been detected during lethal infections (Samuels, 2003; Blackwell *et al.*, 2005; Highet, 2008).

2.8 Burden of respiratory disease and respiratory viruses in children

Even though the specific role of viral infections in the context of SUDI is not well defined, the burden of disease resulting from respiratory illness is the largest cause of morbidity in the

world. ARIs are the leading causes of childhood morbidity and mortality worldwide and young children can have up to 5-8 respiratory infections annually. Although this rate should be comparable throughout the world, a much worse prognosis is found in rural Africa where patients suffer from poor health, malnutrition, high incidences of prematurity, anaemia and other co-morbidities, such as malaria and human immune deficiency virus (HIV) (Tregoning & Schwarze, 2010; O'Callaghan-Gordo *et al*., 2011).

In 2000, about 1.9 million deaths worldwide were attributed to ARI with 70% of these deaths occurring in Africa and Asia. In 2008 about 1.6 million children worldwide died of ARI and in developing countries the major causes of these deaths were due to viral pathogens, such as RSV, HPIV, influenza, HAdV, HCoV and human bocavirus (HBoV) (Kwofie *et al*., 2012). Infection of the respiratory tract was considered to be the third most frequent cause of death in African children younger than five years of age. Furthermore, in South Africa, about 80% of deaths during infancy and early childhood were shown to result from respiratory tract infections (IJpma *et al.*, 2004).

On a smaller scale Ansari *et al*. (2003) studied children from Botswana who died at Nyangabgwe Hospital from 1997 to 1998. They found 83% of the deaths amongst HIV-positive and 67% amongst HIV-negative children at this hospital were attributed to respiratory infections.

It was proposed by Weber & Sebire (2010) that infants succumbing to SUDI may have underlying genetic and biological vulnerabilities and immunodeficiencies. Even though the pathogenicity of respiratory viruses can be low in immunocompetent individuals and are often associated with a good prognosis, this postulated deficiency in the ability of the infant to deal with mundane infectious agents can predispose them to sudden and unexpected death in the presence of a mild viral infection, an unregulated immune response and/or other externally established risk factors (Weber & Sebire, 2010).

HRV seems to be very common in young African children with acute wheezing. A recent study found HRV in 58.2% of children below five years of age, of whom 72% were younger than two years and a further 50% of these occurred in infants younger than 12 months. Co-infections occurred with HMPV, HBoV and HCoV in 6.3%, 4.7% and 0.8% of infants respectively. In the infants that tested negative for HRV, 5.4%, 10.9% and 3.3% were positive for HMPV, HBoV and for HCoV respectively (Smuts *et al*., 2011).

A prospective study during the winter months of 2002 identified HMPV as the sole pathogen in 5.8% of hospital-referred children with URTI and LRTI at Tygerberg Hospital, Cape Town,

South Africa. Other infections were due to RSV (15%), influenza B (15%) and influenza A (13%) (IJpma *et al.,* 2004).

In Ghana viruses detected in children under five years of age that were hospitalised with severe pneumonia in 2008 included RSV (14.1%), HAdV (10.2%), HPIV 1, 2 and 3 (3.1%) and influenza B (0.8%). There was one co-infection of RSV and HPIV 1 and another co-infection of RSV with influenza B and HAdV. These viruses were isolated in 25.7% of the children and bacterial pathogens were only isolated in 9.4% of the children. From this study the majority of children infected were between the ages of six months to two years, and the majority of infections were of viral origin; 61.1 % were isolated in the rainy season, with RSV predominating (Kwofie *et al*., 2012).

In Mozambique viruses isolated from 1999-2000 from infants younger than 12 months with ARIs included HRV (26%), HAdV (14%), HMPV (7%), HPIV (5%) and human enterovirus (3%). Co-infections with two viruses occurred in 9% of the cases and 2% had three viruses detected, with the majority of co-infections involving HAdV and HMPV. This study did not screen for RSV and the true frequency of viral infections might have been underestimated as a result (O'Callaghan-Gordo *et al*., 2011).

In Cape Town, South Africa, viruses isolated from infants between the ages of two months and one year who were admitted to the paediatric intensive care unit for ARI in 2009 included HRV (39%), RSV (27.7%), HAdV (15.4%), influenza A (13.3%), HPIV 1, 2 and 3 (11.8%), HMPV (6.2%) and influenza B (0.5%). The mortality rate of 11.4% was associated with HIV infection or exposure and nosocomial and influenza A infections. In this study the overall viral detection was 86%, with 13.5% having viral co-infection with two viruses and 0.5 % with three viruses (Ghani *et al*., 2012).

In another study in Cape Town, South Africa, done by Zampoli *et al.* (2011), the isolated viruses included CMV (66%), RSV (13%) and HAdV (9%) in 202 children admitted to the Red Cross Children's Hospital suffering from severe pneumonia. Of these children, 62% were HIV infected. In 17% of the children co-infections occurred with two or more pathogens identified. In 43% of the children with severe pneumonia admitted to the Red Cross Children's Hospital, no pathogens could be detected, which indicates that better viral screening and detection methods are required to diagnose the causes of pneumonia. For this study, the in-hospital mortality rate was 25%. The majority of deaths occurred in HIV-infected children suffering from CMV-associated pneumonia, accompanied by high CMV viral loads. The cut-off viral load that can predict severe or fatal CMV disease still needs to be established (Zampoli *et al*., 2011). This high prevalence of CMV in South African children suffering from pneumonia

(66%) was similar to the CMV prevalence in lung autopsy samples from Zambia and Botswana (22.8% and 20.2% respectively) in the 1990s (Chintu *et al.*, 2002; Ansari *et al*., 2003). CMV infection generally results from opportunistic infections in patients that are immunocompromised, in which case, it may cause fatal lung disease. This virus has also been shown to cause pneumonia in infants believed to be immunocompetent. CMV is often detected alongside other causes of lung disease, therefore its individual role in fatal infection is questionable (Chintu *et al.*, 2002).

2.9 Diagnostic tests for respiratory infections

Several important factors may help in the identification of different viral isolates in SUDI cases. These factors include the selection of viruses tested, viral load, and isolation and detection techniques used. Molecular PCR techniques use specific primer sequences and immunohistochemistry uses monoclonal antibodies that are extremely specific for the virus in question. Furthermore, Patrick *et al*. (1989) stated that many viruses may not be detected using current viral isolation methods and individuals immune systems may also respond differently to viral infection. For viral culture detection methods that highly rely on viral viability a long PMI may be important for viral viability. Weber *et al*. (2010a) speculated that long PMIs where degradation of viral particles occur, may also affect viral nucleic acid detection assays. Although the viability of viruses and the composition of nucleic acids may decline over time, no time-limit had been specified. According to Patrick *et al*. (1989) a prolonged PMI should not be a contraindication for viral investigations, because 18% of their viral positive cot death cases confirmed by culture methods and immunology assays had a PMI longer than 24 hours. Using non-culture methods for bacterial and viral detection assays, a PMI of four hours to four days still yielded significant bacterial and viral isolates in 85% of SUDI cases (Rambaud *et al*., 1999).

Prior to the 1900s viral growth was mainly achieved by propagation in embryonic eggs and experimental animals, but since then the achievement of growing human cells *in vitro* viral isolations have largely replaced *in vivo* laboratory diagnosis. Viruses grown in susceptible cell cultures can yield high viral concentrations. In addition, culture tubes facilitate manipulation and allow the addition of antibiotics to prevent contamination, which is not possible in animal hosts. Cell cultures were first used to culture the vaccinia virus in 1913 and later smallpox and yellow fever in the 1930s. These *in vitro* cultures facilitated the development and production of vaccines (Leland & Ginocchio, 2007).

Cell culture methods have always been designated as the “gold standard” for the detection of viable viruses, but with conventional cell culture techniques it can take eight to ten days for

sufficient viral growth, which limits the diagnostic value of such methods. On the other hand centrifugation-enhanced SVC greatly reduces the turn-around time to 1-2 days, which may further be decreased with the use of mixed cell lines, such as a combination of Hep-2 and Madin-Darby Canine Kidney (MDCK) cell lines (Leland & Ginocchio, 2007; Mahony, 2008).

Faster, alternative techniques, such as NAATs were developed and can provide results in less than three hours (Loeffelholz & Chonmaitree, 2010). NAATs have higher positivity rates than SVC (Lee *et al*., 2010) and have superior diagnostic sensitivity and specificity for detection of respiratory viruses, to the point where interpretation of positive results becomes challenging. NAATs does not indicate viral viability and therefore challenges arise when viruses are detected in persons without any clinical symptoms of infection or once the persons have already completely recovered from illness. Prolonged shedding of HRV can occur up to three weeks after initial infection (Hendley *et al*., 1973; Gwaltney, 1995). These techniques also frequently detect multiple viruses in the same sample, which can complicate interpretation of results. Real-time PCR methods allow amplification and analysis of viral targets in one step and conventional multiplex PCR methods can detect larger range of viruses in a single reaction, but require an additional step for post-amplification detection (Pabbaraju *et al*., 2008; Gadsby *et al*., 2010; Loeffelholz & Chonmaitree, 2010; Gharabaghi *et al*., 2011).

The detection of a virus in autopsy samples may not necessarily indicate a specific cause of death or an active infection, because CMV has been found to be present in numerous infants without any pathological manifestations. The contribution of a viral positive PCR result to events leading to death cannot be excluded, but require further evidence that an active infection was present through the detection of viral specific immune responses (Blood-Siegfried, 2000; Álvarez-Lafuente *et al.*, 2008). The sensitivity of NAATs and the latency of some viruses, such as CMV and other herpes viruses, means that positive tests need to be interpreted carefully by trained personnel and in parallel with histology and other results (Fernández-Rodríguez *et al.*, 2006; Howatson, 2006). Techniques that have commonly been used in viral investigations from sudden infant death specimens include culture techniques (Balduzzi & Greendyke, 1966; Urquhart & Grist, 1972; Weber 2010a), immunofluorescence (Urquhart & Grist, 1972; Zink *et al.,* 1987; Weber 2010a), immunohistochemistry (Dettmeyer *et al.*, 2004), PCR and gel electrophoresis (Grangeot-Keros *et al.*, 1996; Bajanowski *et al.*, 2003; Dettmeyer *et al.*, 2004; Weber 2010a), *in situ* hybridization (An *et al*., 1993) and immunoglobulin (Ig) M and IgG immunoassays (Grangeot-Keros *et al.*, 1996; Williams *et al.*, 1984; Zink *et al*., 1987). Specimens collected for viral investigations in sudden infant death studies included fresh, frozen and paraffin-embedded formalin-fixed samples from lung

tissue, serum, lumbar and CSF, colon, stool, suprarenal fat, myocardium, brain, thymus and nasal and tracheal swabs. Although formalin-fixed samples have been used to detect viruses Gazziero *et al*. (2009) found significant degradation of RNA and DNA in formalin-fixed cells compared to fresh cells and cells fixed in formalin-free media. Formalin fixation can thus cause fragmentation of RNA and DNA, reducing the nucleic acids suitable for molecular amplification (Gazziero *et al*., 2009; Desmond *et al*., 2013). CMV and VZV were only detected from frozen tissue and not formalin-fixed tissue in a SUDI case report by Desmond *et al*. (2013).

2.10 Respiratory immune defence

The principle defence mechanism of the host against respiratory infections is the physical and cellular components of the immune system and immune mechanisms of the respiratory tract. Breathing is a life-preserving function of the lungs and provides oxygen to the cells of the body, but during the breathing process, harmful microorganisms can also gain entry into the nasopharynx and the lungs (Nicod, 2005).

In the URT a cough generates shearing forces that drives foreign material up and out of the bronchi and trachea, assisted by the beating of cilia on AEC lining the URT. The secretion of mucus-containing IgA allows the neutralisation of foreign pathogens and prevents the attachment of microorganisms to the epithelium. The AECs secrete chemokines, such as IL-8 and IL-16, when stimulated by viral infections, cigarette smoke and other microorganisms, which are established risk factors for SUDI. The expression of these cytokines is followed by an increased expression of adhesion proteins during inflammation. Adhesion proteins slow down the immune cells in circulation by permitting adherence of neutrophils, monocytes, macrophages and lymphocytes to blood vessels and facilitate their migration from the blood stream to the area of inflammation. The recruited alveolar macrophages serve as a first line of defence in the lungs by neutralising foreign particles and recruiting more neutrophils and mononuclear cells to the site of infection and inflammation. The inflammatory response is enhanced by the secretion of IL-1α, IL-1β and TNF-α, followed by secretion of adhesion molecules on epithelial surfaces, as well as chemokines, which form part of the innate immune response. During RSV bronchiolitis, necrosis and sloughing of AECs occur accompanied by increased mucus production, which reduces airflow (Hall, 2001).

The respiratory mucosa houses DCs that are able to detect, capture and transport foreign microorganisms to the lymph nodes of the body, where the adaptive immune system can be stimulated. The DCs phagocytose epithelial cells that are infected with viruses. They then display viral particles on the outer membrane receptors to attract and stimulate naïve T

lymphocytes into effector T cells, present at the lymph nodes in the lungs. These effector T cells play an important role in viral clearance. Lymphocytes form 10% of the immune cells of the alveoli of the lungs, consisting of 50% cluster of differentiation (CD) 4^+ T helper and 30% $CD8^+$ cytotoxic T cells. A further 10-15% of the immune cells of the alveoli consist of NK cells and 5% consist of B lymphocytes. Both $CD4^+$ and $CD8^+$ T cells are vital role players in the adaptive immune defence against viral pathogens. The main Ig subtype produced by B cells in the alveoli is IgG, of which subtypes IgG1 and IgG2 are predominant (Nicod, 2005).

2.11 Functional immaturity of the infant immune system

Despite the respiratory immune defence mechanisms, the immune system of infants is functionally immature, compared to that of older children and adults. Before birth, the uterine cavity offers little antigen exposure, due to the sterility of that environment (Palmeira *et al.*, 2012). Infants are thus initially dependent on their innate immune mechanisms, maternal antibodies that were transferred across the placenta from the mother to the baby during pregnancy and the protective immune components of breastmilk for protection against pathogens (Cáceres *et al.*, 2000; Jackson & Nazar, 2006; PrabhuDas *et al*., 2011). Some aspects of the infants' immune systems may be fully functional at birth, but the majority are not (Palmeira *et al.*, 2012).

Neutrophils function as part of the first line of defence during the innate immune response and their primary function is to phagocytose and subsequently kill and digest bacterial and fungal pathogens through the release of enzymes from cytoplasmic granules (Segal, 2005). Fewer neutrophils are present in infants, compared to older children and adults. Furthermore, neutrophils of infants respond suboptimally to chemo-attractants when compared to adult neutrophils, which means that recruitment of immune cells to sites of infection may be inefficient (Palmeira *et al.*, 2012). Macrophages also forms part of the innate immune system and circulate as monocytes in the blood. Monocytes are recruited from the blood to tissue sites of infection, through chemo-attractants, during illness or cellular damage. At the site of tissue damage or infection, monocytes reside as resident macrophages to phagocytose and destroy invading pathogens, and clear the body of cells that have undergone normal apoptosis (Geissmann *et al.*, 2010). Similar to neutrophils, the monocytes and macrophages in infants are also less sensitive to chemo-attractants, even though these cells are reported to be fully functional. As part of the innate immune system, infant monocytes generate lower levels of IFN-α and IFN-γ, than those of older children and adults. These two cytokines are key components of the regulation of both the innate and adaptive immune responses to viral infections through interference with viral entry and replication (Schroder *et al.*, 2004; Palmeira *et al.*, 2012).

In the cellular branch of the adaptive immune system, T cells are important regulatory cells to viruses and other intracellular pathogens and many of their functions are mediated by the expression and secretion of cytokines, such as IFN-α and IFN-γ. T cell populations in neonates are predominantly expressing the naïve phenotype and the expression of memory T cells only develops as the neonates grow older. As most cytokines are predominantly expressed by memory T cells, higher cytokine levels are found in older children compared to the infants that mainly express naïve T cells. The development of memory T cells with increasing age in the first to the second year of life is demonstrated by an increased expression of these effector T cell cytokines, IFN-α and IFN-γ (Wiegering *et al.*, 2009). Infants are capable of mounting a T cell response to infectious agents, but such responses may differ in quality and magnitude compared to adults. Furthermore, $CD4^+$ T cell responses take longer to develop upon intracellular viral exposure and infection with CMV and HSV. Neonates have also been shown to have a smaller thymus if they were exposed to inadequate nutrition *in utero*. This could delay the development of T cells and affect their functioning, further enhancing the immaturity of the immune system in infancy (PrabhuDas *et al*., 2011).

The humoral branch of the adaptive immune system, composed of B cells and antibodies, is also immature in terms of the onset, duration and quantity of the Igs produced in early infancy. The Igs are also of a different isotype, where infants have lower levels of IgG2 with lower affinities for antigens, compared to the Ig in adults. It has also been found that certain defects occur in the formation of the germinal centre in B cells in neonatal humans and mice. These defects seem to only resolve when infants are about four months old, which is just outside the 2-4 month peak window period for SUDI and SIDS. This is therefore another immaturity of the immune systems that leaves infants vulnerable during the SUDI and SIDS window period. With repeated exposure to foreign antigens in the infant's environment and once placental transferred IgG antibodies have declined, B cells start to produce antigen-specific antibodies. With the maturation of the humoral immune system during late infancy, and as a result of repeated antigen stimulation, the infant's Ig levels begin to rise (Wiegering *et al.*, 2009; Palmeira *et al.*, 2012).

2.12 The vulnerable period in infancy and SIDS peak

Immaturities of the innate immune system, that direct functioning of adaptive immune responses, leave infants vulnerable in their first year of life, characterised by a greater susceptibility to developing infections (Palmeira *et al*., 2012; Reikie *et al*., 2012). Exposure to cigarette smoke, poor nutrition, a mild viral infection, excessive heat while sleeping in the prone position, an immature immune system due to a critical period in development and

other medical conditions all pose enough risk factors to fit the triple-risk model for SIDS (Filiano JJ & Kinney HC, 1994; Samuels, 2003; Bajanowski *et al*., 2007; Weber *et al*., 2010a; Weber & Sebire, 2010). According to this model the risk for SUDI is the highest when an underlying vulnerability in homeostatic control and an exogenous stressor coincide at a critical developmental period. One of the most likely environmental or exogenous triggers is viral infections and some of the proposed underlying vulnerabilities are immune deficiencies and poor control of over expression of inflammatory mediators (Samuels, 2003; Opdal & Rognum, 2004; Bajanowski *et al*., 2007; Weber *et al*., 2010a; Weber & Sebire, 2010). In addition, this critical developmental period could also coincide with the loss of protection of maternally derived antibodies (Balduzzi & Greendyke, 1966; du Toit-Prinsloo *et al.*, 2011).

While the immune system of the infant takes time to mature, a degree of humoral immune protection would have been provided from placental transfer of IgG from the mother during pregnancy (Cáceres *et al.*, 2000). Mothers infected with HIV (Farquhar *et al*., 2005; Reikie *et al.*, 2013) or with vaccine-induced immunity (Zhao *et al*., 2010) transfer lower levels of maternal IgG antibodies for specific vaccine preventable infections to their infants. Furthermore immune protection from placental transfer of IgG is short-lived and confers only limited protection against pathogens (Staines *et al*., 2010). Maternally derived IgG levels decrease as infants grow older. The decline in levels occur rapidly within the first six weeks after birth and markedly decline by six months, at which stage the maternal IgG would no longer offer protection. The maternally derived IgG levels become negligible as early as nine months of age, but usually towards 12 months, by which time infants are solely reliant on their own immature immune system for protection against pathogens (Gans *et al.*, 1998; Nicoara *et al.*, 1999; Thaithumyanon *et al.*, 2000; Gans *et al.*, 2004; Tapia *et al*., 2005). Since SIDS is documented to be higher in the 2-4 month age group, this could represent a critical developmental period that coincide with a loss of immune protection due to waning maternally-derived antibodies (Balduzzi & Greendyke, 1966; du Toit-Prinsloo *et al*., 2011).

Human breastmilk provides nutrition to infants and contains immune system components protective against infections. It also contains large quantities of secretory IgA specific to infectious agents that the mother was exposed to, which can neutralise pathogens and prevent pathogen attachment to cells in the infant hosts. Other factors in breastmilk, such as lysozyme and lactoferrin, can limit microbial growth by disrupting bacterial cell walls and removing iron, which is essential for bacterial survival (Jackson & Nazar, 2006). IgM and IgG antibodies are also present, but at lower levels than to IgA. Breastmilk also contains cytokines, macrophages, neutrophils, T and B cells, offering cell-mediated immune protection in infancy (Jackson & Nazar, 2006; Chirico *et al*., 2008).

Due to the functionally immature immune system infants may respond differently to infections, compared to adults and older children, which can complicate diagnosis of infections. Signs of clinical progression of illness may not be so prominent during an examination of the clinical history of a suspected SUDI case (Dempers *et al.*, 2011). All infants start off with a functionally immature immune system and can therefore not be regarded as an independent risk factor, because not all infants are predisposed to SIDS. Yet, the suggestion that certain clinical symptoms may be missed in some infants may be what sets healthy infants apart from those predisposed to SUDI, especially when a subset of infants have additional exposure to external modifiable risk factors. In these instances, and in line with the triple-risk model, the functionally immature immune system could play a contributory role by increasing infant vulnerability.

2.13 Inflammation and possible contribution to infant demise

The frequency of sudden unexpected death, although considered the predominant cause of death within the first year of life, declines drastically after the age of one year (Côté, 2010). By the ages of one to two years, when unexpected death in infants and children are rare, the majority of the immune responses and mechanisms have matured and are fully functional. Because sudden unexpected childhood deaths (SUCD) are rare after two years, it supports the theory that the immature immune system in infants may play a significant contributory role in SUDI and SIDS.

It is important to thoroughly investigate all SUDI cases, because this can explain the cause of death in a noteworthy proportion of cases. The most common findings include infections, and to a lesser extent congenital abnormalities, such as metabolic disorders and cardiovascular diseases. A review of SUDI literature from 1966-2009 showed that 51% of the explained deaths were due to infections, followed by congenital malformations and unintentional injuries (Nicod, 2005; Côté, 2010). For the diagnosis of SIDS, specific criteria have to be met and most importantly, thorough investigations should not yield a cause of death, even though some cases may not display the typical pattern of SIDS (Blair *et al*., 2012).

To establish a definite cause of death in SUDI cases can be quite challenging when only minor pathological signs are present and the boundaries between normal pathology and a subtle disease state can be indistinguishable. A positive virology test result from a SUDI case without histological evidence of inflammation becomes very difficult to interpret (Weber *et al*., 2010a). The mere presence of viral pathogens in lung tissue from SUDI cases does not necessarily mean that it was the cause of death and other factors must also be considered in the final diagnosis. The significance of isolating potential pathogens from sites of the body

that are normally considered sterile also remains debatable, especially in the absence of immunopathology and inflammation. This was demonstrated by Krous *et al*. (2009), where cardiac inflammatory infiltrates had been detected from both SUDI and control cases. The mean numbers of necrotic cardiomyocytes and inflammatory infiltrates, including lymphocytes and macrophages, were found to be similar in 24 SUDI cases and 25 infants that suffocated. This questions whether such findings should not actually rather be considered normal autopsy findings in many infant deaths. The study also included a third group of four infants that died of myocarditis. Krous *et al*. (2009) argued that histological evidence of severe myocardial inflammation can easily be attributed to myocarditis, but at the other end of the spectrum when very mild myocardial inflammation is detected, the significance of the possible contribution to death remains questionable. The degree of myocardial inflammation, cellular necrosis and viral load that can be severe enough to cause death in infants, or be sufficient for a diagnosis attributable to viral infections, must still be determined. Krous *et al*. (2009) does not regard the presence of a small number of scattered inflammatory cells, in the absence of pathogenic agents, as significant to be lethal. Similarly, the absence of HAdV and human enterovirus which they were screening for from cases with evidence of lymphocytic infiltration into the myocardium did not necessarily mean that viral aetiology could completely be excluded, because other unrecognised or undetected viruses might have been involved. Present knowledge on the interaction between viruses and the immune system in the myocardium is restricted to experimental mouse models and require further investigation.

Krous *et al*. (2003) found similar lymphocytic infiltration in the LRT (bronchi, bronchioles and the pulmonary interstitium) in SIDS and control cases (inflicted injuries, accidents, infanticides) in California from 1991 to 2000. In the URT (proximal and distal trachea) lymphocytic infiltration was significantly more severe in the control cases compared to SIDS cases. In their study histology signs of URT pathology observed were similar in SIDS and control cases (39% and 40% respectively). They concluded that microscopic signs of inflammation in SIDS *are not necessarily lethal* and can therefore not be a marker to indicate lethal infections. Clinically, bronchiolitis and pneumonitis severe enough to be lethal is normally preceded by clinical signs of illness including apnoea, lethargy, feeding difficulties and respiratory distress (Krous *et al*., 2003).

Recent studies suggest the possibility of SUDI infants having primary immune abnormalities. This is in parallel with the hypothesis that infants succumbing to SUDI could have inherent vulnerabilities (Krous *et al*., 2009; Weber & Sebire, 2010). Primary immunodeficiencies from defects in the cellular and humoral immune response systems can lead to increased

susceptibility to infections. Examples of primary immunodeficiencies include X-Linked agammaglobulinaemia where B cell maturation is impaired and Ig production is reduced and Zap-70 deficiency where the concentration of $CD8^+$ T cells is reduced and the $CD4^+$ T cells are non-functional. Primary immunodeficiencies can lead to recurrent viral infections, and in the case of severe combined immunodeficiencies, the outcome may be fatal unless bone marrow transplantation occurs (Lim & Elenitoba-Johnson 2003).

Defective control of inflammation, due to an underlying vulnerability in the host has been proposed as a relevant component in a subset of SUDI cases (Blood-Siegfried, 2000; Staines *et al*., 2010). The primary function of the immune system is to provide the host with a defence mechanism against infections, such as viral respiratory pathogens. The functions of a tightly regulated inflammatory response to infections include the migration of immune cells to the site of infection to prevent further pathogen replication, the creation of a barrier which can prevent the spreading of the invading pathogens and lastly the stimulation of stimulate tissue repair (Gotsch *et al*., 2007).

