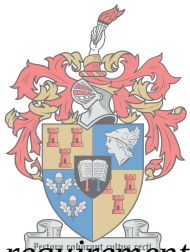
Profiling Enterovirus and Parvovirus B19 in sudden and unexpected death in infancy (SUDI) at the Tygerberg Medico-legal Mortuary and the role of myocarditis as a possible cause of death

by
Jamie Saayman



Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Medicine and Health Science at Stellenbosch University

Supervisor: Dr Corena de Beer
Co-supervisor: Prof. Johan Dempers

March 2018

DECLARATION

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

_____ _____

Signature Date

ABSTRACT

Background: Sudden infant death syndrome (SIDS) remains one of the leading causes of death among infants. The Triple-Risk Model has contributed to identifying modifiable risk factors that may lead to a reduction of SIDS occurrences. Cardiovascular infection contributes significantly to mortality and morbidity in children and adults. Acute myocarditis affects infants more severely than adults and has a known association with Coxsackie-B, Adeno-, parvo- B19, Epstein Barr-, Cytomegalo-, Human herpes-6 viruses. These viruses have been explored in sudden unexpected death in infancy (SUDI) and shown some association between SIDS and myocardial infection.

Aim: This study aimed to describe two cardiovascular viruses in SUDI cases and to determine the association of myocarditis to these deaths.

Methodology: Heart swab and tissue samples were prospectively collected from SUDI cases at the Tygerberg Medico-legal Mortuary over a one year period. The samples were collected in parallel with routine heart swabs for microbiology analysis and peripheral blood for HIV screening. The SUDI samples were additionally screened for enterovirus and parvovirus B19 by polymerase chain reaction assays. The heart tissue was processed for histological analysis. Sociodemographic information, medical history and final cause of death were obtained during the initial interview with family / caregiver(s) and from case files respectively, and potential risk factors in the SUDI population were identified from the data by statistical analysis.

Results: Heart swab and tissue samples were collected from 168 and 161 SUDI cases respectively. The SUDI population consisted of 81 males and 87 females. Majority of deaths (64%) were in infants younger than 14 weeks and 67% occurred during the colder months of the year. In more than half of the cases an infectious cause of death was confirmed, while in 40% death was attributed to SIDS. There was a higher frequency of death among black infants, which is consistent with the literature, however it is not clinically relevant as it is not a representation of the general population profile in the Western Cape. The heart tissue for histology was within normal limits in all but 10 of 161 SUDI cases examined for morphological change associated with viral myocarditis, and 1 of these 10 cases was diagnosed as myocarditis as the final cause of death. The only significant risk factor identified in this population was ethnicity, but the finding was not clinically relevant.

Conclusion: The results obtained from this study support the Triple-Risk Model of SIDS. The high proportion of deaths that remained unexplained (i.e. SIDS) emphasizes the need to introduce additional testing, such as molecular based tests which provide significant value when establishing a final cause of death. SIDS research in South Africa is limited and would be valuable in the forensic environment.

OPSOMMING

Agtergrond: Skielike sterfte in babas (SIDS, SUDI of "wiegiedood") is steeds een van die hoofoorsake van dood by babas. Die *Triple-Risk Model* het bygedra tot die identifisering van sekere risikofaktore wat kan lei tot 'n vermindering in wiegiedood gevalle. Kardiovaskulêre infeksie dra aansienlik by tot mortaliteit en morbiditeit in kinders en volwassenes. Akute miokarditis affekteer babas meer ernstig as volwassenes en het 'n bekende assosiasie met Coxsackie-B, Adeno-, parvo-B19, Epstein Barr-, Cytomegalo-, Human herpes-6 virusse. Hierdie virusse is ondersoek in skielike sterftes in babas en het 'n assosiasie tussen wiegiedood en miokardiale infeksie getoon.

Doel: Hierdie studie het twee kardiovaskulêre virusse in SUDI-gevalle ondersoek en die bydrae van miokarditis tot hierdie sterftes geëvalueer.

Metodes: Deppers en weefsel van die hart is prospektief versamel van SUDI-gevalle by die Tygerberg Mediesgeregtelike Lykshuis oor 'n een jaar tydperk. Hierdie monsters is versamel bo en behalwe die roetine hart deppers vir mikrobiologiese kweking en perifere bloed vir MIV-sifting. Die SUDI monsters is addisioneel getoets vir enterovirus en parvovirus B19 deur middel van 'n polimerase kettingreaksie metode. Die hartweefsel is geprosesseer vir histologiese analise. Sosiodemografiese inligting, mediese geskiedenis en finale oorsaak van dood is verkry tydens die oorspronklike onderhoud met familie / versorger(s) en gevalle lêers onderskeidelik, en potensiële risikofaktore in die SUDI studiegroep is met statistiese analise geïdentifiseer.

Resultate: Deppers en weefsel van die hart is onderskeidelik van 168 en 161 SUDI gevalle versamel. Die studiegroep het 81 seuntjies en 87 dogtertjies ingesluit. Die meerderheid sterftes (64%) het voorgekom in babas jonger as 14 weke en 67% van die gevalle het gedurende die kouer maande van die jaar voorgekom. In meer as die helfte van die gevalle is 'n infektiewe oorsaak van dood bevestig, terwyl die oorsaak van dood in 40% aan SIDS toegeskryf is. Daar was meer swart babas wat gesterf het en hoewel hierdie neiging ooreenstem met die literatuur, is dit nie verteenwoordigend van die algemene bevolking in die Wes-Kaap nie en dus nie klinies relevant nie. Morfologiese veranderinge in die hartweefsel van die gevalle was binne normale perke in 151 van 161 gevalle. In die 10 gevalle met abnormale histologie, het die veranderinge gedui op virale miokarditis, maar die finale oorsaak van dood is in slegs 1 geval as miokarditis gediagnoseer.

Gevolgtrekking: Die bevindings van die studie ondersteun die *Triple-Risk Model* van SIDS. Die hoë persentasie onverklaarbare sterftes (d.w.s. SIDS) beklemtoon die noodsaaklikheid om addisionele analises, soos molekulêre toetse in te sluit in die bepaling van die oorsaak van dood om meer lig te werp op die tipe sterftes. Sulke navorsing in Suid Afrika is uiters beperk en sal 'n waardevolle bydrae kan lewer in die forensiese veld.

ACKNOWLEDGEMENTS

I would like to thank God Almighty for granting me the strength, knowledge, ability and opportunity to undertake this research project and to persevere and complete it to the best of my ability.

I would like to acknowledge the following individuals:

My supervisor, Dr Corena de Beer, for her constant support, expert guidance and motivation during the conduction and completion of my research project; and for encouraging me to grow as a young researcher.

My co-supervisor, Prof Johan Dempers, for his assistance in completing and understanding the histology aspect of my project.

Mr André du Toit, Marshall Hartzenberg, John Segole for their assistance and expertise during the histological processing of my samples.

Mrs Riley, Prudence Flaendorp and Rameez Sulaiman for notifying me about SUDI cases, and the staff and Forensic pathologists at Tygerberg Medico-legal Mortuary for their assistance with sample collection.

Dr Janette Verster for her valuable time with helping me capture data from the case files and for her encouragement during the course of and up to completion of my project.

Ms Mathilda Claassen for her expertise in the laboratory, Bronwyn Kleinhans, Danielle Cupido, Landry Kabego, Ndapewa Ithete, Shahieda Isaacs, the staff and students of the Division of Medical Virology for their assistance and guidance during the course of my project.

My mom, dad, sister, brother and Jarryd Lamour for their constant support and encouragement.

Harry Crossley, Poliomyelitis Research Foundation and National Research Foundation for funding this project.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
Amp	Ampicillin
ATP	adenosine triphosphate
B19	parvovirus B19
bp	base pairs
cDNA	complementary DNA
CI	confidence interval
CMV	cytomegalovirus
COD	cause of death
CTP	cytidine triphosphate
CVB	coxsackievirus B
DNA	deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ds	double-stranded
DSI	death scene investigation
EBV	Epstein-barr virus
E. coli	*Escherichia coli*
EV	enterovirus
FN	false negative
FP	false positive
GTP	guanosine triphosphate
H&E	haematoxylin and eosin
HAdV	human adenovirus
HHV	human herpesvirus
HIV	Human immunodeficiency virus
HREC	Health Research Ethics Committee
HS	Hot Start
HSV	Herpes simplex virus
Ig	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IQR	interquartile range
LB	Lysogeny broth
LR	likelihood ratio

LRTI	Lower respiratory tract infection
$MgCl_2$	magnesium chloride
NHLS	National Health Laboratory Service
OR	Odds ratio
PCR	polymerase chain reaction
PM	post-mortem
PMI	post-mortem interval
QCMD	Quality control for molecular diagnostics
RE	restriction enzyme
RNA	ribonucleic acid
rpm	Revolutions per minute
RSV	respiratory syncytial virus
RT	reverse transcription
S. aureus	*Staphylococcus aureus*
SD	standard deviation
SIDS	Sudden infant death syndrome
ss	single-stranded
SUDI	Sudden unexpected death in infancy
SUID	Sudden unexpected infant death
SVC	shell vial culture
Ta	Annealing temperature
TN	true negative
TNA	Total nucleic acid
TP	true positive
U&C	urea and creatinine
UK	United Kingdom
USA	United States of America
UTM	universal transport media
UTP	uridine triphosphate

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Sudden infant death syndrome (SIDS) is a devastating event that may torment affected families for a long time. SIDS is a common cause of death in infants previously thought to be healthy (Byard & Marshall 2007). SIDS is defined as the sudden and unexpected death of an infant during their first year of life which remains unexplained even following the conduction of a thorough post-mortem (PM) investigation, death scene and review of the clinical history (Beckwith 2003). Sudden unexpected death in infancy (SUDI) is a broader term used to define the death of infants younger than one year old that show no prior history of fatal illness or injury and is used to classify all cases of sudden infant death, whether explained, unexplained or unascertained. SIDS is one of the branches of SUDI (Weber *et al.* 2008a).

In South Africa, children under one have the highest mortality rate within the pediatric range (Statistics South Africa 2015), and SIDS accounts for 3.41/1 000 live births in Cape Town – one of the highest rates in the world (Kinney & Thach 2009).

Three factors have been highlighted in the literature that jointly trigger SIDS: a critical developmental period, a vulnerable infant, and exogenous stressors (Filiano & Kinney 1994). This has led to the identification of various risk factors commonly observed in SIDS cases, including a male predominance, peak during colder months, ethnicity, poverty, low birth weight and infection to name a few (Byard & Krous 2003).

Infection is believed to be an important contributor to SIDS. Both viral and bacterial pathogens have been associated with SIDS, either as mediators of abnormal systemic immune response or as external stressors. Multiple pathogens have been identified in SUDI cases, although no single infectious agent has been consistently found to cause SIDS (Alfelali & Khandaker 2014; Weber *et al.* 2008a).

Cardiovascular infection in SIDS remains controversial. However, there is an increase in the number of reports of heart disease, especially myocarditis, in SUDI (Dancea *et al.* 2002). Although clinical myocarditis is rare in infants, asymptomatic cases are more common and have been reported in SIDS. Weber *et al.* (2008b) and Dettmeyer *et al.* (2004) have shown the prevalence of myocarditis in SIDS, and identified coxsackievirus B (CVB), human adenovirus (HAdV), parvovirus B19 (B19) and human herpesvirus (HHV) 6 and Epstein-barr virus (EBV).

PM investigations are extremely important in SUDI cases, especially to ascertain an exact cause of death and/or rule out SIDS in these cases. What is alarming is that the great majority of SUDI cases are ultimately classified as SIDS (Weber *et al.* 2008a). In South Africa, according to the South African Inquests Act (Act 58 of 1959), deaths considered to be from other than natural causes are investigated by the South African Police Service and referred to a medical practitioner who may, if necessary, perform an autopsy to ascertain the cause of death; these include SUDI cases. For diagnosis of SUDI cases, a proper protocol needs to be followed. However, no nationally accepted guideline for sudden infant death exists in South Africa; and therefore, different guidelines for such cases are followed by different institutions in South Africa and around the world (Livesey 2005; Bajanowski *et al.* 2007; du Toit-Prinsloo *et al.* 2011; du Toit-Prinsloo *et al.* 2013). At Tygerberg Medico-legal Mortuary all cases are investigated as per the standard facility protocol, which may include a death scene investigation (DSI) and medical history review, autopsy and limited laboratory investigations. All SUDI cases are also subjected to viral culture screening, specifically for HAdV, Cytomegalovirus (CMV), Influenza virus A and B, human parainfluenza virus 1, 2 and 3 and respiratory syncytial virus (RSV) from lung and liver tissue (la Grange *et al.* 2014).

Apart from South Africa, limited research is conducted in Africa regarding SIDS (Ibeziako *et al.* 2009), and no research has thus far investigated cardiovascular pathogens in SUDI cases. This introduces a special field of interest in SIDS by researchers in Africa.

Therefore, the aim of this project was to investigate two cardiovascular pathogens in SUDI cases and to assess whether the pathogens play a significant role in the final cause of death (COD).

CHAPTER TWO

LITERATURE REVIEW

2.1 A historical background of sudden infant death

Sudden death in infants is not a new disease but has been around for many years and was first described in the Bible where an infant had died as a result of an accidental suffocation event (Byard & Krous 2003). SIDS was first described in 1969 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". However, due to the ambiguity in the 1969 definition, it was amended in 1989 to "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" (Beckwith 2003).

2.2 The evolution of sudden infant death

SIDS is a branch of SUDI, sometimes referred to as sudden unexpected infant death (SUID). SUDI, used as a more circumstantial term for any sudden and unexpected death in an infant (Berkowitz 2012), defines the sudden and unexpected death of any infant, generally while asleep, that is between 7 days and 1 year old, showing no history of fatal injury or illness (Weber *et al.* 2008a). SUDI is used as a multifarious term for the diagnosis of all cases of sudden death in infants. Three categories of SUDI have previously been described, namely explained death, unascertained death and unexplained death. These classifications are established after a thorough PM investigation has been conducted, which includes reviewing of the death scene and clinical background. Death as a result of a known COD, such as infection, infanticide, cardiac defects, and previously undiagnosed metabolic disorders, is known as *explained SUDI*, while death where the COD is perhaps difficult to establish due to inadequate information, such as in a case of possible accidental suffocation, bed sharing or asphyxia, is known as *unascertained SUDI*. When the COD remains unexplained after all the investigation procedures have been followed, it is classified as *SIDS* (Krous 2010; Schnitzer *et al.* 2012; Kennedy 2016).

SIDS is known as a complex disease of which the exact cause is still unclear. The major theory associated with the events leading to SIDS has been defined as the Triple-Risk Model (Filiano & Kinney, 1994). Spinelli *et al.* (2017) summarized the emergence of the multifaceted hypothesis first theorized by Wedgwood (1972) as the Multiple Contingency Theory, and just over a decade later formulated by Filiano and Kinney as the Triple-Risk Model (Figure 2.1). The development of this

theory has led to the notion that SIDS may result when there is an overlap of three main factors, namely: a) an infant that is innately weak, b) experienced a critical development phase in homeostatic control and c) exposed to some sort of external stressor. The susceptibility of an infant may not manifest until the infant crosses into the critical developmental phase and is subject to an external stress (Filiano & Kinney 1994). The Triple-Risk Model does not provide information on the mode or mechanism of death leading to SIDS, however it has assisted in identifying some of the risk factors associated with SIDS cases.

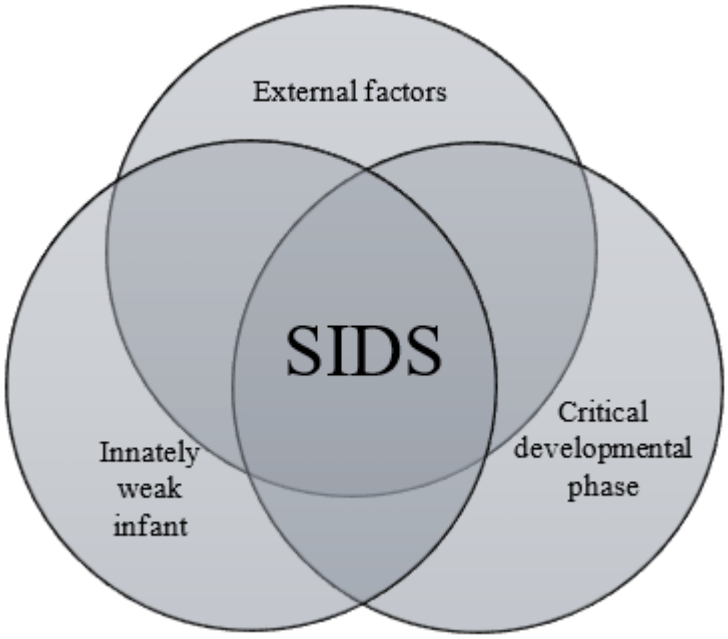


Figure 2.1. Triple-Risk Model (adapted from Filiano & Kinney 1994).

2.3 Incidence and risk factors in SUDI and SIDS

Infant mortality in South Africa is a major problem, particularly due to the high rates which are increased by the burden of human immunodeficiency virus (HIV)/AIDS (Sartorius *et al.* 2011). Incidence rates for SIDS in South Africa are poorly reported, with greater emphasis placed on the rates of overall infant death. A national report on the mortality and causes of death in South Africa in 2014 (Statistics South Africa, 2015) indicated that infant mortality (<1 year old) remains highest in the pediatric population (1 to 14 years, as classified by the Department of Health in 2012). In terms of geographic location, most infant deaths occurred in North-West province. Respiratory and cardiovascular disorders specific to the perinatal period (20th week of pregnancy to 7 days after birth, as classified by the Department of Health 2012) were the leading causes of death in infants within the neonatal period (birth to 28 days, as classified by the Department of Health 2012), followed by intestinal infection and respiratory (influenza and pneumonia) infections within the postneonatal period (between 4 weeks and 1 year old, including neonatal period). Malnutrition and other acute

respiratory infections were among the top ten leading causes of death among infants (Age Definitions 2012; Statistics South Africa, 2015).

SIDS is a crucial topic of interest and poses a great threat to infants worldwide, specifically because no single cause has been identified yet that may aid in preventing these deaths from occurring. Globally, SIDS was attributed to approximately 15 000 infant deaths in 2013 (Vos *et al.* 2015), and persists as a leading cause of death in the postneonatal period after congenital malformation, conditions related to low birth weight and maternal complications in the United States of America (USA) (Matthews *et al.* 2015). Trends in SIDS rates were shown to be highest in New Zealand with a rate of 0.80/1 000 live infants, followed by the USA and United Kingdom (UK) with 0.57/1 000 and 0.41/1 000 respectively, and Japan and The Netherlands with 0.09/1 000 and 0.01/1 000 respectively (Kinney & Thach 2009).

Since the recognition of the Triple-risk Model, identifiable risk factors (Figure 2.2) have been discussed in the literature that have contributed to a better understanding of SIDS. These risk factors all form part of Triple Risk Model.

Figure 2.2. Some of the common risk factors of SIDS (adapted from Byard & Krous 2003).

Infant vulnerability may be attributed to prematurity, low birth weight, self-reported pre- or postnatal exposure to parental tobacco, pre-existing metabolic disorders or common mutations that increase susceptibility to specific environmental factors and infection (Fleming *et al.* 2015). Cigarette smoke in particular is extremely detrimental to the developing infant (i.e. prenatal exposure); and affects development and maturation of the infant's autonomic nervous system as well as respiratory function (Blood-Siegfried 2009). A recent study showed a strong association between brainstem abnormalities and SIDS, where abnormally high total serotonin neurons were observed in SIDS cases compared to non-SIDS control cases (Bright *et al.* 2017). Alcohol exposure during pregnancy is especially dangerous as it can pass freely across the placenta and will remain in the fetal system longer than in the maternal circulation, increasing the risk to the infant. The Western Cape has one of the highest incidences of Fetal Alcohol Spectrum Disorders in South Africa and previous studies have shown that a high percentage of women admitting to regularly consume alcohol during pregnancy (May *et al.* 2014).

Males have a greater risk to SIDS than females, with an incidence rate of 2:1 (Kinney & Thach 2009). Elhaik (2016) proposed the allostasis model to explain the major risk factors of SIDS being associated with brain function. Increased *in utero* exposure to different stressors, pain and trauma induces allostatic overload, i.e. a maladaptive effect in the developing nervous system that may affect later adaptability to environmental or iatrogenic stress. He postulated that neonatal male circumcision may increase allostasis, as the surgical procedure constitute intra- and postoperative risks, such as intense pain, cardiorespiratory shock and infection, among others. This can lead to increased vulnerability of male infants, also predisposing them to an increased risk for SIDS, however this theory is still to be proven.

The first six months of life may be regarded as a critical developmental period during which the infant immune system is still developing, and SIDS is common during this period with a peak incidence between two and four months old (Kinney & Thach 2009, Blackwell *et al.* 2015).

External factors such as maternal parity, low socioeconomic background, mothers with low educational background; as well as young mothers, particularly those younger than 20 years old are also associated with increased risk to SIDS (Byard & Krous 2003; Mitchell & Krous 2015; Ndu 2016). Furthermore, seasonality also plays an important role as an external risk factor. SIDS rates are higher during the colder months during which infants are highly susceptible to infection, particularly respiratory infection (Kinney & Thach 2009). Major differences in SIDS incidence are also observed in social and racial groups, specifically within the different ethnic groups (Matthews *et al.* 2015). SIDS rates in the USA (Hunt & Hauck 2006) and New Zealand are higher in black infants than in

white infants, which has been associated with such socially- and culturally-related risk factors as listed above (Mitchell *et al.* 1993; Blair *et al.* 2006).