The immune response to respiratory viruses often induces pro-inflammatory mediators. Due to the changes in cytokine levels favouring an exaggerated immune response, it has been proposed that the fatal outcome in SUDI cases may not necessarily be specific to a certain type of infection or pathogen, but may instead be a result of a failure in the immuno-regulatory mechanisms in the infants. It was further suggested that a seemingly insignificant infection could trigger an inflammatory response exaggerated beyond normal, especially at a developmental stage when infants are less able to control such a response, which may lead to septic shock and the demise of such infants (Blood-Siegfried, 2000; Staines *et al*., 2010). Inflammation can also lead to the obstruction of bronchioles which can impair breathing and often, especially in the case of RSV infection, an exaggerated immune response occurs (Gotsch *et al*., 2007; Openshaw, 2005). One of the main anti-inflammatory cytokines is IL-10, which is considered an inhibitory cytokine that can dampen exaggerated inflammatory responses. A significantly lower IL-10 production was found in infants than in adults. Infants may thus be less able to compensate for inflammatory responses and may be more susceptible to the damaging effects that pro-inflammatory cytokines exert on organs in the host during infections (Schultz *et al*., 2004). Mutations that cause lower expression of anti-inflammatory cytokines have been detected in some SUDI cases, together with increased pro-inflammatory cytokines, such as IL-6 and TNF-α (Staines *et al*., 2010). During the course of infection the levels of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α and IFN-γ, are increased. IL-1 has been shown to induce drowsiness and sleep apnoea, a possible mechanism contributing to SUDI in both humans and animals. Surprisingly, despite a

functionally immature immune system early in infancy, the production of pro-inflammatory cytokines can be detected from very early in life. The pro-inflammatory cytokine IL-6 is considered a marker for inflammation and tissue damage. It is the principal cytokine secreted during the acute-phase inflammatory responses to damage in tissues. This cytokine has been found in high levels in SUDI cases and infants who died of bacterial sepsis (Blood-Siegfried, 2000; Gotsch *et al*., 2007), which highlights the similarities between SUDI cases and deaths due to infections and supports a possible contributory role of infections and the subsequent immune response to such infections in SUDI.

Even though it is agreed that SIDS is a diagnosis by exclusion after a thorough medico-legal investigation and autopsy, a range of pathologic features have been found in such cases. Mild abnormalities that are categorised as non-specific findings include petechiae in the thymus, lungs and heart, congestion of the small veins and capillaries and intra-alveolar haemorrhage. These findings are considered non-specific because such minor microscopic discoveries cannot be considered to significantly contribute to or sufficiently explain the demise of the infant (Weber *et al.*, 2008a). Other observed pathologic features include signs of mild inflammation, especially in the respiratory tract with increased IgG and IgM concentrations and T and B cell numbers in the lungs, which support the hypothesis of a prior respiratory tract infection possibly contributing to SUDI (Blood-Siegfried, 2000).

2.14 Validity of positive bacterial findings in sudden infant death cases

Prior to death, the internal organs of the body are considered sterile, but post-mortem, when the host's immune system ceases to function, epithelial barriers lose their integrity and bacteria migrate (Tuomisto *et al*., 2013). Some of the positive bacterial isolates that have been detected from SIDS cases include Staphylococcus species, *Escherichia coli*, *Streptococcus* species, *Haemophilus influenza*, *Klebsiella* species and *Neisseria meningitidis* (Rambaud *et al*., 1999; Weber *et al.*, 2008b; Lobmaier *et al.*, 2009; Pryce *et al*., 2011).

It has been suggested that positive bacterial results obtained from autopsy samples can be acquired in four different ways with the first as a result of a true positive bacterial event, the second being agonal spreading of bacteria, the third being post-mortem migration and the fourth being contamination (Tuomisto *et al*., 2013). Firstly, a true positive event is defined when there is pure growth of a single species. It is suggested that such a species, if present during life, can therefore also be detected at death (Tuomisto *et al*., 2013). Periods of bacteraemia can often occur in life in the absence of any signs of damage and inflammation, but when the isolated species is a pure growth of a recognised pathogen (e.g. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*,

Listeria monocytogenes, *Neisseria meningitidis, Neisseria gonorrhoea, Haemophilus influenza and Cryptococcus neoformans*, *Enterobacter* species, *Enterococcus* species, *Klebsiella* species, *Proteus* species, *Salmonella* species, *Serratia marcescens, Streptococcus pneumonia, Streptococcus pyogenes, Streptococcus agalactiae*, group A beta-haemolytic streptococcus and group B beta-haemolytic streptococcus), its presence at death should be seen as a contributory factor. More significance may be placed on a positive autopsy result if it can be corroborated with histological signs of disease (Morris *et al.*, 2006). Secondly, agonal spreading of bacteria is hypothesised to occur during dying and resuscitation attempts when mucosal integrity is compromised under ischaemic and hypoxic stress (Morris *et al*., 2006; Tuomisto *et al*., 2013). Such positive results comprising of mixed growth of commensals and potential pathogens would not be considered a cause of death, but rather a consequence of it (Morris *et al*., 2006). Whether agonal spread is a true phenomenon is still uncertain and no evidence has yet shown that it occurs in practise (Morris *et al.,* 2006; Tuomisto *et al*., 2013). Thirdly, post-mortem migration or translocation of bacteria occurs when species migrate into the blood from the gut after circulation has ceased. This occurs during the normal process of putrefaction (Tuomisto *et al*., 2013). In a review Morris *et al*. (2006) suggested that post-mortem translocation may be a rare event, especially when samples are collected soon after death and if bodies are refrigerated prior to autopsy. Fourthly, during specimen collection contaminants can be introduced into the organs or samples at autopsy from external sources. When mixed species are isolated from autopsy findings it generally indicates contamination of the specimens or post-mortem migration of species (Tuomisto *et al*., 2013). Krous *et al*. (2003) found organisms in 80% of SIDS cases and 89% of control cases (unnatural deaths) in California between 1991 and 2000. Their results were not interpreted as pathogenic because of the high percentage of polymicrobial growth suggesting post-mortem contamination.

In a SUDI systemic retrospective case review study in the UK Weber *et al*. (2010b) found that a PMI of five or more days resulted in a reduction of microorganisms isolated. They recommended that microbial sampling should be taken from all SUDI cases, even those with a PMI of five or more days. During their study they could not find an increased risk of normal bacterial translocation as the PMI increased. Therefore results may still be valid with longer PMIs. They also found a significant decrease in the number of positive single bacterial cultures and polymicrobial growth isolated from samples taken after a PMI of five or more days, compared to those from samples 24 hours after death (Weber *et al*., 2010b).

From 33 autopsies performed on out-of-hospital adult male deaths from 2009-2010 in Tampere, Finland, liver specimens had a higher sterility rate compared to other sites (portal

vein blood and mesenteric lymph node specimens) up until five days post mortem. In contrast to Weber *et al*. (2010b) who did not find an association with an increased PMI and mixed or positive isolates after a PMI of five days, Tuomisto *et al*. (2013) found that both single and mixed bacterial isolates from liver specimens increased.

2.15 Other risk factors for SUDI and SIDS

Apart from viral infections, other risk factors for SUDI include but are not limited to socio-demographics, sleeping position and -environment, genetics and the cardiovascular system, (Table 2.2) (Athanasakis *et al*., 2011).

A poor socioeconomic status is considered a risk factor for SUDI and SIDS. This includes multiple social factors, such as resources, financial status, birth weight, parental smoking and sleeping position amongst others. These factors may act in combination with a range of other co-morbidities throughout life and contribute to morbidity and mortality in infants (Spencer & Logan, 2004). Other factors include low level of maternal education, multiple pregnancies, male gender, recent infection(s), lack of breastfeeding, and not using a pacifier (Byard & Krous, 2003; Samuels, 2003; Hunt, 2005; Vennemann *et al*., 2007; Highet, 2008; Adams *et al*., 2009; Weber *et al*., 2010a; Athanasakis *et al*., 2011; Blair *et al*., 2012).

Poor antenatal care and substance abuse affect foetal development and growth, increasing the risk of prematurity and low birth weight, which are known risk factors for SUDI. After birth the sleep environment plays a crucial role in SUDI, where bed-sharing, heavy comforters, soft bedding, covering of the infant's head, wedging between objects, warm room temperatures, induction of sweating and fever, parental smoke in the sleeping environment and sleeping in the prone position, have all been identified as risk factors (Athanasakis *et al*., 2011). The prone position has been suggested to increase the temperature in the nose, especially during viral infections, which could facilitate bacterial colonisation and toxin production (Blood-Siegfried, 2000). Cigarette smoke can enhance the susceptibility to viral infections and in animal models it enhances the lethality of toxins produced by bacteria (Blood-Siegfried, 2000).

Table 2.2: A few well-established risk factors for sudden infant death cases

Risk factors	References
Male gender	Bergman *et al*., 1972; Patrick *et al*., 1989; An *et al*., 1993; Vennemann *et al*., 2005; Vennemann *et al*., 2007; Brookfield *et al.,* 2011; du Toit-Prinsloo *et al.*, 2011; Hutchison, *et al*., 2011; Trachtenberg *et al*., 2012; du Toit-Prinsloo *et al*., 2013
Cold months of the year	Bergman *et al*., 1972; An *et al*., 1993; Nunes *et al,.* 2001; Vennemann *et al*., 2005; du Toit-Prinsloo *et al.*, 2011; du Toit-Prinsloo *et al*., 2013
Maternal and/or paternal smoking	Nunes *et al*., 2001; Vennemann *et al*., 2005; Vennemann *et al*., 2007; Ostfeld, *et al*., 2010; Brookfield *et al*., 2011
Non-supine sleeping position	Nunes *et al.,* 2001; Paterson *et al*., 2006; Vennemann *et al*., 2007; Ostfeld, *et al*., 2010 Hutchison, *et al*., 2011; Trachtenberg *et al*., 2012
Bed-sharing	Hutchison, *et al*., 2011;
Age peak near the two-four month period	Balduzzi & Greendyke, 1966; Bergman *et al*., 1972; Nunes *et al*., 2001; Vennemann *et al*., 2005; du Toit-Prinsloo *et al.*, 2011; Hutchison, *et al*., 2011; Trachtenberg *et al*., 2012; du Toit-Prinsloo *et al*., 2013

Avoidance of specific risk factors, such as sleeping prone and exposure to cigarette smoke, resulted in a drastic decrease in the prevalence of SUDI, especially after the launch of the Back-to-Sleep campaign in 1994 (Willinger *et al*., 1994; Trachtenberg *et al*., 2012). Ultimately, SUDI is most likely the culmination of a variety of risk factors, mechanisms and pathophysiological responses (Byard & Krous, 2003; Randall *et al*., 2009).

Table 2.2 highlights certain factors that regularly occur in SUDI and SIDS cases. Some of these factors are considered risks, despite the lack of comparison to age-matched control infants dying of known causes. Risk factors were included if it was present in more than half of the SUDI cases in these studies, irrespective of the population size or a suitable control group. If a control group was present, then the risk factors were included if the difference between the cases and controls were statistically significant, or much more prevalent. Most of these risk factors are well established in the literature (Nunes *et al*., 2001; Athanasakis *et al*., 2011).

SUDI cases with none or one risk factor in isolation were found to be extremely rare (0.8%-18%) in a population-based New Jersey study from 1996-2000. The majority of cases (78.3%) displayed between two and up to seven risk factors (Ostfeld, *et al*., 2010).

Therefore, SUDI rarely occurs in the presence of risk factors that are generally not considered life-threatening, but rather require multiple risk factors to create the sequence of events that could lead to SUDI.

2.16 Developmental and genetic disorders

Another leading hypothesis is that a developmental disorder of the brainstem may lead to impaired protective responses against life-threatening stressors while an infant is asleep, such as asphyxia, hypercarbia and hypoxia, including epileptic-seizure-induced hypoxaemia (Samuels, 2003; Randall *et al*., 2009). Proposed genetic risk factors include polymorphisms in the serotonin transporter (Hunt, 2005; Athanasakis *et al*., 2011) or immunoregulatory genes (Highet, 2008). Kinney *et al*. (2003) investigated serotonergic receptor binding and found that tritiated-lysergic acid diethylamide binding to 5-hydroxytryptamine (5-HT) receptors was significantly lower in SIDS cases compared to infants who died suddenly but in whom a cause of death was revealed after the autopsy. Paterson *et al*. (2006) used frozen medulla oblongata specimens from SIDS and control cases between 1997 and 2005 in California to investigate the 5-HT serotonergic neurons, which are involved with regulation of autonomic and respiratory systems. These neurons are situated in the brain stem and spinal cord and are responsible for regulation of homeostasis during sleep. Abnormal 5-HT receptor binding has previously been demonstrated in a subset of SIDS and it has been suggested that such abnormalities may be associated with SIDS through a failure to respond to homeostatic stressors while sleeping. Abnormalities included higher 5-HT neuron count and density in SIDS cases and a lower density in 5-HT binding sites. In addition, a significantly lower density in 5-HT binding sites were observed in male SIDS cases compared to female SIDS cases, which could contribute to the male predominance in SIDS cases. They concluded that some SIDS cases may thus have dysfunctions in their neuron firing, synthesis, release and also clearance of 5-HT, which can influence autonomic and respiratory responses (Paterson *et al*., 2006). They found that 87% of the SIDS cases had 5-HT abnormalities and an exogenous stressor such as prone sleeping and bed-sharing. In another study in California between 2004 and 2008, SIDS cases had lower levels of serotonin and also confirmed reduced 5-HT receptor binding in SIDS cases compared to controls (Jhodie *et al*., 2010). In a Norwegian study between 1984 and 2007 SIDS cases had significantly higher expression of the IL-6 Receptor (IL-6R) compared to control cases (Rognum *et al*., 2009). The IL-6R is expressed by 5-HT neurons in the arcuate nucleus, which is where neurons aggregate in the hypothalamus. The SIDS group also had slightly elevated levels of IL-6 in the CSF compared to the control group. Rognum *et al*. (2009) showed a potential link between the 5-HT medullary system and cytokines. They concluded that possible interactions between IL-6 and the 5-HT medullary system could play a contributory role in impaired homeostatic responses towards hypercapnoea induced by infections or sleeping in the prone position in some SIDS cases. In support of the “triple-risk model” (Filiano & Kinney, 1994), Paterson *et al*. (2006), Rognum *et al*. (2009) and Jhodie *et*

al. (2010) showed links between external stressor risk factors, 5-HT abnormalities of the brain and the immune system.

Ackerman *et al*. (2001) found mutations in the sodium channel gene (SCN5A) in 2% of the SIDS cases from 1997-1999 in Arkansas, USA. Both mutations detected in this gene cause a persistent inward sodium current similar to defects reported in patients with the long QT syndrome (LQTS), leading to increased cardiac events. URTIs could act as potential triggers that are typically characterised by manifestations that include irritation of the receptors in the URT, which can lead to apnoea or arrhythmia in infants with predisposing respiratory or cardiac malfunctions, such as malignant arrhythmia from LQTS (Vorontsov & Kelmanson, 1990; Samuels, 2003). A cardiac channelopathy such as the LQTS may lead to lethal ventricular arrhythmias that affect both the rhythm and rate of the heartbeats (Schwartz *et al*., 1998). Other cardiac channelopathies such as Brugada syndrome and catecholaminergic polymorphic ventricular tachycardia have also been observed in SIDS and could lead to sudden cardiac death (Van Norstrand & Ackerman, 2010). In addition, certain inborn metabolic errors have also been identified as risk factors for some SIDS cases, more specifically the deficiency in the medium-chain acyl coenzyme A dehydrogenase has been found in SIDS (Van Norstrand & Ackerman, 2010).

2.17 Aims and objectives

All SUDI cases in the Tygerberg FPS area, South Africa, are admitted to the Tygerberg FPS Mortuary and investigated according to the Inquests Act 58 of 1959. All the SUDI cases are subsequently screened for HAdV, CMV, RSV, influenza A and B, HPIV 1, 2 and 3 and HMPV as the standard procedure in an effort to identify the possible cause of death, but exploring alternative methods that will improve the accuracy of diagnosis and include a broader range of viruses seem justified.

The specific aims and objectives of this study were:

- To prospectively collect tracheal and lung swabs from SUDI cases through the Division of Forensic Pathology, Tygerberg FPS Mortuary, to assess respiratory tract pathology.
- To collect SUDI cases presenting at Tygerberg FPS Mortuary and include one full year to assess possible seasonal peak incidences of SUDI and SIDS cases
- To use the Seeplex® RV15 Ace detection PCR to establish the frequency of 13 RNA respiratory viruses (influenza A & B, HPIV 1-4, HCoV [OC43, 229E/NL63], HRV A/B/C, RSV A/B, human enterovirus and HMPV) from extracted RNA samples collected during the autopsy performed on SUDI cases

- To compare viruses detected with lung histology signs of infection
- To collect demographic data to investigate possible risk factors for SUDI
- To determine the viruses circulating in living hospital infants at Tygerberg, during the study period.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER CONTENTS

3.1 Study approach and design

This was a prospective descriptive study designed at profiling respiratory viruses from cases presented as SUDI at Tygerberg FPS Mortuary in the Western Cape. Autopsy samples were collected from SUDI cases over a one year period from May 2012 to May 2013 and included four consecutive seasons; autumn, winter, spring and summer. The methodology subsections to follow consist of the ethical considerations for the study, and detailed descriptions of the sample collection, processing and detection methods used to investigate respiratory pathogens from SUDI cases collected.

3.2 Ethical considerations

The study was approved for one year by the Health Research Ethics Committee of Stellenbosch University on 7 March 2012 (protocol number N12/02/007) and renewed for a second year on 25 March 2013 (Addendum 1).

The samples that were collected during the study period did not differ substantially from the current autopsy protocol used for SUDI investigations at Tygerberg FPS Mortuary. Therefore, a waiver of consent was granted for collection and subsequent laboratory testing of specimens. As the tissue was retained and tested with the aim of reporting the results to the Division of Forensic Pathology to assist in the process of determining the cause of death, consent was provided for by section 3(a) of the Inquests Act (58 of 1959) and the Criminal Procedure Acts (1977). The tests conducted on these specimens only included methods for testing for respiratory pathogens. All specimens were marked with the death register number only. No personal identifiers were available to laboratory personnel or the research team. The identity of the deceased and the link to the specimen was protected by a liaison officer in the Division of Forensic Pathology (Mrs Riley), as was the case with the routine specimens sent to the virology and microbiology laboratories.

3.3 Study population demographics

During the one year study period samples from 148 SUDI cases were collected from the Tygerberg Forensic Mortuary. All these cases were included in the study, provided that they were between the ages of at least three days and one year. All the cases that initially presented as SUDI, but were subsequently classified by the pathologists as unnatural causes of death, such as child abuse, homicide and poisoning either during the autopsy procedures or after further histological examinations of relevant organs and ancillary investigations, were excluded from the study. All known natural causes of infant death, where

life-threatening disease was noted by physicians prior to death, or where it included hospitalisation of the infants prior to the day of death, were also excluded from the study.

Seasonal distribution of SUDI cases was based on a publication by Hussey *et al.* (1997) where the seasons in Cape Town were classified as follows: winter (June to August), spring (September to November), summer (December to February) and autumn (March to May).

Eighty-nine (60.1%) of the cases were male and 59 (39.9%) female. Tracheal and lung swabs were collected from every SUDI case except the first case, where only tracheal swabs were collected.

3.4 Sample collection and processing

This section describes the type of autopsy specimens that were collected from SUDI cases both as part of routine mandatory microbiological and virological testing by the NHLS, Tygerberg Hospital, and those for additional viral investigations for this study. All specimens collected were labelled with the unique Western Cape (WC) Tygerberg (14) case number starting at 0000, followed by the year (e.g. WC14/0000/2012).

3.5 Routine NHLS sample collection and processing

To begin the autopsy a Y-shaped incision was made by a forensic pathologist or forensic pathology officer (FPO) assisting with the body dissection. Two incisions started behind the ears and met at the top of the sternum. The cut was then extended all the way down to the pubic bone. Next, the sternum and ribs were cut open to lift out the chest plate and expose the chest cavity.

3.5.1 Routine heart blood sample collection

For routine blood sampling, the pericardial sac was first opened. Thereafter the apex of the heart was partially lifted out of the chest cavity, using clean forceps so that blood could be drawn from the base of the heart at the right atrium and inferior vena cava. Blood was drawn using a 5 ml sterile syringe capped with a 15 gauge (g) needle into a red top chemistry glass tube. The blood tubes were sent with request forms for HIV testing or chemistry analysis to the NHLS laboratories at Tygerberg Hospital, depending on the pathologists' requests and the conditions surrounding the infant's death.

3.5.1.1 Routine heart blood sample testing (HIV)

The majority of the HIV testing from the SUDI blood specimens was performed by a rapid third generation VISITECT® HIV1/2 test (Omega Diagnostics, UK; Davies Diagnostics (Pty) Ltd, Gauteng, South Africa) which is a point-of-care, qualitative immunoassay for the detection of antibodies to HIV-1/2 from human whole blood (specificity = 99.6% and sensitivity = 100%). Although this test is designed for non-haemolysed specimens free of contaminants, virtually all the autopsy blood specimens were notably haemolysed with platelet formation, but no other test was available at this centre. The test device consists of a membrane containing HIV-1/2 specific recombinant antigens and rabbit IgG. On the Test Line of the device are HIV-1/2 specific recombinant antigens and on the Control Line are goat anti-rabbit IgG. When serum and buffer are dispensed into the device, the HIV-1/2 specific antigens form complexes with HIV antibodies in samples that are positive for HIV. This complex then travels down the membrane to the Test Line where the HIV-1/2 specific recombinant antigens are coated on the membrane causing the sample-HIV-1/2 specific antigen complexes to immobilise. A pink line can then be visualised at the Test Line. The rabbit IgG continues moving down the membrane to the Control Line, where it becomes immobilised by the goat anti-rabbit IgG coated onto the membrane at this region (Figure 3.1). Testing of the blood specimens were performed by the routine Medical Virology NHLS laboratory, Tygerberg Hospital.

The process of the rapid third generation VISITECT® HIV-1/2 test was as follows: A drop (50 µl) of serum was dispensed into the sample well using a disposable plastic dropper supplied by the kit. Five drops of the diluent buffer was dispensed into the buffer well. After a 15-30 minute incubation period the results were read. Negative tests showed only the pink line represented by the validation positive control at the Control Line, whereas a positive test result for HIV-1/2 showed the pink line represented by the positive control at the Control Line, including a second pink line by the Test Line (Figure 3.1). If the positive control line did not appear, the tests would have been regarded as invalid and repeated.

If the blood was not severely haemolysed, as was the case with only a few of the specimens, an automated fourth generation HIV-1/2 Ag/Ab Combo (Alere Determine™, Japan; Abbott Molecular, Roodepoort, South Africa) test was performed on the AXSYM™ system (Abbott Laboratories, USA). The test is a microparticle enzyme immunoassay test for detection of antigen and antibodies to HIV-1 and HIV-2 (specificity = > 99% and sensitivity = 100%).

A **B**

Figure 3.1: Examples of a positive and negative VISITECT® HIV1/2 tests from three SUDI cases collected in 2013

A: a test showing a line at the Control Line the farthest down the membrane and another line is present at the Test Line, closer to the sample well, indicating an HIV positive test result, confirming HIV1/2 specific antibodies in the test serum.

B: two negative tests showing a red line each only at the Control Line.

3.5.1.2 Routine heart blood sample testing (Chemistry)

The chemistry analysis included detecting and reporting of urea and creatinine levels in the serum on an automated platform using the ADVIA® SIEMENS 1800 Clinical Chemistry system (Siemens Healthcare, Germany). Urea was measured using the ADVIA® Urea Nitrogen protocol based on the Roch-Ramel enzymatic reaction, using urease and glutamate dehydrogenase. Creatinine was measured using the Creatinine_2 method based on the reaction of picric acid with creatinine in an alkaline medium. The serum urea and creatinine tests are used for clinical investigation and diagnosis of metabolic and biochemical disorders, such as kidney and renal failure or dehydration. Unlike many chemical substances that are altered during post-mortem autolysis, both urea and creatinine levels are relatively stable up until about four days post-mortem, which makes the diagnosis of nitrogen retention possible. The testing of urea and creatinine were performed by the routine Chemical Pathology NHLS laboratory, Tygerberg Hospital.

3.5.2 Routine swab autopsy sample collections

For the heart swab collection at autopsy the surface of the heart was scorched with a red hot spatula heated in a direct flame. This was to avoid the subsequent culture of post-mortem flora or contaminants, and an incision was made into the organ with a sterile surgical blade. The swab specimen was then taken from the incision in the heart. The same process was followed for the lower lobes of both lungs. Request forms were completed for the routine

swab samples and sent to the routine Medical Microbiology NHLS laboratory, Tygerberg Hospital, for routine microbiological culture.

3.5.2.1 Routine swab autopsy sample testing

The media used for routine bacterial culture was prepared at the Media laboratory, NHLS, Green Point, South Africa, before transportation to Tygerberg Hospital. The Quality Control (QC) department, Media laboratory, NHLS, Green Point tested all the prepared media on a daily basis and the test results were verified by the QC department. The routine QC procedure includes viewing prepared media for contaminants or precipitation, confirming the appropriate volume and distribution of agar and the pH and colour of the media. Agar plates are then inoculated with American Type Culture Collection (ATCC) bacterial strains to identify lactose fermentation, haemolysis and antibiotic sensitivity. The media is then transported to the Medical Microbiology NHLS laboratory at Tygerberg Hospital.

For this study, the material on each SUDI swab specimen was spread across a 4% blood agar plate, a MacConkey agar plate and a Chocolate agar plate permitting clinically relevant growth of Gram-positive, Gram-negative and non-selective bacteria respectively (MERCK, Germany). This was followed by an 18-24 hour incubation period at 35°C in the presence of carbon dioxide (CO_2).

3.5.3 Routine lung and liver autopsy sample collection

For routine virological centrifugation-enhanced SVC a 5x5 mm section of the margin of the left lower lobe of the lung was placed in a red top chemistry tube using a clean forceps and a sterile blade. A small piece from the margin of the left lower lobe of the liver was placed in a separate red top chemistry tube. The specimens were transported to the Division of Forensic Pathology, where 1 ml of viral transport media (VTM) (Highveld Biological (Pty) Ltd, Gauteng, South Africa) was added to the tubes. The routine laboratory request forms were completed and the specimens were sent to the Division of Medical Virology, for routine SANAS (South African National Accreditation System) accredited virology SVC.

3.5.3.1 Tissue (lung and liver) routine autopsy sample testing

A Respiratory Panel 1 Viral Screening and Identification Kit (Light Diagnostics™ Millipore, United Kingdom; Davies Diagnostics (Pty) Ltd, Gauteng, South Africa) was used for SVC of the lung tissue samples. It is an indirect immunofluorescence assay for detecting HAdV, influenza A and B, HPIV 1-3 and RSV in addition to CMV and HMPV. The liver samples were screened for CMV and HAdV. CMV was cultured on human fibroblasts (HF). HAdV was also

cultured on HFs and in a separate flask on a combination (combi) of Hep-2 and MDCK cells. The Hep-2/MDCK combi was also used to simultaneously culture the viruses included in the Respiratory Panel 1 Viral Screening and Identification Kit in addition to HMPV.

3.6 Sample collection and storage for virological studies

3.6.1 Tracheal swab sample collection

The neck tissue was dissected by the pathologist or the assisting FPO to expose the trachea. An incision was then made in the trachea between the sternal notch and the thyroid gland using a sterile blade. The trachea was pulled upwards to expose the lumen and a flocked swab (FLOQSwabs™, Copan Flock technologies, Italy LasecSA, Cape Town, South Africa) was inserted and pushed downwards into the bronchi and rotated several times before retrieving the sample. The same process was repeated for a second swab to ensure sufficient sample collection from this site. The flocked swabs are designed with a solid plastic shaft coated at the end with Nylon® fibres. Unlike cotton swabs, flocked swabs do not have an internal core and thus volumes of specimen entrapment are virtually negligible allowing a better sample yield. The perpendicular arrangement of the swab fibres allows the sample to remain near the surface of the swab, facilitating sample recovery. After tracheal specimen collection both flocked swabs were placed in a single 16x100 mm tube containing 2 ml universal transport media - room temperature (UTM-RT) and three glass beads (COPAN Transystem® Innovating Together™, Italy; LasecSA, Cape Town, South Africa). The UTM-RT system is designed to keep viral and bacterial samples stable at room temperature, but once the swabs are collected the screw cap tubes should be kept at 2-8°C during transit. The media is isotonic and contains antimicrobials to inhibit growth of certain bacteria and yeasts. The media also contains sucrose which enables the cryopreservation of the specimens during prolonged storage at –80°C. The UTM-RT tube was labelled with the case number and the type of specimen. Further processing of the UTM-RT tube and swabs required the samples to be vortexed to enable maximum sample recovery from the flocked swab, which was facilitated by three glass beads in each tube.

3.6.2 Lung sample collection

The lower lobes of both lungs were excised using clean scissors and scalpel blades and placed in dry plastic screw cap 90 ml specimen containers without any media (Bio-Smart Scientific, Cape Town). The containers were labelled with the case number and marked as left or right lung. Samples for virological studies were transported to the Division of Forensic Pathology on the 6th floor of the Clinical Building at Tygerberg Campus.

3.6.2.1 Histology of lung tissue sections

The lower left and right lung lobes were handled with forceps (CJ Labs, Cape Town, South Africa), individually weighed and the information were recorded and sent to the pathologists. Next, the lungs were dissected through the middle to obtain a smooth flat interface, using a Tissue-Tek® Accu-Edge® trimming knife handle and disposable blades (Sakura Finetek Europe B.V., The Netherlands). From each of the lower left and right lung lobe tissue samples, a 1-3 mm thick slice was dissected across the broad lung surface and placed into separate histology cassettes and submerged in formalin (10% formalin, 90% tap water). The cassettes were labelled with the case number, and "2X L" (left lung) or "2Y R" (right lung), e.g. WC14/2222/2013, 2XL.

3.6.2.2 Lung swab sample collection

After cutting the tissue sections for histology, four flocked swab samples were taken from the freshly cut lower left and right lung lobe tissue. The swabs were rotated several times on the lung surface to ensure collection of small traces of lung tissue. The two swabs from the left lower lung lobes were placed in a single tube containing 2 ml UTM-RT and the same was done for the two swabs collected from the lower right lung lobe. The remainder of the lung tissue was placed back into the original screw cap tubes and stored at -80°C.

3.6.3 Lung and tracheal swab sample storage

The samples for virological studies were transported to a biosafety level 3 (BLS3) laboratory with restricted access, at the Division of Medical Virology, where the tissue samples were stored at -80°C. The UTM-RT tubes containing the tracheal and lung swabs were vortexed for one minute after which the lysate and swabs were divided into microcentrifuge tubes and stored at -80°C.

3.7 Histology preparation and analysis process

After at least 24 hours of fixation in 10% formalin to ensure tissue and morphology preservation, the histology cassettes containing the left and right lower lung lobe sections were removed from the formalin. The tissue sections were then manually processed for embedment in wax blocks and facing / trimming, cutting and subsequent haematoxylin and eosin (H&E) staining performed by Mr André Du Toit, medical technologist at the Division of Forensic Pathology.

3.7.1 Embedment (wax block preparations)

The Tissue-TeK® VIP™ 5 Jr. Vacuum Infiltration Processor (Sakura Finetek Europe B.V., The Netherlands) was used to prepare the wax blocks. This is an automated tissue processor that employs pressure and vacuum cycles to facilitate penetration of the formalin, ethanol and xylene into tissue sections. The Tissue-TeK® VIP™ 5 Jr. can contain a maximum of 150 cassettes and undergoes a series 14 one hour automated solution stations. The process starts with 10% formalin (BASF, The chemical company, Germany) and continues with an increasing series of ethanol (Illovo, Durban, South Africa) concentrations starting at 70%, 80%, and 96% and then remaining cycles at an ethanol concentration of 100%. The increasing ethanol concentrations prevent precipitation of salts from the formalin and serves as a dehydrating solution to remove both water and residual formalin from the tissues in the cassettes. The last stations contain xylene (BASF, The Chemical Company, Germany), a clearing agent used to allow mixing of the tissue sections with wax, because ethanol and water are not soluble in paraffin wax. The Tissue-TeK® TEC embedding console system (Sakura Finetek Europe B.V. The Netherlands) was used to create wax blocks of the tissue. The Tissue-Tek® Base Moulds were filled with hot Tissue-Tek® paraffin wax (Sakura Finetek Europe B.V. The Netherlands). Forceps were used to correctly place the tissue into the hot wax in the embedding mould and left to set for 20 minutes. Once hardened, facing was performed during which the excess wax was trimmed from the block. This was followed by cutting on the Microm HM 335E microtome Rotary (Thermo Fisher Scientific, USA) to acquire a 4-5 µm sized tissue section that was placed in a water bath containing tissue adhesive at 45°C. The water bath keeps the 5 µm tissue sections warm and straightens out any folds, after which the sections were mounted on glass microscope slides (Starfrost®, United Kingdom). The slides were then placed in an incubator to allow them to dry and facilitate attachment of tissue to slide.

3.7.2 Haematoxylin and Eosin staining

During the preparation for H&E staining the tissue had to be rehydrated by placing it in xylene for three cycles of five minutes each to ensure penetration of the H&E aqueous solutions. The slides were then placed in a series of decreasing ethanol concentrations starting at 100% then 96% and lastly 70% ethanol for five minutes each. The slides were then placed under running tap water to remove the excess ethanol prior to placing it in Mayers-Haematoxylin (MERCK, Germany). Haematoxylin is a basic dye that stains the nuclei of the cells a dark purple-blue to facilitate visualisation under a light microscope. The slides were rinsed again under running tap water and placed in Scott's solution, which is a blueing agent. Next the slides were placed in eosin (MERCK, Germany), followed by another

rinse in running tap water. Eosin is an acidic dye that stains the cell cytoplasm in various shades of pink while staining red blood cells an orange-red colour to facilitate visualisation and identification. Following this, the slides were placed in increasing concentrations of ethanol to dehydrate the tissue again and lastly in xylene. Next, cover slips were mounted onto the slides using non-aqueous mounting medium (Personal communication. February 2013. Mr André Du Toit, Chief Forensic Technologist at the Division of Forensic Pathology).

3.7.3 Histology analysis

Histological evaluations were conducted under the supervision of Dr Johan Dempers, the Principal Specialist Forensic Pathologist, at the Division of Forensic Pathology, Faculty of Medicine and Health Sciences, University of Stellenbosch and the findings were recorded. Findings were categorised into the following sections:

- Oedema
- Congestion
- Interstitial Pneumonitis (IP) as defined by Krous *et al*. (2003):

  Grade 1: "Very mild (barely present, often focal, and with no overall alteration of the alveolar septa)"
  Grade 2: "Mild (relatively diffuse and mild thickening of alveolar septa)"
  Grade 3: "Moderate (diffuse involvement and associated with interstitial oedema)"
  Grade 4: "Severe (diffuse infiltration associated with significantly widened alveolar septa)"
- Bronchopneumonia
- Focal collapse
- Pigment (iron / formalin)
- Alveolar debris
- Alveolar haemorrhage
- Bronchus associated lymphoid tissue (BALT)
- Bronchiolitis
- Comments (any unusual observations)

3.8 RNA extraction and complementary DNA synthesis for virological studies

RNA was extracted for the identification of thirteen respiratory RNA viruses and subtypes (Table 3.1). There were only two DNA viruses and because the prevalence of RNA and DNA viruses in SUDI and SIDS cases and their contribution are not defined yet, the DNA viruses were excluded. There were more RNA viruses in the Seeplex® RV15 Ace detection kit and because funds were limited focus was laid on the RNA viruses only.

RNA was extracted from lung and tracheal swabs using the protocol for the purification of total RNA from animal and human cells from the Qiagen® RNeasy Plus Micro kit (Whitehead Scientific, Cape Town, South Africa) according the manufacturer's instructions. Minor modifications were introduced to the protocol to facilitate the swab samples. A negative control containing UTM-RT was included with every RNA swab extraction.

In brief, the process was as follows: To lyse the samples, 350 µl Buffer RLT Plus and 5 µl carrier RNA (4 ng/µl) was added to each sample (swab in 200ul UTM). This was transferred to a QIAshredder spin column to disrupt and homogenise the sample. After homogenisation the lysate was transferred to a genomic DNA Eliminator spin column to remove genomic DNA. Then 350 µl of 70% ethanol was added and the lysate was transferred to an RNeasy MinElute spin column. Several wash steps were then performed, starting with 700 µl Buffer RW1, then 500 µl Buffer RPE and lastly 500 µl 80% ethanol to remove residual contaminants. Lastly the purified RNA was eluted twice into the same tube using two separate volumes of 14 µl RNase-free water.

RNA viruses and subtypes are diagnosed using complementary DNA (cDNA), which is more stable than RNA. The RNA samples were therefore reverse-transcribed to cDNA using a RevertAid™ Premium Reverse Transcriptase enzyme (Fermentas, USA). Due to the discontinuation of this enzyme during the study period, a Maxima H Minus Reverse Transcriptase enzyme was used for some of the samples. Random hexamers were used as primers (Fermentas, USA), and a deoxynucleotide triphosphate (dNTP) mix (Fermentas, USA), Thermo Scientific RiboLock™ RNase Inhibitor (Fermentas, USA), Qiagen® nuclease free water (Whitehead Scientific, Cape Town, South Africa) and 5X reverse transcriptase (RT) Buffer (Fermentas, USA) were also used for the cDNA synthesis process. A non-template control (NTC) containing nuclease free water was included to control for cDNA synthesis reagent contamination.

Table 3.1: The panel of 15 viruses and subtypes included in the Seeplex® RV15 Ace detection kit, however only the RNA viruses were investigated in this study (HAdV and HBoV are excluded).

Virus Name	Family	Nucleic acid
HRV	Picornaviridae	(+)ssRNA
HCoV OC43 HCoV 229E/NL63	Coronaviridae	(+)ssRNA
Influenza A Influenza B	Orthomyxoviridae	(-)ssRNA
PIV 1 PIV 2 PIV 3 PIV 4	Paramyxoviridae	(-)ssRNA
Human enterovirus	Picornaviridae	(+)ssRNA
RSV A RSV B	Paramyxoviridae	(-)ssRNA
HMPV	Paramyxoviridae	(-)ssRNA
HAdV*	Adenoviridae	dsDNA
HBoV*	Parvoviridae	ssDNA

*HAdV and HBoV were not investigated in the study because they are deoxyribonucleic acid (DNA) viruses and because funding did not allow for testing of the DNA viruses.

In brief, cDNA synthesis was done as follows: 8 µl of sample RNA was added to a 0.2 µl Quality Scientific Plastics (QSP®) PCR tube (Whitehead Scientific, Cape Town, South Africa) followed by the addition of a master mix, containing 1µl random hexamers, 1 µl dNTP mix and 4.5 µl nuclease free water (Whitehead Scientific, Cape Town) per sample. The samples were then incubated at 65°C for five minutes. A second master mix was added, consisting of 4 µl of 5X RT Buffer, 0.5 µl RiboLock and 1 µl RevertAid™ Premium Reverse Transcriptase enzyme / Maxima H Minus Reverse Transcriptase enzyme per sample. cDNA synthesis was done on a Gene Amp® PCR system 9700 (Applied Biosystems, California). The reverse transcription was initiated with an incubation of ten minutes at 25°C, and continued with a 30 minute incubation at 60°C and ending with a five minute incubation at 85°C.

After DNA and RNA extraction and again after cDNA synthesis the nucleic acid concentration was measured on a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies, USA)

3.9 Multiplex polymerase chain reaction for virological studies

After extraction and cDNA synthesis, the nucleic acid product from the swab samples were screened for 13 of the respiratory viruses contained in the Seeplex® RV15 Ace detection assay (Seegene, South Korea) according to the manufacturer's instructions. The two DNA

viruses contained in the kit (HAdV and HBoV) were not analysed. The kit is designed to qualitatively detect multiple respiratory viruses in a multiplex PCR assay. Each sample was simultaneously amplified in three separate reactions.

The kit contained internal, negative and positive controls that were included every time the PCR was performed and procedures were followed according the manufacturers' instructions. The internal control (IC) was designed to identify inhibiting substances in the samples that could impede nucleic acid amplification. If the IC was not detected in any of the experiments, that assay's results would have been invalid and the procedures would have been repeated. The IC contains a DNA plasmid designed to ensure simultaneous amplification of the IC and respiratory viruses. The kit positive controls contain a mixture of the 15 respiratory viruses, including clones of the IC. The negative controls contain sterilised water and received all the other relevant reagents, to control for any form of contamination in the PCR process. According to the manufacturers the limit of detection for the RV15 Ace detection assay is 100 copies per 3 µl cDNA.

In brief, a master mix, containing 4 µl 5X RV15 Ace PM, 10 µl, 2x Multiplex Master Mix and 3 µl 8-Mop solution per sample was prepared for PCR amplification. Seventeen microliter of the master mix and 3 µl of the sample cDNA were added to each sample tube. The PCR reaction was conducted on the Gene Amp® PCR system 9700 (Applied Biosystems, California) with one cycle at 94°C for 15 minutes followed by 40 cycles at 94°C for 0.5 minutes, 60°C for 1.5 minutes and 72°C for 1.5 minutes and ending with one cycle at 72°C for 10 minutes.

For visualisation of the PCR products, a 2% gel was prepared by heating and dissolving 2 g Lonza SeaKem® LE agarose (Whitehead Scientific, Cape Town) and 100 ml Tris-acetate (TAE) 1x working concentration buffer. A stock of 50x TAE buffer was first prepared consisting of 242 g crystallised Tris (hydroxymethyl) aminomethane base (Boehringer Mannheim, USA), 57.1 ml AnalaR® acetic acid (BDH, South Africa) and 100 ml 0.5 m Ethylene diamine tetra acetic acid (EDTA) (Sigma-Aldrich®, USA) at a pH of 8.0 (Sambrook, *et al*., 1989). The warm gel was poured into 16 and up to 51 well gel moulds and allowed to set. Subsequently, 5 µl of the PCR product or molecular markers or 2 µl of the positive control supplied by the kit, were mixed with 1 µl GeneDirex® novel juice (Biocom Biotech, Cape Town) and loaded onto the 2% agarose gel. Novel juice aids visualisation of DNA bands on agarose gel once illuminated. The gels were run at 60 volts for 30-40 minutes using a BG Power6000 electrophoresis system (BayGene®, China) with gentle shaking on a lab rotator (Digisystem laboratory instruments Inc. Taiwan). The gels were analysed using an UVitec computer based gel documentation system (UVitec, United Kingdom) by first

adjusting the scale under white light and then visualising the PCR bands under the transilluminator.

3.10 Validation of the extraction and multiplex Seeplex® RV15 Ace Detection Assay

Validation of the extraction kits included slight modifications to the protocols to assist maximum sample recovery, including extraction and cDNA synthesis of confirmed positive respiratory samples from the diagnostic laboratory to ensure that the procedures were working. Validation of the multiplex Seeplex® RV15 Ace detection assay also included the use of confirmed positive respiratory samples from the diagnostic laboratory and QC samples from Quality Control for Molecular Diagnostics (QCMD, United Kingdom). The DNA and RNA were simultaneously extracted using the QIAamp® MinElute® Virus Spin kit for the validation processes (Whitehead Scientific, Cape Town, South Africa).

In brief, the process was as follows: To lyse the samples, 25 µl QIAGEN® Protease was added to 1.5 ml microcentrifuge tubes, followed by the addition of 200 µl sample and 200 µl Buffer AL (containing 28 µg/ml of carrier RNA). Samples were then incubated at 56°C for 15 minutes. The lysate was applied to a QIAamp MinElute column and centrifuge at 8 000 rpm for one minute. Several wash steps were then performed starting with 500 µl Buffer RW1, then 500 µl Buffer RW2 and lastly 500 µl ethanol (96–100%), in order to remove residual contaminants. The purified nucleic acids were eluted using 60 µl Buffer AVE. The viruses that were successfully detected from diagnostic respiratory samples and the QCMDs included the two DNA viruses, HAdV and HBoV, the RNA viruses, HRV, HCoV OC43/HKU1 and 229E/NL63, influenza A, HPIV 1-3, RSV A and B and HMPV. Influenza B, HPIV 4 and human enterovirus could not be detected during the validation procedures.

3.11 Validity and interpretation of results

The Seeplex® RV15 Ace detection results were considered valid if:

- The IC was detected for every sample and control
- The NTC and negative controls were free of any viral products

The positive results were interpreted according to the template provided in Table 3.2, although HAdV and HBoV were excluded from the results because only RNA extracts and the subsequent cDNA products were used for the viral screening.

Table 3.2: Interpretation of results according to amplified PCR product size compared to the molecular markers

RV15 ACE Detection (A set)	Size in agarose gel (base pairs)
IC	850
HAdV	534
HCoV 229E/NL63	375
HPIV 2	264
HPIV 3	189
HPIV 1	153
RV15 ACE Detection (B set)	**Size in agarose gel (bp)**
IC	850
HCoV OC43/HKU1	578
HRV A/B/C	394
RSV A	269
Influenza A	206
RSV B	155
RV15 ACE Detection (C set)	**Size in agarose gel (bp)**
IC	850
HBoV	579
Influenza B	455
HMPV	351
HPIV 4	249
Human enterovirus	194

3.12 Reviewing of case files and collection of routine laboratory results

Demographic and epidemiological data were recorded from the case files from all SUDI and SIDS cases included in the study and captured on a MS Office® Excel spreadsheet. Information collected included birth, death and autopsy dates, home address, sleeping position, type of blankets used, bed-sharing, illnesses in the days preceding death, alcohol use or smoking habits of the parents and the final concluding diagnosis and comments of the pathologists assigned to the cases.

3.13 Data mining to collect virology results from hospitalised infants at Tygerberg

Official data mining was done through the NHLS to acquire anonymous data from infants admitted to Tygerberg Hospitals during the study period. The data mining form is presented as Addendum 2. The gender and ages of hospitalised infants from May 2012 to May 2013

were retrieved, as well as the specimen types and test results of routine SVC and routine RV15 PCR. This data was used to compare to the SIDS and SUDI cases.

3.14 Statistical analysis

Statistical analysis was conducted using STATISTICA StatSoft® version 11 under the supervision Dr Justin Harvey from the Centre for Statistical Analysis at Tygerberg Campus (Stellenbosch University). For data that was not normally distributed, the Kruskal-Wallis and Pearson Chi-square tests were used.

CHAPTER FOUR

RESULTS

CHAPTER CONTENTS

4.1 Overview

During this prospective descriptive study, SUDI cases were collected over a one year period from Tygerberg FPS Mortuary to investigate potential respiratory infections. A multiplex PCR was used to investigate the presence of 13 respiratory RNA viruses (influenza A and B, HPIV 1-4, HCoV [OC43, 229E/NL63], HRV A, B and C, RSV A and B, human enterovirus and HMPV). We also captured routine bacterial and viral culture results as part of the routine screening according to the facility protocol. All cases were microscopically examined for histological signs of immune cell infiltrations. All available epidemiological data was also collected to investigate possible risk factors that may contribute to SUDI.

4.2 Characteristics of study population

A total of 148 SUDI cases were collected at Tygerberg FPS Mortuary during the one year study period. The male : female ratio of the study group was 1.5:1 (60.1% males and 39.9% females).

The seasonal pattern and monthly distribution for the cases collected is shown in Figure 4.1 where more than half of the SUDI cases presented at Tygerberg during the autumn and winter months.

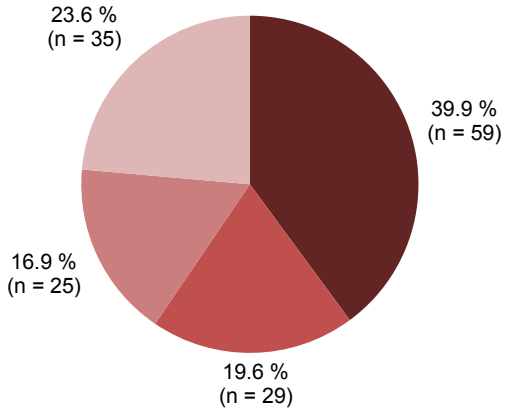


Figure 4.1: The seasonal distribution of the SUDI cases from May 2012 to May 2013. There was an increase of SUDI cases during autumn and winter.
■ Winter (Jun-Aug) ■ Spring (Sep-Nov) ■ Summer (Dec-Feb) ■ Autumn (Mar-May).

Of the 148 SUDI cases the medico-legal investigations were only completed and the files signed out in 105 cases. The epidemiological data only includes information from these 105 case files. The medico-legal investigations for the other 43 cases were still incomplete at the time of writing the thesis because the pathologists still needed to sign out those cases.

The median age of the cases was two months (mean 2.3 ± 2.32 months; range 3 days-12 months). The majority of cases were between zero and three months old (84/105; 80%) and the largest peak was observed in the one to two months group (51.4%; 54/105) (Figure 4.2). Although the original inclusion criteria was an age between one week to one year, one three-day-old infant, two of five days old and one six-day-old infant were included, because they fitted the profile of sudden and unexpected death. Of the 105 case files completed and signed out by pathologists, 104 files specified the date of death and the date of autopsy. The median PMI was three days (mean 3 ± 1.97 days; range 1-13 days). Up to 83.7% (87/104) of the autopsies were performed within 2-5 days of the infant's death.

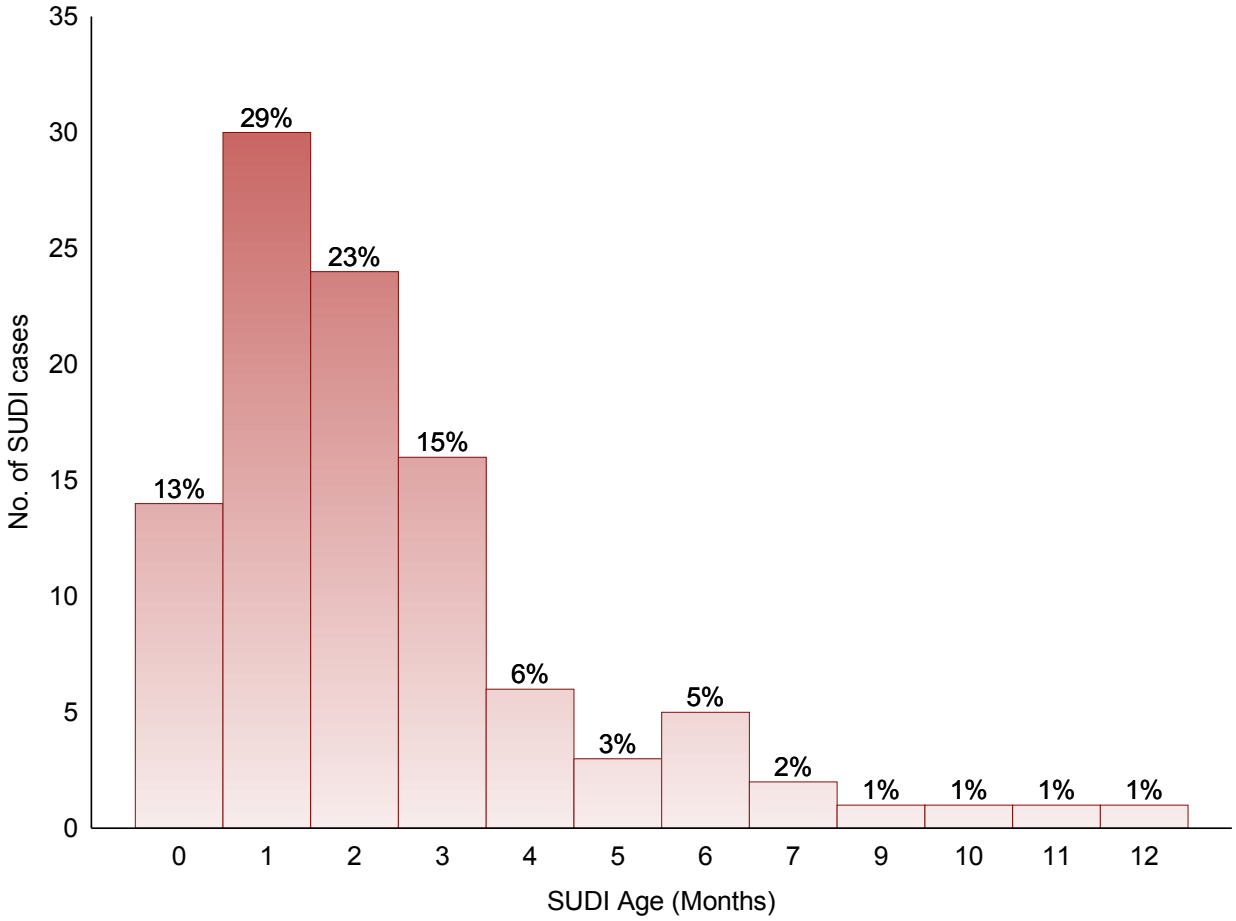


Figure 4.2: Age distribution of the SUDI cases during the one year study period at Tygerberg FPS Mortuary. The highest prevalence was found in the zero to three month age group.