Sleep-related risk factors include prone sleeping, bed sharing and excessive bedding/clothing (including soft bedding or objects). These factors can either play an independent role in the pathogenesis of SIDS, or can function as a combination of one or more factors potentiating asphyxia (Hunt *et al.* 2015). Bed sharing specifically is common practice within some ethnic groups and individuals characterized by poor maternal education, socioeconomic deprivation, as well as premature or very young and vulnerable infants (Colson *et al.* 2013).

From the first appearance of SIDS in the Bible, and in its definition, sleep-related suffocation or accidental death has been a major contributing factor for SIDS. This dates as far back to the 1940s when Abramson (1944) showed an extreme incidence of infant death due to accidental mechanical smothering, which was the highest mortality rate due to accidental death in infants in New York City at the time of the study. The mechanisms by which accidental suffocation may occur are described in Table 2.1. The way in which an infant is put to bed plays an important role as a risk factor for SIDS. An infant placed to sleep in the prone (faced-down) position will be at a higher risk for suffocation or smothering than when placed on the side or back (supine). Although the supine sleeping position is regarded as the safest, many infants are placed on their side. This position can also be risky at times, as an infant is more likely to roll over to the prone than supine position when placed on their side (Katwinkel *et al.* 2000). Previous studies have highlighted the risk of SIDS in infants that are placed to sleep in the prone position (Abramson 1944; Ponsonby *et al.* 1993; Dwyer & Ponsonby 1996; Schnitzer *et al.* 2012). Since prone sleeping was identified as one of the leading risk factors associated with SIDS, New Zealand first initiated the implementation of back-to-sleep campaigns during the 1980s where awareness was raised about the risk of placing infants on their stomach and recommendations were made to place infants on their backs or side to sleep. Such campaigns have subsequently also shown great success in countries such as the USA and UK (Katwinkel *et al.* 2000; Gilbert *et al.* 2005). During the 1980s and 1990s, significant decreases of 50-90% in SIDS incidences were observed, where much of this decline was attributed to the use of the supine sleeping position (Moon *et al.* 2007).

Although prone sleeping is known to increase the risk of asphyxia in infants, it may also increase their susceptibility to infection (Blackwell *et al.* 1999). Respiratory symptoms are often observed in SIDS cases prior to death, suggesting some type of infection. Toxigenic bacteria have been isolated from the gastrointestinal tract in SIDS cases and infants who slept in the prone position have been

associated with increased colonization of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) isolated from the nasopharyngeal ducts (Goldwater 2004).

Table 2.1. Mechanisms for accidental suffocation (adapted from Abramson 1944 and Shapiro-Mendoza *et al.* 2014).

Mechanism	Definition
Overlay	When sharing a bed or sleep surface with an infant and the person rolls over or against the infant, blocking the airway or compressing the neck/chest region of the infant causing the infant to stop breathing
Soft bedding	Soft blankets, duvets, pillows, mattresses, stuffed toys, etc. on the bed that may block the airway of the infant causing the infant to stop breathing
Wedging or entrapment	When an infant is entrapped between two objects, e.g. mattress and wall, or even against the mother's breasts or upper limb that may block the airway of the infant causing the infant to stop breathing
Other	Any other sleep-related factor that may block the airway of the infant causing the infant to stop breathing.

The role of infection in the pathogenesis of SIDS remains a crucial topic of interest in the field as a variety of pathogens are constantly found in these cases. Histopathological features, specifically in the upper respiratory tract, often show mild inflammatory changes in SUDI cases and may suggest an active infection at the time of or immediately prior to death (Weber *et al.* 2010a). Several studies have investigated the association of viral and bacterial infections and toxins to SIDS. Burger *et al.* (2014) explored the presence of CMV, HAdV and RSV in the lungs of SUDI cases in South Africa. Parainfluenza virus type 2 was detected in bone marrow and liver tissue in a case of sudden infant death in Japan (Kashiwagi *et al.* 2004). *S. aureus* and *S. enterotoxins* (Harrison *et al.* 1999; Highet *et al.* 2009), *Streptococcus agalactiae* (Highet *et al.* 2014), *E. coli* (Pearce *et al.* 2010), and *Bordetella pertussis* (Heininger *et al.* 2004) have all previously been detected in SIDS cases.

2.4 Viral infection in SUDI

The role of viral infections in SIDS is not yet fully understood. Viruses are thought to cause either straightforward infection which elicits a cytokine response, or can work synergistically with bacteria by attracting pathogenic bacteria to colonize the region and cause infection when toxins are produced. The presence of respiratory pathogens have most commonly been associated with either an infection, or just being present in infants during the postneonatal period, and not necessarily resulting in infection. Acute infections related to respiratory viruses are the major cause of death globally in children younger than 5 years. Mild respiratory infections are also common in about 80% of SIDS cases (Cutz and Jackson 2001), and present as flu-like symptoms prior to demise. Although frequently

isolated, no specific respiratory or any other virus has been found to have a self-sufficient lethal effect in SIDS (Bajanowski *et al.* 2003; Highet 2008).

Cardiovascular viruses in SUDI

Evidence on the role of viruses in heart disease has been well documented between the 18th and 19th century. Cardiopathies associated with viral infection are frequently related to enteroviruses (EV), especially in the case of myocarditis. Such cardiopathies may be caused by direct infection with the virus or by maternal transmission during pregnancy. Coxsackieviruses also have a known role in the etiology of cardiovascular infection (Kawana 1985). Myocarditis affects the heart muscle as a result of inflammation, either focal or diffuse, and with or without myocytolysis, which is normally triggered by an infectious agent – although it may be accompanied by autoimmune disease, hypersensitive reactions and toxins. It has been associated with the parasite, *Trypanosoma cruzi*, but in children it is more commonly associated with viruses. EV, specifically CVB, is the most common cause of pediatric myocarditis. With the use of molecular-based techniques such as polymerase chain reaction (PCR), other viruses including HAdV, B19, CMV, HHV6 and EBV have now also been identified and implicated in myocarditis (Uhl 2008; Canter & Simpson 2014) (Figure 2.3).

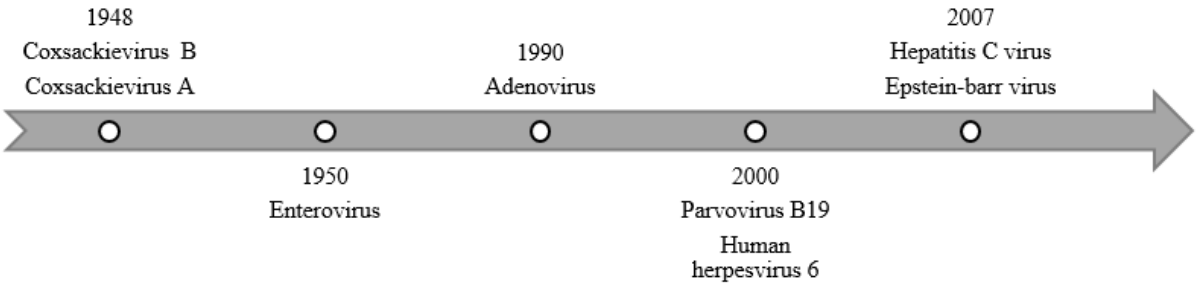


Figure 2.3. Viruses implicated in myocarditis (adapted from Shauer *et al.* 2013).

Myocarditis has been recognized as a cause of sudden and unexpected death in both children and adults. It is often characterized as a subclinical disease; however it may lead to fatal heart failure and even sudden cardiac death. Acute myocarditis is one of the challenging diseases to diagnose, as the clinical presentation is often variable and non-specific. Presentation of the disease varies between asymptomatic and mild to severe illness and acute or cardiac arrest, and symptoms, if clinically identified, may include mild fever, and respiratory- or gastrointestinal-associated infection (Shauer *et al.* 2013). Diagnosis of the disease remains controversial due to the inconsistency in the methods of detection. Diagnosis of myocarditis relies primarily on histological (histopathological or immunohistochemical) analysis of the myocardial tissue (usually an endomyocardial biopsy), although other diagnostic methods, such as PCR and in-situ hybridization, are more frequently being used for viral detection (Mahfoud *et al.* 2011). Histologic examination, the cornerstone for diagnosis

of acute myocarditis, generally follows the Dallas criteria, i.e. the presence of inflammatory infiltrates with or without myocytic necrosis. Discrepancies exist with regards to tissue sampling and the way in which medical professionals interpret histologic results, questioning its sensitivity. Controversy regarding the reliability of the Dallas criteria for diagnosis of myocarditis has been discussed previously, which highlighted the fact that other methods should be included in diagnosis to affirm the COD as myocarditis (Baughman 2006). Variation in the clinical presentation and histological interpretation of myocarditis could explain why the exact frequency of myocarditis as a cause of paediatric sudden death is still uncertain and the natural history of acute myocarditis remain controversial (Dennert *et al.* 2008; Weber *et al.* 2008b).

An association of sudden infant death and acute myocarditis has been shown in the literature. The number of reports of heart disease is significantly increasing in sudden and unexpected death in the infant population (Dancea *et al.* 2002), especially viral myocarditis (Weber *et al.* 2008a). Due to the sporadic occurrence of fatal myocarditis in SUDI, the true incidence remains unknown. One of the largest descriptive studies in the USA in children under 19 years old showed a significant incidence of acute myocarditis especially among infants (Figure 2.4). A prospective study in Germany investigating the presence of myocarditis-associated viruses (CVB, HAdV, EBV, B19, HHV6) in SIDS cases showed a high incidence of 43.5% for one of the viruses (Dettmeyer *et al.* 2004); compared to 16.8% shown in a previous study where myocardial involvement detected by histology was observed (Rambaud *et al.* 1992).

EV belong to the *Picornaviridae* family and include polio- and non-polioviruses (CVB, echoviruses and EV), which are some of the largest ribonucleic acid (RNA) viruses in humans and animals. These single-stranded (ss) RNA viruses are responsible for high mortality in newborns, and are known to cause subclinical infection. They have been associated with sepsis, meningoencephalitis, hepatitis and myocarditis (Inwald *et al.* 2004; de Crom *et al.* 2016). The spread of non-polio EV occurs via transplacental and feco-oral routes and respiratory aerosols (Hawkes & Vaudry 2005). EV infection is more common during the first two weeks of life, with a minimal chance of full recovery in newborns, and in the majority of cases, maternal infection was present prior to or shortly after birth (Freund *et al.* 2010). Jenista *et al.* (1984) observed an increased frequency of neonatal EV infection during the late summer/early autumn (June to October in USA). Most infants in their study presented no symptoms of infection. They suggested that there may be an association between breast-feeding and protection from infection.

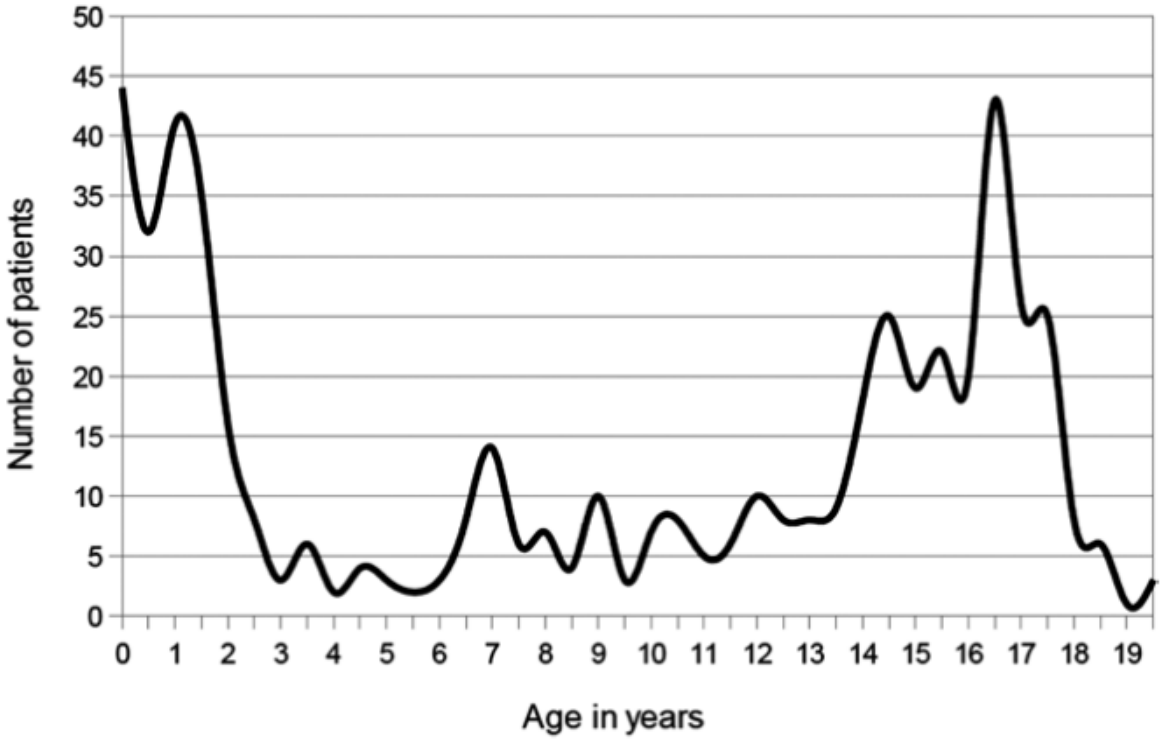


Figure 2.4. Incidence of acute myocarditis in a pediatric population in the USA - 2006 to 2011 (Ghelani *et al.* 2012).

B19 belongs to the *Parvoviridae* family and is spread via respiratory aerosols, blood, household contacts and nosocomially. Infection may be asymptomatic, mild or severe; in children, it is associated with fifth disease (or erythema infectiosum) marked by a rash on the cheeks. B19 may display clinical features relating to flu-like symptoms and later on during viremia may present with antiviral antibodies (immunoglobulin [Ig] M and IgG). B19 infection is ubiquitous in the human population and pregnant women are known to have increased susceptibility to B19 infection. In pregnant women infected with B19, the virus may be transmitted to the fetus via the placenta and infect the liver of the fetus. It is also involved in the production of erythrocytes during early stages in development and as such may cause acute anemia or myocarditis (Young & Brown 2004; Tschöpe *et al.* 2005). B19 viral deoxyribonucleic acid (DNA) found in heart tissue have been associated with viral myocarditis and inflammation in the tissue was also evident (Pankuweit *et al.* 2003). Although EV and HAdV have been commonly associated with myocarditis in the past, B19 is emerging in the field of viral myocarditis (Shauer *et al.* 2013). B19 infection, especially in the myocardium of infants, may cause fatal acute myocarditis. Co-infection of B19 with other cardiovascular viruses is common and co-infection with HHV6 has been reported to cause fatal myocarditis. Transactivation of HIV and CMV by HHV6 has been observed previously (Alfaro *et al.* 2016).

HAdV is a well-known pathogen responsible for respiratory infections in the pediatric population. This double-stranded (ds) DNA virus may cause sporadic infection throughout the year, and HAdV

epidemics have been reported. Clinical symptoms of HAdV infection may include wheezing, fever and hypoxia (Calvo *et al.* 2015). Previous studies have reported incidence rates of HAdV infection in children of up to 18% (Carballal *et al.* 2002; Jin *et al.* 2013; Ferone *et al.* 2014). HAdV infection is often associated with pneumonia, especially in patients with immunosuppression (Chen *et al.* 2013) and viral myocarditis (Bowles *et al.* 2003).

HHV belonging to the *Herpesviridae* family, are known for their ability to remain latent in the body, as well as to be reactivated in immunocompromised individuals (Deback *et al.* 2008). HHV, CMV, HHV6 and EBV have been associated with myocarditis and sudden death (Dettmeyer *et al.* 2008; Mahfoud *et al.* 2011), moreover human herpes simplex virus (HSV) and EBV are clinically known to be cardiopathogenic when present in heart tissue samples (Dettmeyer *et al.* 2009). EBV, HHV6 and CMV have been studied in a group of SIDS cases and compared with a control group of natural deaths. Significantly higher prevalence of EBV and HHV6 were detected in SIDS cases than in the controls, suggesting a possible association of these viruses with SIDS. The prevalence rates of CMV in the SIDS and control cases were not very different in this study (Álvarez-Lafuente *et al.* 2008), however it has been reported to cause severe infection in infants.

EBV, also known as HHV4, is a dsDNA virus that is ubiquitous in the human population and is known to cause asymptomatic infection. Primary infection is more common in children and transmission is via the oral route (Linde & Falk 2007; Griffiths 2009). Condon *et al.* (2014) evaluated the prevalence of EBV infection in children under 20 years old in USA. They observed a seroprevalence of 31% in children under 5 years old in their population.

CMV, also known as HHV5, is an opportunistic virus that frequently causes asymptomatic infections, but may present with non-specific respiratory-related symptoms and lead to severe illness, such as interstitial pneumonia. CMV infection is not uncommon during the first year of life, and may be associated with poor living conditions. CMV infection may result in significant fatality in immunosuppressed individuals, especially vulnerable infants, such as those born prematurely or with other pre-existing infection (i.e. congenital). Thus, infants in the perinatal and neonatal period are at a higher risk of disease progression upon initial infection as maternal antibodies against CMV have a protective role against *in utero* transmission and the infant immune systems are still developing/immature. The virus can be transferred to infants by infected mothers during delivery (via vaginal secretion), breastfeeding or via aspiration of maternal blood (Gandhi & Khanna 2004). A prospective study by Yagmur *et al.* (2016) investigated the prevalence of CMV infections in SUDI cases over a two-year period. They found that CMV-DNA was frequent in salivary glands and other tissue using molecular-based and histological techniques. They also found other viruses, such as EV

and HAdV in some samples. Histopathological findings showed features of CMV pneumonia and sialadenitis.

HHV6 is a dsDNA virus which is present in a significant proportion of the population. Transmission of HHV6 may occur perinatally, even if exposure to the virus occurred earlier in life and reached latency; and via the oral route. The incidence of HHV6 ranges from 10-66% in infants. Patients with an immune vulnerability, such as HIV, are more likely to experience reactivation. Common symptoms of infection in infants are marked by fever, respiratory and gastrointestinal symptoms and seizures (Kasolo *et al.* 1997; Leach 2016).

CVB3 (Jin *et al.* 1990; Dettmeyer *et al.* 2002), HAdV, B19, EBV and HHV are frequently confirmed in SIDS cases by molecular methods, such as PCR, in situ hybridization and serology (Towbin *et al.* 1993; Martin *et al.* 1994).

2.5 Viral and bacterial screening in SIDS

A significant proportion of SUDI is attributed to infection. Of the remaining cases, more than 60% remains unexplained (SIDS) following PM investigation, highlighting the urgency of additional, more sensitive diagnostic testing especially when addressing the infectious contribution in SIDS (Weber *et al.* 2008a). Conventionally, virus culture was used for virus isolation, but present difficulty and has been shown to provide low sensitivity and specificity for viral detection (Dennert *et al.* 2008). The low success rate of virus isolation associated with PM samples may be related to the quantity of virus in the sample, the demanding conditions of viral culture, the integrity and/or viability of viral RNA (or DNA) (Bajanowski *et al.* 2003). Alternative methods of screening for infection is emphasized in SUDI cases, especially molecular-based methods. Infection in SUDI cases has previously been identified by use of histology in conjunction with microbiology (Arnestad *et al.* 2002; Weber *et al.* 2008a). Since discrepancies, such as contamination, may occur during the autopsy, it is imperative to perform histopathological analysis as microbiology or virological results can be misinterpreted. However, risk of contamination during PM sampling can be reduced by the use of good aseptic practices. Currently, SUDI investigation protocols recommend that routine virological and bacteriological (sometimes toxicology and genetic screening) testing be included in PM investigations. Although no standardized guidelines exist that detail the major SUDI-related viruses or screening methods to be used, recommendation to improve detection of common viruses encountered in SUDI cases have been discussed. Molecular techniques allow for more targeted screening that is more specific and sensitive; hence strengthening the precision of diagnosis associated with infection (Freymuth *et al.* 2006; Weber & Sebire 2010; Weber *et al.* 2010a).

2.6 PM investigation in SUDI cases

The role of PM investigation is imperative to understand why the rates of SIDS still remain high in developing countries and to try to contribute towards reducing the mortality rates. This can be achieved by implementing strategies or lifestyle changes for risk-related infants or households. One of the biggest challenges currently is the lack of a systematic protocol for investigation of SUDI cases; no nationally or internationally accepted guidelines exist. Controversies and inconsistencies in investigation approaches have been discussed previously which have led to differential diagnosis in SUDI cases (du Toit-Prinsloo *et al.* 2013; Brooks & Gill 2015).

Bajanowski and colleagues (2007) discussed some recommendations that can contribute towards the implementation of a standardized diagnostic protocol for SUDI investigations (Table 2.2). DSI, as stated in the 1989 definition of SIDS, is important. Generally, this is done by police officials and requires specific training for SUDI investigations. In reality, forensic specialists, trained for such investigations, rarely assist with DSI. Radiology and histological investigation should also be included - especially assessment of the brain, lungs and heart, although other main organs should also be assessed to identify possible signs of injury or morphological changes (e.g. skeletal abnormalities, old bruises, lethal lesions, inflammation, hemorrhage, edema, etc.) that may aid in establishing a COD. Additional tests should include metabolic or genetic testing, microbiology and virology testing and toxicology (Bajanowski *et al.* 2007).

Table 2.2. Recommended investigation approach for SUDI cases (adapted from Koehler 2010).