The sleeping environment and parental information are shown in Figures 4.3 and 4.4. There were 32 (30.5%) SUDI cases that were put to sleep on their stomachs, 54 (51.4%) were wrapped in thick heavy blankets, 45 (42.9%) lived in informal settlements and the majority of the infants slept in a room with a closed window and shared a bed with up to four parents and/or siblings. Parents smoked and/or used alcohol in 41 (39.0%) and 39 (37.1%) SUDI cases respectively and the majority of cases had a single mother who was unemployed. The

median maternal age was 24 years but 13.3% were ≤ 20 years of age (mean 26.7 ± 7.30 years; range 13-50 years).

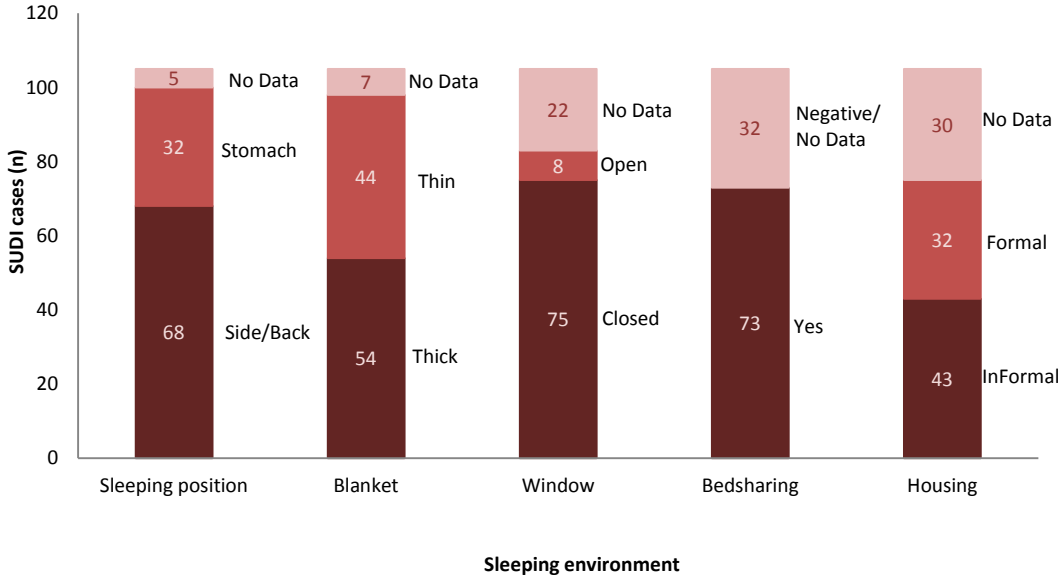


Figure 4.3: Factors relating to the sleeping environment.

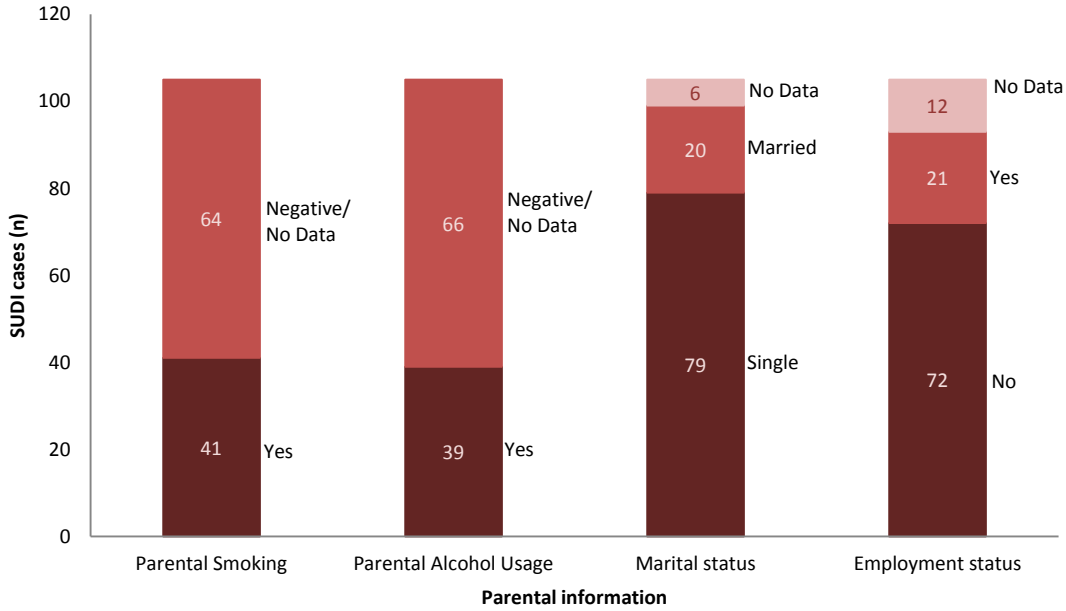


Figure 4.4: Parental information of the SUDI cases.

4.3 *Cause of Death Classification*

The final *Cause of Death Classification* was assigned by pathologists after the completion of the medico-legal investigation and autopsy for 101 cases only (Figure 4.5). To determine the

final cause of death classifications the pathologists had access to the routine virology and bacteriology results prior to signing out their SUDI cases, but the PCR viral results were not available to them. The data for the other 47 cases was not completed in the case files. Half of all the SUDI cases were assigned a *Cause of Death Classification - Infection*, 33.7% were classified as *SIDS* where evidence failed to reveal a cause of death and the remaining 15.8% died of other causes (congenital cardiac abnormalities in seven cases; aspiration of foreign material or gastric contents and starvation and dehydration in three cases each; unable to exclude accidental suffocation, prematurity and peritonitis as a complication of Meckel's diverticulum or malformation of gastrointestinal tract in one case each).

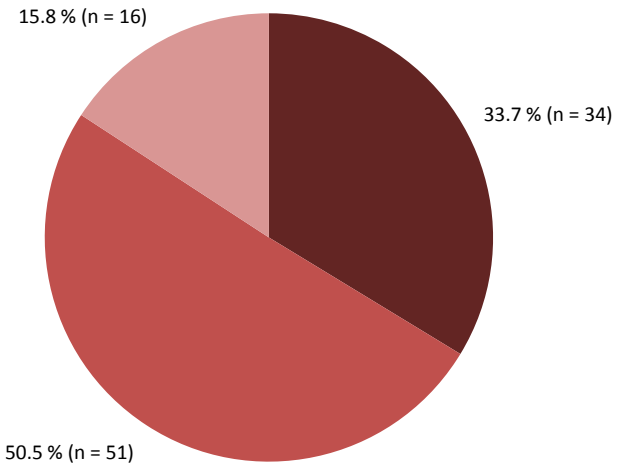


Figure 4.5: The final *Cause of Death Classification* as determined by the pathologists after the medico-legal investigation. Pathologists were blinded to the PCR virology results, when they determined the final cause of death.
■ SIDS ■ Infection ■ Other.

4.4 Respiratory pathogen detection

Laboratory results were obtained for all 148 cases in the study. Bacterial and/or viral pathogens were detected in 86.5% (128/148) of all cases. Positive viral PCR and culture results, regardless of whether some of those cases were also positive for bacteria, were found in 62.2% (92/148) of cases. Positive bacterial results were found in 56.8% (84/148) of cases, regardless of whether some of those cases were also positive for viruses. In 44.5% (57/128) of the cases a single infectious agent was detected and co-detections of between two and six viruses and/or bacteria occurred in 55.5% (71/128) of cases.

4.5 Viruses detected in the study

4.5.1 Multiplex polymerase chain reaction for RNA viruses

With the Seeplex® RV15 Ace detection assay between one and three viruses were detected in 59.5% (88/148) of cases. Of the 88 cases that had viruses detected, 75% (66/88) had one virus and 25% (22/88) had co-detections of two to three viruses (Table 4.1). Figure 4.6 shows distribution of viruses detected from the 88 PCR positive SUDI cases. The most common viruses detected were HRV in 77% (68/88) of cases, RSV in 18% (16/88) of cases and HCoV in 14% (12/88) of cases. Most of the HRV, RSV and HCoV cases were positive from tracheal and lung samples. Histology signs of infection were observed in 64% (44/68) HRV-positive cases, 62.5% (10/16) RSV-positive cases, 58% (7/12) HCoV-positive cases, 16.7% (1/6) human enterovirus-positive cases, 80% (4/5) HMPV-positive cases, 75% (3/4) PIV 3-positive cases and 25% (1/4) Influenza-positive cases.

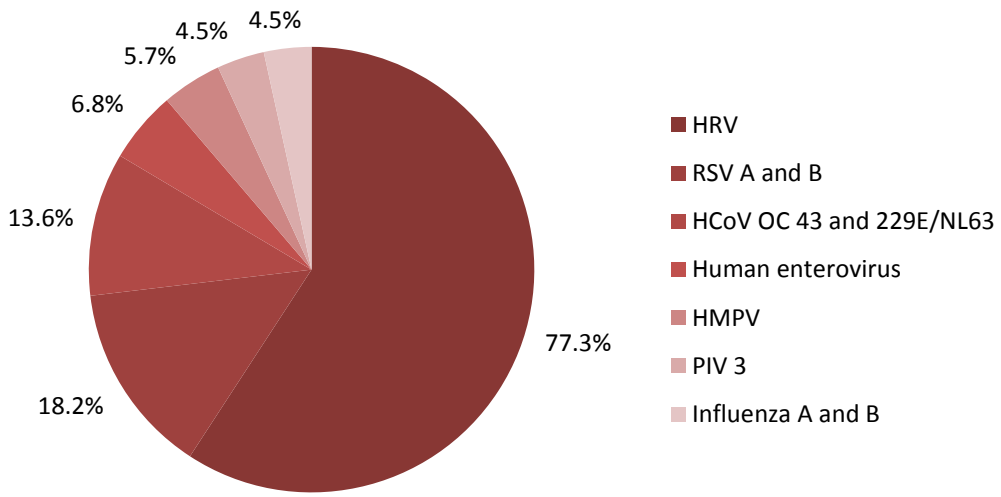


Figure 4.6 The distribution of viruses detected from the 88 PCR positive SUDI cases. HRV was by far the most frequently detected virus.

The details of all PCR results are summarised in Addendum 3. In summary, HRV was detected most frequently in this study, followed by RSV A, HCoV OC43, human enterovirus, HMPV, HCoV 229E/NL63, HPIV 3, influenza A, RSV B and influenza B. In 62.5% the of positive cases, viruses were detected in all three specimens (tracheal swab, lower left lung swab and lower right lung swab), while 8% and 28.4% of cases produced positive results in two or one specimens respectively. Tracheal swab specimens were more frequently positive for viruses (86/88; 97.7%) than the lung swabs (63/88; 71.6%).

Table: 4.1: Prevalence of the respiratory viruses detected from the SUDI cases in the study using the Seeplex® RV15 Ace detection PCR assay.

Virus	Prevalence in the study / (n)	Positive in Trachea	Positive in Lungs	Positive in all three specimens	Number of cases with one or more co-detections	Viruses co-detected in one or more cases
HRV	**46%** (68/148)	68	45	38	19	RSV A RSV B HCoV OC43 HCoV 229E/NL63 Human enterovirus HMPV PIV 3 Influenza A
RSV A and B	9.5% (14/148) and 1.4% (2/148) **Total: 10.8%**	13	15	8	11	HRV HCoV OC43 HCoV 229E/NL63 HMPV PIV3
HCoV OC43 and 229E/NL63	5.4% (8/148) and 2.7% (4/148) **Total: 8.1%**	10	7	3	9	HRV RSV A RSV B OC43 HCoV 229E/NL63 PIV3
Human enterovirus	**4.1%** (6/148)	6	5	3	4	HRV
HMPV	**3.4%** (5/148)	5	4	3	2	HRV RSV A
PIV3	**2.7%** (4/148)	1	4	1	4	HRV RSV A HCoV OC43
Influenza A and B	2.0% (3/148) and 0.7% (1/148) **Total: 2.7%**	4	2	1	1	HRV

The most PCR-positive cases occurred in autumn with an equal percentage of cases in winter and spring and a decline in summer (Table 4.2). Influenza PCR-positive SUDI cases only occurred in winter and HMPV PCR-positive cases occurred in winter and spring. No clear seasonal pattern was observed for any of the other viruses. There were more HRV PCR-positive cases in spring and summer compared to autumn and winter. Both RSV and HCoV had the most PCR-positive cases occurring in autumn and winter.

Table 4.2: The seasonal pattern for the respiratory RNA viruses detected in the study.

Virus Name	Autumn: 80% of the cases were PCR-positive (28/35)	Winter: 79.7% of the cases were PCR-positive (47/59)	Spring: 79.3% of the cases were PCR-positive (23/29)	Summer: 64% of the cases were PCR-positive (16/25)
HRV	53.6% (15/28)	53.2% (25/47)	73.9% (17/23)	68.6% (11/16)
RSV A and B	21.6% (6/28)	17% (8/47)	0	12.5% (2/16)
HCoV OC43 and 229E/NL63	21.6% (6/28)	8.5% (4/47)	4.3% (1/23)	6.3% (1/16)
human enterovirus	3.6% (1/28)	4.3% (2/47)	8.7% (2/23)	6.3% (1/16)
HMPV	0	6.4% (3/47)	8.7% (2/23)	0
PIV 3	0	2.1% (1/47)	8.7% (2/23)	6.3% (1/16)
Influenza A and B	0	8.5% (4/47)	0	0

4.5.2 Unexpected Multiplex PCR results

HAdV was detected in 3.4% (5/148) of cases; in the trachea in four cases and the trachea and both lungs in the 5th case. HAdV was detected as the sole virus in two cases and co-detected with viruses such as HRV, RSV B, HCoV and HPIV 3 in three cases (data excluded from Tables and Addendum three because we focused on the 13 RNA viruses only and not the DNA viruses).

4.6 Routine viral culture results

SVCs were positive in 9.5% (14/148) of all cases. CMV was detected in 13 cases, of which one was in the liver tissue, two in the liver and lung tissue and ten in the lung tissue only. HAdV was detected in one case from the liver. Examples of SVC negative and positive results are shown in Figure 4.7. Histology signs of infection were noted in 78.6% (11/14) of

cases with a positive SVC result. Three cases wherein CMV was detected did not show any histological evidence of infection.

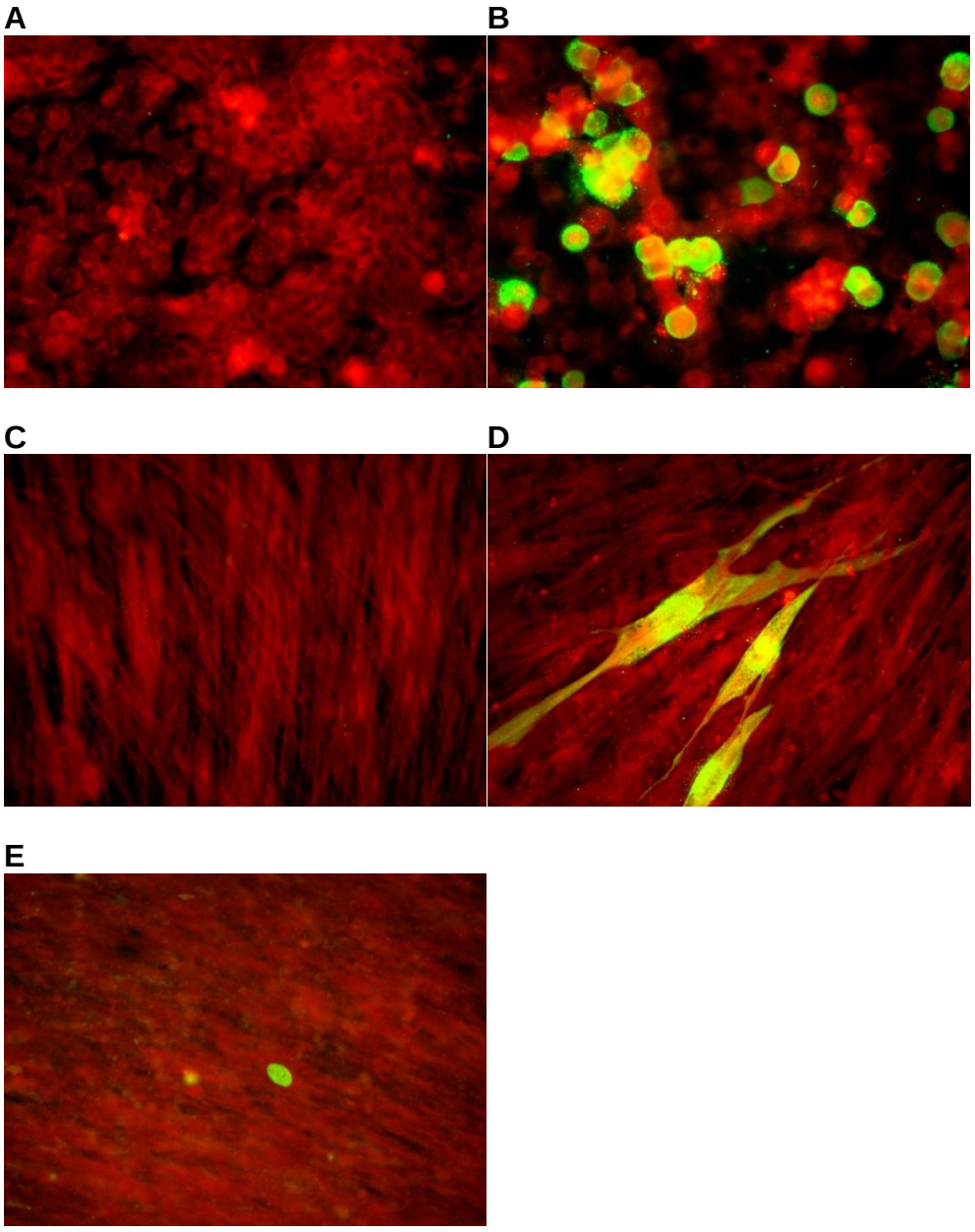


Figure 4.7: Images of negative and positive SVC results

A: an example of a negative SVC result from combi Hep-2 and MDCK cells.

B: an example of a HAdV positive SVC result from combi Hep-2 and MDCK cells.

C: an example of a negative SVC from HF cells.

D: an example of a HAdV positive SVC result from HF cells.

E: an example of a CMV positive SVC result from HF cells.

4.7 Routine bacterial culture results

Bacterial culture results were positive in 56.8% (84/148) of cases. Of these, 83.3% (70/84) had one species detected, 15.5% (13/84) had two species detected and 1.2% (1/84) had four species detected. An equal number of cases (32/148; 21.6%) had either detection of post-mortem flora or no growth on the culture plates. Histology signs of infection were observed in 60.7% (51/84) of the SUDI cases that were bacterial culture-positive. Of the bacterial culture-positive cases, 57.1% (48/84) also produced PCR-positive viral results and 58.3% (28/48) had histological evidence of infection. More lung specimens were positive for bacteria. The least likely specimen type to produce bacterial growth and post-mortem contaminants was the heart swab. The lung specimens more often cultured post-mortem flora and bacterial isolates (Table 4.3).

The percentages of bacterial species detected during routine bacterial culture from SUDI cases are shown in Table 4.4. *Klebsiella pneumonia* and *Escherichia coli* were the most frequently detected pathogens.

Table 4.3: The routine bacterial culture results according to sample site.

	Heart Swab (n)	Left Lung Swab (n)	Right Lung Swab (n)
Bacterial species	25	57	46
Post-mortem Flora	15	63	70
No Growth	108	28	32

Table 4.4: The prevalence of bacterial species found during routine culture

Bacterial species	Prevalence in the study / (n)
Klebsiella pneumoniae	16.9% (25/148)
Escherichia coli	16.2% (24/148)
Staphylococcus aureus	5.4% (8/148)
Beta-Haemolytic Streptococci	4.7% (7/148)
Klebsiella oxytoca	4.1% (6/148)
Raoultella planticola	2.7% (4/148)
Haemophilus influenzae	2.0% (3/148)
Candida albicans	2.0% (3/148)
Streptococcus pneumoniae	1.4% (2/148)
Enterococcus faecalis	1.4% (2/148)
Enterobacter cloacae	1.4% (2/148)
Enterobacter aerogenes	0.7% (1/148)
Raoultella ornithinolytica	0.7% (1/148)
Enterococcus species	0.7% (1/148)
Pseudomonas species	0.7% (1/148)
Serratia liquefaciens	0.7% (1/148)
Lactococcus garvieae	0.7% (1/148)
Leuconostoc citreum	0.7% (1/148)
Salmonella	0.7% (1/148)
Enterobacter species	0.7% (1/148)
Acinetobacter species	0.7% (1/148)
Staphylococcus epidermidis	0.7% (1/148)
Coagulase negative *Staphylococcus*	0.7% (1/148)

4.8 Histology results

Microscopic signs of infection from the left and right lower lung lobes were detected in 59.5% (88/148) of cases. Signs of possible infection included IP, bronchopneumonia, bronchiolitis and possible viral infections characterised by findings such as neutrophil and lymphocyte infiltrations and viral inclusion bodies (■ Figure 4.8). In 40.5% (60/148) of the cases non-specific changes were observed in the lungs that were generally not considered to be indicative of infections (■ Figure 4.9). Examples of lung histology from the SUDI cases are shown in Figures 4.10 – 4.13.

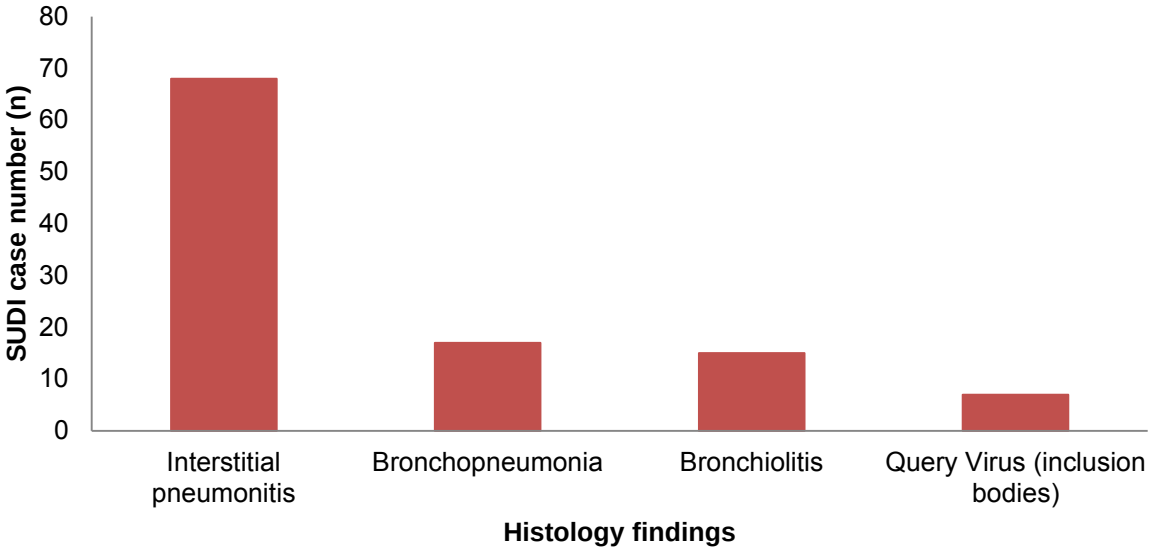


Figure 4.8: Specific histology findings for the 148 SUDI cases.

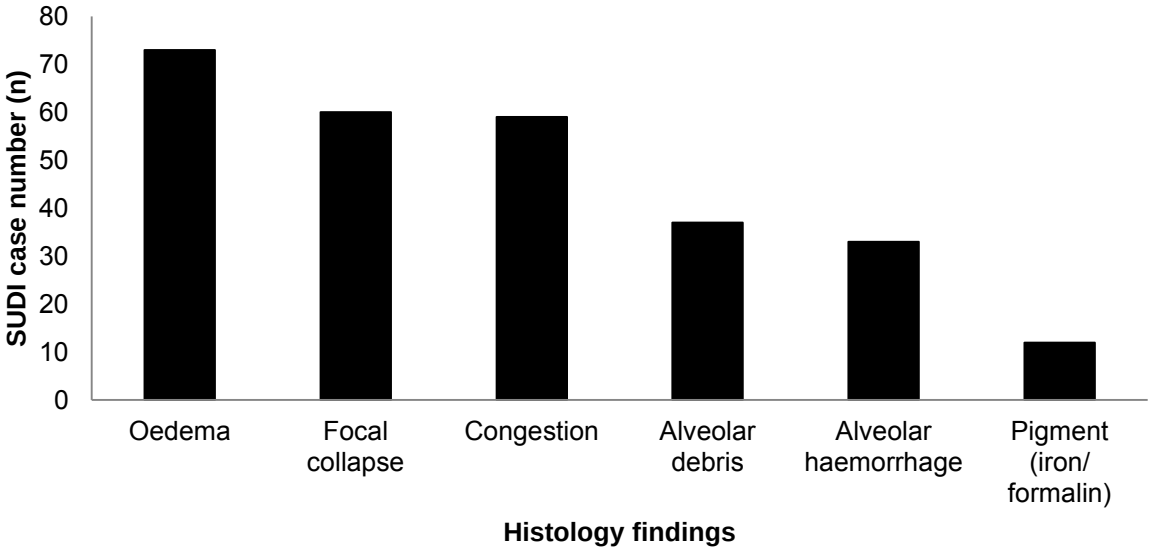
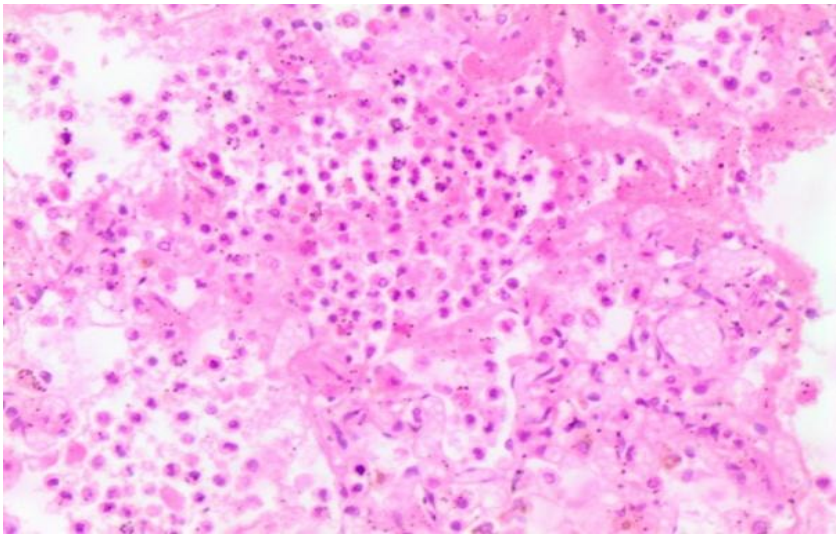


Figure 4.9: Non-specific histology findings for the 148 SUDI cases.

A



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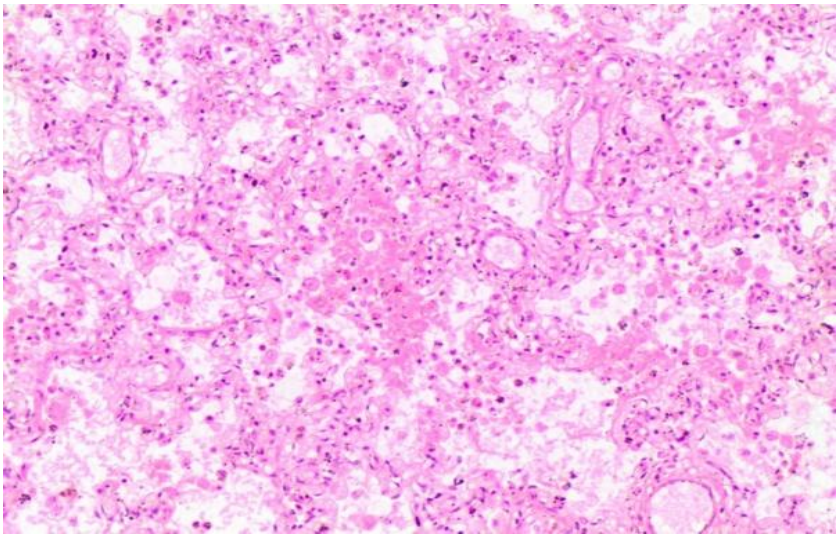


Figure 4.10: Example of a SUDI case with pulmonary bronchopneumonia

A: fibrin present in the lungs.

B: confluent pneumonia with neutrophil infiltrations.

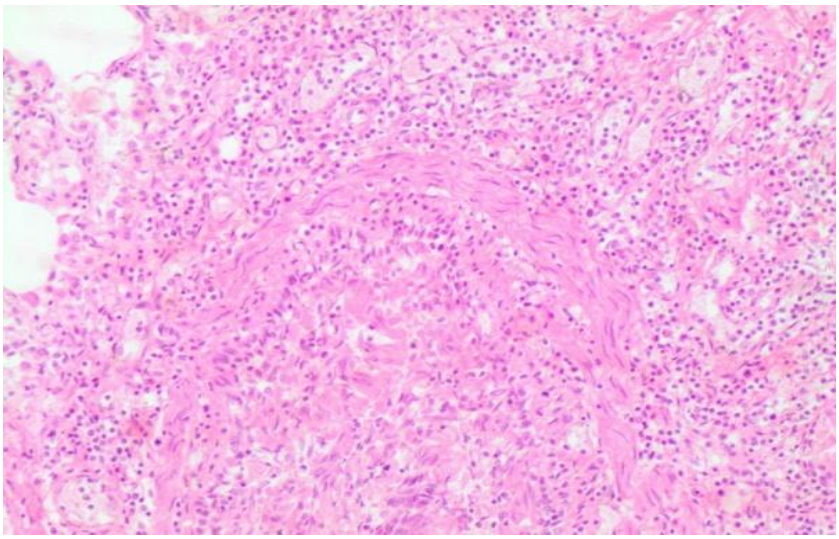
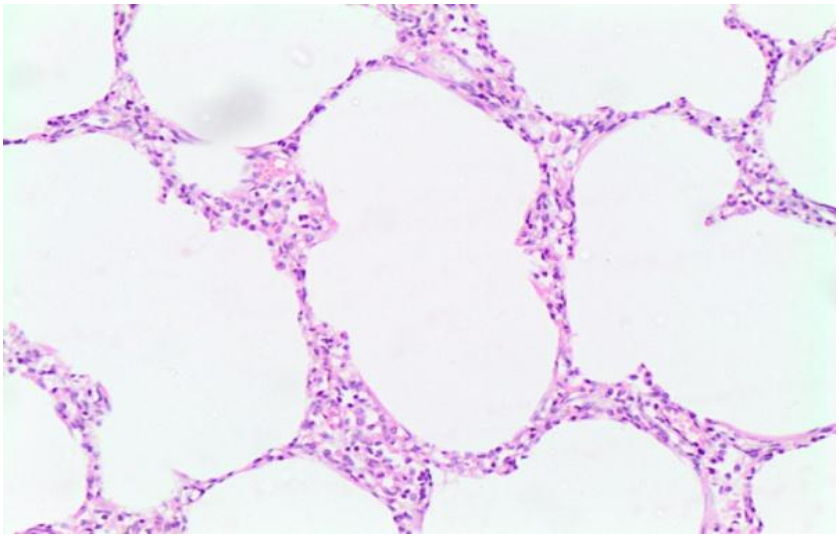


Figure 4.11: Example of a SUDI case with histology signs classified as pulmonary bronchitis. Lymphocyte infiltrations are present in the lungs.

A



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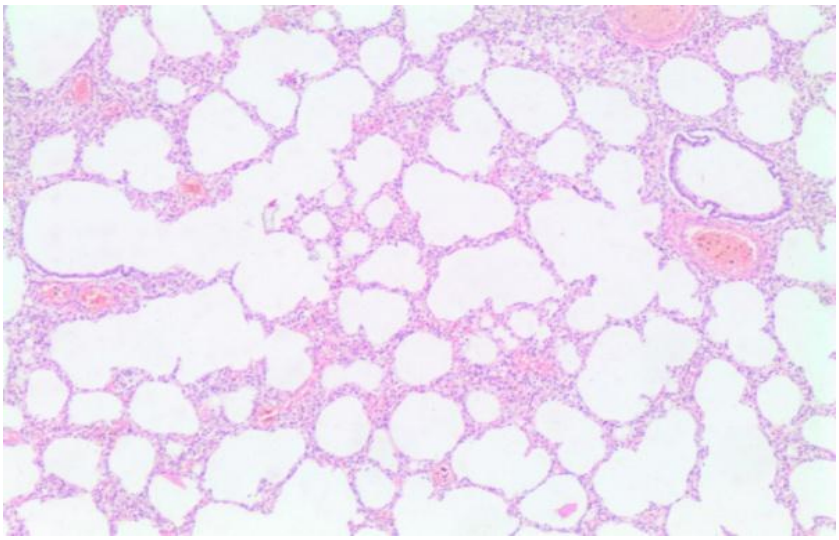

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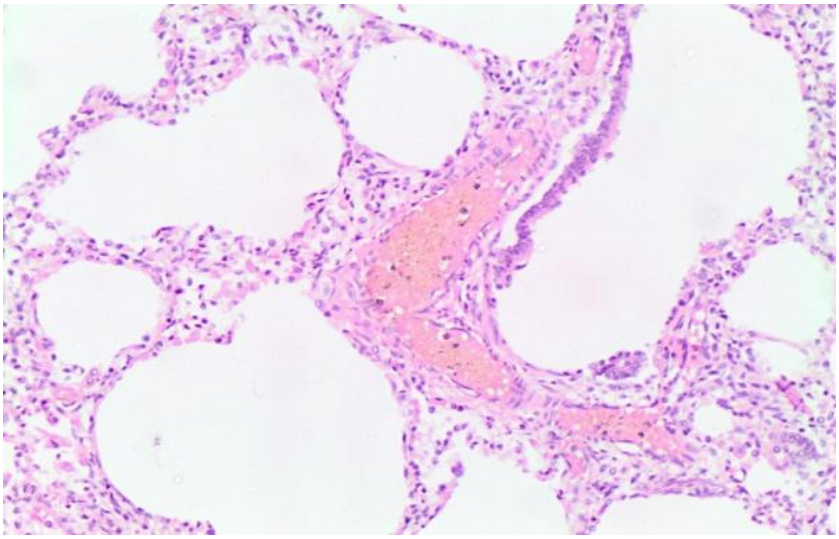


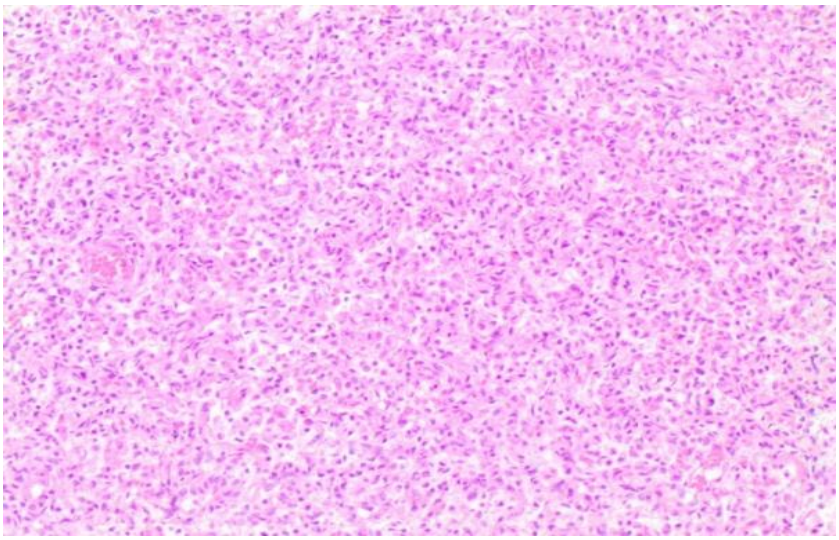
Figure 4.12: Example of one SUDI case classified as having normal lung histology

A: healthy alveolar structures in the lungs of a SUDI case.

B: signs of congestion in the blood vessels.

C: congestion.

A



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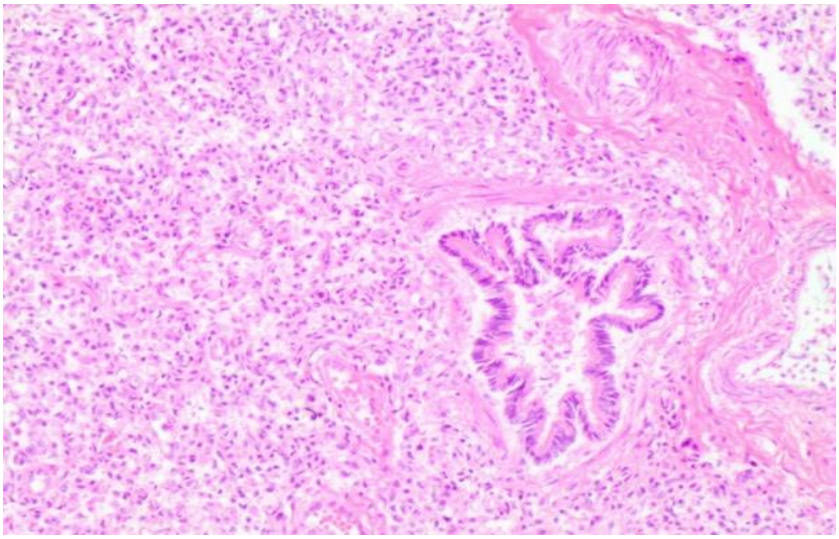

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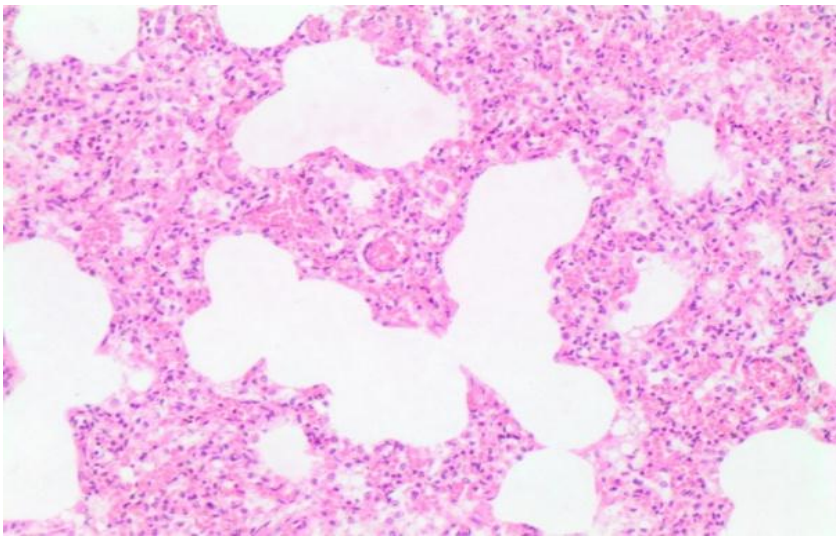

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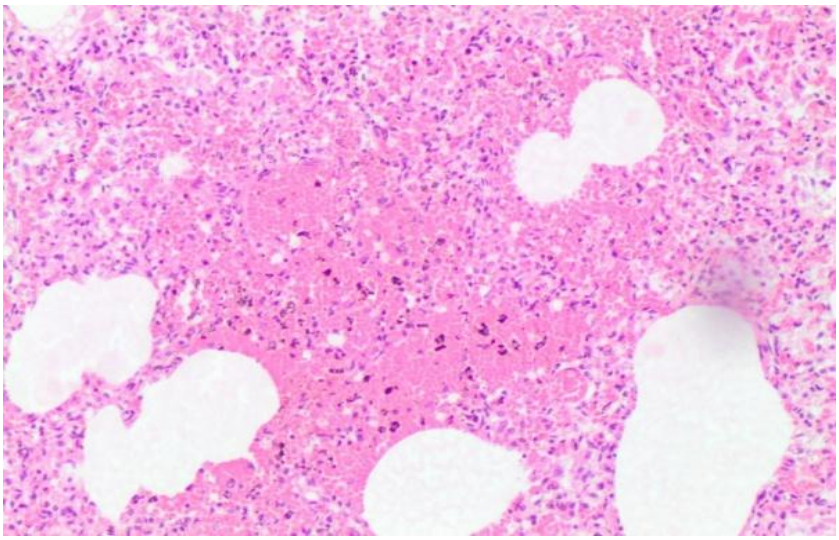

Figure 4.13: Examples of two SUDI cases classified as pulmonary IP
A and B: a case with grade 3-4 IP.
C: a case with grade 1 IP.
D: signs of intra alveolar haemorrhage in the same case shown in C.

Of the cases that were positive for signs of infection on histology, 89.8% (79/88) had one or more infectious agents detected with PCR and culture. In contrast, 81% (49/60) of the cases with negative histology had one or more infectious agents detected with PCR and culture.

4.9 Routine blood results

Of the cases that had blood sent for HIV testing, 29.6% (40/135) were reactive as recorded by the laboratory, indicating the presence of HIV-1/2 antibodies in these serum samples.

4.10 Medical history

A medical history was recorded in all cases and in 26.7% (28/105) of cases, parents reported some clinical symptoms prior to the infant's death. The main symptoms recorded included fever, caughing or a runny nose.

4.11 Statistical analysis and comparisons

4.11.1 *Cause of Death Classification* and pathogen detection

In a 2-way summary table comparing *Cause of Death Classification* and respiratory viruses detected by PCR alone, an association was found between positive PCR results and a *Cause of Death Classification - Infection* (Pearson Chi-square, p = 0.0096) (Table 4.5).

Pathologists were blinded to the viral PCR results when assigning a *Cause of Death Classification*. The same number of cases classified as *SIDS* was PCR positive and PCR negative and details are presented in Table 4.6.

Cases with a final *Cause of Death Classification - Other* had the lowest PCR-positive detection rate at 37.5% as shown in Table 4.4. Details of the six PCR-positive SUDI cases with a *Cause of Death Classification - Other* are shown in Table 4.7.

When both PCR and culture results were compared to the *Cause of Death Classification* in a 2-way summary table, an association was found again between the *Cause of Death Classification* and respiratory pathogens detected (Pearson Chi-square, p = 0.0226). In all three *Cause of Death Classification* groups (*Infection, SIDS* and *Other*) more cases had one or more pathogens detected compared to cases with no pathogens detected (Table 4.8).

Table 4.5: 2-way summary table comparing *Cause of Death Classification* and respiratory viruses detected by PCR.

PCR Viruses detected	*Cause of Death Classified - Infection*	*Cause of Death Classified - SIDS*	*Cause of Death Classified - Other*
No	25.5% (n = 13)	50% (n = 17)	62.5% (n = 10)
Yes	**74.5% (n = 38)***	50% (n = 17)	37.5% (n = 6)

*** - p = 0.0096**

Table 4.6: Details about the 17 PCR-positive SUDI cases classified as SIDS

Case No.	PCR results	Histology signs of infection	Medical History	*Cause of Death Classification*	Routine Culture Results
009	•HRV •RSV B •HCoV OC43/HKU1	Query RSV	Stomach virus and cold	*SIDS*	
023	•HCoV OC43/HKU1	Focal collapse, alveolar debris	None	*SIDS*	
029	•HRV	Congestion, IP grade 4, alveolar haemorrhage	None	*SIDS*	
039	•HRV •RSV A	IP grade 1, focal collapse	Coughing and fever	*SIDS*	•*Escherichia coli* •*Pseudomonas* spp •*Serratia liquefaciens* •*Lactococcus garvieae*
045	•HRV	Oedema, focal collapse, pigment/formalin	None	*SIDS*	•*Staphylococcus aureus* •*Klebsiella pneumoniae*
051	•HRV	Unremarkable	None	*SIDS*	•*Leuconostoc citreum*
053	•Influenza B	Oedema, focal collapse, congestion	Flu-like symptoms	*SIDS*	•*Klebsiella pneumoniae*
062	•HRV	Unremarkable	Coughing	*SIDS*	•*Klebsiella pneumoniae*
069	•HRV	IP grade 1, focal collapse, alveolar haemorrhage	None	*SIDS*	•*Klebsiella oxytoca*
072	•RSV A	Congestion, IP grade 2, focal collapse, bronchiolitis, alveolar haemorrhage	No	*SIDS*	•*Escherichia coli*
083	•HRV	Oedema, IP grade 2, alveolar debris	No	*SIDS*	•HAdV
085	•HRV	Oedema, congestion	Throat infection, seven days antibiotics	*SIDS*	•*Haemophilus influenzae*
102	•HRV	Oedema	No	*SIDS*	•*Klebsiella pneumoniae*
104	•PIV 3 •HCoV OC43/HKU1	Oedema, bronchopneumonia, Alveolar debris, bronchiolitis	Lethargic at crèche	*SIDS*	
139	•HRV	Oedema, IP grade 2	Slight cold symptoms	*SIDS*	
145	•HRV •HCoV 229E/NL 63	Oedema, IP grade 2	No	*SIDS*	
147	•HCoV 229E/NL 63	Oedema, IP grade 3, alveolar debris	No	*SIDS*	

Table 4.7: Details about the six PCR-positive SUDI cases with a *Cause of Death Classification - Other*

Case No.	PCR results	Histology signs of infection	Medical History	*Cause of Death Classification - Other*	Routine Culture results
005	RSV A	Oedema, congestion, focal collapse, pigment iron/formalin, alveolar debris	Yes	Congenital cardiac abnormalities	
013	RSV A HCoV OC43/HKU1	Congestion, pigment iron/formalin, alveolar haemorrhage	None	Aspiration of foreign material	
015	RSV A	Focal collapse, alveolar debris	Was not breathing right	Congenital endocardial fibroelastosis and the consequences thereof	
043	HRV	Oedema, congestion, IP grade 1, focal collapse, pigment iron/formalin	None	Not determined by autopsy alone, but accidental suffocation could not be excluded	
064	HRV	Oedema, congestion, alveolar debris, alveolar haemorrhage, viral inclusion bodies query RSV	None	Pulmonary embolism: natural	
073	HRV	Oedema, IP grade 2, alveolar debris, alveolar haemorrhage, viral inclusion bodies query RSV/HAdV, foreign material in airways	None	Aspiration of stomach contents and the consequences thereof	*Klebsiella pneumoniae*

Table 4.8: 2-way summary table comparing death classification and respiratory pathogens detected by PCR and culture.

PCR and culture pathogens	*Cause of Death Classification- Infection*	*Cause of Death Classification - SIDS*	*Cause of Death Classification - Other*
No	3.9% (n = 2)	23.5% (n = 8)	18.8% (n = 3)
Yes	**96.1% (n = 49)***	76.5% (n = 26)	81.3% (n = 13)

*** - p = 0.0226**

4.11.2 Polymerase chain reaction viruses, and seasonality and gender

Although more viruses were detected in the SUDI cases during autumn and winter (Figure 4.14), the association between seasonality and positive PCR results was not significant (Pearson Chi-square, p = 0.975). There was also no significant association between PCR positive cases and gender, but 64% of the males were PCR positive compared to 55% of the females (Figure 4.15) (Pearson Chi-square, p = 0.263).

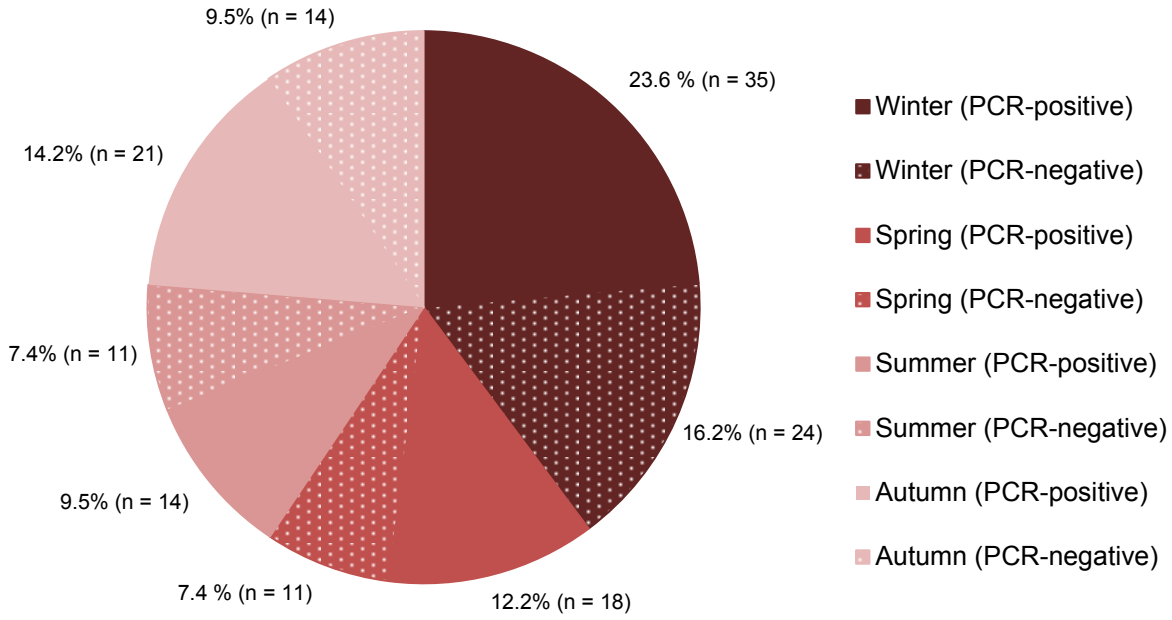

Figure 4.14: The SUDI cases that were PCR-positive and PCR-negative across the four seasons.

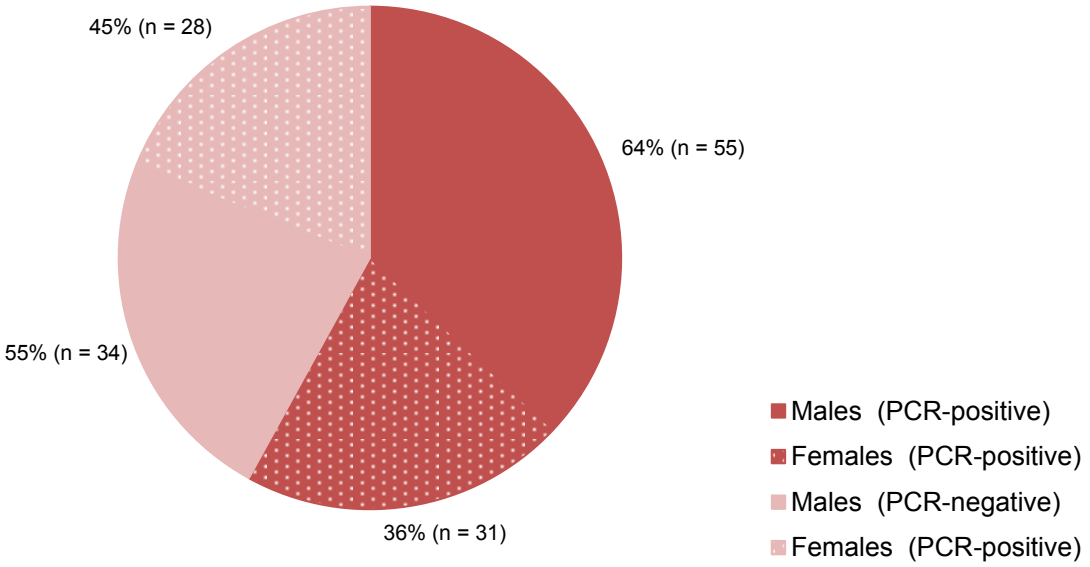

Figure 4.15: Gender according to PCR-positive and PCR-negative SUDI cases.

4.11.3 Histology signs of infection and pathogen detection

Although more SUDI cases that were PCR-positive had histology signs of infection compared to PCR-negative cases, no significant association was found between histology signs of infection and detection of PCR viruses (Pearson Chi-square, p = 0.8177).

We further found no association between histology signs of infection and the detection of PCR and culture pathogens (Pearson Chi-square, p = 0.264), or the detection of bacteria alone (Pearson Chi-square, p = 0.909).