Assessment	Description
DSI	Secure and photograph death scene, conduct witness interview/s, re-enact scene, compile death scene report
Pre-autopsy investigation	Review medical history (infant and mother if possible), DSI, case information, first responder and police reports
Autopsy: external examination	Photograph infant, assess appearance of infant (including signs of trauma/injury)
Autopsy: internal examination	Removal and assessment of organs Collect specimens, such as blood, tissue, etc. for histology, microbiology, toxicology, virology etc.
Additional (may not be available in all cases)	Collect epidemiological information

At the Tygerberg Medico-legal Mortuary (Cape Town) all cases are investigated according to the standard facility protocol, which may include a DSI, autopsy and selected laboratory investigations. Shell vial culture (SVC) screening, specifically for HAdV, CMV, Influenza virus A and B, human parainfluenza virus 1, 2 and 3 and RSV from lung and liver tissue, bacterial isolation from the lungs

and heart (pus swabs), HIV analysis on blood and chemical pathology (urea and creatinine [U&C]) testing on serum are routinely performed on all SUDI cases admitted to the institution (la Grange *et al.* 2014).

2.7 Where are we now with SIDS in Southern Africa

SIDS research used to be underinvestigated in Africa (Ndu 2016), however more research is emerging in South Africa, particularly at academic institutions. SIDS mortality in mixed-race infants in Cape Town is considered among the highest, with an incidence of 3.41/1 000 live births (Kibel *et al.* 2005; Kinney & Thach 2009), seven times higher than the national average in USA (Goldstein *et al.* 2016), and 1.06/1 000 live births in white infants in Cape Town (Kibel *et al.* 2005). This may be representative of the population demographics within the Western Cape.

In South Africa, all deaths due to unnatural causes (sudden and unexpected or unexplained included) are investigated according to the Inquests Act (Act 58 of 1959), where the body is admitted to a medico-legal mortuary for investigation. The investigation procedure at various medico-legal mortuaries in South Africa is similar to that outlined in section 2.6. This includes reviewing of the clinical history of the case admitted, DSI, macroscopic assessment, radiology, histology of organ blocks, toxicology, microbiology, virology and genetic tests. The macroscopic assessment is performed either by complete evisceration of the body with dissection of organ blocks for macro- and microscopic analysis, partial assessment with or without evisceration due to COD becoming apparent, or no autopsy due to history displaying known underlying condition.

The prevalence of SUDI in South Africa was investigated at two medico-legal mortuaries, Pretoria and Tygerberg (Cape Town) between 2000 and 2004. Over the four-year period 813 infant cases were admitted to the two centres, of which 99 (Pretoria) and 413 (Tygerberg) were identified as SUDI. Similar trends in these deaths were observed in the two study populations, such as male predominance, peak age of 2 to 4 months, high frequency in black and coloured infants and a peak in colder months (June to August, with another peak in December). Differences in additional laboratory analyses (such as histology, bacteriology, virology and toxicology) requested at autopsy were identified between the two centres. No DSI was performed for any of the cases. A total of 171 cases (161 Tygerberg, 10 Pretoria) remained unexplained (SIDS) following PM investigation, 34 were unascertained, 158 died due to infection (pneumonia) and 129 due to other causes (du Toit-Prinsloo *et al.* 2011). A study by Dempers *et al.* (2016) in the Eastern Metropole of Cape Town reported similar findings.

The burden of cardiovascular infection is perhaps underdetermined in the field of sudden infant death due to a paucity of research to investigate the exact prevalence of the condition. In terms of cardiovascular disease in children (described as 'ill-defined cardiovascular disease' in children under 5 years old) (Nannan *et al.* 2012), determining the true incidence in South Africa seems to be challenging, perhaps due to the difficulty in diagnosis of such disease (Figure 2.5).

As part of the current study, a retrospective analysis was carried out to investigate the mortality rates attributed to cardiovascular diseases (either myocarditis or cardiovascular-related causes such as congenital cardiac conditions) at Tygerberg Medico-legal Mortuary between 2006 and 2015 (Figure 2.6). Only the diagnosis of the final COD was used for this analysis. The incidence seemed to be much lower than what the literature suggests. The reason for this is not currently known, however, this introduced the need for more research in this field especially in South Africa - where such controversy could be addressed.

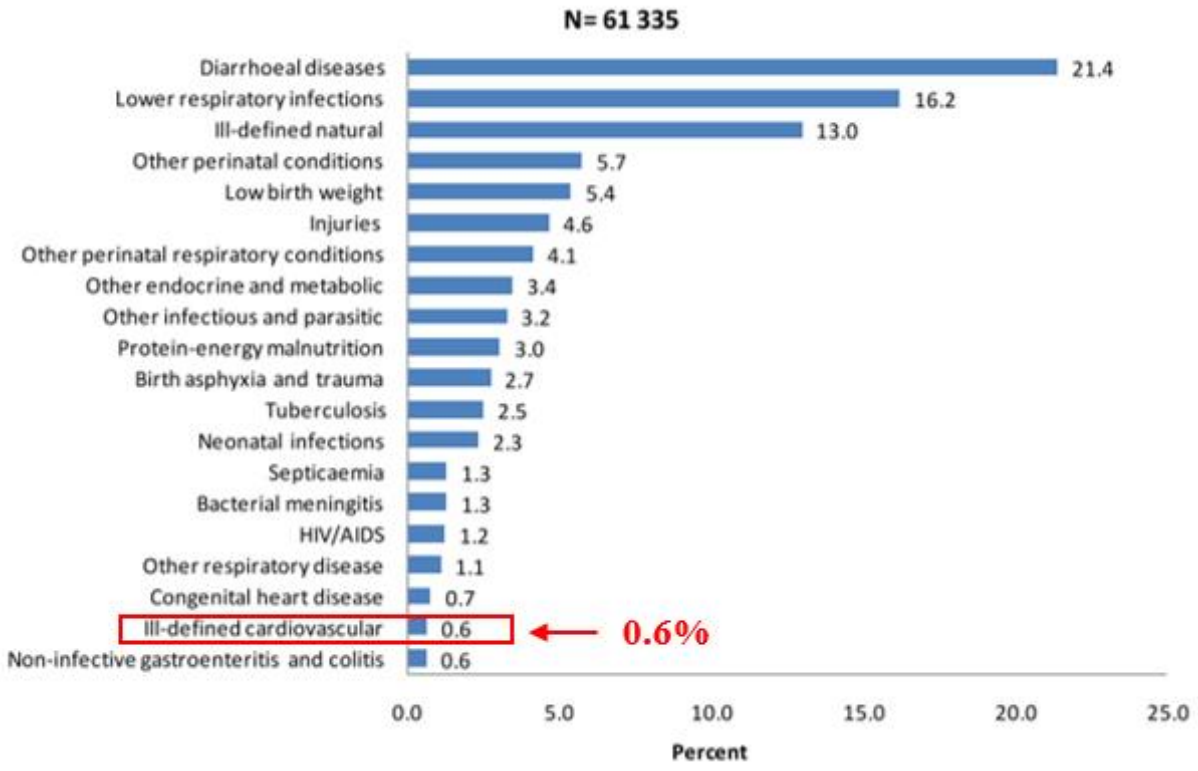


Figure 2.5. Ranking of mortality rates for children under 5 years of age in 2007 (Nannan *et al.* 2012).

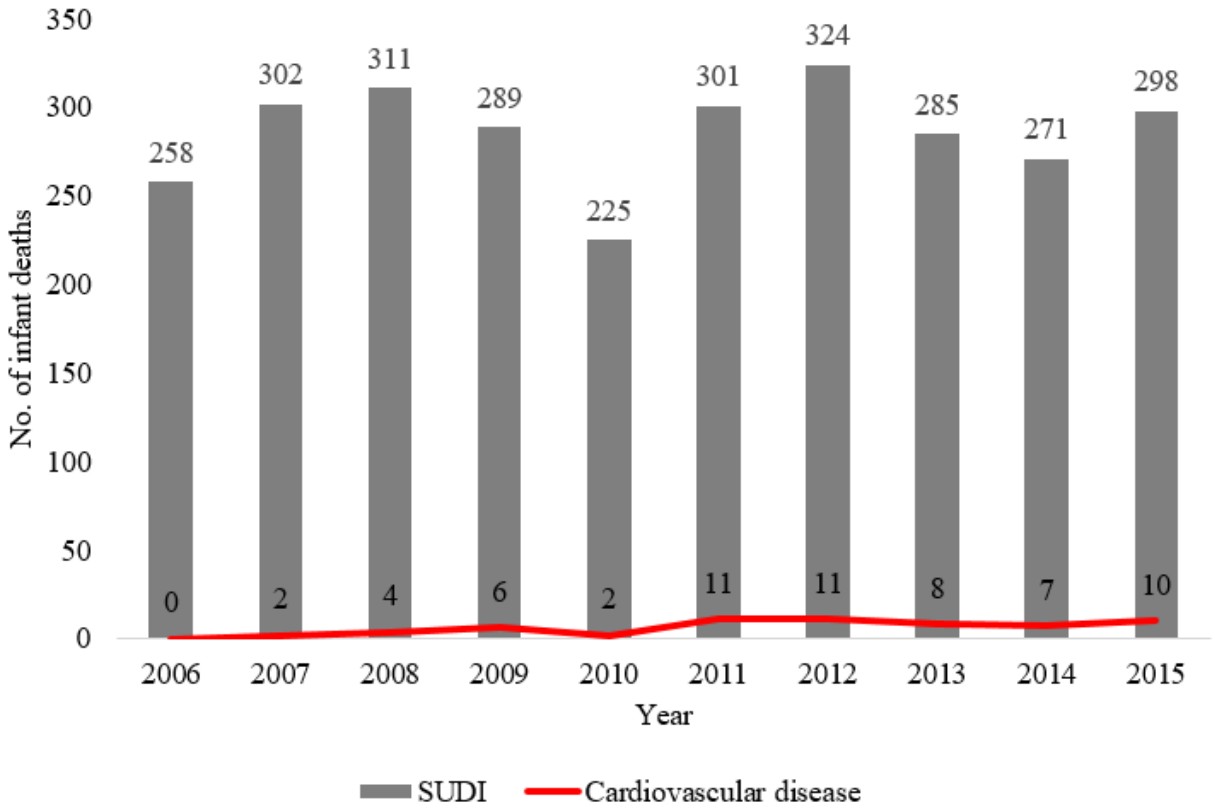


Figure 2.6. Number of infant deaths over 10 years at Tygerberg medico-legal mortuary.

2.8 Research aims and objectives

- **Aim I:** To develop PCR-based screening assays for specific viruses often associated with myocarditis
  - Objective I: To generate plasmid DNA as positive controls from known virus samples by cloning a fragment of the viral DNA into a suitable vector
  - Objective II: To optimize PCR assays for virus detection with the positive controls generated
- **Aim II:** To profile EV and B19 in SUDI cases using molecular techniques
  - Objective III: To screen for EV and B19 viruses in SUDI heart swab samples using the PCR assays developed and optimised as part of Aim I
  - To analyse heart tissue samples to determine whether infection could be implicated in the final COD

CHAPTER THREE

METHODOLOGY

3.1 Study design

SUDI cases admitted to the Tygerberg Medico-legal Mortuary, Eastern Metropole of the Western Cape, were prospectively investigated from June 2016 to June 2017. Heart swab samples were collected for viral screening and heart tissue samples were collected for histological examination for each case, which supplemented the routine diagnostic tests conducted by the Division of Forensic Medicine. Samples were collected from infants who died suddenly and/or unexpectedly, aged between 7 days and 1 year old. The first week of life is generally regarded as the neonatal period where the immune system of the infant may still be adapting to the extrauterine life and death during this time may often be as a result of birth defects or respiratory distress). All samples were collected at the time of autopsy under the auspices of the Inquests Act (Act 58 of 1959).

3.2 Ethical consideration for study

The protocol for this project was reviewed and approved by the Health Research Ethics Committee (HREC) at Stellenbosch University, Tygerberg Campus (Ref. number N12/02/007) and a waiver of consent was granted. All SUDI cases are required by law to be investigated in order to find a COD and as long as our study objectives did not deviate from the objectives set in the Inquests Act (Act 58 or 1959), i.e. finding a COD, we were not required to obtain informed consent from the parents when admitting their infant to the Tygerberg Medico-legal Mortuary.

3.3 Sample acquisition and storage of known virus samples

Quality control for molecular diagnostics (QCMD) samples, EVRNA16C1-03 and B19DNA16C1-02, were collected from the National Health Laboratory Service (NHLS) Virology laboratory (Tygerberg campus) for EV and B19 respectively. EV and B19 QCMD samples were stored at -20°C upon collection for later construction of positive controls for the viral screening assays.

3.4 Nucleic acid extraction and quantitation of EV and B19

Total nucleic acid (TNA) extractions were done for EV and B19 QCMD samples using the QIAmp® cador® Pathogen Mini Kit (QIAGEN®, Cape Town) following the manufacturer's instructions. Nucleic acid extractions were done at room temperature in a laminar flow cabinet.

For lysis of cellular debris, 20 µl of proteinase K and 200 µl virus-containing samples were added to 350 µl Buffer ACB, pulse vortexed and incubated at room temperature for 15 minutes. Lysates were applied to QIAmp Mini columns provided in the kit and centrifuged at 8 000 revolutions per minute (rpm) for 1 minute. The samples were washed twice with 600 µl Buffer AW1 and centrifuged at 8 000 rpm for 1 minute. A final centrifugation step was included to remove traces of ethanol from the wash buffer. Elution of the DNA was done in a nuclease-free 1.5 ml microcentrifuge tube with 40 µl Buffer AVE and the DNA was stored at -80°C to ensure increased stability.

The NanoDrop ND-1000 spectrophotometer was used for the determination of the quantity and purity of the TNA extracted. Briefly, the upper and lower surfaces of the microspectrophotometer sample retention system were cleaned using clean deionized water and wiping it down with paper towel. The software system was opened and the nucleic acid module was selected. The spectrophotometer was initialized by placing 1 µl clean water onto the lower optic surface, lowering the arm of the device and selecting "initialize". The upper and lower optical surfaces were cleaned with paper towel and 1 µl of AVE Buffer was placed on the lower optic surface to blank the system. Both optical surfaces were again cleaned with paper towel. Depending on whether DNA or RNA was being analysed, the option was selected on the software prior to starting the sample analysis. To quantify the sample, 1 µl of the sample (RNA/DNA) was placed onto the lower optical surface, the arm of the spectrophotometer was lowered and the selection to "measure" was made on the software. The readings were saved into a Microsoft® Excel® spreadsheet for analysis.

3.5 Reverse transcription (RT) assay

After the TNA was recovered from the EV QCMD sample, complementary DNA (cDNA) was generated using the Maxima Reverse Transcriptase kit (Thermo Scientific™, Randburg) following the manufacturer's instructions. The RT protocol followed consisted of a two-step process, and was performed using random hexamers, instead of universal primers, as the random primers can bind to any RNA species in a sample i.e. no template specificity required. The reaction was performed in a final volume of 20 µl followed by the modified instructions below:

First step:

Nuclease-free water	6.5 µl
Random Hexamers (Primer)	2 µl
dNTP mix	1 µl
RNA template	5 µl
Total volume in tube	**9.5 µl**

Second step:	
5 X RT Buffer	4 µl
RNase inhibitor	0.5 µl
Maxima RT	1 µl
Total volume	**5.5 µl**

Thermal cycling parameters:		
Denaturation, first step reagents	65°C	5 minutes
Annealing	4°C	1 minutes
Addition of second step reagents		
cDNA synthesis	25°C	10 minutes
	50°C	30 minutes
Enzyme inactivation	85°C	5 minutes
Holding	4°C	∞

The RT products were stored at -20°C.

3.6 Pre-cloning PCR assay

Primers were selected from literature (Table 3.1) to establish a PCR protocol for this study. The primers were analysed using the online database BLAST (https://blast.ncbi.nlm.nih.gov/) to confirm the binding sites to our viruses of interest. Each primer sequence was entered into the query box on the webpage and the "go" button was selected. The output generated a list of various binding sites corresponding to the virus of interest.

Table 3.1. Primer sequences used for the amplification of EV and B19.

Virus	Primer sequence (5'- 3')	Reference	Expected product size (bp)
EV (a/b) *	a - TCC GGC CCC TGA ATG b - ACA CGG ACA CCC AAA GT	Martin *et al*. 1994	151*
EV (a/c) **	c - CAC CGG ATG GCC AAT CCA		196**
PVB19 *	AGC ATG TGG AGT GAG GGG GC AAA GCA TCA GGA GCT ATA CTT CC	Bültmann *et al*. 2003	290*
PVB19 **	GCT AAC TCT GTA ACT TGT AC AAA TAT CTC GTC GGG GTT GAG		173**

* First reaction, pre-nested primers

** Second reaction, nested/hemi-nested primers

The cDNA/DNA for EV and B19 QCMD samples were detected by individual pre-nested PCR assays using a chemically modified recombinant *Taq* DNA polymerase, Maxima Hot Start (HS) *Taq* DNA Polymerase (Thermo Scientific™, Randburg). Pre-nested PCR reactions were performed in a final volume of 25 µl following the instructions below:

Maxima HS Buffer	2.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
$MgCl_2$	2.5 µl
dNTP mix	0.5 µl
Maxima HS Taq	0.1 µl
cDNA template	5 µl
Nuclease-free water	9.4 µl
Total volume	**25 µl**

The reactions were placed into the GeneAmp® PCR System 9700 following parameters below:

Initial Denaturation	95°C	4 minutes
Denaturation **Annealing** **Extension**	95°C Variable (see Table 2) 72°C	30 seconds (40 cycles)
Final extension	72°C	7 minutes (20 minutes for pre-cloning PCR)
Holding	4°C	∞

Table 3.2. Annealing temperatures of the primers used for the amplification of EV and B19.

Primers	Annealing temperature (Ta)
EV*	54.7°C
EV**	57.3°C
PVB19*	51.4°C
PVB19**	47.5°C

* First reaction, pre-nested primers

** Second reaction, nested primers

3.7 Gel electrophoresis

The PCR products were separated using a 2% agarose gel electrophoresis protocol. The agarose was prepared in a microwave safe glass flask using 2 g agarose powder in 100 ml 1X SB Buffer (Appendix A). The flask was placed in the microwave for 2 minutes or until the gel was completely dissolved (clear) and left to cool for a few minutes. While the gel was left to cool, the combs were inserted onto the casting plate and the routes leading to the electrical wires were sealed with molten agarose. Once the gel was cooled, 7 µl Ez-Vision® Blue Light DNA Dye (Inqaba Biotec™, Pretoria) was added to the agarose and the gel was carefully poured into the casting plate and left to set. Once the gel was set, the combs were removed from the gel and 1X SB Buffer was poured onto the set gel until the entire gel was covered with Buffer. The samples were loaded onto the gel by mixing 5 µl of the PCR product with 1 µl 6X DNA Loading Dye Buffer Orange and Blue (Separations Simply Spectacular, Cape Town). The gel casting plate was connected to the electricity supply and run at 90V for 30 minutes. Following gel electrophoresis, the gel was visualized on the Gel Doc system.

3.8 Purification of PCR products

The PCR products were purified using the MiniElute PCR Purification Kit (QIAGEN®, Cape Town) following the manufacturer's instructions. Briefly, five volumes of Buffer PB was added to 1 volume of the PCR reaction, pulse vortexed, applied to MiniElute column provided and centrifuged at 13 000 rpm for 1 minute. The sample was washed with 750 µl Buffer PE and centrifuged at 13 000 rpm for 1 minute. An additional centrifugation step was included at maximum speed for 1 minute to remove residual ethanol from Buffer PE, and the purified PCR product was eluted into sterile 1.5 ml microcentrifuge tube with 10 µl Buffer EB. The DNA eluted was stored at -20°C for further analyses.

3.9 Molecular cloning assay

A 30 µl overnight ligation reaction was set up for the EV and B19 purified PCR products using an InsTAclone™ PCR Cloning Kit (Thermo Scientific™, Randburg) following the manufacturer's instructions. Briefly, 3 µl Vector pTZ57R/T, 6 µl Ligation Buffer, 1 µl T4 DNA Ligase, 17 ng and 50 ng respectively (Table 3.3) were added and brought to a final volume of 30 µl with nuclease-free water. The reactions were placed into the GeneAmp® PCR System 9700 for incubation as follows:

Ligation	25°C	2 hours
	4°C	2 hours (8 cycles)
Enzyme inactivation	75° C	5 minutes
Hold	4°C	∞

The ligation reactions were stored at -20°C for further processing.

Lysogeny broth (LB) and agar plates were prepared for transformation with 100 mg/µL Ampicillin (Amp) (Appendix B). Zymo research premade *Mix & Go* Competent JM109 *E. coli* cells, 50 ml aliquots, (Zymo Research Corporation) were used for the transformation procedure to enable blue/white screening. These premade chemically competent cells ensure highly efficient and simple transformation, do not require heat shock procedures for the foreign DNA to enter the cells and allow for the foreign DNA to be added directly into the cells that can be directly spread onto the agar plates. Briefly, LB/Amp agar plates were set to pre-warm at 37°C for 30 minutes, then supplemented with X-gal (Thermo Scientific™, Randburg), 100 mg/µl Amp and 100 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG), and the plates were left to dry at 37°C. *Mix & Go* cells were thawed on ice and 2.5 µl of the ligation product was added to the cells. The cells were gently mixed and incubated on ice for 5 minutes. The transformation mix was spread onto the LB agar plates and the plates were incubated at 37°C overnight.

For bacterial culture, a single colony/transformant was picked from the plates and mixed in 5 ml LB/Amp broth. The transformants were incubated at 37°C overnight shaking at 225 rpm.

The cells were harvested by centrifugation at 4 000 rpm for 5 minutes. The pelleted cells were purified using the GeneJET Plasmid Mini Prep Kit (Thermo Scientific™, Randburg) following the manufacturer's instructions. Briefly, the cells were resuspended in 250 µl Resuspension solution, thoroughly mixed and 250 µl of the Lysis solution was added to the samples. The samples were mixed until the solution was viscous and slightly clear and 350 µl of the Neutralization solution was added to the samples, and mixed again. To pellet the cellular debris, the samples were centrifuged at 13 000 rpm for 5 minutes and the clear supernatant was applied to the GeneJET spin columns provide and centrifuged at 13 000 rpm for 1 minute. The samples were washed with 500 µl of Wash solution and centrifuged at 13 000 rpm for 1 minute. An additional centrifugation step was included to remove residual wash solution, and the plasmid DNA was eluted into a sterile 1.5 ml microcentrifuge tube with 50 µl Elution buffer (pre-warmed to 70°C). The eluted plasmid DNA was stored at -20°C for further analyses.

The following amount of PCR product was used for the ligation reaction according to the ligation protocol (adapted from the Thermo Scientific™ InsTAclone™ PCR Cloning Kit):

Table 3. 3. Recommended amount of PCR product for the ligation reaction.

Length of PCR product for the ligation reaction	Optimal PCR product quantity for ligation reaction, (0.52 pmol ends)
100	17 ng
300	51 ng
500	86 ng
1000	172 ng
2000	343 ng
3000	515 ng
4000	686 ng
5000	858 ng

3.10 Sequencing of clones generated

The purified plasmids were sequenced using the BigDye® Terminator 3.1 Cycle Sequencing Kit (Thermo Scientific™, Randburg) with M13 forward and reverse primers provided in the kit. The sequencing reaction was set up for each primer (forward and reverse) per template following the modified details below:

BigDye® Terminator v3.1 sequencing buffer	1.5 µl
BigDye® Terminator v3.1 cycle sequencing RR	0.5 µl
Primer (M13 forward/reverse)	1 µl
Template (300 ng)	1 µl
Water	1 µl
Total volume	**5 µl**

The reactions were briefly mixed and centrifuged to collect the contents at the bottom of the wells, and the sequencing PCR was set up following the parameters below:

Initial denaturation	96°C	1 minute	
Denaturation	95°C	10 seconds	30 cycles
Annealing	54°C	5 seconds	
Extension	60°C	4 minutes	
Hold	4°C	∞	

Following sequencing, the clean-up was done using the BigDye XTerminator™ Purification Kit (Thermo Scientific™, Randburg). Briefly, a master mix was prepared with 49 µl SAM™ solution and 11 µl XTerminator™ solution. The master mix was thoroughly mixed and 60 µl was added to each sequencing reaction. The reactions were mixed by shaking for 30 minutes and placed in the 3130*xl* Genetic Analyzer system for capillary electrophoresis, selecting the suitable run module ("sequencing").

The raw data (sequenced reads/reads) obtained from the 3130*xl* Genetic Analyzer was imported into Geneious R10 software for sequence analysis which generated nucleic acid sequences for each plasmid. Portions of each sequence was analysed using the online database, BLAST, to determine or confirm the identity of the clone. Once the sequences were confirmed as containing the insert (i.e. virus cloned into the vector), contigs were generated with the forward and reverse reads per sample against the primer sequence used by selecting the "De Nova Assemble" command in Geneious R10 and trimming the sequence with the M13 Forward and M13 Reverse primers. The contig generated resembled that of the insert.

3.11 Linearization and in vitro transcription of EV plasmid DNA

The EV plasmid DNA was linearized with *EcoRI* in an *EcoRI* restriction enzyme (RE) digestion as outlined below:

10 X RE Buffer	4 µl
Acetylated BSA	0.4 µl
Template	1 µg
Nuclease free water	to 40 µl
EcoRI (10 U/µl)	1 µl
Total volume	**40 µl**

The reaction was incubated as outlined below:

Linearization	37°C	2 hours
Enzyme inactivation	85°C	5 minutes
Hold	4°C	∞

The linearized EV plasmid DNA was purified using the MiniElute Reaction Cleanup Kit (QIAGEN®, Cape Town) following the manufacturer's instructions. Briefly, the sample was mixed with 300 µl Buffer ERC, spun down and applied to the MiniElute columns provided. The samples were

centrifuged 13 000 rpm for 1 minute then washed with 750 µl Buffer PE and centrifuged at 13 000 rpm for 1 minute. An additional centrifugation step was included at maximum speed for 1 minute to remove traces of Buffer PE. The linearized plasmid was eluted into a sterile 1.5 ml microcentrifuge tube with 10 µl Buffer EB and the purified product was stored at -20°C for further analyses.

High yield *in vitro* transcription was performed on the linearized EV plasmid DNA using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific™, Randburg) following the manufacturer's instructions. Modifications for the protocol are outlined below:

DEPC-treated water	up to 40 µl
5 X TranscriptAid Reaction Buffer	8 µl
ATP/CTP/GTP/UTP mix	8 µl
Template	1 µg
TranscriptAid Enzyme mix	2 µl
Total volume	**40 µl**

The reaction tubes were thoroughly mixed, briefly spun down and incubated at 37°C for 2 hours. Following incubation, a DNase treatment was done with 2 U DNase I, RNase-free and the reactions were incubated at 37°C for 15 minutes.

The RNA generated was purified using the Pure Link® RNA Mini Kit (Life Technologies™, Johannesburg) following the manufacturer's instructions. Briefly, one volume of Lysis Buffer (prepared with 2-mercaptoethanol) was mixed with one volume of RNA, applied to the spin column provided and centrifuged at 12 000 rpm for 15 seconds. The sample was then washed with 500 µl Wash Buffer II and centrifuged at 12 000 rpm for 1 minute. An additional centrifugation step was included at 12 000 rpm 1 minute. The purified RNA was eluted into a sterile 1.5 ml microcentrifuge tube with 50 µl RNase-Free Water and stored at -80°C for further analyses.

Determination of viral DNA/RNA copy number

The concentrations of the EV plasmid RNA and B19 plasmid DNA were quantified using the Qubit® RNA Assay Kit (Life Technologies™, Johannesburg) and Qubit® dsDNA HS Assay Kit (Life Technologies™, Johannesburg), respectively, following the manufacturer's instructions. Briefly, the reactions were set up with two standards for each sample assay. The Qubit® working solution was prepared in a 1:200 dilution with Qubit® RNA/DNA (specific for the kit) in Qubit® RNA/DNA Buffer. The modifications for the assays are outlined below:

	Standard 1	Standard 2	RNA/DNA sample
Working solution	190 µl	190 µl	198 µl
Standard	10 µl	10 µl	
Plasmid RNA/DNA			2 µl
Total volume	**200 µl**	**200 µl**	**200 µl**

Once the concentration for each plasmid was obtained, the copy number was calculated using the online tool, ENDMEMO (http://endmemo.com/bio/dnacopynum.php). The data for each virus such as sequence length (number of base pairs [bp]), weight (ng/µl) and genome structure (ssDNA, dsDNA, ssRNA, dsRNA) was entered into the required query field and the output generated the copy number per µl DNA/RNA.

3.12 Optimization of PCR assays for viral detection using the known virus clones generated

10-Fold serial dilutions were prepared from the highest copy number down to the lowest (i.e. $1x10^1$) for each plasmid in order to determine the sensitivity of the assay. Each dilution was subjected to PCR amplification in order to determine the limit of detection for each assay. The Maxima HS *Taq* DNA Polymerase (Thermo Scientific™, Randburg) kit was used with minor modifications.

The pre-nested master mix for 9 reactions per assay:

Pre-nested	
Maxima HS *Taq* Buffer	23.6 µl
Forward primer	23.6 µl
Reverse primer	23.6 µl
$MgCl_2$	23.6 µl
dNTP mix	4.7 µl
Maxima HS *Taq* polymerase	1.2 µl
Nuclease-free water	88.6 µl

20 µl of the master mix was mixed with 5 µl of each dilution (as the template) and subjected to the following cycling parameters:

Initial Denaturation	95°C	4 minutes
Denaturation **Annealing** **Extension**	95°C Variable (see Table 3.2.) 72°C	30 seconds (40 cycles)
Final extension	72°C	7 minutes (20 minutes for pre-cloning PCR)
Holding	4°C	∞

The nested/hemi-nested master mix for 9 reactions per assay:

Nested/Hemi-nested	
Maxima HS *Taq* Buffer	47.3 µl
Forward primer	47.3 µl
Reverse primer	47.3 µl
$MgCl_2$	47.3 µl
dNTP mix	9.5 µl
Maxima HS *Taq* polymerase	4.4 µl
Nuclease-free water	252.8 µl

48 µl of the master mix was added with 2 µl of pre-nested PCR product and subjected to the same cycling conditions outlined above. The nested PCR products were visualized by gel electrophoresis as described in section 3.7.

3.13 Sample collection and processing of study samples

Heart swab samples were collected from each case during the PM investigation. Upon exposure of the heart, swab samples were collected from the right ventricle of the heart. This was done by singeing the heart surface with a spatula heated in a direct flame to create an aseptic environment, making an incision on the singed surface using a sterile scalpel blade and collecting the swab by rotating on the inside surfaces of the incision. The swab samples were stored in a tube containing universal transport media (UTM) upon collection. Heart tissue samples were collected from the left ventricle using a sterile technique, as the routine swab was collected from the right ventricle. The tissue was collected by making a transverse incision approximately 5 mm into the heart muscle using an ethanol sterilized scalpel blade and forceps. A second incision was made approximately 2 mm away from the first incision at the same depth, and the tissue between the two incisions was collected and placed into a

Tissue-Tek Uni-Cassette. The cassette was then stored in a sealed container with 10% formalin. The swab and tissue samples collected were transported to the Medical Virology and Forensic Pathology laboratories, Tygerberg campus, respectively.

3.14 Nucleic acid extraction and quantitation

TNA was extracted from 168 heart swab samples collected and the purity and concentration of the TNA were determined following the methodology described in section 3.4. Briefly, a spin column method was incorporated using the QIAmp® cador® Pathogen Mini Kit (QIAGEN®, Cape Town). The method utilizes a 15-minute lysis step, application of the lysate to the spin column for the nucleic acids to bind, a wash step to remove impurities and an elution step.

3.15 Pre-nested, nested and hemi-nested PCR assay and visualization

cDNA was generated from 5 µl TNA for all samples following the methodology described in section 3.5. The cDNA generated and the TNA for each sample were subjected to EV and B19 viral screening using the Maxima Hot Start *Taq* DNA Polymerase (Thermo Scientific™, Randburg) kit. The EV plasmid RNA and B19 DNA were used as positive controls for each assay. Each positive control was used at its most sensitive dilution (i.e. $1x10^1$) and a negative control containing only water as the template was also included. The pre-nested assays were first performed for all samples, followed by the nested and hemi-nested assays as outlined below:

Pre-nested	
Maxima HS *Taq* Buffer	2.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
$MgCl_2$	2.5 µl
dNTP mix	0.5 µl
Maxima HS *Taq* polymerase	0.125 µl
Nuclease-free water	9.4 µl
Template	5 µl
Total volume	**25 µl**

The values above are per sample reaction.

Nested/hemi-nested	
Maxima HS *Taq* Buffer	5 µl
Forward primer	5 µl
Reverse primer	5 µl
$MgCl_2$	5 µl
dNTP mix	1 µl
Maxima HS *Taq* polymerase	0.3 µl
Nuclease-free water	26.7 µl
Pre-nested PCR product	2 µl
Total volume	**50 µl**

The cycling parameters for the assays:

Initial Denaturation	95°C	4 minutes
Denaturation	95°C	
Annealing	Variable (see Table 3.2.)	30 seconds (40 cycles)
Extension	72°C	
Final extension	72°C	7 minutes (20 minutes for pre-cloning PCR)
Holding	4°C	∞

The nested PCR products were visualized by gel electrophoresis as described in section 3.7.

3.16 Histological analysis

3.16.1. Fixation and tissue processing

Chemical fixation of the heart tissue was done with 10% formalin as outlined in section 3.13. Briefly, the tissue was placed into individual Tissue-Tek Uni-Cassettes and immersed in 10% formalin for 2-24 hours to allow for hardening of and preservation of the tissue. Following chemical fixation, the cassettes were removed from the formalin and the tissue was processed using the Tissue-Tek® VIP™ 5 Vacuum Infiltration Processor (Sakura® Finetek, Europe). This instrument functions to fixate, dehydrate, clear and paraffin infiltrate various types of tissue as automated system utilizing a vacuum and pressure source. Briefly, the Tissue-Tek Uni-Cassette containing the hardened tissue was placed into the resort of the instrument. The tissue was immersed in a series of alcohol solutions during the process of dehydration to remove most of the water from the tissue prior to wax infiltration. The parameters for this process are outlined below.

Alcohol concentration	Time	
70%	1 hour	
80%	1 hour	
90%	1 hour	
100%	1 hour	3X

Following the procession of dehydration, the tissue was immersed into xylene, a clearing agent that is miscible with alcohol and paraffin wax, for two cycles of 1 hour each. The clearing process ensures that the alcohol is completely displaced by the xylene and that large amounts of fat in the tissue is removed for ease of the wax to infiltrate the tissue. The final step in the process is for molten paraffin wax to infiltrate the tissue. During this process, the tissue is infused with hot wax in a series of four cycles consisting of 3 minutes each to allow the molten wax to penetrate the tissue to displace the xylene from the clearing process.

3.16.2. Wax block embedding

Once the tissue was completely infiltrated with wax, a mould (block) was formed that can be clamped into a microtome for the sectioning of the tissue. The tissue specimen was placed into the mould and molten wax was poured over it. A cassette was placed over the mould, covered with more molten wax and left to solidify for 20 minutes. The wax block was clamped to a microtome and sectioned/cut at 3-4 µm thickness. The section was floated out in a water bath (set between 50-55°C, i.e. below the melting point of the wax) and picked up on a positive charged glass slide. The slide was placed in an incubator at 70°C for 15 minutes to melt down excess wax around the tissue and for the tissue to adhere to the slide.

3.16.3. Haematoxylin and eosin (H&E) staining

H&E staining was done using the Stainer coverslipper which is an automated Tissue-Tek film machine. The first process allowed for rehydration of the tissue. The slide was immersed into xylene for a series of three cycles of 5 minutes each, followed by a series of immersion in alcohol solutions as outlined below.

Alcohol concentration	Time	
100%	1 hour	3X
96%	1 hour	
70%	1 hour	

The slides were immersed in tap water for a rinsing step and then immersed in Mayers Haematoxylin for 10 minutes to stain the nuclei of the cells. Another rinse step was included. The slide was immersed in 1% Lithium carbonate for 1 minute to intensify the colour of the haematoxylin. The slide was then counterstained in Eosin-yellowish solution, an acidic solution that stains basic parts of the cell, such as the cytoplasm. A final wash step was included. The final step included the dehydration of the tissue on the slide by immersion in a series of alcohol and xylene cycles as outlined below.

Reagent	**Time**	
70% alcohol	5 minutes	
96% alcohol	5 minutes	
100% alcohol	5 minutes	3X
Xylene	5 minutes	3X

Histological processing was conducted in the Division of Forensic Medicine by Mr André du Toit and Mr John Segole, Chief Forensic Technologist and Medical Technologist, respectively. The protocol for histological processing was obtained by direct communication from Mr André du Toit.

3.17 Reviewing of case file information and routine laboratory results

Available sociodemographic data and clinical history for each SUDI case were obtained from the case files and questionnaires, Appendix C, (answered by the parent, family member or friend at the time of admitting a case to the Tygerberg Medico-legal Mortuary) for the cases included in this study. The data was captured onto a Microsoft® Excel® spreadsheet for statistical analyses. Information such as age, race, birth weight, date of birth and death, infant sleeping position, parental smoking and drinking habits, COD classification as assigned by the forensic pathologist based on a thorough PM investigation (which may have included DSI, autopsy and clinical history review), etc. were included in the data analysis sheet.

3.18 Statistical analysis

Statistical analysis was performed using R software version 3.4.1. Categorical variables were summarized using proportions and frequencies. To measure the strength of the association between groups, odds ratio and its 95% confidence interval (CI) were computed using simple logistic regression analysis.

The dataset was stratified according to gender, season (warm and cold) and COD (only Infection and SIDS) as major risk factors according to the literature. Categorical variables were compared between

the two groups using the Chi-square test or the Fisher exact test. Numerical variables were compared between the same groups using the two sample Wilcoxon test or student t-test when required. Sensitivity and specificity tests were used to evaluate the EV and B19 PCR assays developed against histology as the gold standard in the diagnosis of myocarditis.

CHAPTER FOUR

RESULTS

4.1 Introduction

This study aimed to profile EV and B19 in prospective SUDI samples collected over a period of one year (July 2016 – July 2017). Two PCR-based assays were designed for the detection of EV and B19 nucleic acid in heart swab samples and were compared to microscopic features of infection in heart tissue samples. Routine microbiology of the heart and HIV screening results were also obtained and incorporated into the study. Clinical and sociodemographic data were obtained from the case files and assessed to identify possible risk factors associated with these SUDI cases.

4.2 Population characteristics

A total of 168 SUDI cases were included in the study over the one-year period with a male to female ratio of 1:1.07. This sample number was lower than those shown in section 2.7 (Figure 2.6) as it did not include ALL the SUDI cases (N=210) admitted to the mortuary over this study period and the selection criteria used in this study was different to that of the cases in section 2.7. During the study period, only black and coloured infants were admitted to the Tygerberg Medico-legal Mortuary as SUDI, with the majority of cases being black (n=107, 64%). The majority of deaths occurred during the colder months of the year (75 in winter and 37 in autumn; or 67% combined) (Figure 4.1). The mean birth weight of the infants was 2 463.98 g (standard deviation [SD]: 809.06 g). The median age of the infants at the time of death was 10.9 weeks (interquartile range [IQR]: 6.25 - 17.32). The majority of infants were aged between 1 and 13 weeks at the time of death (n=107, 64%), with a peak between 9 and 13 weeks old (n=44, 26%) (Figure 4.2). The median post-mortem interval (PMI), which is the interval between the date of death and the date of autopsy, was observed as 5 days (IQR: 3 - 7).

A total of 29 (18%) and 50 (31%) infants were exposed to alcohol and tobacco smoke during pregnancy respectively, and 15 (11%) were exposed to tobacco smoke postnatally.

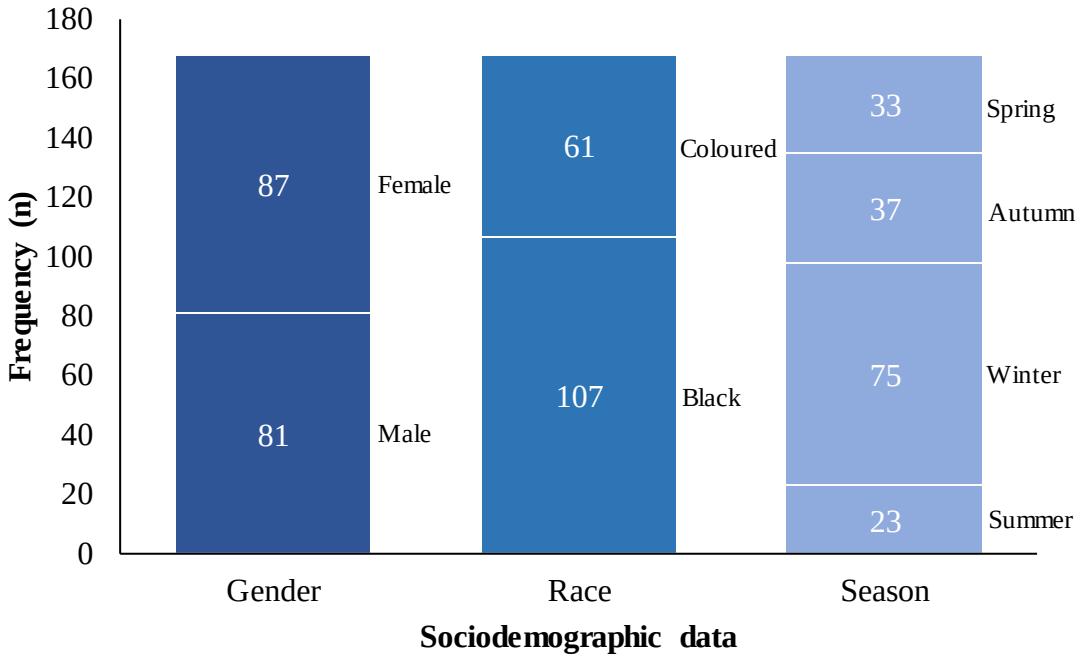


Figure 4.1. Sociodemographic data in the SUDI population.