4.11.4 *Cause of Death Classification* and age in months

Infants in the *Cause of Death Classification - Infection* group were significantly older with a median age of two months (mean 2.75 ± 2.25) compared to infants in the *Cause of Death Classification - Other* group with a median age of one month (mean 1.31 ± 1.35) (Kruskal-Wallis Test: p = 0.015), but not compared to the *Cause of Death Classification - SIDS* group with a median age of 1.5 months (mean 2.32 ± 2.25) (Kruskal-Wallis Test: p = 0.40) (Figure 4.16). Although infants in the *Cause of Death Classification - SIDS* group seemed to have a higher mean age in months compared to infants in the *Cause of Death Classification - Other* group, the difference did not reach statistical significance (Kruskal-Wallis Test: p = 0.36).

4.11.5 Pathogen detection and age in months

The SUDI cases that were positive for viruses using PCR only were significantly older with a median age of two months (mean 2.73 ± 2.17) than infants who were PCR-negative with a median age of one month (mean 1.88 ± 2.48) (Kruskal-Wallis Test: p = 0.003) (Figure 4.17).

No statistical significance was found between the ages of cases that were culture positive with a median age of two months (mean 2.61 ± 2.58), compared to cases that had a flora result and/or a culture negative result with a median age of two months (mean 2.11 ± SD 1.91) (Kruskal-Wallis Test: p = 0.39).

Cases that were positive using the routine SVC method were significantly older with a median age of 2.5 months (mean 3.13 ± 2.03) compared to SVC-negative cases that had a median age of two months (mean 2.34 ± 2.34) (Kruskal-Wallis Test: p = 0.0021) (Figure 4.18).

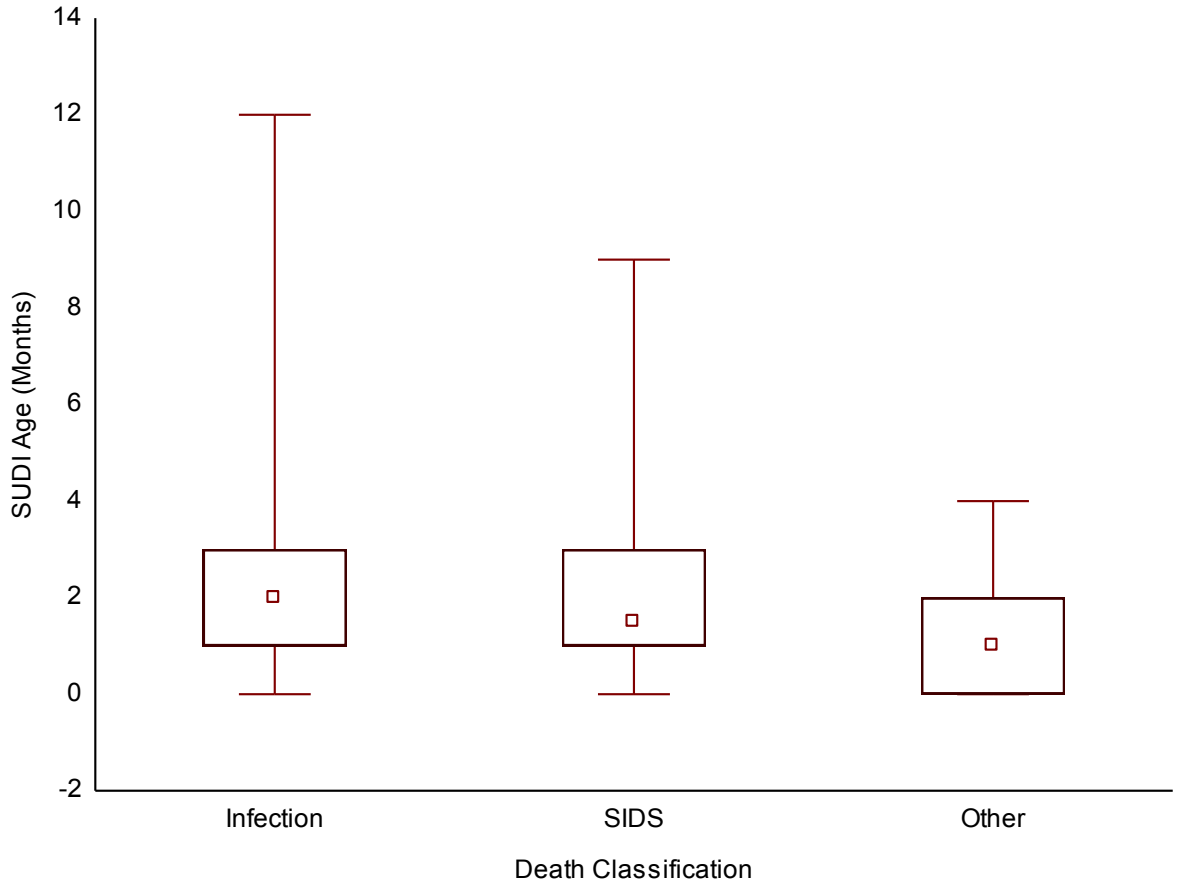

Figure 4.16: A box and whisker plot showing the median age in months for SUDI cases in the three *Cause of Death Classification* groups. Infants in the *Infection* group were significantly older than those in the *Other* group (p = 0.015), but was similar to the *SIDS* group. The median value is represented by the middle box. The outer box represents the interquartile range (IQR) spanning the middle 50% of the data set. The whiskers indicate the minimum and maximum age values.

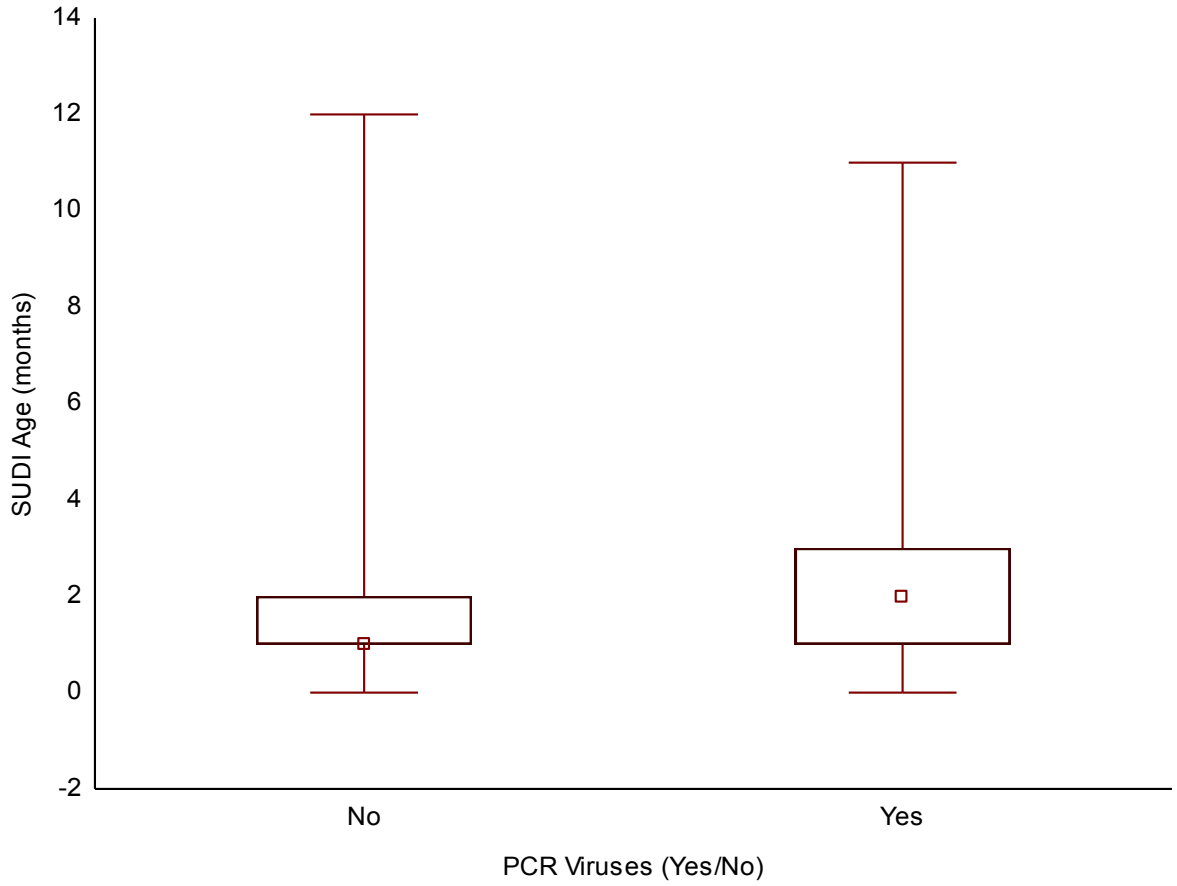

Figure 4.17: The box and whisker plot shows the median age of SUDI cases that were PCR-positive (Yes) and those that were PCR-negative (No) for respiratory viruses. Cases with viruses detected were significantly older than those cases that were PCR-negative (p = 0.003). The median value is represented by the middle box. The outer box represents the IQR spanning the middle 50% of the data set. The whiskers indicate the minimum and maximum age values.

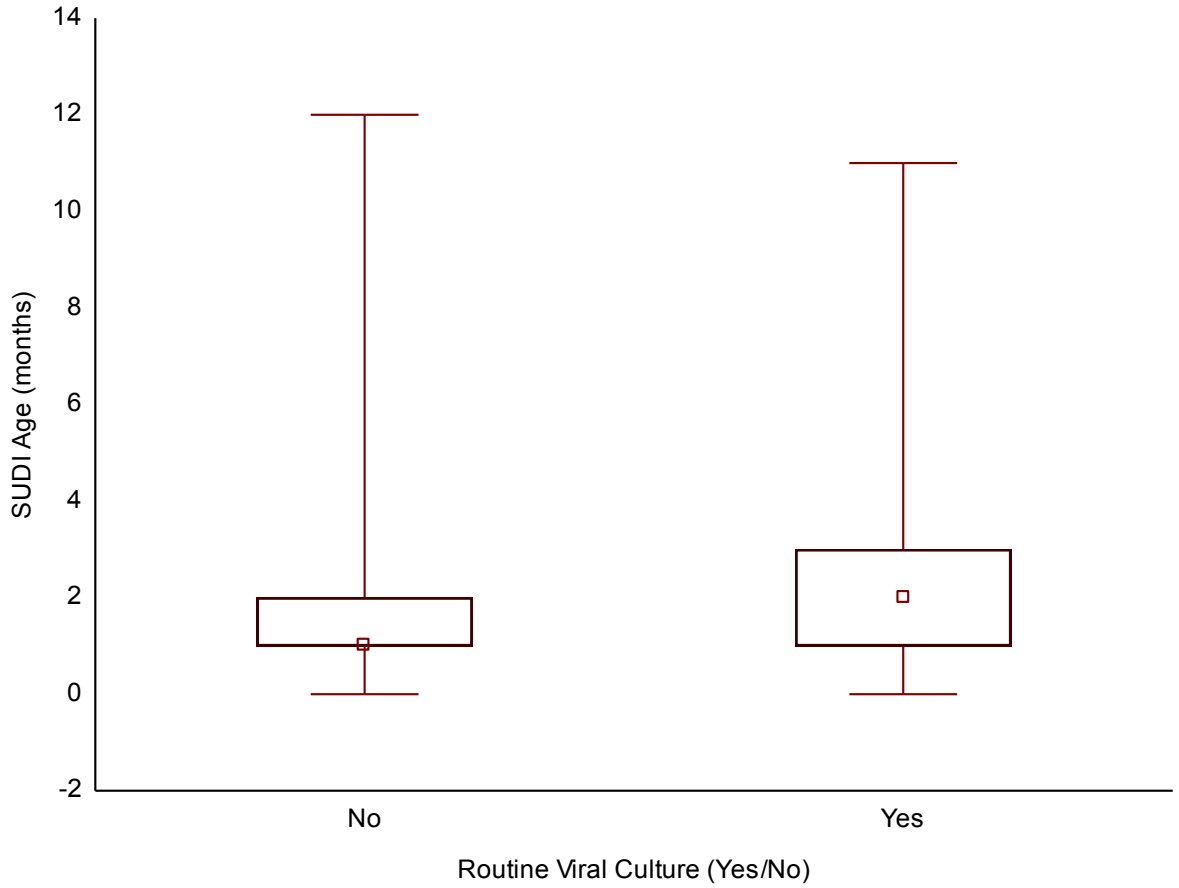

Figure 4.18: The box and whisker plot shows the median age of SUDI cases that were routine SVC-positive (Yes) and those that were SVC-negative. SVC-positive SUDI cases were significantly older than cases where no viruses were cultured (p = 0.0021). The median value is represented by the middle box. The outer box represents the IQR spanning the middle 50% of the data set. The whiskers indicate the minimum and maximum age values.

4.11.6 Post-mortem interval and pathogen detection

There was no difference between the median PMI (4 days) and PCR-positive cases (mean 5.10 ± 3.25) compared to PCR-negative cases (mean 4.56 ± 3.36) (Kruskal-Wallis Test: p = 0.19).

No association could be found between the median PMI of 3.5 days in SUDI cases with bacterial culture positive results (mean 4.42 ± 3.23) or the median PMI of four days for those with no growth or mixed growth and post-mortem flora / contaminants (mean 5.57 ± 3.31) (Kruskal-Wallis Test: p = 0.84).

Cases where viruses were cultured using routine SVC had a significantly higher median PMI of 4.5 days (mean 5.5 ± 3.07) compared to the median PMI of three days for cases with no viruses cultured (mean 3.5 ± 1.79) (Kruskal-Wallis Test: p = 0.025) (Figure 4.19).

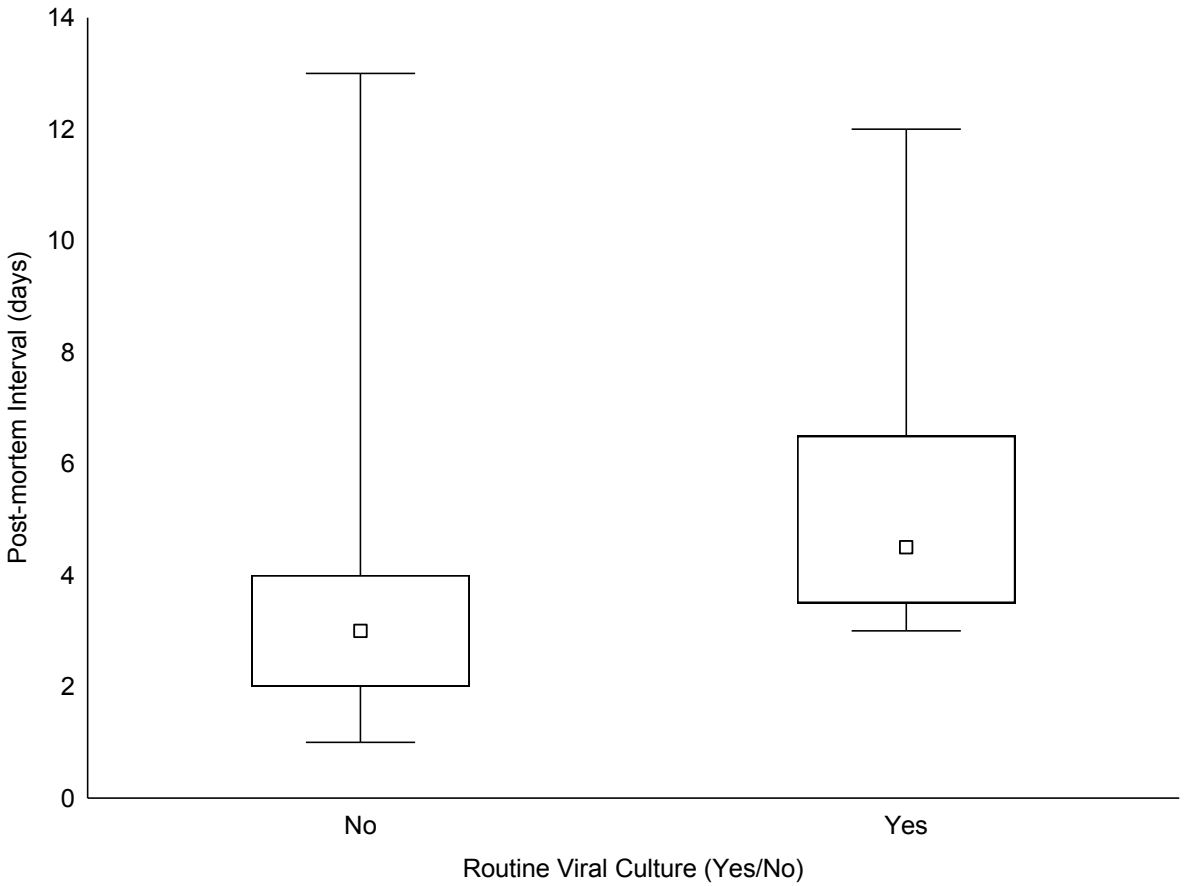

Figure 4.19: The box and whisker plot shows the median PMI for SUDI cases that were SVC-positive (Yes) and those that were SVC-negative. Cases that were SVC-positive had a higher PMI compared to cases that were SVC-negative (p = 0.025). The median value is represented by the middle box. The outer box represents the IQR spanning the middle 50% of the data set. The whiskers indicate the minimum and maximum age values.

Cases with sterile autopsy samples with no bacterial culture growth had a similar median PMI of three days (mean 4.09 ± 2.52) compared to the median PMI of 3.5 days in cases with bacterial growth (mean 4.42 ± 3.23) and the median PMI of three days for cases with post-mortem flora cultured (mean 3.35 ± 1.69).

4.11.7 Demographics and potential SIDS risk factor investigations

The median number of people sharing the same bed with infants (n=2) was the same for all three *Cause of Death Classification* groups; *Infection* (mean 2.0 ± 0.84), *SIDS* (mean 1.83 ± 0.76), as well as *Other* (mean 1.67 ± SD 0.65).

The median number of people per household (n=4) did also not differ between the *Cause of Death Classification* groups; *Infection* (mean 4.5 ± 2.38), *SIDS* (mean 4.6 ± 2.16), and *Other* (mean 4.75 ± 2.60) (Kruskal-Wallis Test: p = 1.000).

The mothers of all cases had a similar median age, regardless of the *Cause of Death Classification* assigned to the infant; *Infection*, 26 years (mean 26 ± 7.47), *SIDS,* 25 years (mean 27 ± 7.74), and *Other,* 23 years (mean 25 ± 5.51) (Kruskal-Wallis Test: p = 1.000).

No significant associations were found when comparing *Cause of Death Classification* to factors such as sleeping position, the use of thick or thin blankets, gender, type of housing, maternal employment status and seasonality. The only significant difference was found for positioning of the window (open/closed) (p = 0.0506) and the maternal marital status (p = 0.001) (Table 4.9).

4.12 Viruses confirmed in living infants admitted to Tygerberg hospital

For comparison purposes, routine SVC and PCR results from 553 infants (53% males and 47% females) that were admitted to Tygerberg Hospital from May 2012-May 2013 were retrieved. Of these, 88 were tested using SVC with 21.6% (19/88) found to be positive for 1-2 viruses (Table 4.10) and 465 were tested by PCR with 50.5% (235/465) found to be positive for 1-3 viruses (Table 4.11). HRV and HCoV are not included in the routine SVC test. The Virology laboratory, NHLS, Tygerberg Hospital recommends PCR for routine analysis of infant specimens, due to higher detection rates of viral pathogens, but physicians still requested SVC in some of these cases.

Table 4.9: Comparison of *Cause of Death Classification* with various epidemiology factors

Sleeping position	***Cause of Death Classification - Infection***	***Cause of Death Classification - SIDS***	***Cause of Death Classification - Other***	**Pearson Chi-square**
Side	71.4% (n = 35)	51.6% (n = 16)	53.3% (n = 8)	**p = 0.221**
Stomach	26.5% (n = 13)	41.9% (n = 13)	33.3% (n = 5)	
Back	2.1% (n = 1)	6.5% (n = 2)	13.3% (n = 2)	
Type of blanket used				
Thin light blanket	36.2% (n = 17)	51.6% (n = 16)	53.3% (n = 8)	**p = 0.297**
Thick heavy blanket	63.8% (n = 30)	48.4% (n = 15)	46.7% (n = 7)	
Gender				
Male	56.9% (n = 29)	61.8% (n = 21)	50.0% (n = 8)	**p = 0.370**
Female	43.1% (n = 22)	38.2% (n = 13)	50.0% (n = 8)	
Window				
Closed	71.4% (n = 35)	51.6% (n = 16)	53.3% (n = 8)	**p = 0.0506**
Open	26.5% (n = 13)	41.9% (n = 13)	33.3% (n = 5)	
No window	2.1% (n = 1)	6.5% (n = 2)	13.3% (n = 2)	
Housing				
Informal	58.8% (n = 20)	51.9% (n = 14)	44.4% (n = 4)	**p = 0.705**
Formal	41.2% (n = 14)	48.1% (n = 13)	55.6% (n = 5)	
Maternal Employment status				
Unemployed	81.4% (n = 35)	80.0% (n = 24)	60.0% (n = 9)	**p = 0.213**
Employed	18.6% (n = 8)	20.0% (n = 6)	40.0% (n = 6)	
Maternal marital status				
Single	93.8% (n = 45)	64.5% (n = 20)	60.0% (n = 9)	**p = 0.001**
Married	6.2% (n = 3)	35.5% (n = 11)	40.0% (n = 6)	
Seasons				
Autumn	23.5% (n = 12)	20.6% (n = 7)	25.0% (n = 4)	**p = 0.936**
Winter	35.3% (n = 18)	41.2% (n = 14)	50.0% (n = 8)	
Spring	23.5% (n = 12)	23.5% (n = 8)	12.5% (n = 2)	
Summer	17.7% (n = 9)	14.7% (n = 5)	12.5% (n = 2)	

Table 4.10: Viruses detected using SVC from 19 infants at Tygerberg hospital from May 2012-May 2013.

Virus name	Number of infants (n)	Prevalence in 19 positive cases	Prevalence in 88 cases tested
RSV	11	57.9%	12.5%
CMV	3	15.8%	3.4%
Influenza A	2	10.5%	2.27%
PIV 3	2	10.5%	2.27%
PIV 1	1	5.3%	0.9%
HAdV	1	5.3%	0.9%

Table 4.11: Viruses detected using PCR from 235 infants admitted at Tygerberg hospital from May 2012-May 2013.

Virus name	Number of infants (n)	Prevalence in 235 positive cases	Prevalence in 465 cases tested
HRV	93	39.6%	20%
RSV A	85	36.2%	18.3%
HBoV	85	36.2%	18.3%
HAdV	23	9.8%	4.9%
Influenza A	8	3.4%	1.7%
RSV B	8	3.4%	1.7%
PIV 4	8	3.4%	1.7%
HMPV	7	3.0%	1.5%
Human enterovirus	6	2.6%	1.3%
HCoV OC43/HKU1	4	1.7%	0.9%
PIV 1	3	1.3%	0.6%
PIV 2	2	0.9%	0.4%
HCoV 229E/NL63	1	0.4%	0.2%

CHAPTER FIVE

DISCUSSION

CHAPTER CONTENTS

5.1 Overview

Attempts at identifying a cause of death for SUDI cases are still partially unsuccessful, leaving some cases classified as SIDS, with the cause of death remaining a medical mystery (Koehler, 2010). SIDS cases still make up a large proportion of SUDI cases, despite many current regional protocols including a review of the clinical history and the circumstances surrounding the event, as well as an autopsy, laboratory investigations and occasional investigation of the death scene (du Toit-Prinsloo *et al.*, 2011; du Toit-Prinsloo *et al*., 2013). In this prospective descriptive study we aimed to investigate the prevalence of 13 respiratory RNA viruses (influenza A and B, HPIV 1-4, HCoV [OC43, 229E/NL63], HRV A, B and C, RSV A and B, human enterovirus and HMPV) in SUDI and SIDS cases at Tygerberg FPS Mortuary over a one year period. Many of these viruses have been detected from infants that died suddenly and unexpectedly and from living infants with ARIs and pneumonia (Weber *et al.*, 2010a; Kwofie *et al*., 2012). We wanted to explore possible viral infections in SUDI and SIDS cases and aimed to determine the usefulness of PCR in detecting viruses from SUDI cases that may contribute to death. The virology results were interpreted alongside a clinical history of the presence or absence of infection prior to death and histology signs of respiratory infection. We also collected routine laboratory results and epidemiology data. In keeping with the study objectives, we showed that more than half of the SUDI cases had positive PCR results for viruses and half of the cases in the final *Cause of Death Classification - SIDS* group were positive for viruses. In this setting at Tygerberg, a high percentage of viruses were detected by PCR from SUDI and SIDS cases. Many of these viruses remain mostly undetected by the current routine SVC method used in the SUDI laboratory investigation protocol at this facility. Using PCR we detected viruses that might have contributed to infant death in a portion of cases, especially where histology signs of infection were observed. Incorporating this PCR method into the facility protocol could potentially alter the final diagnostic categories for SUDI cases at Tygerberg, or at the least, highlight the possibility of viruses contributing to fatal events. In the discussion sections to follow, we will cover demographic risk factors and further lay emphasis on the respiratory viruses detected from PCR-positive cases, and specifically more detail on the cases with a final *Cause of Death Classification - SIDS* where viruses were detected.

5.2 Characteristics of the study population

Out of the 148 SUDI cases we collected, 60.1% were males. Our percentage of males was very similar to Trachtenberg *et al*. (2012) who found 60.6% of SIDS cases to be male (1996-2008) and slightly higher than du Toit-Prinsloo *et al*. (2013) who found 56% of SUDI cases to be male (2005-2009). The male gender has long been established as a risk factor for SIDS

(Vennemann *et al*., 2005). In our study no significant association was found between the male gender and cases with a final *Cause of Death Classification* of *Infection, SIDS* or *Other*. More males in our study were virus-positive (55/89; 61.8%) compared to females (31/59; 52.5%), but there was no significant association between the male gender and respiratory viruses. The male predominance seems to span over infections, prematurity and infant mortality in general and is therefore not limited to SIDS. The cause for male predominance in SIDS is still unclear, but it has been suggested to have a biological origin (Drevenstedt *et al*., 2008). Moscovis *et al*. (2013) found that adult male peripheral blood mononuclear cells produced less pro-inflammatory cytokines compared to females. They suggested that a reduced pro-inflammatory response could limit damage during pathogenic stimulation, but could also lead to a higher susceptibility to initial invasion of pathogens. They also found that the pro-inflammatory response was reduced further upon exposure to a surrogate for cigarette smoke (Moscovis *et al*., 2013). In SIDS cases specifically, Paterson *et al*. (2006) found more males with medullary abnormalities than females, which could reduce the ability to respond to homeostatic stressors while sleeping. Thus, the male predominance may stem from inherent vulnerabilities, which seems to be multifactorial, as is the case with SIDS.

The median age of three months for the SUDI cases in our study was consistent with two to four month peak age for SIDS reported in the literature (Balduzzi & Greendyke, 1966). The peak age for the infants in our study was between zero to three months, and although the one to two month age peak was the second largest in our study, our findings were still close to the two to four month age peak found by Trachtenberg *et al*. (2012) and the three to four month age peak found by du Toit-Prinsloo *et al*. (2013). The first few months of life are considered a particularly vulnerable period for infants because their immune protective responses to pathogens are still developing, their maternally transferred IgG antibodies have begun to wane and all vaccinations have not yet been completed (Gans *et al.*, 2004; Palmeira *et al*., 2012; Reikie *et al*., 2012; Reikie *et al*., 2013). The number of SUDI and SUCD cases are negligible by the age of one to two years when the immune system of infants should be fully functional (Wiegering *et al.*, 2009; Côté, 2010; Palmeira *et al.*, 2012), which suggests that immature immune responses play a contributory role in infant susceptibility to SIDS in early life. SUDI cases with a *Cause of Death Classification - Infections* were significantly older than those in the *Cause of Death Classification – Other* group, but still fell within the vulnerable period before vaccinations have been completed and when maternal antibodies have already drastically declined (Reikie *et al*., 2013). The median age for our *SIDS* cases also falls within the vulnerable period although they were not significantly older than the mean age of infants in the *Other* group.

The median age of two months for our SUDI cases who were PCR-positive were significantly higher than the median age of one month for PCR-negative cases. Patrick *et al*. (1989) also found that older infants (>16 weeks / four months) had significantly more viruses compared to younger infants with the least viruses detected in the zero to two months age group, but explanations for this were not offered in their article. Williams *et al*. (1984) also detected significantly more viruses from SIDS cases older than three months (39%), compared to cases younger than three months (14%). They suggested that some respiratory viruses, and specifically RSV, may play an important role in sudden death in infants older than three months, but that this still could not explain the cause of death. More recent studies do not specifically compare older to younger infants in terms of viruses detected from SUDI or SIDS cases (Weber *et al*., 2010a; du Toit-Prinsloo *et al*., 2013). We did not measure antibody levels for the infants or their mothers, but despite early infant vulnerability, maternal IgG offer partial protection in very young infants. We could only speculate that maternal antibodies circulating in young infants may protect them from infections and could possibly explain why PCR-negative cases were more prevalent in younger cases. In addition, breastmilk contains high levels of certain immune factors that could protect young infants against infections, but the levels of immune components in breastmilk decrease over time. This may also explain why younger infants could possibly show less infections compared to older infants. Sadeharju *et al*. (2007) found that infants in Finland (1995-1996) that were breastfed exclusively for more than two weeks had higher maternal enterovirus specific IgA antibodies in breastmilk and decreased enterovirus infections throughout their first year of life compared to infants that were breastfed for less than two weeks. After three months the enterovirus specific IgA levels in breastmilk samples declined. Ladomenou *et al*. (2010) found reduced episodes of ARIs and common infections, including hospital admissions in infants exclusively breastfed for at least six months compared to non-breastfed or partial breastfed infants in Crete (2004-2005). Gordon *et al*. (1999) also found human breastmilk to contain IgA antibodies specific for bacterial toxins which could neutralise these toxins and possibly contribute to protection against SIDS in breastfed infants. Breastfeeding has been shown to decrease the risk of SIDS where Vennemann *et al*. (2009) suggested that breastfeeding for the first six months should be incorporated into the risk-reduction messages. During our study we did not collect data regarding breastfeeding because it was not included in the case files. We were therefore also unable to determine if infants were exclusively breastfed or also given formulas. It is clear that breastfeeding is pivotal for infant immune maturity and this data should be incorporated in future research studies.

In our study the infants with a final *Cause of Death Classification - infection* seem to more closely resemble infants with a *Cause of Death Classification - SIDS*, in terms of age and

PCR-positive cases, compared to infants in the *Cause of Death Classification - Other* group. The latter group was the only group where more than half of the cases were PCR-negative. Another possible explanation why infants in this specific group were so much younger could be because they died of explained causes that included cardiac abnormalities, aspiration of foreign material or gastric contents, starvation and dehydration and prematurity amongst others and thus did not fit the general profile of SUDI or SIDS.

It has been confirmed in the literature that SIDS cases rise in winter months when the number of infections also increase (Grangeot-Keros *et al*., 1996). A very early study from Nelson *et al*. (1975) found a significant rise in SIDS cases during influenza outbreaks over a 42 month study period. They also found a significant association between a rise in temperatures and SIDS, but infections with influenza A was independent of temperature. We found a high percentage of SUDI cases in the winter and autumn months. Weber *et al*. (2008a) found no relationship between the seasons and the frequency of SUDI cases. In agreement with our findings, Vennemann *et al*. (2005) and du Toit-Prinsloo *et al*. (2013) also found more cases in the colder months of the year. In our study, more cases were PCR-positive than PCR-negative for viral pathogens throughout all four seasons and we therefore could not demonstrate a significant association between the detection of viruses and the colder seasons. It is possible that our study period was not long enough to identify possible trends. We were able to observe that Influenza PCR-positive SUDI cases only occurred in winter and HMPV PCR-positive cases occurred in winter and spring but no clear seasonal pattern could be determined because the numbers were so small.

5.3 Demographics

Different factors in the infant's sleeping environment have been considered risks for SUDI and SIDS, including the sleeping position, heavy comforters, warm room temperatures and bed-sharing (Athanasakis *et al*., 2011; Hutchison, *et al*., 2011). In our study only a third of the cases for which data was provided were put to sleep on their stomachs. It is now several years since the National Institute of Child Health and Development introduced the "Back-to-sleep" campaign in the USA, recommending that infants be placed on their backs to sleep rather than the stomach or so-called prone sleeping position (Trachtenberg *et al*., 2012). The prone sleeping position has also been associated with increased density of bacterial colonisation in the nasopharyngeal tract in infants (Goldwater, 2004). It was also hypothesised that infants could ingest bacteria that are present on their sleeping surfaces and that an increased temperature could enhance bacterial toxin production when sleeping in the prone position (Goldwater, 2004).

More than half of the infants in our study were found with thick heavy blankets and slept in rooms with closed windows. In the literature, warm home environments and overheating due to excessively wrapping infants in bedding and warm clothing are considered risk factors for SIDS (Sullivan *et al*., 2001). The majority of our cases had single parents in their mid 20s who were unemployed and lived in informal settlements. SIDS has been associated with young mothers below the age of 20, including populations with a poor socio-economic status and single and unemployed parents (Sullivan *et al.*, 2001). Many of our cases also shared a bed with between one to four other adults and children. Parents overlaying their infants have long been considered a risk factor for SIDS. Bed-sharing could also lead to overheating and re-breathing of CO_2 from others in the same bed (Sullivan *et al*., 2001). Parental alcohol usage and smoking were recorded in many of our cases, both of which are known risk factors for SIDS. O'Leary *et al*. (2013) found a high risk of SIDS when mothers used alcohol during or one year after pregnancy, but it also increased the risk of non-SIDS infant deaths. Exposure to alcohol *in utero* can have negative effects on the brainstem development which in turn could affect homeostatic control. Zhang & Wang (2012) found a significant association between both pre- and postnatal smoking and SIDS. Exposure to cigarette smoke could adversely affect the neurotransmitter systems required for homeostatic control. The cases within our study population were thus exposed to multiple known demographic risk factors.

5.4 Polymerase chain reaction viruses detected and histology signs of infection

Using the Seeplex® RV15 Ace detection assay on cDNA from tracheal and lung swab samples, we detected between one and three viruses in the majority of cases. More than half of our PCR-positive cases suggest a viral aetiology, confirmed by the *Cause of Death Classification – Infection* and histological signs of infection. Conversely, we found viruses in several cases in the *Cause of Death Classification – Other* group. Weber *et al*. (2010a) suggested that if viral detection occurs in the absence of clinical or morphological signs of infection, it may indicate asymptomatic carriage or an incidental finding with no contribution to the cause of death. They stated that the majority of pathologists would be in agreement that a positive viral result could only be deemed significant if it is supported by significant signs of inflammation. They further admitted that it is a challenge even in the presence of inflammation, to distinguish between a virus that may be incidental or have a contributory role and those viruses that were a direct cause of death (Weber *et al*., 2010a). We propose that a contributory role of viruses to the cause of death cannot be ignored, even in the absence of histological evidence of infection as seen in 60.2% of our SUDI cases. Inflammation can be demonstrated histologically by signs of tissue infiltration of the innate (i.e. macrophages and neutrophils) and adaptive (i.e. lymphocytes) immune systems. In

order for these cells to be observed in the injured or infected tissues, the appropriate chemo-attractant signals need to be present to facilitate migration of these cells to the tissues. The existence of a window period was therefore suggested during which time the molecular signals of inflammation may already be evident before the manifestations of inflammation can be observed histologically (Gotsch *et al*., 2007). In infancy, this window period may be further prolonged, because their macrophage and neutrophil cell populations are less sensitive to chemo-attractants (Palmeira *et al.*, 2012).

5.4.1 Human rhinovirus

HRV was detected the most frequently in our cases. In all our HRV-positive cases the virus was detected from the trachea and in two-thirds it was detected from one or both lung samples as well. The presence of histological evidence of infection supported the detection of the virus by PCR in the majority of our cases. HRV has long been considered an aetiological factor for the common cold in the URT (Hendley *et al*., 1973; Gwaltney, 1995), but in a more recent study it has also been identified to cause more severe LRTI and wheezing (Louie *et al*., 2009). In addition, Miller *et al*. (2012) confirmed HRV as the predominant pathogen in premature infants, which is already a recognised risk factor for SUDI.

HRV has not been investigated in SUDI in recent studies, but Patrick *et al*. (1989) detected the virus in only 16% of viral-positive SIDS cases which is much lower than the prevalence found in our study. When such discrepancies arise, it could be attributed to differences in the study populations and/or detection techniques, which most probably played a major role in this instance, as molecular techniques were not readily available in 1989. Recent studies demonstrated higher HRV detection rates in living paediatric patients with clinical symptoms of infection and wheezing (Piotrowska *et al*., 2009; Smuts *et al*., 2011). These results were comparable to the number of HRV-positive cases we found in the comparison group of hospitalised infants in our study, suggesting that HRV rarely seem to occur in asymptomatic patients. In our study HRV was co-detected in a low number of cases with RSV, HCoV, human enterovirus, HPIV, HMPV and influenza A. Smuts *et al*. (2011) also co-detected HRV with very low frequency with HMPV, HBoV and HCoV. Thus similar to our findings, a low co-detection rate occurs, with the majority of PCR-positive results yielding HRV as the sole pathogen.

Our HRV-positive results need to be carefully interpreted. Although more than half of the cases showed histological evidence of infection, HRV has been known to result in prolonged viral shedding after the initial establishment of infection (Hendley *et al*., 1973; Gwaltney,

1995). The actual contribution of the virus to the infant's demise is questionable in such cases, especially when HRV was found in the trachea alone in about a third of our cases. The majority of the cases had the virus in both the trachea and the lungs. Almost half of the HRV-positive cases in our study were assigned a *Cause of Death Classification – Infection*, with much less in the *SIDS* and only a few cases in the *Other* categories. We detected HRV in case 046 with a *Cause of Death Classification – Other*, but this infant was said to have died from a pulmonary embolism. Pulmonary embolisms are considered very rare in infants (Byard & Cutz, 1990), but a case report has shown that it can be a possible cause for sudden infant death (de la Grandmaison & Durigon 2002). The contribution of HRV to death in this case seems unlikely in the absence of histology signs of infection.

5.4.2 Respiratory syncytial virus A and B

RSV A and B were only detected in a few cases in our study, predominantly from the lungs. RSV A seemed to be accompanied by histological evidence of infection in the majority of cases, while RSV B was not. We could not demonstrate a correlation between the presence of RSV and *Cause of Death Classification*. Our findings seem to be in agreement with the literature where very low prevalence rates of RSV had been described in SUDI cases (Weber *et al*., 2010a), as well as premature infants with ARIs (Miller *et al*., 2012). We also found a higher prevalence of RSV A than RSV B in the hospitalised infants, which agrees with the findings of Miller *et al*. (2012). In contrast to Miller *et al*. (2012) and da Silva *et al*. (2013) where more RSV was detected in living infants in Brazil, we found more HRV than RSV in both our SUDI cases and hospitalised infants.

None of our cases with RSV A and a *Cause of Death Classification – Other* showed significant histological evidence of infection and their deaths were ascribed to congenital cardiac abnormalities, aspiration of foreign material and congenital endocardial fibroelastosis. In a review by Ogra (2004) it was indicated that congenital cardiac disease is a well-established risk factor for developing severe infection with RSV, as it is associated with the need for increased ventilator support and requires more periods of intensive care compared to infants who do not have congenital cardiac disease.

RSV is recognised as the most significant cause of LRTI in early childhood (Ong *et al*., 1982). Therefore the presence of RSV should at least be considered as playing an important contributory role to infant death in our study, even if not a direct cause.

5.4.3 Human coronavirus

We detected HCoV in a small percentage of cases, but the majority also showed histological evidence of infection. We confirmed co-detection with HCoV OC43/HKU1 and HCoV 229E/NL63 in a few cases but could not identify a trend towards an anatomical site because the virus was detected from the trachea alone, from lungs alone and from both sites. No association with the *Cause of Death Classification* could be found either. Kuypers *et al.* (2007) and Smuts *et al*. (2011) found a low prevalence in paediatric patients, either associated with severe LRTI or co-infections with other respiratory viruses. In other studies investigating respiratory viruses in SIDS cases, HCoV was not investigated (Patrick *et al*., 1989; Weber *et al.*, 2010a)

5.4.4 Human enterovirus

Human enterovirus was found in only a small percentage of our cases from the trachea and lungs, either in isolation or in combination with HRV. Human enterovirus provided the lowest level of agreement with histological evidence of infection. We found more cases with human enterovirus than Weber *et al*. (2010a), but due to the low numbers, no significant conclusions can be drawn from this. The detection of the virus in the absence of histological evidence of infection could possibly be linked to oral polio vaccine antigen shedding (Buonagurio *et al*., 1999).

5.4.5 Human metapneumovirus

HMPV was detected from trachea and lung samples of our cases, but at a very low prevalence. We found histological evidence of infection in the majority of positive cases, including bronchopneumonia and IP grade 2-3. Despite the very low prevalence, all cases were assigned to the *Cause of Death Classification – Infection* group. HMPV was co-detected with HRV and RSV A, but the numbers were inadequate to draw any conclusion from this. We cannot compare the prevalence of HMPV in our cases to those of other studies because this virus does not seem to be included when investigating respiratory viruses in SIDS (Patrick *et al*., 1989; Weber *et al.*, 2010a).

5.4.6 Human parainfluenza virus

HPIV 2 was detected in one SIDS case in a study from the UK by Ann *et al*. (1993) at a prevalence of 2.2% which is similar to our four cases (2.7%) of HPIV 3 detected in the trachea and lung samples. We did not detect HPIV 1, 2 or 4. Histological evidence was present in most of the cases and included bronchopneumonia, bronchiolitis, and IP grade 2-

3. Co-detections with other PCR viruses occurred in all the cases, but the significance is unclear due to the limited numbers.

5.4.7 Influenza A and B

Influenza A had been reported in the literature at a very low prevalence (Patrick *et al*., 1989; Weber *et al*., 2010a). Our study also detected Influenza A and B in a very small percentage from trachea and lung samples. We did not find associations with regard to histological evidence of infection, co-infection with other viruses or *Cause of Death Category*.

5.4.8 Human adenovirus

We did not expect to detect HAdV in our study, but a few cases were PCR-positive for HAdV. Although the multiplex PCR included primers for this virus, we used an RNA extraction kit and did not expect DNA to be present in the sample after extraction. After we validated the extraction procedures with a confirmed positive HAdV sample, it seemed as if DNA was indeed preserved during the RNA extraction procedures with the QIAGEN RNeasy Plus Micro Kit. We thus kept these unexpected results separate from the other virology results.

5.5 The value of polymerase chain reaction viruses detected

The PMI did not seem to influence whether or not respiratory viruses were detected by PCR. Viruses detected by the RV15 Seeplex® PCR from hospitalised infants during our study were similar to those detected from our SUDI cases, although at a lower prevalence. There did not seem to be a link between the HPIV strains circulating in the hospital and those we found in SUDI cases during the same period. The first most noticeable difference between the viruses confirmed in living infants and our SUDI cases was HPIV, where only HPIV 3 was detected from SUDI cases, and only HPIV 1, 2 and 4 from the hospitalised infants at Tygerberg Hospital. HPIV 1, 2 and 4 were circulating in the hospital setting at Tygerberg, but did not seem to be contributing to SUDI cases in our small study. The second difference was the detection of HBoV, which is a DNA virus, and was detected the third most frequently from hospitalised infants, but not present in our SUDI cases. This is partly because we used extracted RNA and not DNA. Although the PCR method picked up HAdV in some of the samples, it is possible that the HBoV DNA was eliminated during RNA extraction by the genomic DNA eliminator spin column. If this is the case, HBoV may have been present in some of our SUDI cases, but remained undetected because we specifically used an RNA extraction procedure that eliminated some, if not all, HBoV DNA. The PCR-positive HAdV cases we detected still needs to be clarified. HBoV might actually not have been present in

the SUDI cases in our study, but at present the reason for the absence of HBoV remains unanswered.

The majority of our cases in the *Cause of Death Classification – Infection* group were PCR-positive and this association was significant. Thus, even without the PCR results, the routine laboratory tests and histology were able to assist the pathologist with determining the final cause of death. The PCR test is still important, as this can identify an aetiological agent, because most of the cases classified as infection are not specific for an aetiological agent. In the *Cause of Death Classification – SIDS* group, the PCR results could assist with determining a possible cause of death in 50% of our cases. It may also be relevant in cases where histology does point to a clear infectious cause of death, especially when routine culture does not reveal any aetiological agents. In 39.8% of our SUDI cases that were PCR-positive, histological evidence of infection severe enough to cause death was not noted. This percentage of cases seems too high to indicate asymptomatic carriage, a past viral infection or just an incidental finding. It also questions how good a predictor of viral infections the H&E lung histology analysis really is. Viral infections are frequently detected from children that died of causes other than infections, therefore Samuels (2003) stated that it does not imply that the viral infections resulted in death. The detection of a pathogen must be substantiated with pathology changes indicative of infections, otherwise a cause of infection cannot be supported (Fernández-Rodríguez *et al*., 2006; Weber *et al*., 2010a).

To evaluate the significance of PCR in detecting viruses and their possible contributions to SUDI, especially in the absence of histological evidence of infection, additional research is required. The specific viral immune response needs to be characterised in SUDI cases, both in terms of the histological picture, but also the serum antibody levels. If the systemic response to specific viruses could be further investigated in our SUDI cases, it may help to assist in determining the significance of viruses in causing active infection severe enough to contribute to death. For instance, primary infection with RSV leads to elevated levels of IgM which remains detectable one to three months after initial infection. The IgG levels increase from initial onset of symptoms to maximum serum levels in about 20-30 days and thereafter serum levels decrease and remain at low levels after a year. After re-exposure or re-infection IgG levels rise and can be detected at high serum levels within five to seven days after antigen stimulation (Ogra, 2004). Thus, IgM levels indicate recent infection and possible active infection which could be a valuable tool to corroborate viral PCR results. IgG levels are more difficult to interpret, because it can indicate primary infection, past infection or even a re-infection/re-exposure where the latter would result in a less severe infection than primary infection.

Fernández-Rodríguez *et al*. (2006) detected IgM and IgG levels against EBV, CMV, RSV, influenza A and HHV-6 from sudden death in children using serology. In a two-year-old child, HAdV was detected with viral culture, but serology was negative and they suggested that this was because the onset of the illness was too rapid for the immune response to become detectable. High RSV-specific antibody levels determined by complement fixation were regarded as indicative of recent infection in light of pathology findings indicating epiglottitis and laryngitis. They considered high HHV-6 specific IgM levels to indicate a recent infection in a three-year-old child, but a positive serology result for influenza A antibodies in a new-born was interpreted as maternal antibody levels that had been transferred across the placenta to the infant. The same group acknowledged the limitations of serology, especially in the case of children younger than three years, where the immune response to infections take time to develop and could thus be delayed. Also, careful interpretation is required to distinguish between recent and past infections, including maternal antibodies in the infant's circulation.

Another approach is to investigate the cellular immune response in SUDI cases. Howat *et al*. (1994) detected increased eosinophils and T and B lymphocytes in the lungs of SIDS cases compared to controls using immunocytochemistry when autopsies were performed within 72 hours after death. They provided evidence of inflammation characterised by abnormal T lymphocyte responses in the lungs of SIDS cases and further suggested that the products from degranulation of eosinophils could lead to airway obstruction and hypoxia (Howat *et al*., 1994). Gaaloul *et al*. (2012) used paraffin tissue blocks from the myocardium of sudden unexpected death cases (18-42 years of age) with suspected inflammatory heart disease and detected significant CD3 T cells and CD19 B cells considered as markers of inflammation, compared to control cases that died from traffic accidents. $CD4^+$ and $CD8^+$ T cells play a major role in viral clearance during infections and the antigen-specific $CD4^+$ T cell response and the RSV-specific $CD8^+$ T cell responses had been investigated by Arnold *et al*. (2002) and Heidema *et al*. (2004). It may therefore be worthwhile to also investigate the viral specific cellular immune responses in SUDI cases.

In our study 58.3% of PCR-negative SUDI cases presented with histological evidence of infection. Although some of these were positive for routine culture tests, we cannot exclude the possibility of other undetected pathogens contributing to the observed histology. Other viruses that were not included in this study, but that had been implicated in SUDI and SIDS must also be considered, such as VZV (Desmons *et al*., 2013), rotavirus and echovirus (Patrick *et al*., 1989), EBV (Álvarez-Lafuente *et al.*, 2008), CMV, HSV (Weber *et al.*, 2010a) and Ljungan virus (Niklasson *et al*., 2009). These viruses require further investigation. It is

possible we may be underreporting the prevalence of respiratory viruses from our SUDI cases as we did not look for DNA viruses and we also did not investigate viruses from liver or heart tissue, post-nasal swabs, bowel, recto-sigmoid loop or suprarenal fat specimens.

We also cannot exclude the possibility that the microscopical picture for the PCR-positive cases with histological evidence of infection, may have been caused by other unknown events, since histological signs of inflammation have been detected from cases dying from unnatural causes and in cases where no specific infectious aetiology could be identified (Krous *et al*., 2003).

5.6 Cases with a *Cause of Death Classification - SIDS*

We detected HRV, RSV A and B, HCoV (OC43/HKU1 and 229E/NL), influenza B and HPIV from half of our cases SIDS.

5.6.1 Human rhinovirus-positive SIDS cases

5.6.1.1 Cases 029 and 139

Two of our cases had HRV as the only aetiological agent detected from both trachea and lung specimens. Case 029 had histological evidence of IP grade 4, and case 139 had histological evidence of IP grade 2 together with a medical history of slight cold symptoms prior to death. In light of the histological evidence of infection, a specific aetiological agent identified from the URT and LRT tract and a medical history of cold symptoms indicates that this virus should be considered a significant finding, which in turn could alter the diagnostic category of death from SIDS to infection. A diagnosis of SIDS is only valid when all the evidence excludes any possible cause of death. In these cases, although HRV might not have been the definite cause of death, it may still have contributed to it.

5.6.1.2 Cases 045, 051, 062, 069, 085, 102 and 083

In six cases HRV was detected in combination with positive routine bacterial culture results. In case 045 *Staphylococcus aureus* and *Klebsiella pneumonia* were cultured, in case 051 *Leuconostoc citreum* was cultured and in case 102 *Klebsiella pneumonia* was cultured, but no histological evidence of infection or a medical history was noted. Case 062 had a medical history of coughing prior to death and a positive bacterial culture for *Klebsiella pneumonia* and case 085 had a medical history of throat infection and was on antibiotics for seven days prior to death, but histological evidence of infection was not observed in either of the two cases. In case 069 *Klebsiella oxytoca* was cultured and histology showed signs of IP grade 1. Only one case showed mild histology indicative of an IP grade 1, which complicates

interpretation of results in favour of a possible infection from HRV or the bacteria that were co-detected. The last case (083) was HAdV-positive by routine SVC and histology showed IP grade 2. HRV was only present in the trachea of this case and HAdV was detected from the liver. This case was further complicated because the aetiological agents were not detected in the lungs where the IP was observed. In this case and the ones above, the PCR result would not have altered the final diagnostic category.

5.6.2 Viruses in the other SIDS cases

Case 009 was positive for three PCR viruses, i.e. HRV, RSV B and HCoV OC43/HKU1 from tracheal and lung specimens. The histology picture showed evidence of viral inclusion bodies, and a medical history of a stomach virus and a cold prior to death were noted. Da Silva *et al*. (2013) speculated that co-detection of HRV and RSV could result in an additive effect leading to more severe infection in infancy. In this case the PCR results may alter the final *Cause of Death Classification* from *SIDS* to *Infection*. Case 039 had co-detections with HRV and RSV A, and was culture positive for *Escherichia coli*, *Pseudomonas* species, *Serratia liquefaciens* and *Lactococcus garvieae*. A history of coughing and fever prior to death were noted, and an IP grade 2 was seen from the histology. In Case 145 HRV was detected from the trachea and HCoV 229E/NL from the lungs, along with histology signs of IP grade 2. Although HRV was not the sole pathogen in all HRV-positive SIDS cases, its possible contribution to death cannot be excluded in at least five SIDS cases in light of the histological evidence of infection, co-detection with other viruses and presence of the virus in the LRT, especially since Louie *et al*. (2009) and Miller *et al*. (2012) have associated this virus with more severe disease than previously thought.

In case 023 HCoV OC43/HKU1 was detected as the sole pathogen in the absence of other signs of infection and in 147 HCoV OC43/HKU1 and HCoV 229E/NL 63 were detected as the sole pathogens in the trachea and lungs with histological evidence of IP grade 3. The detection of HCoV in the latter case should be considered significant in light of the histology picture.