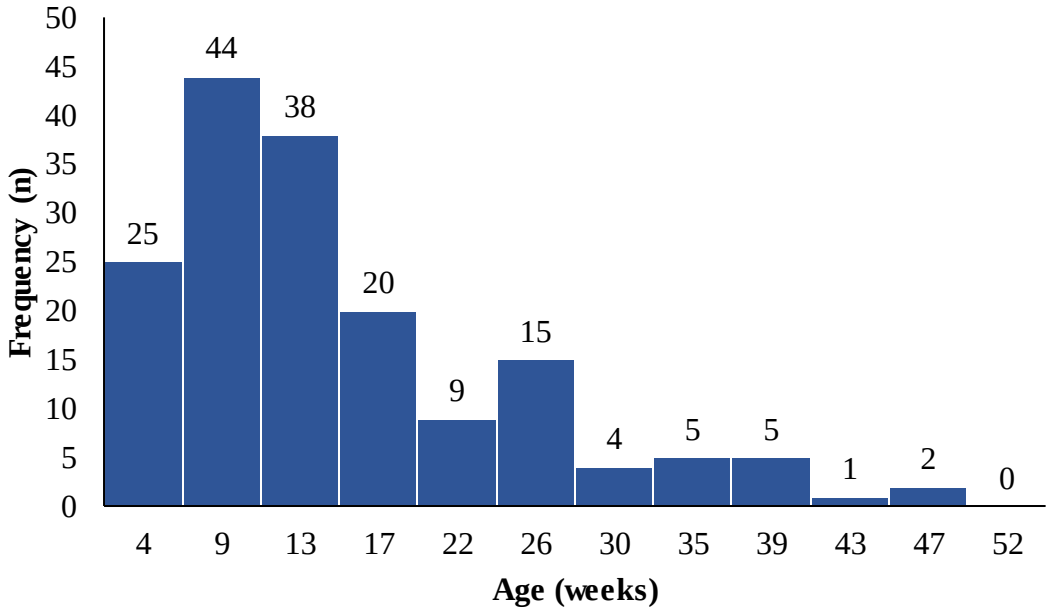


Figure 4.2. Age distribution of infants at time of death.

Details about the infants' sleep enviroment are denoted in Figure 4.3. The majority of infants (n=88, 56%) resided in informal housing enviroments and a significant proportion (n=144, 96%) slept in a bed with one or more individuals. A total of 33 (21%) infants sleep in non-ventilated rooms. The majority of infants were put to sleep (n=90, 64%) and found dead (n=67, 49%) on their sides.

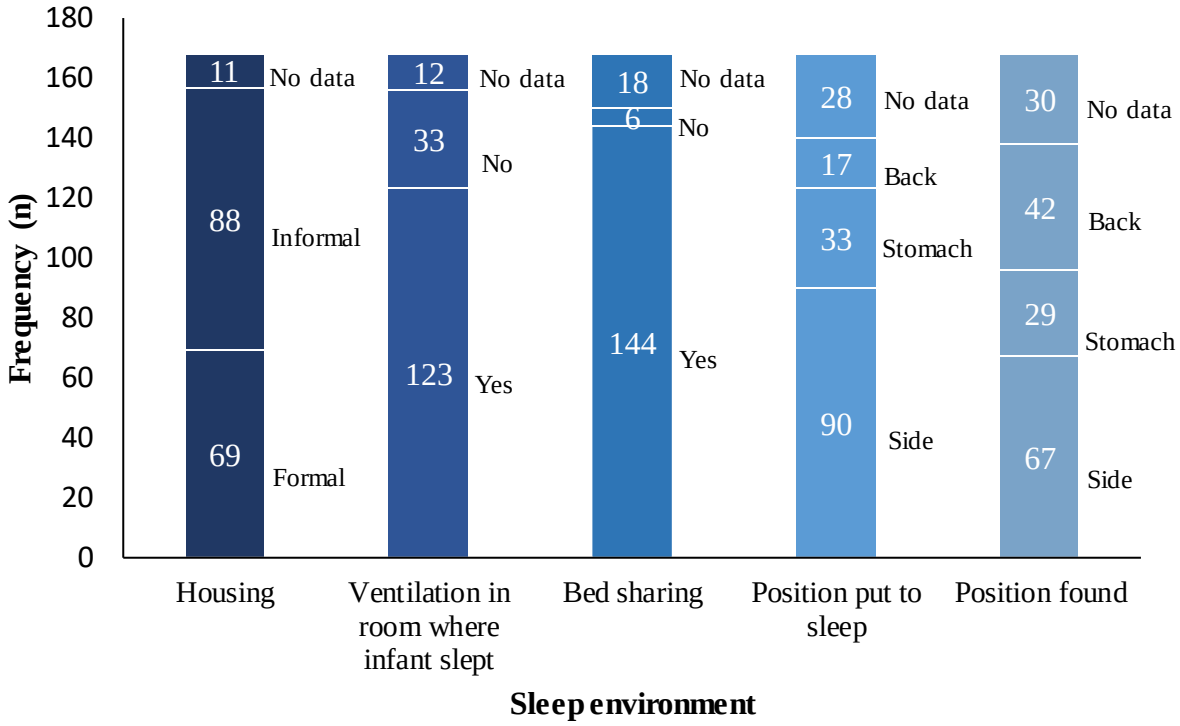


Figure 4.3. Housing and sleep-related practices in the study population.

Out of a total of 168 cases, clinical symptoms prior to demise were observed in 36 cases (23%). Microscopic signs of myocardial infection were observed in 10 cases (6%), in which 1 (20%) case showed macroscopic abnormalities (Case 122) where the coronary arteries were patent and the left ventricular wall of the heart displayed a mottled appearance. Specific bacteria and viruses were confirmed in 39 (23%) and 162 (96%) SUDI cases respectively.

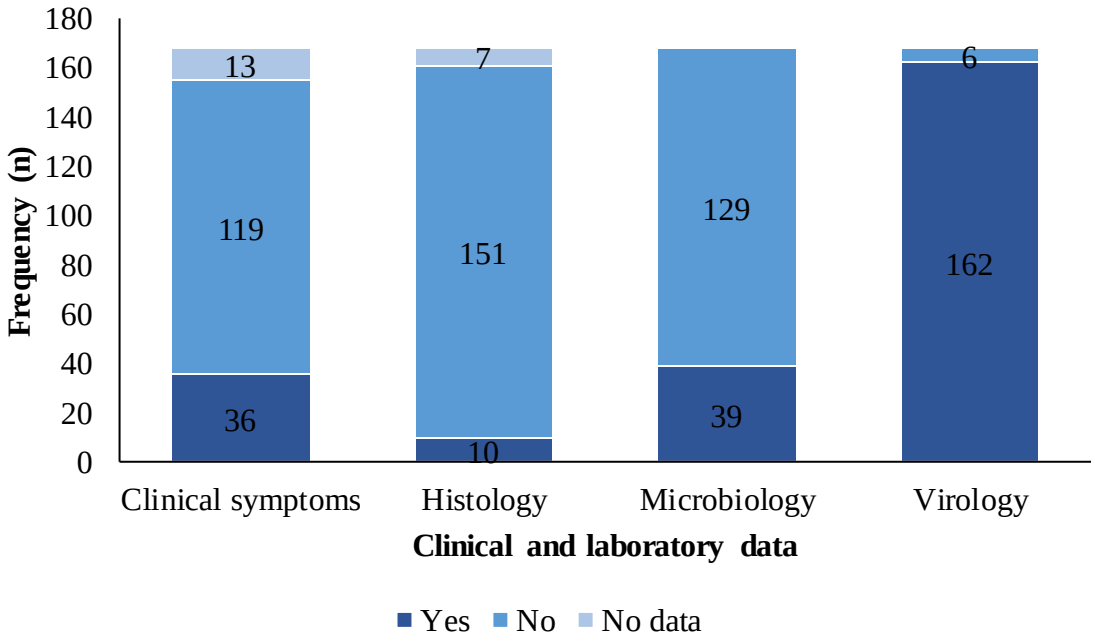


Figure 4.4. Clinical and laboratory findings for the SUDI cases.

4.3 COD classification

The final COD for the SUDI cases was assigned by the Forensic pathologists at the Tygerberg Medico-legal Mortuary following the completion of the PM investigation, which included reviewing of the clinical history and death scene (may not have been performed in all cases) and a full autopsy examination. Only a total of 121 (72%) SUDI cases were assigned a final COD at the time of statistical analyses, while the remainder are still under investigation. The final COD was classified into three groups (Figure 4.5), namely infection (n=64, 53%), SIDS (n=48, 40%) and other (n=9, 7%). Other constituted death due to acute aspiration (n=2), asphyxia (n=1), congenital heart conditions (n=2) and undetermined by autopsy i.e. where an unnatural cause could not be ruled out or where there was insufficient information to ascertain a definite cause of death (n=4).

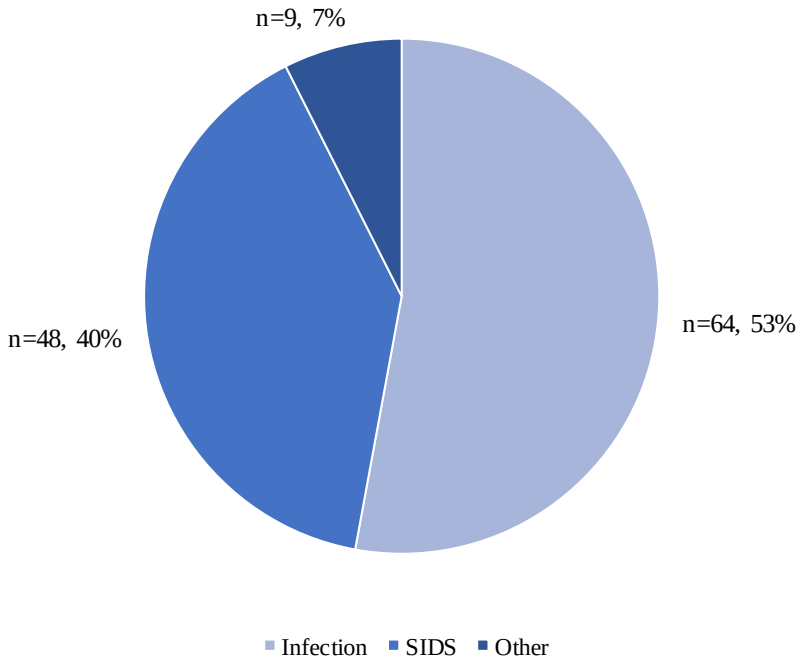


Figure 4.5. The final COD classification for the SUDI cases.

4.4 Routine microbiology analysis of the heart

Bacterial culture on the specimens collected from the heart, one of the routine tests for SUDI cases at Tygerberg Medico-legal Mortuary, was included in the study. Bacteria was detected in only 39 (23%) SUDI cases. *E. coli* and *S. aureus*, pathogens commonly identified in SUDI cases, were detected in 7 (4%) and 3 (2%) SUDI cases respectively. Post-mortem and normal skin flora were detected in 12 (7%) and 5 (3%) cases respectively (Table 4.1). *Streptococcus pyogenes* (1 case), PM flora (1 case), *E. coli* (2 cases), *Streptococcus pneumoniae* (1 case), *Klebsiella pneumonia* (1 cases), *Enterococcus facieum* (1 case) and *Enterococcus faecalis* (1 case) were detected in the cases in which clinical

symptoms were observed prior to death. Positive microbiology results were found in 16 (41%) cases where infants were exposed to tobacco smoke either in utero and/or postnatally, 8 (21%) cases where infants slept in non-ventilated rooms, 30 (77%) cases in which the practice of bedsharing was observed and 10 (26%) cases in which infants were put to sleep or found dead on their stomach. In 27 (69%) cases where the PMI exceeded 4 days (median 6 days, range 5 – 12 days), PM flora was confirmed in 8 cases, *E. coli* in 5 cases, normal skin flora in 4 cases, *Klebsiella pneumonia* in 2 cases and *S. aureus*, *Enterococcus species*, *Streptococcus agalactiae*, group B *Streptococcus*, *Serratia liquefaciens*, *Enterococcus faceuim* and *Enterococcus faecalis* in 1 case each.

Table 4.1. Bacteria isolated from heart.

Microbiology	Frequency (n)	Percentage (%)
Coagulase negative *Staphylococcus*	1	0.6
Enterobacter cloacae	2	1.1
Enterococcus faceuim	1	0.6
Enterococcus faecalis	1	0.6
Enterococcus species	1	0.6
E. coli	7	4.1
Klebsiella pneumoniae	2	1.1
No growth	128	76.1
Normal skin flora	5	2.9
Post-mortem flora	12	7.1
Serratia liquefaciens	1	0.6
S. aureus	3	1.7
Streptococcus agalactiae	1	0.6
Group B *Streptococcus*	1	0.6
Streptococcus pneumoniae	1	0.6
Streptococcus pyogenes	1	0.6

4.5 Routine HIV testing

Peripheral blood was available for only 160 SUDI cases and sent to the NHLS Medical Virology laboratory for the rapid detection of antibodies against HIV 1/2. A total of 36 (23%) of these SUDI cases was positive for HIV 1/2 antibodies from the whole blood samples.

4.6 Histology

A total of 161 SUDI cases were included for histologic analysis of the heart tissue collected and processed, while no tissue samples were available for the other 7 cases. In the majority of the cases (n=151, 94%) no remarkable signs of infection were observed in the myocardial tissue and in these cases the histology was classified as "normal". Signs of infection were considered when features of inflammation and/or myocytolysis (such as infiltration of inflammatory cells, abnormal myocytes and eosinophils) were observed (Table 4.2). An example of the microscopic features of myocardial infection is depicted in Figure 4.6, where infiltration of inflammatory cells and myocytolysis were observed in the myocardial tissue for one of the SUDI cases.

Table 4.2. Microscopic features observed in the heart tissue for the SUDI cases.

SUDI case	Histology
Case 015	Diffuse inflammation consisting of chronic inflammatory cells including lymphocytes and plasma cells, myocytolysis
Case 032	Mild chronic inflammation
Case 039	Focus of chronic inflammation with very mild and focal myocytolysis
Case 040	Area of myocytolysis, not considered artefact
Case 045	Very focal acute inflammatory cells, neutrophils and scant eosinophils in myocardium associated with blood vessels, with virtually no manifestation of myocytolysis
Case 050	Cellular interstitium consisting of mixed inflammation, interstitial edema. Relatively focal inflammation, and myocardium relatively spared
Case 122	Chronic inflammation, myocytolysis
Case 123	Mixed inflammation, but predominantly chronic
Case 127	Chronic inflammation, mild myocytolysis
Case 144	Focal parenchymal hemorrhage, myocytolysis

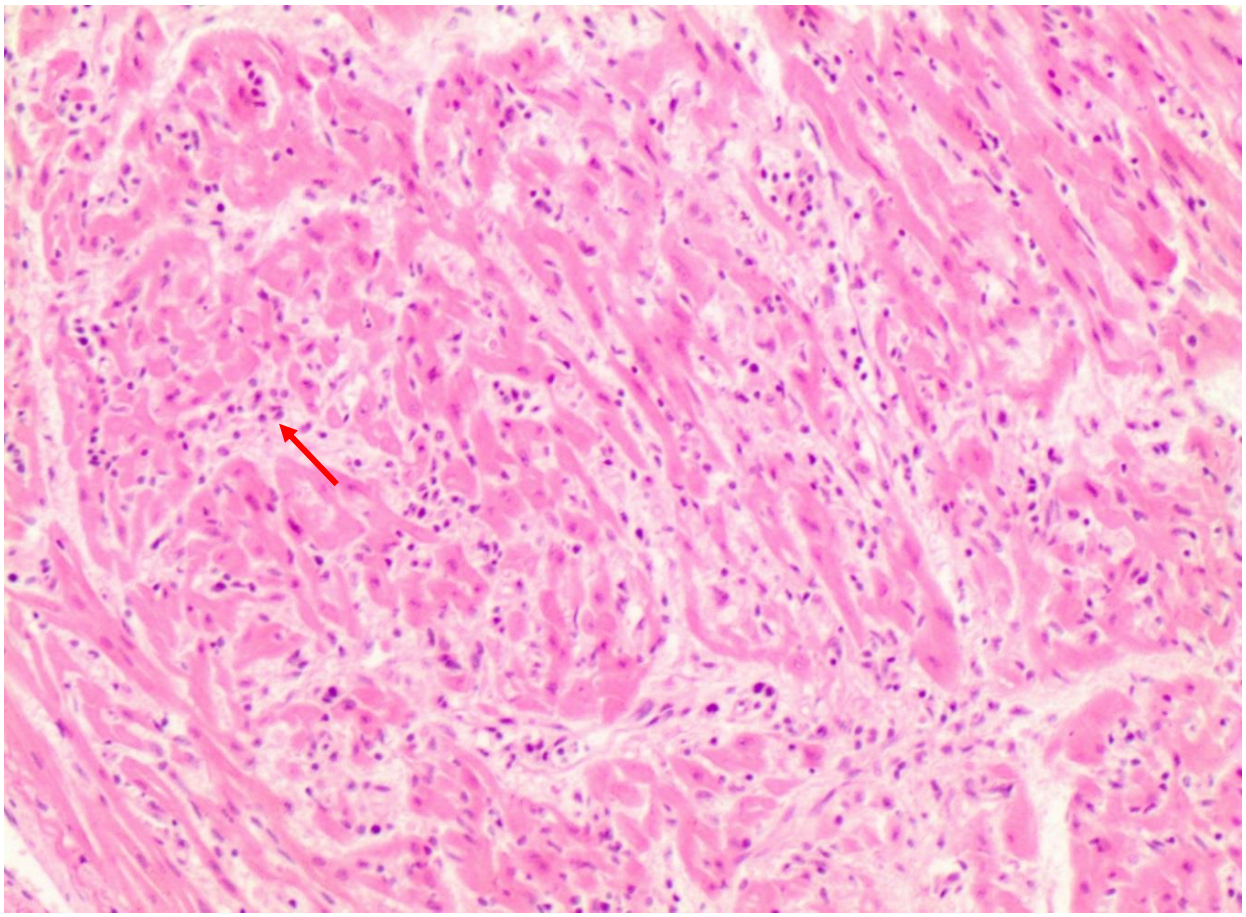


Figure 4.6. Microscopic features of inflammation in the myocardium. Red arrow shows infiltration of chronic inflammatory cells and myocytolysis (SUDI Case 122; magnification ×100)

4.7 PCR assays

Two PCR assays were designed for the detection of B19 and EV nucleic acids respectively using the generated plasmids described in Chapter 3 as positive controls for the assays. The copy numbers for the EV and B19 plasmids were determined as 133.65 and 145.33 copies/µl, respectively, at the lowest detection limit. The B19 DNA and EV cDNA for 168 SUDI cases were amplified by nested/hemi-nested PCR and separated by 2% agarose gel electrophoresis (Figure 4.7) to produce bands of 173 bp and 196 bp respectively (indicated by the red arrows).

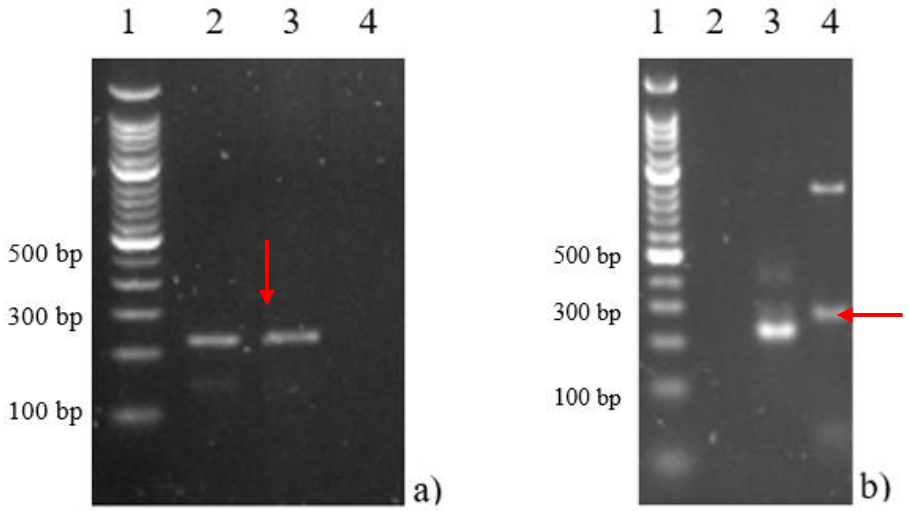


Figure 4.7. Detection of B19 and EV by Nested PCR.
(a) EV PCR, Lanes 1: 50bp DNA ladder, 2: positive control, 3: SUDI case sample, 4: negative control. (b) B19 PCR, Lanes 1: 50bp DNA ladder, 2: negative control, 3: positive control, 4: SUDI case sample.

The complete PCR results are listed in Appendix D. In summary, a total of 161 (96%) and 50 (30%) positive amplicons were detected for B19 and EV respectively. Both B19 and EV were detected in 49 (29%) cases.

Sequencing of 8 random gel purified (Appendix E) B19 positive amplicons were performed following the protocol in section 3.11 using 3.2 µl forward and reverse B19 primers. Contigs were generated for each sequenced amplicon and the contigs were aligned to the sequence of the positive control in a multiple sequence alignment in Geneious R10 software. No exact match was observed in any of the sequences and the identity was confirmed as highly related to B19 isolates using the BLAST online software tool.

4.8 Statistical analyses

Categorical variables were summarized using proportions and frequencies. Median and IQR were used to summarize numerical data where kurtosis or skew distribution was evident. Mean and SD were used for numerical data displaying a normal distribution. The strength of an association between two groups were measured by Odds Ratio (OR) and its 95% CI.

4.8.1. Sensitivity and specificity tests for PCR assays

The sensitivity and specificity for the two PCR assays designed were calculated to determine the diagnostic value of the assays against histological analysis as the gold standard for diagnosis of myocardial infection. The sensitivity was calculated using the true positive (TP) and false negative (FN) values for each assay and the specificity was calculated using the true negative (TN) and false

positive (FP) values for each assay (Tables 4.3 and 4.4). Sensitivity of an assay refers to its false positive rate i.e. the probability that the assays will yield a positive result in the presence of a certain pathogen, while specificity is the false negative rate or the probability of the assay to yield a negative result in the absence of a certain pathogen. Sensitivity and specificity of the PCR assays developed were calculated as 44% and 73% respectively for B19 and 89% and 3% respectively for EV using the equations below. The sensitivity and specificity of each assay was used to calculate the likelihood ratio (LR) in order to determine the likelihood that the PCR result is correct to the likelihood that the PCR result is incorrect using the equation below (LR + is the likelihood of a positive PCR result). The positive LR for B19 assay according to the calculation is +1.65, which would indicate that an infant is about 1.65 times more likely to have a positive B19 PCR result when myocardial infection is present than in the absence of myocardial infection. Similarly, for the EV assay, an infant is about 0.92 times more likely to have a positive EV PCR result when myocardial infection is present than in the absence of myocardial infection.

Table 4.3. Two-table contingency test for B19 assay against histology.

PCR (as test in question)	Histology (as golden standard)	
	Positive	**Negative**
B19 positive	4	41
B19 negative	5	111

Sensitivity $= \frac{TP}{TP+FN} = \frac{4}{4+5} = 44.44\%$

Specificity $= \frac{TN}{FP+TN} = \frac{111}{41+111} = 73.03\%$

LR+ $= \frac{sensitivity}{1-specificity} = \frac{0.44}{1-0.73} = 1.65$

Table 4.4. Two-table contingency test for EV assay against histology.

PCR (as test in question)	Histology (as golden standard)	
	Positive	**Negative**
EV positive	8	147
EV negative	1	5

Sensitivity: $\frac{TP}{TP+FN} = \frac{8}{8+1} = 88.89\%$

Specificity: $\frac{TN}{FP+TN} = \frac{5}{147+5} = 3.29\%$

LR+ $= \frac{sensitivity}{1-specificity} = \frac{0.88}{1-0.03} = 0.92$

4.8.2. Histology, clinical history and viruses detected by PCR

The clinical history was compared to the viruses detected in the 10 SUDI cases with histological features of infection. Clinical symptoms of infection, such as fever, runny nose and vomiting prior to death, were present in 4 of these cases (Table 4.5).

4.8.3. Comparison of histology, final COD, virology, microbiology and HIV status

Although features of myocarditis were observed microscopically in 10 SUDI cases, the final COD as myocarditis was assigned in only 1 SUDI case (Case 015). Both B19 and EV DNA were detected in this case. In another SUDI cases, Case 123, B19 and EV were absent although features of inflammation in the myocardium were present (Table 4.6).

4.8.4. Gender, seasonality and final COD and the viruses detected by PCR

In contrast to the male predominance in SUDI observed in the literature, no significant association between a positive PCR result and the gender of the infants was observed in this study (Figure 4.8). Similar to the literature, a higher proportion of viruses were detected during the cold months (n=74, 44% in winter and n=35, 21% in autumn; combined n=109, 65%; Figure 4.9) and in infants that died as a result of infection (n=64, 53%; Figure 4.10), however neither were statistically significant.

Table 4.5. Clinical history and viruses detected in the ten SUDI cases with histological features of myocarditis.

SUDI case	Histology (features of infection)	Clinical history	Virus
Case 015	Inflammation, myocytolysis	Fever, coughing, seizures	B19, EV
Case 032	Inflammation		B19
Case 039	Inflammation, myocytolysis		B19, EV
Case 040	Myocytolysis		B19, EV
Case 045	Inflammation	Runny nose	B19, EV
Case 050	Inflammation		B19, EV
Case 122	Inflammation, myocytolysis	History of lung infection	B19
Case 123	Inflammation		
Case 127	Inflammation, myocytolysis	Vomiting when fed	B19
Case 144	Myocytolysis		B19

Table 4.6. Summary of SUDI cases with histological features of myocarditis as determined by microscopic analysis of myocardial tissue and COD, viral, bacterial and HIV screening.

SUDI case	Histological signs of infection	COD assigned by pathologist	Virology	Microbiology	HIV
Case 015	Yes (I, M)	Myocarditis	++	No growth	Negative
Case 032	Yes (I)	Pneumonitis	+	No growth	Positive
Case 039	Yes (I, M)	SIDS	++	No growth	Negative
Case 040	Yes (M)	Under investigation	++	No growth	Positive
Case 045	Yes (I)	Interstitial pneumonia	++	No growth	Negative
Case 050	Yes (I)	LRTI	++	No growth	Negative
Case 122	Yes (I, M)	Under investigation	+	*E. coli*	Negative
Case 123	Yes (I)	Under investigation	-	No growth	Positive
Case 127	Yes (I, M)	LRTI	+	No growth	Negative
Case 144	Yes (M)	SIDS	+	PM flora	Insufficient specimen

I – inflammation, **M** – myocytolysis, **LRTI** – Lower respiratory tract infection, ++ (B19 & EV positive), + (B19 positive)

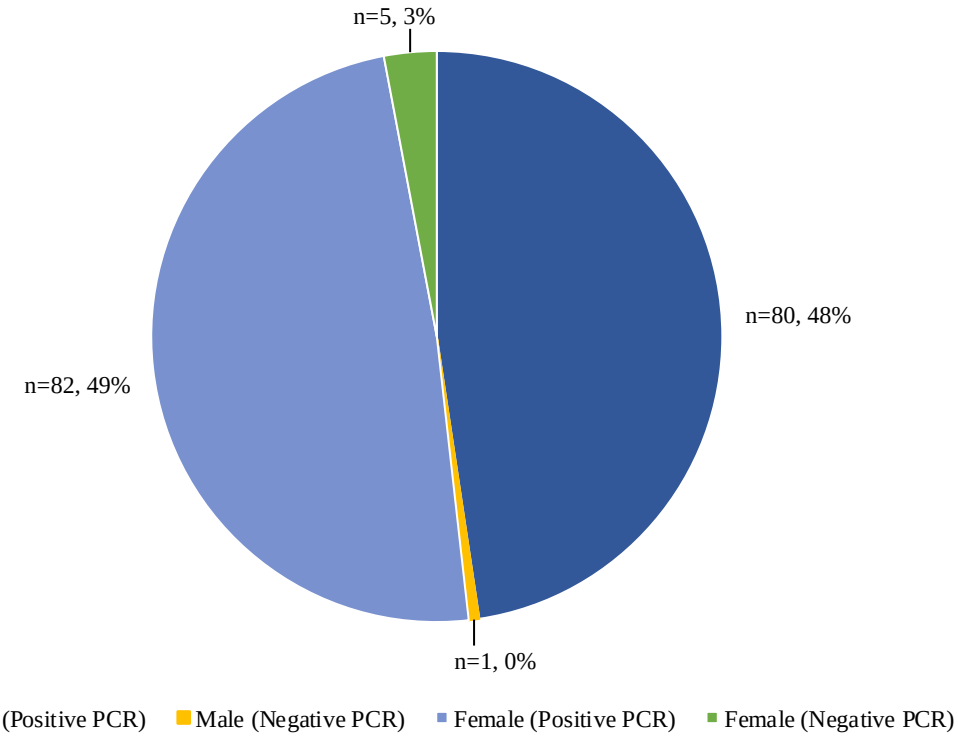


Figure 4.8. Viruses detected by PCR between male and female infants.

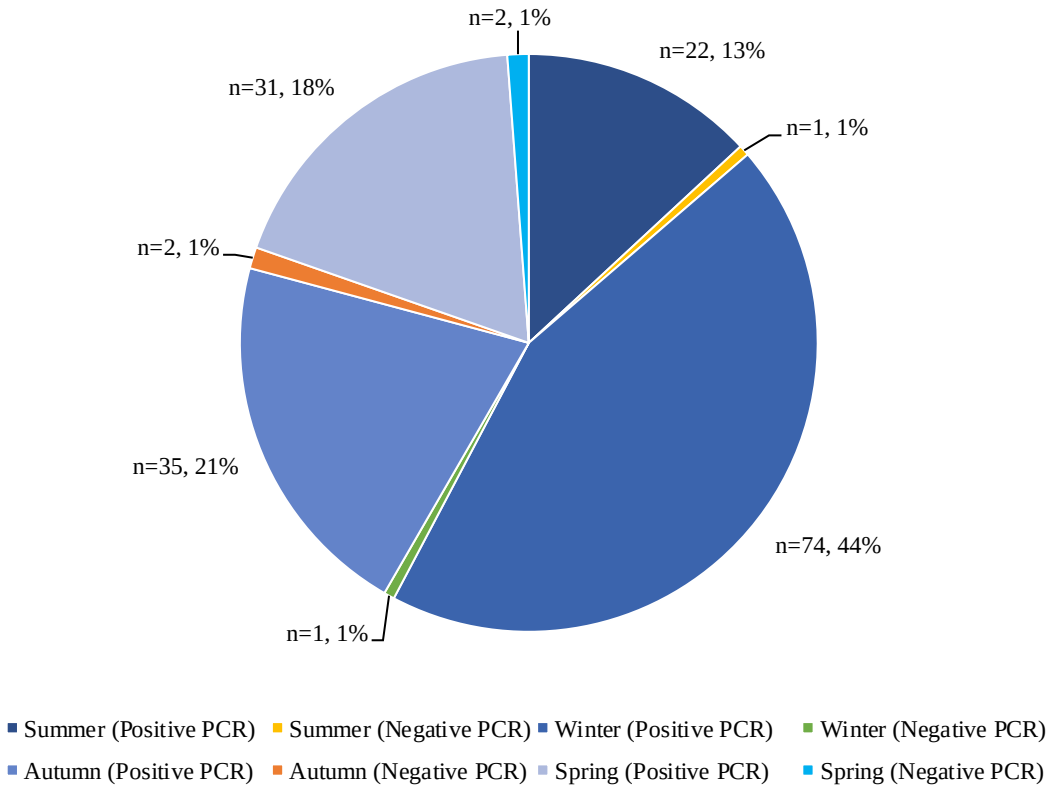


Figure 4.9. Viruses detected by PCR across the different seasons of the year.

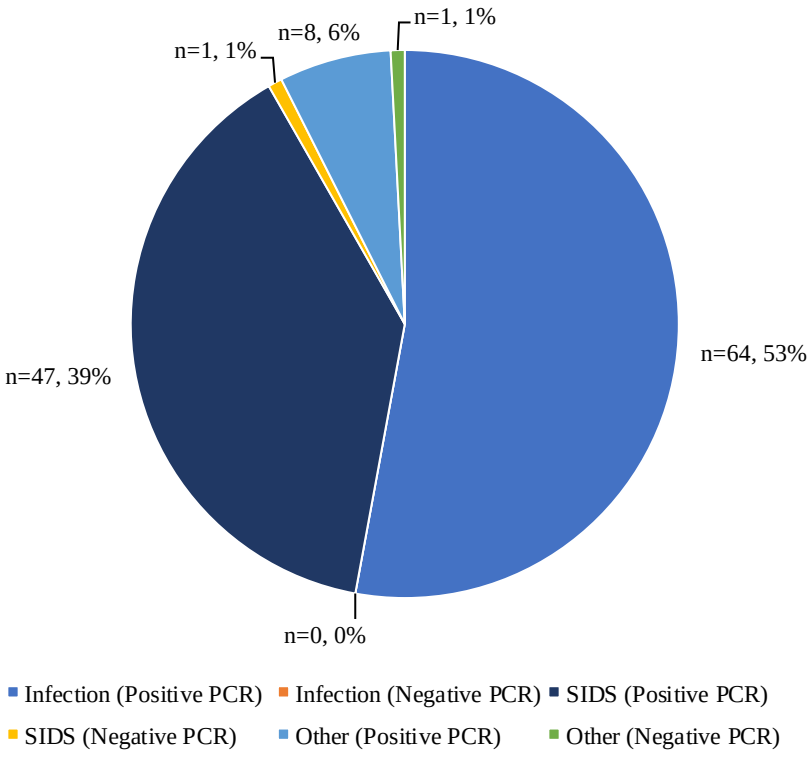


Figure 4.10. Viruses detected by PCR within the final COD category.

4.8.5. Population characteristics categorized by final COD (excluding Other)

Risk factors associated with SUDI in the literature were compared between SIDS and infection for categorical (Table 4.7) and numerical data (Table 4.8). A p-value less than 0.05 was regarded as statistically significant. The results identified black infants to have a 2.36 odds of SUDI being attributed to infection (p = 0.028). This is however not clinically relevant because the referral area of the Tygerberg Medico-legal Mortuary does not represent the true demographic distribution in the general population. Furthermore, no other differences that may show statistical significance were identified in the COD categorization.

Table 4.7. Population characteristics categorized by COD.

Characteristics	SIDS, N (%)	Infection, N (%)	p-value	OR	95% CI
Gender					
Male	21 (43.8)	35 (54.7)	0.251	1.55	0.73 – 3.32
Female	27 (56.2)	29 (45.3)			
Race					
Black	24 (50.0)	45 (70.3)	0.028	2.36	1.09 – 5.22
Coloured	24 (50.0)	19 (29.7)			
Season					
Autumn	9 (18.8)	12 (18.8)	0.769	1.33	0.34 – 5.29
Spring	10 (20.8)	10 (15.6)		1.00	0.25 – 3.97
Winter	22 (45.8)	35 (54.7)		1.59	0.48 – 5.25
Summer	7 (14.6)	7 (10.9)			
Housing					
Informal	21 (45.7)	38 (62.3)	0.080	1.96	0.90 – 4.32
Formal	25 (54.3)	23 (37.7)			
Ventilation in bedroom					
Yes	39 (84.8)	46 (78.0)	0.377	0.63	0.21 – 1.71
Bed sharing					
Yes	41 (97.6)	56 (93.3)	0.646	0.34	0.01 – 2.41
Position put to sleep					
Back	4 (9.5)	6 (10.9)	0.524	0.79	0.17 – 3.78
Side	29 (69.0)	32 (58.2)		0.58	0.21 – 1.48
Stomach	9 (21.4)	17 (30.9)			
Position found (dead)					
Back	12 (29.3)	9 (18.0)	0.398	0.45	0.13 – 1.46
Side	20 (48.8)	26 (52.0)		0.78	0.27 – 2.12
Stomach	9 (22.0)	15 (30.0)			
In utero tobacco exposure					
Yes	18 (38.3)	20 (32.8)	0.552	0.78	0.35 – 1.74
Postnatal tobacco exposure					
Yes	4 (10.8)	6 (11.3)	1.000	1.05	0.27 – 4.39
In utero alcohol exposure					
Yes	9 (19.1)	11 (18.0)	0.882	0.92	0.34 – 2.51

Table 4.8. Population characteristics categorized by COD.

Characteristics	SIDS	Infection	p-value
Birth weight*	2458.02	2625.31	0.279
Age (weeks)†	9.65	11.15	0.726
Maternal age (years)†	27.50	28.00	0.599

* Mean

† Median

CHAPTER FIVE

DISCUSSION

5.1. Introduction

SUDI has been an important topic of interest for centuries, and there is very limited information available in Africa (Ibeziako *et al.* 2009). SIDS is one of the main causes of infant death worldwide, alongside congenital abnormalities, prematurity and low birth weight (Goutas *et al.* 2011). Infection remains a common contributor to SUDI, but SIDS rates remain high, especially among infants in developing countries (Ndu 2016). Our findings agree with that of Weber *et al.* (2008b) where 63% of their SUDI cases remained unexplained and of the 37% explained deaths, more than half (58%) were attributed to infection.

We used a cross-sectional study to investigate the association between 168 SUDI cases and myocarditis. A small proportion of cases showed histological signs consistent with myocardial infection, but a final diagnosis or COD of myocarditis was only made in one case.

5.2. Population characteristics and demographics

Male gender is an important risk factor frequently reported in SIDS. Mage and Donner (2014) reported that males are more susceptible to respiratory infection during infancy, possibly contributing to the overall male predominance found in SIDS. Mage and Donner (2009) reported a male to female ratio of 1:0.61 for SIDS cases in the USA, which is slightly lower than 1:0.82 observed in South Africa between 2005 and 2009 (du Toit-Prinsloo *et al.* 2013). However, the male to female ratio in our study (1:1.07) did not agree with trends in the literature. Instead, we observed slightly more females than males, which was similar to a previous study (unpublished), 1:0.03, done at the Tygerberg Medico-legal Mortuary between 2015 and 2017. The reason for this difference is not known.

Male circumcision might be associated with the male predominance in previous SIDS studies (Elhaik 2016), however, this hypothesis needs to be tested in a non-biased setting (e.g. where circumcision is observed in both male and female infants). We do not consider circumcision as a possible risk factor for SIDS in our setting, as circumcision of males is a traditional and cultural practice that occurs much later in life (teenage period) and none of the male infants included in this study were circumcised at the time of autopsy (personal observation). Therefore, the gender discrepancy found between our

cases and the literature needs to be further investigated in future prospective studies or extensive retrospective case file audits.

Two-thirds of our cases occurred during the colder months, consistent with the published peak incidence during winter and decrease in the warmer months. Respiratory infections are common in winter and circulation of respiratory pathogens can easily be transmitted to infants by household contacts or in close contact environments, such as day care (Mage 2004; Mage *et al.* 2016).

The 2 to 4 month peak age reported in the literature (Hakeem *et al.* 2015) is similar to the 9 to 13 week peak found in our study. An association between the peak age in SIDS and the age at which maternally-acquired antibodies are decreased or completely lost have previously been described, indicating increased susceptibility to infection, leading to increased infant vulnerability. Although infant immunization is initiated around this period of vulnerability (6 weeks), infants are not fully vaccinated against common illnesses encountered in childhood and the infant immune system is still regarded as functionally immature (Hoffman *et al.* 1987; Waaijenborg *et al.* 2013).

Several studies have reported on bed sharing, such as Hauck *et al.* (2003) who reported a 50% incidence and Blair *et al.* (2009) who also reported more than half of their study population sharing a bed with another person. However, this is much lower than the 96% observed in our study. We postulate that this might be a result of poverty (56% of infants reside in informal living conditions) and also high parity found in our study, where the number of beds in the household might be lower than the number of individuals in the household (Ball *et al.* 2012). An early study conducted in New Zealand found that bed-sharing was present in both Maori and non-Maori populations, however only among Maori infants was bed-sharing related to increased risks in SIDS (Mitchell *et al.* 1993). This finding needs further elucidation in the broader South African population to investigate possible correlations between specific ethnic groups and the risk of SUDI.

The most common sleeping position in which infants were *placed* was on their sides, however, fewer cases were *found* on their sides. It has been proposed that this is in fact the most unstable position (Ibeziako *et al.* 2009), because infants can easily roll over to another position during the night and succumb to SUDI due to other external stressors. Despite this theory, the side sleeping position is still considered safer than the prone position.

According to the surveys in the case file reports in our study, only 18% and 31% of infants were exposed *in utero* to alcohol and tobacco respectively. May *et al.* (2014) showed that 88 - 91% of mothers who have infants with Fetal Alcohol Spectrum Disorders consumed alcohol during pregnancy and up to 70% reported binge drinking, which is regarded to be much more harmful to the

developing infant. Additionally, 74% of mothers smoked during pregnancy and 67% reported a combination of smoking and drinking, which are both known risk factors for SUDI. The differences between our data and that of May *et al.* (2014) can partly be ascribed to the fact that their survey was done in mothers with living infants. However, *in utero* exposure to alcohol and tobacco increases the risks of SIDS (Hakeem *et al.* 2015) by jeopardizing fetal development and thereby increasing the vulnerability of the infant after birth (Blair *et al.* 2009). Although smoking and drinking during pregnancy are some of the most obvious modifiable lifestyle changes that should be advocated, it would require much larger case-control studies in order to confirm smoking and drinking during pregnancy as independent risk factors for SUDI. However, due to ethical constraints, we are not able to include any control cases at this stage.

5.3. Routine microbiology testing

PM microbiology analysis is important in SUDI investigation as it provides valuable information about whether infection can be implicated in death and can also describe the role and presence of nosocomial bacteria in a specific hospital environment (Tsokos & Püschel 2001). However, possible contamination with PM flora often challenges the value of microbiological culture in PM cases. The presence of bacteria in an organ should not necessarily be regarded as the COD, as it often only indicates the presence of these pathogens at the time of death (Eisenfeld *et al.* 1983; Aranda *et al.* 1998; Rambaud *et al.* 1999). It is therefore imperative to supplement these results with ancillary tests or investigations.

In our study, although bacterial growth was observed in 39 SUDI cases, the majority were attributed to PM flora, followed by a few cases of *E. coli*. Similar to the findings of Weber *et al.* (2010b), we could not demonstrate any association between increased PMI (> 4 days) and positive bacterial culture.

The risk of prone sleeping, excessive bedding/wrapping and presence of viral infection have previously been associated with colonization by *Streptococcus pyogenes* and release of toxins (Blackwell *et al.* 1995). Our study also identified *Streptococcus pyogenes* in one of our cases and we postulate that other factors, such as a non-ventilated bedroom, bed-sharing and the presence of B19 and EV DNA observed in this case, might have potentiated the growth of this bacterium. The histology in this case was normal and a final COD has unfortunately not yet been assigned by the pathologist at the time of statistical analyses. It would be important to follow up on this case once it is finalized and incorporate the findings in the development of a standardized institutional investigation protocol for SUDI at the Tygerberg Medico-legal Mortuary.

5.4. Viral detection by PCR

A number of reports in the literature demonstrate the association between SUID and viruses often implicated in myocarditis (Bowles *et al.* 2003; Dettmeyer *et al.* 2004, Dettmeyer *et al.* 2008; Uhl 2008; Dettmeyer *et al.* 2009). In the past, EV has been reported as an important cause of viral myocarditis, however B19 is currently also emerging as a causative agent (Canter & Simpson 2014).