In case 104 HPIV 3 and HCoV OC43/HKU1 were detected with a histology picture of bronchopneumonia and bronchiolitis and a medical history of being lethargic prior to death. In this case the significance of HPIV 3 and HCoV possibly contributing to death requires further investigation, but the detection of bronchopneumonia may indicate an underlying infection or inflammatory response. This case therefore did not fit the normal definitional criteria for SIDS.

Influenza B was detected as the sole virus along with *Klebsiella pneumonia* in case 053 with a history of flu-like symptoms, but no histology signs of infection were observed. In this case, the medical history may be significant in light of the PCR result in the events that lead to the sudden death.

RSV A was detected in case 072 along with *Escherichia coli* and the histology picture showed signs of IP grade 2 and bronchiolitis. In light of the histology findings RSV should be considered significant in the infant's death.

HRV has been detected repeatedly from children with wheezing and LRT infection (Louie *et al*. 2009; Smuts *et al*., 2011). RSV has also been associated with bronchiolitis, wheezing and reduced lung function in hospitalised children (Zomer-Kooijker *et al.* 2014). Asthma has been known to result from respiratory viral bronchiolitis in paediatric populations (Tregonong & Schwarze 2010). Asthma is characterised by inflammation in the respiratory tract, contraction of bronchioles and airway remodelling and symptoms include shortness of breath and wheezing (Dhariwal *et al*. 2013). We detected HRV, RSV and HCoV the most frequently from our SUDI cases, with most of the lung and tracheal samples positive for these three viruses and the majority showing histology signs of inflammation was observed in more than half of all HRV, RSV and HCoV SUDI cases. Detecting these viruses in the LRT along with histology evidence of infection strongly suggest that these viruses played a role in the infectious events leading to death. Inflammation can also lead to the obstruction of bronchioles which can impair breathing (Gotsch *et al*., 2007; Openshaw, 2005) and thus the role of these viruses in asthma exacerbation may contribute to SUDI in some cases who were predisposed to asthma or who had reduced lung function.

5.7 The routine diagnostic tests used for SUDI investigations at Tygerberg

5.7.1 Rapid third generation VISITECT® HIV1/2 test

During routine autopsies on SUDI cases at Tygerberg FPS Mortuary heart blood is collected and sent for HIV testing. We found 29.6% of the blood specimens to be reactive with the HIV1/2 specific recombinant antigens used in the rapid third generation VISITECT® HIV1/2 test. Little value is placed on this test when pathologists review all the laboratory evidence to determine a final cause of death, but it is still part of the facility protocol for SUDI investigations at Tygerberg. Firstly, it is not a PCR test and does not detected HIV antigens, but rather HIV1/2 specific antibodies. Within the first 12-18 months of infancy it is thus not possible to distinguish between vertical transmission of HIV-specific maternal antibodies or the infant's own HIV1/2 specific antibodies (Graham & Gibb, 2002). Secondly, infants infected with HIV often experience secondary respiratory infections and thus HIV in itself is

not responsible for increased mortality in HIV infected populations. HIV is therefore not regarded as the aetiological agent responsible for death.

5.7.2 Routine shell vial culture

At the Tygerberg FPS Mortuary lung and liver specimens from every SUDI case are sent for routine SVC to screen for HAdV, influenza A and B, HPIV 1-3, RSV, CMV and HMPV. We only detected viruses using SVC in 9.5% of the cases. We detected HAdV in only one case and CMV in 13 cases. We know that some of the viruses included in the SVC method, such as influenza A and B, HPIV 3, RSV and HMPV, are indeed much more prevalent in our SUDI cases, because we detected these viruses along with others using the multiplex PCR. This questions whether some of the viruses we detected with the PCR may be false positive results. A high rate of false positivity is unlikely, given that the Seeplex® RV15 Ace detection assay is SANAS accredited for routine diagnostic practise and has a sensitivity of 100 copies/3 µl cDNA. A more likely explanation may be the integrity of autopsy specimens and possible viral loads too low for SVC detection and the delay between taking the sample and getting the specimens to the lab, as well as the PMI and adequate refrigeration of the body.

Through data mining we acquired the SVC results from 88 specimens sent from living infants admitted at Tygerberg Hospital during our study period. Of these 21.6% (19/88) were positive for viruses that included RSV, CMV, influenza A, HPIV 1 and 3 and HAdV. This questions why we did not detected any viruses other than CMV and HAdV from SVC conducted routinely on all SUDI cases. This also may point back to sample integrity. Specimens at Tygerberg Hospital are often taken upon admittance and transported to the lab where testing begins immediately. SVC may thus not be adequate to detect sufficient respiratory viruses from autopsy specimens, while there are time delays between specimen collection and testing. In our study we showed that all the viruses we detected using the Seeplex® RV15 Ace detection assay were missed with the SVC method. While only this method is used, we may be missing important infections that could contribute to SUDI and could lead to an inaccurate diagnosis of SIDS in some cases at the Tygerberg FPS Mortuary.

One could still argue that SVC is important because we detected HAdV from liver tissue in a case that was PCR-negative for this virus. Liver tissue was not collected for PCR and thus the fact that we missed HAdV from one case could be ascribed to specimen type, rather than an inability of the PCR to detected HAdV. In addition, SVC was not directly matched to the Seeplex® RV15 Ace detection assay because SVC does not include screening for HRV and HCoV, the most frequent and third most frequent viruses detected in our study, respectively.

Although we acknowledge that sample integrity must play a role in the differences in SVC viruses detected from hospitalised infants and the SUDI cases at Tygerberg during our study period, the SUDI cases that were SVC positive had a significantly higher PMI than the SVC-negative cases. Despite this higher PMI in SVC-positive cases, the viruses detected on SVC from our cases were DNA viruses (CMV and HAdV) which are considered more stable than RNA viruses and may thus have been less likely to undergo degradation of nucleic acids and thus viral viability might have been preserved. In addition, the significance of the association between the SVC-positive cases and a longer PMI is underpowered, because the sample size for the positive cases is so small. More research on bigger study groups is necessary to elucidate this finding.

5.7.3 Routine Bacterial Culture

Although the focus of this study was not the detection of bacterial pathogens, it forms an integral part of the routine laboratory investigations in SUDI cases at Tygerberg FPS Mortuary. Bacterial pathogens have also frequently been detected and considered to contribute to SIDS (Pryce *et al*., 2011). We detected potential bacterial pathogens in 56.8% of our SUDI cases. Histology correlated with the detection of bacteria in 60.7% of the cases. In a review Goldwater (2004) described that viral co-infections could increase bacterial toxin production and lethality and thus pose a true risk for SIDS. We found that 57.1% of our cases were co-infected with viruses and bacteria, with 58.3% of these showing histological evidence of infection.

In the literature emphasis has been placed on the PMI and how it could affect the validity of culture results. As part of the natural process of putrefaction the body's epithelial barriers lose integrity, which causes migration of bacteria (Tuomisto *et al*., 2013). Organs that possibly were sterile in life may become colonised with bacteria after death. The isolation of mixed bacterial species are considered post-mortem contaminants or post-mortem flora (Tuomisto *et al*., 2013) and therefore should have no bearing on the cause of death (Krous *et al*., 2003), as is the case at Tygerberg FPS Mortuary. The routine culture test results from the laboratory would not even identify the pathogens and only state post-mortem contaminants or post-mortem flora on the test result. In our study we did not find a significant association between the PMI of cases in which post-mortem flora was identified compared to cases where bacterial species were isolated and identified. Weber *et al*. (2010b) found that a longer PMI of up to five or more days caused a reduction in the amount of positive bacterial samples. In contrast, Tuomisto *et al*. (2013) found that a longer PMI caused liver specimens to be positive for bacteria more often, possibly representing bacterial migration or post-mortem contamination.

5.8 Strengths

To our knowledge, this is the first study that investigated 13 RNA respiratory viruses in SUDI cases in South Africa, a country where research into SIDS seems to be limited. In our study we were able to characterise the prevalence of RNA viruses in SUDI cases at Tygerberg FPS Mortuary over a one year period and included all four consecutive seasons. We also used a superior detection method to SVC. The PCR is more sensitive in detecting viruses from lung tissue in our SUDI cases and does not seem to be affected by the PMI.

5.9 Limitations and future research

One of the limitations to our study was the use of a qualitative assay and thus the inability to determine the viral load in our samples. Viral load is important to predict disease severity, and especially in the case of viruses with latent life cycles, such as CMV, a high viral load could distinguish active from infection when viral loads are low. Very low viral loads, especially in the case of HRV, could merely present past infections and viral shedding. Thus, if we were able to determine viral load in our study, we might have had a better idea of how important a role the viruses played in the events leading to these sudden deaths.

A second limitation to our study, which is very common, is the lack of a suitable control group. Ethical approval for specimen collection from infants who died of unnatural causes is very hard to acquire. If we had a control group we could have compared the PCR viruses in that group to our SUDI cases, to determine whether the viruses and histological evidence of infection are also present in infants dying from causes other than infections. We used the laboratory results from living hospitalised infants during the same period to compare our results to and although the optimal control group would have been infants who died from unnatural causes, we feel that the information collected from the living infants is valuable in describing viral prevalence rates in this vulnerable population.

A third limitation to our study is that we only included cases during one study year. If we collected data over a longer period, epidemiological risk factors might have emerged more significantly and a larger sample size over consecutive seasons could provide a better profile of respiratory viruses with seasonal variation in our setting and aid in determining patterns between viruses circulating in hospitalised infants and those in SUDI cases.

A fourth limitation is that only the left and right lower lung lobes were assessed for histology signs of infection. If more representative histology sampling was conducted which included the upper and lower left lung lobes and the upper, middle and lower right lung lobes, it could ensure that underlying disease processes were not missed.

Lastly, no immunological data was collected that could shed light on specific viral immune responses in the form of virus specific IgG or IgM antibodies, nor about the cellular immune response and thus we had to rely on general infectious histology signs in the lungs to interpret our findings.

The indicated limitations warrant additional inspection and should be further investigated in a much larger study.

CHAPTER SIX

CONCLUSION

We aimed to determine the prevalence of 13 RNA respiratory viruses from SUDI cases at Tygerberg FPS Mortuary during a one year period in order to investigate possible infectious causes of death. To determine the significance of viruses in our cases, we assessed different signs of infection present in the respiratory tract.

We found that more than half of the SUDI cases were PCR-positive for one or more respiratory viruses. Many of the viruses we detected are included in the routine SVC test that forms part of the medico-legal laboratory investigation for all SUDI cases at Tygerberg FPS Mortuary. None of the viruses we detected using PCR were ever cultured using the routine SVC test. Factors including autopsy sample integrity, the PMI, and viral viability and viral load most probably played a big role in the failure to culture viruses, especially since SVC was able to detect many different viruses in paediatric hospital patients at Tygerberg Hospital during the same study period. This indicates that the SVC method is suboptimal at determining respiratory viral prevalence in the post-mortem setting. We showed that SVC is potentially missing many respiratory viruses that could contribute to death in some of the SUDI cases at Tygerberg FPS Mortuary. In some cases that were classified as *SIDS*, the PCR results cannot be ignored, especially when it is supported by histological evidence of infection. We thus propose that the use of PCR could have altered a *Cause of Death Classification* from *SIDS* to *Infection* in some of these cases.

Further research is needed to determine the importance of detecting viruses from SUDI cases where no significant histological evidence of infection was observed. This questions whether PCR may be too sensitive and is detecting past and latent viral infections that do not play any role in the cause of death. The histological picture also requires further characterisation to determine if it accurately predicts infections or lethal events and can truly support virology findings, especially in young infants whose immune systems are still maturing.

In line with the aims and objectives of our study we showed that a substantial proportion of SUDI cases were PCR-positive for respiratory viruses, but the true clinical significance of detecting these viruses could not be determined within the scope of this study due to the absence of information regarding a viral-specific immune response. SVC routinely included in the SUDI investigations at Tygerberg FPS Mortuary is missing PCR-positive cases and is thus insufficient for autopsy samples in a post-mortem setting. Before the significance of

respiratory viruses can adequately be determined in the context of SUDI and SIDS, protocols need to include sufficient anatomical sampling sites and sufficient laboratory techniques to shed light on the prevalence of viruses. In addition, viral specific immune response needs to be further characterised. Without determining the true prevalence of viruses in SUDI cases and the viral-specific immune response, the contribution of virus-specific infections to this syndrome will remain largely undetermined.

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Addendum 1

Ethical approval and renewal

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Approval Notice
New Application

08-Mar-2012
De Beer, Corena C

Protocol #: N12/02/007

Title: **Investigation of viral respiratory patogens in cases of Sudden Unexpected Death in Infants (SUDI) in the Tygerberg Medico-legal laboratory drainage area of the Western Cape Metropole**

Dear Doctor Corena De Beer,

The **New Application** received on **24-Feb-2012**, was reviewed by members of **Health Research Ethics Committee 2** via Expedited review procedures on **07-Mar-2012** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **07-Mar-2012 -07-Mar-2013**

Please remember to use your **protocol number** (**N12/02/007**) on any documents or correspondence with the REC concerning your research protocol.

Please note that the REC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.
The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number projects may be selected randomly for an external audit.
Translation of the consent document in the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Hine Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard REC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further help, please contact the REC office at 0219389207.

Included Documents:
Application
Declaration
Protocol
Synopsis

CVs
Checklist

Sincerely,

Mertrude Davids
REC Coordinator
Health Research Ethics Committee 2

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Ethics Letter

25-Mar-2013

Ethics Reference #: N12/02/007
Title: Investigation of viral respiratory patogens in cases of Sudden Unexpected Death in Infants (SUDI) in the Tygerberg Medico-legal laboratory drainage area of the Western Cape Metropole

Dear Doctor Corena De Beer,

At a review panel meeting of the Health Research Ethics Committee that was held on 4 March 2013, 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: 4 March 2013 Expiry Date: 4 March 2014

If you have any queries or need further help, please contact the REC Office 0219389207.

Sincerely,

REC Coordinator
Mertrude Davids
Health Research Ethics Committee 2

Addendum 2

Data mining request

TYGERBERG ACADEMIC LABORATORY

TAL.FORM.0101.0

REQUEST FOR DATA MINING

Please use this request form for extractions to be done on the Tygerberg Hospital Disa*Lab Database

REQUEST DETAILS			
Full Name of Requestor	Heleen la Grange		
Contact Address	Division of Medical Virology, Francie van Zijl Avenue, Po Box 19063, Tygerberg, Cape Town, South Africa. 7505	**E-mail**	hlg@sun.ac.za
Date	2nd September 2013	**Tel No**	021 938 9360
Name of Department and/or Organisation	Medical Virology, Stellenbosch, Tygerberg	**Signature**	

Brief description of search to be done (aim) The aim of the project is to detect bacterial and viral pathogens from 148 Sudden Unexpected Death in Infancy (SUDI) cases at the Tygerberg Forensic Mortuary. A Nucleic amplification assay will be used to look for 15 respiratory viruses and this will be corroborated with routine Shell Vial Culture (SVC) and bacterial culture from the SUDI cases at the mortuary.

Full Description of search Hundred-and-forty-eight SUDI cases were collected over one year from May2012-May 2013 (ethics no. N12/02/007) as part of a Masters Degree Thesis in Medical Virology. Routine mandatory specimens were collected at autopsy for viral and bacterial culture. Tracheal and lung swabs were additionally collected for Nucleic acid detection of 15 respiratory viruses. Lung sections were prepared for histology to look for signs of infection and corroborate positive pathogen results. SUDI cases rise in winter when respiratory tract infections rise, therefore we want to compare results from the SUDI cases to those pathogens detected in hospital admitted live babies between the ages of 1 week to 1 yearn (to match the SUDI cases collected from the mortuary) during our one year study period. This will give us an idea of the pathogens circulating in this age group. Through our study we want to show that routine the viral culture from autopsy specimens lack the sensitivity of nucleic acid techniques and that screening for 15 viruses will be better than 9 viruses routinely tested using SVC from SUDI. This nucleic acid detection assay that looks at 15 viruses may be a valuable tool in aiding the search for a diagnosis of infectious deaths in SUDI cases at Tyegrberg Forensic Mortuary.

ADDITIONAL INFORMATION	
Laboratory Data Required	G Ground and A9 Unit PCR15 and SVC results from living babies
Date range of search	May 2012-May 2013
Fields required ie. Patient name, Patient ID etc	• Living babies admitted for respiratory infections between the ages 1week-1year (babies age must please be known) • Baby sex must please be known • Date of PCR15 and SVC test requests (results must at least be identified to the month to allow comparison to mortuary cases)

AUTHORISATION (HOD, Pathologist or Laboratory Controller)			
Authorised by	Dr. J. Maritz		
Date	02/09/2013	**Signature**	

DATA MINING COMPLETED			
Completed by	W Klenthan		
Date	3/9/2013	**Signature**	

Addendum 3

Details of PCR-positive cases (n=88)

Case Number	Virus Detected	Histological Signs of Infection	Medical History	Cause of Death Classification	Routine Culture Results
002	•HRV	Congestion, bronchopneumonia, focal collapse, alveolar haemorrhage	Feverish day before death and two weeks prior to death	Infection	
003	•Human enterovirus	Congestion, focal collapse, pigment iron/formalin			
005	•RSV A	Oedema, congestion, focal collapse, pigment iron/formalin, alveolar debris	Yes	Congenital cardiac abnormalities	
006	•HCoV OC43/HKU1	Congestion, focal collapse		Infection	•*Enterobacter aerogenes*
007	•HRV •RSV B	Oedema, focal collapse		Infection	•*Klebsiella pneumoniae*
009	•HRV •RSV B •HCoV OC43/HKU1	Query RSV	Stomach virus and cold 1 week prior	SIDS	
011	•HRV	Oedema, IP grade 1, focal collapse, pigment iron/formalin, alveolar debris		Infection	
013	•HCoV OC43/HKU1 •RSV A	Congestion, alveolar haemorrhage		Aspiration of foreign material or gastric contents	
014	•HRV	Oedema			•*Raoultella ornithinolytica*
015	•RSV A	Focal collapse, alveolar debris	was not breathing right	Congenital cardiac abnormalities	
017	•HCoV OC43/HKU1 •HCoV 229E/NL63 •RSV A	Oedema, bronchopneumonia, focal collapse, pigment iron/formalin, alveolar debris		Infection	•*Klebsiella oxytoca*
019	•HRV	Focal collapse, alveolar debris, query		Infection	•*Escherichia coli*

		RSV			
023	•HCoV OC43/HKU1	Focal collapse		SIDS	
026	•HRV	Bronchopneumonia	Coughing	Infection	•Beta-haemolytic strep. Group B
027	•HRV	Bronchopneumonia		Infection	
029	•HRV	Congestion, IP grade 4, alveolar haemorrhage		SIDS	
030	•HRV	Oedema, IP grade 1, bronchopneumonia			
032	•HRV	Oedema, IP grade 2		Infection	•*Escherichia coli*
034	•HRV	Unremarkable			•*Raoultella planticola*
037	•HRV •RSV A •PIV 3	Unremarkable			
038	•HMPV	Unremarkable		Infection	•*Escherichia coli* •*Enterococcus* species
039	•HRV •RSV A	IP grade 1, focal collapse	Coughing and fever	SIDS	•*Escherichia coli* •*Pseudomonas* species •*Serratia liquefaciens* •*Lactococcus garvieae*
040	•HRV •Human enterovirus	Unremarkable		Infection	
043	•HRV	Oedema, congestion, IP grade 1, focal collapse, pigment iron/formalin		Unable to exclude accidental suffocation	
044	•HRV •Human enterovirus	Congestion	Breathing difficulties and received antibiotics a week prior to demise	Infection	•CMV •*Staphylococcus aureus*
045	•HRV	Congestion, focal collapse, alveolar debris		SIDS	•*Staphylococcus aureus* •*Klebsiella pneumoniae*
046	•Influenza A	Oedema			•*Escherichia coli*

047	•HRV •RSV A	Oedema, congestion, bronchiolitis			
048	•HRV	Focal collapse	cold/runny nose, diarrhoea and fever 24 hrs before	Infection	
051	•HRV	Unremarkable		SIDS	•*Leuconostoc citreum*
052	•HRV	Oedema, IP grade 2-3			•*Staphylococcus aureus*
053	•Influenza B	Oedema, congestion, focal collapse	Flu-like symptoms prior to death	SIDS	•*Klebsiella pneumoniae*
057	•HRV •Influenza A	IP grade 1, focal collapse	Sick for one week with fever and coughing	Infection	
058	•HRV	Unremarkable			•*Raoultelle planticola*
062	•HRV	Unremarkable	Sick with coughing one day prior to death	SIDS	•*Klebsiella pneumoniae*
063	•Influenza A	Pigment iron/formalin, alveolar haemorrhage			•Beta-haemolytic strep. Group B
064	•HRV	Oedema, congestion, bronchiolitis, alveolar debris, alveolar haemorrhage, query RSV		Congenital cardiac abnormalities	
067	•HRV	IP grade 1, focal collapse			•*Klebsiella pneumoniae*
068	•HMPV	IP grade 2, alveolar haemorrhage			•CMV •*Klebsiella pneumoniae* •*Streptococcus pneumoniae*
069	•HRV	IP grade 1, focal collapse, alveolar debris, alveolar haemorrhage		SIDS	•*Klebsiella oxytoca*
070	•RSV A •HCoV OC43/HKU1	Congestion, bronchopneumonia, focal collapse		Infection	•*Escherichia coli*

071	•HRV •RSV A •HMPV	Oedema, IP grade 2, query RSV/virus		Infection	•*Klebsiella pneumoniae*
072	•RSV A	Oedema, congestion, IP grade 2, focal collapse, alveolar haemorrhage, bronchiolitis, query virus		SIDS	•*Escherichia coli*
073	•HRV	Oedema, IP grade 2, alveolar debris, alveolar haemorrhage, query RSV/HAdV		Aspiration of foreign material or gastric contents	•*Klebsiella pneumoniae*
077	•HCoV OC43/HKU1 •HRV	Oedema, congestion, IP grade 1, bronchopneumonia, focal collapse, alveolar debris, alveolar haemorrhage			•*Streptococcus pneumoniae*
078	•HRV	IP grade 2, focal collapse, alveolar haemorrhage			•*Raoultella planticola*
080	•HRV •HPIV 3	Oedema, congestion, IP grade 2-3, focal collapse, alveolar debris			•*Klebsiella*
081	•HRV •HPIV 3	Oedema, IP grade 2, alveolar debris			
082	•HRV	IP grade 2, focal collapse, alveolar debris, alveolar haemorrhage	Fever on day of death	Infection	
083	•HRV	Oedema, IP grade 2, alveolar debris		SIDS	•HAdV
084	•HRV	Oedema, IP grade 1-2, bronchitis	Ill with vomiting 24 hours prior to death	Infection	•*Enterobacter cloacae*
085	•HRV	Oedema, congestion	Throat infection (7 days on antibiotics)	SIDS	•*Haemophilus influenzae*
086	•HRV •Human enterovirus	Oedema, alveolar haemorrhage			•*Escherichia coli*

087	•HMPV	Oedema, bronchopneumonia, alveolar haemorrhage			•*Klebsiella pneumoniae*
089	•HRV	Congestion, IP grade 4, alveolar debris, alveolar haemorrhage, query virus		Infection	•*Escherichia coli*
090	•HRV	Congestion, IP grade 3, alveolar haemorrhage		Infection	•CMV •*Haemophilus influenzae*
091	•HRV •HMPV	IP grade 3		Infection	•*Klebsiella Pneumoniae*
092	•HRV	Oedema, congestion, IP grade 1, alveolar debris		Infection	•*Klebsiella Pneumoniae* •*Salmonella* group D
093	•HRV	Oedema, focal collapse		Infection	
094	•HRV •Human enterovirus	Oedema		Infection	•*Enterococcus faecalis* •*Klebsiella Pneumoniae*
095	•HRV	Congestion, focal collapse, alveolar debris		Infection	
102	•HRV	Oedema		SIDS	•*Klebsiella pneumoniae*
103	•HRV	Oedema, focal collapse		Infection	
104	•HCoV OC43/HKU1 •HPIV 3	Oedema, bronchopneumonia, alveolar debris, Bronchiolitis	Lethargic at crèche	SIDS	
105	•Human enterovirus	Oedema, congestion, bronchopneumonia			•CMV •*Staphylococcus aureus*
106	•HRV	Congestion, alveolar haemorrhage		Infection	•CMV
108	•HRV	Oedema, focal collapse			
110	•HRV	Oedema, focal collapse, alveolar			

		debris			
111	•HRV	Bronchopneumonia	Breathing problems and diarrhoea	Infection	•*Candida albicans*
112	•HRV	Oedema, IP grade 2, alveolar debris		Infection	•*Candida albicans*
113	•HRV	Congestion, IP grade 1, focal collapse		Infection	•*Klebsiella pneumoniae*
115	•HRV	Oedema, alveolar debris, bronchiolitis, bronchitis			
118	•RSV A	Congestion, IP grade 1, bronchopneumonia			•*Haemophilus influenza*
119	•HRV	Oedema, congestion, IP grade 1-2		Infection	
120	•RSV A •HRV	Congestion, IP grade 1, alveolar debris, alveolar haemorrhage			•*Escherichia coli*
122	•HRV	Oedema, IP grade 2, bronchopneumonia	Diarrhoea, vomiting and listlessness for a week	Infection	•*Klebsiella pneumoniae*
128	•HRV	Oedema, congestion, IP grade 1			
129	•HRV •HCoV 229E/NL63	Oedema, congestion, IP grade 1, alveolar debris			•CMV
131	•HRV	Oedema, congestion		Infection	
132	•HRV	Oedema		Infection	•*Escherichia coli*
135	•HRV •RSV A	Alveolar debris, bronchitis		Infection	
137	•HRV	Oedema, alveolar haemorrhage			•CMV
139	•HRV	IP Grade 2	Slight cold symptoms	SIDS	
142	•RSV A	Oedema, congestion, IP grade 2-3, alveolar debris, bronchiolitis, query RSV			
144	•HRV	IP grade 1, alveolar haemorrhage,		Infection	

		bronchitis		
145	•HRV •HCoV 229E/NL63	Oedema, IP grade 2	SIDS	
147	HCoV 229E/NL63	Oedema, IP grade 3, alveolar debris	SIDS	
148	•HRV	IP grade 1, alveolar haemorrhage	Infection	•CMV