We designed two PCR assays to detect two viruses that are commonly associated with myocarditis, i.e. EV and B19. EV DNA was detected by PCR in 30% of our cases, which was comparable to the 22.5% EV detected in a German SUDI population (Weber *et al.* 2008a), but contrasted the findings of 0% EV detected in SIDS cases from California in the USA (Krous *et al.* 2009). The literature suggests that EV DNA is detected less often in myocardial samples (Mahfoud *et al.* 2011; Nielsen *et al.* 2014) than previously shown (Martin *et al.* 1994). Our study was only able to confirm the presence of both EV DNA and histological signs of inflammation in five cases (Appendix E).

We observed a very high prevalence of B19 DNA in the myocardial swab samples and we confirmed these results TP by sequencing analyses. Schenk *et al.* (2009) also identified a very high prevalence of B19 in their study in myocardial samples with positive serology for B19 infection. However, Dettmeyer *et al.* (2004) detected a much lower frequency for B19 in SIDS cases. B19 is widespread in humans although it may not always be related to active infection. A study done by Nielsen *et al.* (2014) supports this, where they found that B19 viral DNA, detected by PCR, was present in autopsy samples from both myocarditis cases as well as non-myocarditis control heart tissue. They suggested that the presence of B19 in their myocardial samples were rather related to latent or persistent infection that might have occurred earlier in life based on positive IgG titres in 63% of their cases, and positive IgM titres in only one case related to B19 infection. Reports by other authors show similar contradicting findings of B19 prevalence (Bültmann *et al.* 2003; Donoso Mantke *et al.* 2004; Schenk *et al.* 2009; Koepsell *et al.* 2012).

5.5. Final COD in SUDI cases

Diagnosis of the final COD in the SUDI cases in our study were based on the integration of all PM details and autopsy findings, but histological analyses, and specifically evidence of myocarditis, weighed heavily in the determining of the diagnosis. Radiology, SVC in lung and liver tissue for detection of selected respiratory pathogens (as described in section 2.6), bacterial detection (lungs and heart), rapid HIV and U&C testing were additional diagnostic tests that potentially contributed to or supported the diagnosis of the COD.

The findings observed in Table 4.6 challenge the validity of using histology independently in diagnosing the final COD in SUDI cases. Although features resembling myocardial inflammation were apparent microscopically, and either one or both of the viruses investigated were detected in 90% of these cases, there was only one case in which the diagnosis of fatal myocarditis was established based on histopathology alone.

Our study suggested that the reliability and sensitivity of independent histopathological assessment as a solitary tool to diagnose myocarditis, without utilising ancillary techniques such as microbiology or virology, may be questionable. Tissue sampling for histological assessment may affect the outcome of diagnosis of myocarditis, given that the assessment and interpretation is limited to the section of analysis, which may not represent the entire area of inflammation. Gaaloul *et al.* (2016) illustrated the effectiveness of using molecular-based techniques in combination with histology to diagnose viral causes in SIDS. Incorporation of molecular-based techniques has been encouraged to reduce, if not eliminate, suboptimal or incorrect diagnosis of sudden infant death (Arnestad *et al.* 2002; Dettmeyer *et al.* 2002) or any infectious COD.

Interpretation of a positive viral PCR result in the absence of histological signs of active infection is extremely difficult and challenging. For the B19 assay, we observed a relatively higher specificity (73%) than sensitivity (44%). This means that the FN rate of the method was 27% where positive B19 amplicons were not detected. We also observed a fairly high sensitivity (89%) for the EV assay, but an extremely suboptimal specificity (3%), suggesting an unacceptably high FP rate of 97%. Any diagnostic test aims to have sensitivity and specificity rates as close to 100% as possible; so much more for SIDS, for reasons such as feasibility, safety and cost-effectivity. Whether or not high sensitivity or specificity rates are achieved, is determined by the disease in question, the diagnostic standard used for the disease and the implications of the results (Hazra & Gogtay 2017). Comparing the assays to histology suggests that the B19 assay has an advantage over the EV assay at predicting the mere presence of a virus, not necessarily causing infection or death, and highlights the idea that there may be discrepancies in diagnosing myocardial infection – and possibly affecting the final COD.

Respiratory viruses are often isolated in SIDS cases (Raza & Blackwell 1999). In our study, infection was implicated in just over 50% of deaths and respiratory-related infections were the leading COD. In only three of our cases assigned a final COD of respiratory-related infection, features of inflammation and/or myocytolysis and the presence of one or both viruses were also observed.

No other cause of death could be ascertained in a large proportion (40%) of our cases and were being classified as SIDS. This finding agrees with Weber *et al.* (2008a) who found a slightly higher proportion of SUDI (60%) classified as SIDS, although du Toit-Prinsloo *et al.* (2013) only found

between 9% and 15% of cases classified as SIDS. Nonetheless, our findings highlight the need of incorporating alternative or additional tests in SUDI investigations, especially since no standard international guidelines exist.

5.6. Limitations & Recommendations

The main limitation in our study was the absence of a control group for comparison to add secondary value to the findings. Ethical consideration for collection of heart samples from living individuals may not easily be granted, as it is regarded as unethical and unacceptable practice. Additionally, ethical consideration for collection of samples from deaths from non-natural causes is also prohibitively difficult, due to the fact that this study was performed with a waiver of consent. Hence, the only comparisons we could make for the findings observed in our study was to similar findings published in the literature that were able to include control groups into their studies.

Another limitation in our study was that we were unable to profile all of the main viruses (such as CVB, HAdV, EBV, CMV, HHV6) associated with myocarditis due to time and financial constraints. Hence, this is recommended as a future study.

No tests were also conducted to determine the pathogenicity of the bacteria detected, although we know from the literature that *S. aureus* (Zorgani *et al.* 1999; Highet *et al.* 2009) and *E. coli* (Bettelheim *et al.* 1990; Pearce *et al.* 2010) are toxigenic bacteria. No additional tests were also performed to determine whether bacteria (such as *E. coli* for example) consisted of pure or mixed cultures. The additional tests are not part of the standard facility investigation protocol for SUDI cases and financial restraints did not allow further testing. This should also be addressed in future studies.

The timing of this study (when the study and write-up was concluded) served a minor disadvantage in terms of including the final COD for ALL cases into the data analysis, as a few cases (28%) were still under investigation at the time of this writing. This may have limited the interpretation of the results related to COD to some extent. Hence it is recommended to review the cases with a final COD as 'under investigation' and incorporate the results into the statistical analyses for latter publications of this study.

To our knowledge, this was the first study in South Africa to profile B19 and EV in myocardial samples in SUDI cases. The benefit of this study is that:

i) It serves as a foundation for the awareness of myocardial inflammatory and infectious conditions in the South-African medico-legal environment,

ii) It can potentially contribute significantly to the implementation of a national standardized SUDI investigation protocol in which myocardial infectious pathology has a rightful place,

iii) It can potentially focus the sights of the forensic pathology fraternity on the benefits of full testing for myocardial infectious conditions,

iv) The data and knowledge obtained from this study may have significance in the clinical field, as specific findings from PCR screening assays for cardiovascular viruses will be reported at Child Death Review for a where clinicians and epidemiologists in attendance may implement clinical protocols for the early diagnosis and treatment of ill children.

With regards to the B19 PCR assay results, further research is necessary to investigate other factors that may have contributed to the high prevalence of B19 observed in our study.

Other recommendations pertaining to some of the data obtained in this study include investigating:

i) population data and other data for infants within the same age group as in our study in order to investigate possible reason for the slight change from male predominance,

ii) the accuracy of the data reported for the alcohol and tobacco use in our study as it is self-reported usage which may be under- or falsely reported.

CHAPTER SIX

CONCLUSION

This study, to our knowledge, is the first of its kind in South Africa and provided valuable information to the forensic pathology environment. Our aim was to profile EV and B19 in SUDI cases to determine whether myocardial infections, known to be caused by these viruses, may be related to SUDI and can be implicated in the final COD.

We observed that both EV and B19 do in fact circulate in our infant population. We also observed that features of inflammation and/or myocytolysis were found in a small number of cases, which almost all were PCR positive for one of both viruses investigated.

Additionally, this study provides evidence regarding the validity of histology in diagnosis of myocarditis. It highlights the idea that it should be used in conjunction with molecular-based diagnostic tests when assigning COD in SUDI cases, specifically aiming to reduce the proportion of cases that remain unexplained or classified as SIDS. The results obtained from the study should be distributed to the forensic pathologists assigned to the SUDI cases to serve as supplementary tests and included in the case files when defining the final COD.

The association between myocarditis and SIDS remains controversial. Although the literature that shows viruses associated with myocarditis are frequently detected in SUDI cases, and the prevalence of B19 and EV in our myocardial samples correlate with this, it does not seem as though myocardial infections played a significant role in the final COD.

REFERENCES

Abramson H. Accidental mechanical suffocation in infants. *J Pediatr*. 1944; 25(5):404-413.

Age Definitions 2012. 2012 [Online]. Available: http://www.health.gov.za/index.php/shortcodes/2015-03-29-10-42-47/2015-06-10-09-23-36/2015-06-10-09-26-11?download=844:icd-10-definitions.

Alfaro C, Bassols AC, De la Cabada FJ, De la Cabada JB, Rico E, Velazquez Ferrari MA. Parvovirus B19: Past, present and future. *Research Rev J Microbiol Biotech*. 2016;5(1):131-139.

Alfelali M, Khandaker G. Infectious causes of sudden infant death syndrome. *Paediatr Respir Rev*. 2014;15(4):307-311.

Álvarez-Lafuente R, Aguilera B, Suárez-Mier MP, Morentin B, Vallejo G, Gómez J, *et al.* Detection of human herpesvirus-6, Epstein-Barr virus and cytomegalovirus in formalin-fixed tissues from sudden infant death: A study with quantitative real-time PCR. *For Sci Int*. 2008;178(2):106-111.

Aranda M, Martí C, Bernet M, Gudiol F, Pujol R. Diagnostic utility of postmortem fine-needle aspiration cultures. *Arch Pathol Lab Med*. 1998;122(7):650-655.

Arnestad M, Vege A, Rognum TO. Evaluation of diagnostic tools applied in the examination of sudden unexpected deaths in infancy and early childhood. *Forensic Sci Int*. 2002;125(2-3):262-268.

Bajanowski T, Rolf B, Jorch G, Brinkmann B. Detection of RNA viruses in sudden infant death (SID). *Int J Legal Med*. 2003;117(3):237-240.

Bajanowski T, Vege A, Byard RW, Krous HF, Arnestad M, Bachs L, *et al.* Sudden infant death syndrome (SIDS)--standardised investigations and classification: recommendations. *Forensic Sci Int*. 2007;165(2-3):129-43.

Ball HL, Moya E, Fairley L, Westman J, Oddie S, Wright, J. Bed- and Sofa-Sharing Practices in a UK Biethnic Population. Pediatrics. 2012;129(3): e673-e681.

Baughman KL. Diagnosis of myocarditis: death of Dallas criteria. *Circulation*. 2006;113(4):593-595.

Beckwith J. Defining the Sudden Infant Death Syndrome. *Arch Pediatr Adolesc Med*. 2003;15:286-290.

Berkowitz CD. Sudden infant death syndrome, sudden unexpected infant death, and apparent life-threatening events. *Adv Pediatr*. 2012;59(1):183-208.

Bettelheim KA, Goldwater PN, Dwyer BW, Bourne AJ, Smith DL. Toxigenic Escherichia coli associated with sudden infant death syndrome. *Scand J Infect Dis*. 1990;22(4):467-476.

Blackwell CC, Weir DM, Busuttil A. Infectious agents, the inflammatory responses of infants and sudden infant death syndrome (SIDS). *Mol Med Today*. 1995;1(2):72-78.

Blackwell CC, MacKenzie DA, James VS, Elton RA, Zorgani AA, Weir DM, *et al.* Toxigenic bacteria and sudden infant death syndrome (SIDS): nasopharyngeal flora during the first year of life. *FEMS Immunol Med Microbiol*. 1999;25(1-2):51-58.

Blair PS, Sidebotham P, Berry PJ, Evans M, Fleming PJ. Major epidemiological changes in sudden infant death syndrome: a 20-year population-based study in the UK. Lancet. 2006;367(9507):314-319.

Blair PS, Sidebotham P, Evason-Coombe C, Edmonds M, Heckstall-Smith EM, Fleming P. Hazardous cosleeping environments and risk factors amenable to change: case-control study of SIDS in south west England. *BMJ*. 2009; 339:b3666.

Blood-Siegfried J. The role of infection and inflammation in sudden infant death syndrome. *Immunopharmacol Immunotoxicol*. 2009;31(4):516-523.

Bowles NE, Ni J, Kearney DL, Pauschinger M, Schultheiss HP, McCarthy R, *et al.* Detection of viruses in myocardial tissues by polymerase chain reaction: evidence of adenovirus as a common cause of myocarditis in children and adults. *J Am Coll Cardiol*. 2003;42(3):466-472.

Bright FM, Byard RW, Vink R, Paterson DS. Medullary Serotonin Neuron Abnormalities in an Australian Cohort of Sudden Infant Death Syndrome. *J Neuropathol Exp Neurol*. 2017;76(10): 864-873.

Brooks EG, Gill JR. Testing for Infectious Diseases in Sudden Unexpected Infant Death: A Survey of Medical Examiner and Coroner Offices in the United States. *J Pediatr*. 2015;167(1): 178-182.

Bültmann BD, Klingel K, Sotlar K, Bock CT, Baba HA, Sauter M, *et al.* Fatal parvovirus B19-associated myocarditis clinically mimicking ischemic heart disease: an endothelial cell-mediated disease. *Hum Pathol.* 2003;34(1):92–95.

Burger MC, Dempers JJ, de Beer C. Profiling the approach to the investigation of viral infections in cases of sudden unexpected death in infancy in the Western Cape Province, South Africa. *Forensic Sci Int.* 2014;239:27–30.

Byard RW, Krous HF. Sudden infant death syndrome: overview and update. *Pediatr Dev Pathol*. 2003;6:112-127.

Byard RW, Marshall D. An audit of the use of definitions of sudden infant death syndrome (SIDS). *J Forensic Leg Med*. 2007;14(8):453-455.

Calvo C, García-García ML, Sanchez-Dehesa R, Román C, Tabares A, Pozo F, *et al.* Eight Year Prospective Study of Adenoviruses Infections in Hospitalized Children. Comparison with Other Respiratory Viruses. *PLoS One*. 2015;10(7):e0132162.

Canter CE, Simpson KE. Diagnosis and treatment of myocarditis in children in the current era. Circulation. 2014;129(1):115-128.

Carballal G, Videla C, Misirlian A, Requeijo PV, Aguilar Mdel C. Adenovirus type 7 associated with severe and fatal acute lower respiratory infections in Argentine children. *BMC Pediatr*. 2002;2:6.

Chen SP, Huang YC, Chiu CH, Wong KS, Huang YL, Huang CG, *et al.* Clinical features of radiologically confirmed pneumonia due to adenovirus in children. *J Clin Virol*. 2013;56(1):7–12.

Colson ER, Willinger M, Rybin D, Heeren T, Smith LA, Lister G, *et al.* Trends and factors associated with infant bed sharing, 1993-2010: the National Infant Sleep Position Study. *JAMA Pediatr*. 2013;167(11):1032-1037.

Condon LM, Cederberg LE, Rabinovitch MD, Liebo RV, Go JC, Delaney AS, *et al.* Age-specific prevalence of Epstein-Barr virus infection among Minnesota children: effects of race/ethnicity and family environment. *Clin Infect Dis*. 2014;59(4):501-508.

Cutz E, Jackson A. *Airway inflammation and peripheral chemoreceptors. In Sudden Infant Death Syndrome, Problems, Progress and Possibilities ed Byard R and Krous HF*. 2001;London: Arnold.

Dancea A, Côté A, Rohlicek C, Bernard C, Oligny LL. Cardiac pathology in sudden unexpected infant death. *J Pediatr*. 2002;141(3):336-342.

de Crom SCM, Rossen JWA, van Furth AM, Obihara CC. Enterovirus and parechovirus infection in children: a brief overview. *Eur J of Pediatr*. 2016;175(8):1023-1029.

Deback C, Agbalika F, Scieux C, Marcelin AG, Gautheret-Dejean A, Cherot J, *et al.* Detection of human herpesviruses HHV-6, HHV-7 and HHV-8 in whole blood by real-time PCR using the new CMV, HHV-6, 7, 8 R-gene™ kit. *J Virol Methods*. 2008;149(2):285-91.

Dempers JJ, Coldrey J, Burger EH, Thompson V, Wadee SA, Odendaal HJ, *et al.* The Institution of a Standardized Investigation Protocol for Sudden Infant Death in the Eastern Metropole, Cape Town, South Africa. *J Forensic Sci*. 2016;61(6):1508-1514.

Dennert R, Crijns HJ, Heymans S. Acute viral myocarditis. *Eur Heart J*. 2008;29(17): 2073-2082.

Dettmeyer R, Baasner A, Haag C, Bruch S, Schlamann M. Immunohistochemical and molecular-pathological diagnosis of myocarditis in cases of suspected sudden infant death syndrome (SIDS)--a multicenter study. *Leg Med (Tokyo)*. 2009;11 Suppl 1:S124-127.

Dettmeyer R, Baasner A, Schlamann M, Haag C, Madea B. Coxsackie B3 Myocarditis in 4 Cases of Suspected Sudden Infant Death Syndrome: Diagnosis by Immunohistochemical and Molecular-Pathologic Investigations. *Pathol Res Pract*. 2002;198(10):689-696.

Dettmeyer R, Baasner A, Schlamann M, Padosch SA, Haag C, Kandolf R, *et al.* Role of virus-induced myocardial affections in sudden infant death syndrome: a prospective postmortem study. *Pediatr Res*. 2004;55(6):947-952.

Dettmeyer R, Sperhake JP, Müller J, Madea B. Cytomegalovirus-induced pneumonia and myocarditis in three cases of suspected sudden infant death syndrome (SIDS): diagnosis by immunohistochemical techniques and molecularpathologic methods. *Forensic Sci Int*. 2008;174(2-3):229-233.

Donoso Mantke O, Nitsche A, Meyer R, Klingel K, Niedrig M. Analysing myocardial tissue from explanted hearts of heart transplant recipients and multi-organ donors for the presence of parvovirus B19 DNA. *J Clin Virol*. 2004;31(1):32-39.

du Toit-Prinsloo L, Dempers J, Verster J, Hattingh C, Nel H, Brandt VD, *et al.* Toward a standardized investigation protocol in sudden unexpected deaths in infancy in South Africa: a multicenter study of medico-legal investigation procedures and outcomes. *Forensic Sci Med Pathol*. 2013;9(3):344-350.

du Toit-Prinsloo L, Dempers JJ, Wadee SA, Saayman G. The medico-legal investigation of sudden, unexpected and/or unexplained infant deaths in South Africa: where are we--and where are we going? *Forensic Sci Med Pathol*. 2011;7(1):14-20.

Dwyer T, Ponsonby AL. The decline of SIDS: a success story for epidemiology. *Epidemiology*. 1996;7(3): 323-325.

Eisenfeld L, Ermocilla R, Wirtschafter D, Cassady G. Systemic bacterial infections in neonatal deaths. *Am J Dis Child*. 1983;137(7):645-649.

Elhaik E. A "Wear and Tear" Hypothesis to Explain Sudden Infant Death Syndrome. *Front Neurol.* 2016;7:180.

Ferone EA, Berezin EN, Durigon GS, Finelli C, Felicio MC, Storni JG, *et al.* Clinical and epidemiological aspects related to the detection of adenovirus or respiratory syncytial virus in infants hospitalized for acute lower respiratory tract infection. *J Pediatr (Rio J)*. 2014;90(1):42–49.

Filiano J, Kinney H. A perspective on neuropathologic findings in victims of the sudden infant death syndrome: the triple-risk model. *Biol Neonate*. 1994;65 (3-4):194-197.

Fleming PJ, Blair PS, Pease A. Sudden unexpected death in infancy: aetiology, pathophysiology, epidemiology and prevention in 2015. *Arch Dis Child*. 2015;100:984-988.

Freund MW, Kleinveld G, Krediet TG, van Loon AM, Verboon-Maciolek MA. Prognosis for neonates with enterovirus myocarditis. *Arch Dis Child Fetal Neonatal Ed*. 2010;9(3): F206-F212.

Freymuth F, Vabret A, Cuvillon-Nimal D, Simon S, Dina J, Legrand L. Comparison of multiplex PCR assays and conventional techniques for the diagnostic of respiratory virus infections in children admitted to hospital with an acute respiratory illness. *J Med Virol*. 2006;78(11):1498-1504.

Gaaloul I, Riabi S, Evans M, Hunter T, Huber S, Aouni M. Postmortem diagnosis of infectious heart diseases: A mystifying cause of Sudden Infant Death. *Forensic Sci Int*. 2016;262:166-172.

Gandhi MK, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation and emerging treatments. *Lancet Infect Dis*. 2004;4(12):725-738.

Ghelani SJ, Spaeder MC, Pastor W, Spurney CF, Klugman D. Demographics, trends, and outcomes in pediatric acute myocarditis in the United States, 2006 to 2011. *Circ Cardiovasc Qual Outcomes*. 2012;5(5): 622-627.

Gilbert R, Salanti G, Harden M, See S. Infant sleeping position and the sudden infant death syndrome: systematic review of observational studies and historical review of recommendations from 1940 to 2002. *Int J Epidemiol*. 2005;34(4):874-887.

Goldstein RD, Trachtenberg FL, Sens MA, Harty BJ, Kinney HC. Overall Postneonatal Mortality and Rates of SIDS. *Pediatrics*. 2016;137(1).

Goldwater PN. SIDS pathogenesis: pathological findings indicate infection and inflammatory responses are involved. *FEMS Immunol Med Microbiol*. 2004;42(1):11-20.

Goutas N, Konstantinidou MK, Vlachodimitropoulos D, Konstantinidou A, Kontogiannis T, Papadodima S, *et al.* Trends in infant and child mortality. *Open Forensic Sci J*. 2011;4:1-11.

Griffiths PD. Herpesviruses. *Medicine*. 2009;37(12):668-672.

Hakeem GF, Oddy L, Holcroft CA, Abenhaim HA. Incidence and determinants of sudden infant death syndrome: a population-based study on 37 million births. *World J Pediatr*. 2015;11(1):41-7.

Harrison LM, Morris JA, Telford DR, Brown SM, Jones K. The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunol Med Microbiol*. 1999;25(1-2): 19-28.

Hauck FR, Herman SM, Donovan M, Iyasu S, Merrick Moore C, Donoghue E, *et al.* Sleep environment and the risk of sudden infant death syndrome in an urban population: the Chicago Infant Mortality Study. *Pediatrics*. 2003;111(5 Pt 2):1207-1214.

Hawkes MT, Vaudry W. Nonpolio enterovirus infection in the neonate and young infant. *Paediatr Child Health*. 2005;10(7):383-388.

Hazra A, Gogtay N. Biostatistics Series Module 7: The Statistics of Diagnostic Tests. *Indian J Dermatol.* 2017;62(1):18-24.

Heininger U, Kleemann WJ, Cherry JD, Sudden Infant Death Study Group. A controlled study of the relationship between Bordetella pertussis infections and sudden unexpected deaths among German infants. *Pediatrics*. 2004;114(1):e9–15.

Highet AR, Berry AM, Bettelheim KA, Goldwater PN. Gut microbiome in sudden infant death syndrome (SIDS) differs from that in healthy comparison babies and offers an explanation for the risk factor of prone position. *Int J Med Microbiol*. 2014;304(5-6):735–741.

Highet AR, Berry AM, Goldwater PN. Novel hypothesis for unexplained sudden unexpected death in infancy (SUDI). *Arch Dis Child*. 2009;94(11):841-843.

Highet AR. An infectious aetiology of sudden infant death syndrome. *J Appl Microbiol*. 2008;105(3):625-635.

Hoffman HJ, Hunter JC, Damus K, Pakter J, Peterson DR, van Belle G, *et al.* Diphtheria-tetanus-pertussis immunization and sudden infant death: results of the National Institute of Child Health and Human Development Cooperative Epidemiological Study of Sudden Infant Death Syndrome risk factors. *Pediatrics.* 1987;79(4):598-611.

Hunt CE, Hauck FR. Sudden infant death syndrome. *CMAJ*. 2006;174(13):1861-1869.

Hunt CE, Darnall RA, McEntire BL, Hyma BA. Assigning cause for sudden unexpected infant death. *Forensic Sci Med Pathol*. 2015;11(2):283-288.

Ibeziako NS, Ibekwe RC , Ibe BC. Infant Sleeping Environment in South-Eastern Nigeria (Sleeping Place and Sleeping Position): A Preliminary Survey. *J Trop Med*. 2009:283046.

Inwald D, Franklin O, Cubitt D, Peters M, Goldman A, Burch M. Enterovirus myocarditis as a cause of neonatal collapse. *Arch Dis Child Fetal and Neonatal Ed*. 2004;89(5): F461-F462.

Jenista JA, Powell KR, Menegus MA. Epidemiology of neonatal enterovirus infection. *J Pediatr*. 1984;104(5):685-690.

Jin O, Sole M, Butany JW, Chia WK, McLaughlin PR, Liu P, *et al.* Detection of enterovirus RNA in myocardial biopsies from patients with myocarditis and cardiomyopathy using gene amplification by polymerase chain reaction. *Circulation*. 1990;82(1):8-16.

Jin Y, Zhang R, Xie Z, Yan KL, Gao HC, Song J *et al.* Prevalence of adenovirus in children with acute respiratory tract infection in Lanzhou, China. *Virology J*. 2013;10:271.

Kashiwagi Y, Kawashima H, Kanetaka Y, Ioi H, Takekuma K, Hoshika A, *et al.* Sudden infant death syndrome due to parainfluenza virus 2 associated with hemophagocytic syndrome. *J Infect*. 2004;49(4):329–332.

Kasolo FC, Mpabalwani E, Gompels UA. Infection with AIDS-related herpesviruses in human immunodeficiency virus-negative infants and endemic childhood Kaposi's sarcoma in Africa. *J Gen Virol*. 1997;78(4):847-55.

Katwinkel J (Chair), Brooks JG, Keenan ME, Malloy M. Changing concepts of sudden infant death syndrome: implications for infant sleeping environment and sleep position. American Academy of Pediatrics. Task Force on Infant Sleep Position and Sudden Infant Death Syndrome. *Pediatrics*. 2000;105(3 Pt 1):650-656.

Kawana R. Cardiovascular diseases due to viruses. *Heart Vessels Suppl*. 1985;1:91-96.

Kennedy H (Chair). *Sudden unexpected death in infancy and childhood. A multi-agency guideline for care and investigation. The report of a working group convened by The Royal College of Pathologists and endorsed by The Royal College of Paediatrics and Child Health*. 2016;London: The Royal College of Pathologists.

Kibel MA, Molteno CD, De Dekker R. Cot death controversies. *S Afr Med J*. 2005;95(11):853-857.

Kinney HC, Thach BT. Sudden infant death syndrome. *N Engl J Med*. 2009; 361(8): 795–805.

Koehler SA. The importance of a forensics investigation of sudden infant death syndrome: recommendations for developing, low and middle income countries. *Acta Med Acad*. 2010;39(2): 165-174.

Koepsell SA, Anderson DR, Radio SJ. Parvovirus B19 is a bystander in adult myocarditis. *Cardiovasc Pathol*. 2012;21(6): 476–481.

Krous HF. Sudden Unexpected Death in Infancy and the Dilemma of Defining the Sudden Infant Death Syndrome. *Curr Pediatr Rev*. 2010;6(1):5-12.

Krous HF, Ferandos C, Masoumi H, Arnold J, Haas EA, Stanley C, *et al.* Myocardial inflammation, cellular death, and viral detection in sudden infant death caused by SIDS, suffocation, or myocarditis. *Pediatr Res*. 2009;66(1):17-21.

la Grange H, Verster J, Dempers JJ, de Beer C. Review of immunological and virological aspects as contributory factors in Sudden Unexpected Death in Infancy (SUDI). *Forensic Sci Int*. 2014;245:12-16.

Leach CT. *Human Herpesviruses 6 and 7*. Congenital and Perinatal Infections. 2016; Totowa, NJ: Humana Press: 101-109.

Linde A, Falk KI. *Epstein-Barr Virus. In P. R.* Murray (Ed.), Manual of clinical microbiology (9th ed). 2007;Washington D.C:ASM Press:1564-1573.

Livesey A. A multiagency protocol for responding to sudden unexpected death in infancy: descriptive study. *BMJ*. 2005;330(7485):227-228.

Mage DT, Donner EM. A. Unifying Theory for SIDS. *Int J Pediatr*. 2009;2009:368270.

Mage DT, Donner EM. Is excess male infant mortality from sudden infant death syndrome and other respiratory diseases X-linked? *Acta Paediatr*. 2014;103(2):188-93.

Mage DT, Latorre ML, Jenik AG, Donner EM. The Role of Respiratory Infection in Sudden Infant Death Syndrome (SIDS). *Scand J For Sci*. 2016;22(1):15-20.

Mage DT. Seasonal variation of sudden infant death syndrome in Hawaii. *Epidemiol Community Health*. 2004;58(11):912-916.

Mahfoud F, Gärtner B, Kindermann M, Ukena C, Gadomski K, Klingel K, *et al.* Virus serology in patients with suspected myocarditis: utility or futility? Eur Heart J. 2011;32(7):897-903.

Martin AB, Webber S, Fricker FJ, Jaffe R, Demmler G, Kearney D, *et al.* Acute myocarditis. Rapid diagnosis by PCR in children. *Circulation*. 1994;90(1):330-339.

Mathews TJ, MacDorman MF, Thoma ME. Infant Mortality Statistics From the 2013 Period Linked Birth/Infant Death Data Set. *Natl Vital Stat Rep*. 2015; 64(9):1-30.

May PA, Hamrick KJ, Corbin DK, Hasken J, Marais A-S, Brooke LE, *et al.* Dietary intake, nutrition, and Fetal Alcohol Spectrum Disorders in the Western Cape Province of South Africa. *Reprod Toxicol*. 2014;46:31-39.

Mitchell EA, Krous HF. Sudden unexpected death in infancy: A historical perspective. *J Paediatr Child Health*. 2015;51(1): 108-112.

Mitchell EA, Stewart AW, Scragg R, Ford RP, Taylor BJ, Becroft DM, *et al.* Ethnic differences in mortality from sudden infant death syndrome in New Zealand. *BMJ*. 1993;306(6869):13-6.

Moon RY, Horne RS, Hauck FR. Sudden infant death syndrome. *Lancet*. 2007;370(9598):1578-1587.

Nannan N, Dorrington RE, Laubscher R, Zinyakatira N, Prinsloo M, Darikwa TB, *et al.* Under-5 mortality statistics in South Africa: Shedding some light on the trends and causes 1997-2007. 2012; Cape Town: South African Medical Research Council.

Ndu IK. Sudden infant death syndrome: an unrecognized killer in developing countries. *Pediatric Health Med Ther*. 2016;7:1-4.

Nielsen TS, Hansen J, Nielsen LP, Baandrup UT, Banner J. The presence of enterovirus, adenovirus, and parvovirus B19 in myocardial tissue samples from autopsies: an evaluation of their frequencies in deceased individuals with myocarditis and in non-inflamed control hearts. *Forensic Sci Med Pathol*. 2014;10(3):344-350.

Pankuweit S, Moll R, Baandrup U, Portig I, Hufnagel G, Maisch B. Prevalence of parvovirus B19 genome in endomyocardial biopsy specimens. *Hum Pathol*. 2003;34(5):497-503.

Pearce JL, Bettelheim KA, Luke RK, Goldwater PN. Serotypes of Escherichia coli in sudden infant death syndrome. *J Appl Microbiol*. 2010;108(2):731-735.

Ponsonby AL, Dwyer T, Gibbons LE, Cochcane JA, Wang YG. Factors Potentiating the Risk of Sudden Infant Death Syndrome Associated with the Prone Position. *N Engl J Med*. 1993;329(6):377-382.

Rambaud C, Cieuta C, Canioni D, Rouzioux C, Lavaud J, Hubert P, *et al.* Cot death and myocarditis. *Cardiol Young*. 1992;2(3):266-271.

Rambaud C, Guibert M, Briand E, Grangeot-Keros L, Coulomb-L'Herminé A, Dehan M. Microbiology in sudden infant death syndrome (SIDS) and other childhood deaths. *FEMS Immunol Med Microbiol*. 1999;25(1-2):59-66.

Raza MW, Blackwell CC. Sudden infant death syndrome, virus infections and cytokines. *FEMS Immunol Med Microbiol*. 1999;25(1-2):85-96.

Sartorius BK, Sartorius K, Chirwa1 TF, Fonn S. Infant mortality in South Africa--distribution, associations and policy implications, 2007: an ecological spatial analysis. *Int J Health Geogr*. 2011;10:61.

Schenk T, Enders M, Pollak S, Hahn R, Huzly D. High prevalence of human parvovirus B19 DNA in myocardial autopsy samples from subjects without myocarditis or dilative cardio- myopathy. *J Clin Microbiol*. 2009;47(1):106–110.

Schnitzer PG, Covington TM, Dykstra HK. Sudden unexpected infant deaths: sleep environment and circumstances. *Am J Public Health*. 2012; 102(6):1204-1212.

Shapiro-Mendoza CK, Camperlengo L, Ludvigsen R, Cottengim C, Anderson RN, Andrew T, *et al.* Classification system for the Sudden Unexpected Infant Death Case Registry and its application. *Pediatrics*. 2014;134(1):e210-219.

Shauer A, Gotsman I, Keren A, Zwas DR, Hellman Y, Durst R, *et al.* Acute viral myocarditis: current concepts in diagnosis and treatment. *Isr Med Assoc J*. 2013;15(3):180-185.

Spinelli J, Collins-Praino L, Van Den Heuvel C, Byard RW. Evolution and significance of the triple risk model in sudden infant death syndrome. *J Paediatr Child Health*. 2017;53(2):112-115.

Statistics South Africa. Statistical Release P0309.3. *Mortality and causes of death in South Africa, 2014: Findings from death notification*. 2015; Pretoria. Available from: http://www.statssa.gov.za/publications/P03093/P030932014.pdf.

Towbin J, Ni J, Demmler G, Martin A, Kearney D, Bricker JT. Evidence for adenovirus as common cause of myocarditis in children using polymerase chain reaction (PCR). *Pediatr Res*. 1993;33:27A.

Tschöpe C, Bock CT, Kasner M, Noutsias M, Westermann D, Schwimmbeck PL, *et al*. High prevalence of cardiac parvovirus B19 infection in patients with isolated left ventricular diastolic dysfunction. *Circulation*. 2005; 111(7):879-86.

Tsokos M, Püschel K. Postmortem bacteriology in forensic pathology: diagnostic value and interpretation. *Leg. Med (Tokyo)*. 2001;3(1):15-22.

Uhl TL. Viral myocarditis in children. *Crit Care Nurse*. 2008;28(1):42-63; quiz 64.

Vos T, Barber RM, Bell B, Bertozzi-Villa A, Biryukov S, Bolliger I, *et al* (Collaborators). Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. GBD 2013 Mortality and Causes of Death Collaborators. *Lancet*. 2015;385(9963):743-800.

Waaijenborg S, Hahné SJ, Mollema L, Smits GP, Berbers GA, van der Klis FR, *et al*. Waning of maternal antibodies against measles, mumps, rubella, and varicella in communities with contrasting vaccination coverage. *J Infect Dis*. 2013;208(1):10-16.

Weber MA, Ashworth MT, Risdon RA, Hartley JC, Malone M, Sebire NJ. The role of post-mortem investigations in determining the cause of sudden unexpected death in infancy. *Arch Dis Child*. 2008a;93(12):1048-1053.

Weber MA, Ashworth MT, Risdon RA, Malone M, Burch M, Sebire NJ. Clinicopathological features of paediatric deaths due to myocarditis: an autopsy series. *Arch Dis Child*. 2008b;93(7):594-598.

Weber MA, Hartley JC, Ashworth MT, Malone M, Sebire NJ. Virological investigations in sudden unexpected deaths in infancy (SUDI). *Forensic Sci Med Pathol*. 2010a;6(4):261-267.

Weber MA, Hartley JC, Brooke I, Lock PE, Klein NJ, Malone M, *et al*. Post-mortem interval and bacteriological culture yield in sudden unexpected death in infancy (SUDI). *Forensic Sci Int*. 2010b:20;198(1-3):121-5.

Weber MA, Sebire NJ. Molecular Diagnostic Techniques in the Post-Mortem Investigation of Sudden Unexpected Infant Deaths: Current and Future Applications. *Open Pathol J*. 2010;4:110-119.

Wedgwood RJ. *Review of USA experience. In: Camps FE, Carpenter RG, eds. Sudden and Unexpected Death in Infancy (Cot Deaths).* 1972;England: Wright.

Yagmur G, Ziyade N, Elgormus N, Das T, Sahin MF, Yildirim M, *et al*. Postmortem diagnosis of cytomegalovirus and accompanying other infection agents by real-time PCR in cases of sudden unexpected death in infancy (SUDI). *J For Leg Med*. 2016;38:18-23.

Young NS, Brown KE. Parvovirus B19. *N Engl J Med*. 2004;350(6):586-597.

Zorgani A, Essery SD, Madani O, Bentley AJ, James VS, MacKenzie DA, *et al.* Detection of pyrogenic toxins of Staphylococcus aureus in sudden infant death syndrome. *FEMS Immunol Med Microbiol*. 1999;25(1-2):103–108.

APPENDICES

Appendix A: SB buffer composition

20X SB buffer:

- 8 g sodium hydroxide
- 45 g Boric Acid

Add milliQ water up to 1L. Mix well.

Appendix B: Amplicillin composition

100 mg/ml Ampicillin:

- 1 g of sodium ampicillin

Dissolve in sterile nuclease-free water to make a final volume of 10 ml

Store at -20°C in 1 ml aliquots

Appendix C: Sample of SUDI questionnaire

DEPARTMENT of HEALTH
Provincial Government of the Western Cape

FPS006(b)

FORENSIC PATHOLOGY SERVICE

SUDI (Complete If A Baby Should Suddenly And Unexpectedly Die)

FPS laboratory_____ WC _____

Name of baby _____

Part 1: Scene Questionnaire and Observations

Date: Time: Name of Forensic officer:

Section A. **Who gives the history/ information in this case e.g. mother/father/granny/grandpa/other relative(give details)**	
Name:	Relationship:
Address:	Contact telephone number:
ID Number:	
Infants full name:	
Home Address:	
Age of Baby	Date of birth:
Race:	Sex:

Section B **Person(s) at/called to the scene and relationship**		
Name/relationship	Date	Time
Name/relationship	Date	Time
Name/relationship	Date	Time
Police response/name	Date	Time
Paramedic response/name	Date	Time
When was the death certified/by whom	Date	Time
If the baby was taken to hospital		
Name of hospital		

WC_____

Date of arrival: Time of arrival:			
Name of doctor seen / declared death:			
Comment: Get copies of doctors notes			
Was resuscitation done on the baby by the paramedic or the doctors at the hospital?			
Section C **Household environment:**			
Place where baby lives: house shack other –			
Number of bedrooms Is the room in which the baby is found well ventilated?			
Odour(s) present in the room the baby slept in?		Yes	No
Peeling paint in the room the baby slept in?		Yes	No
Fungal growth (mould) in the room the baby slept in?		Yes	No
Did people smoke cigarettes in the room the baby slept?		Yes	No
Are there pets in the house? If yes – type and number:		Yes	No
Did caregiver use alcohol or drugs on the night baby died?		Yes	No
Was there a heater or open fire or galley blik or other heating device in room where baby slept?		Yes	No
In what position was the baby found lying? Has the baby been moved? Were there any covers/ clothing etc over the baby's head?			
Was the baby squashed/wedged between anything (object)?		Yes	No
Was there overlaying (someone lay on top of the baby)?		Yes	No
Comments from forensic officer who attended the scene:			

2

WC_____

Part 2: Facility Questionnaire

Date: ***Time:*** ***Name of Forensic officer:***

Section D
Circumstances of death / details about events before death

1. When was the baby last seen alive				Date	Time
2. Who last saw the baby alive					
3. When was the baby found dead				Date	Time
4. Who found the baby dead at the scene					
5. Was the baby ill?				Yes	No
a) If yes – What was wrong and for how long?					
b) Was the baby taken to the doctor or pharmacy or clinic or traditional healer for the illness? When (date and time)?				Yes	No
c) If not, why not:					
d) Was the baby admitted to a hospital or clinic for the illness: When (date and time)?				Yes	No
e) If not, do you know why not?					
f) What medication was given (names please					
6. Where was the baby found dead	Bed	Couch	Cot	Floor	Other
Other:					
7. Did the baby sustain any injuries – eg by falling or being hit: If yes:				Yes	No
a) When did it happen?					
b) How did it happen?					
c) Where did it happen?					
d) What did the caretaker do about it?					

WC_____

8. a) On what was the baby placed to sleep	Bed with a pillow	Bed without a pillow	Couch with a pillow	Couch without pillow	Cot with pillow
	Cot without pillow	Floor with pillow	Floor without pillow	Other	
b) If placed on a bed/cot, what was the mattress type			Foam rubber	Inner spring	Other
c) Was the mattress covered with a blanket or sheet				Yes	No
d) What position was the baby placed when put to sleep?		Back	Stomach	Side	Other
Other -					
e) what was used to cover the baby: List items					
e) What position was the baby found dead?		Back	Stomach	Side	Other
Other –					
f) Has the baby been moved?				Yes	No
g) Face position when the baby was found dead			To the left	To the right	Face down
			Face up	Unknown	
h) Face and or chest squashed / wedged between any object(s) when the baby was found dead?			Yes	No	Unknown
If yes – details please –					
i) Was the nose and mouth of the baby covered by anything – eg blankets or anything else			Yes	No	Unknown
j) Were there other items in contact with the baby – eg pillow			Yes	No	Unknown
k) Did the baby use a Dummy (pacifier)?				Yes	No
l) Did the baby sleep in the same bed as the mother?				Yes	No
m) Did the baby sleep in her arms?				Yes	No
n) Did the baby sleep on her chest?				Yes	No
o) Did the baby sleep with the mother on a couch?				Yes	No
p) How many other people slept on the same bed as the baby at the time the baby died?					
q) Was anyone found on top of the baby while in the bed (Overlaying)?				Yes	No
r) Was the window where the baby slept on the day /night the baby died				Open	Closed
s) Did the mother or anyone in the house smoke while the baby slept on the night/day of death?					
t) When was the baby last fed?				Date	Time

WC_____

u) Did the mother/caregiver use alcohol before going to bed with the baby on the night/day the baby was found dead? If yes, how much?			Yes	No
v) Did the mother/caregiver use drugs before going to bed with the baby on the night/day the baby was found dead? If yes, what drugs?			Yes	No
w) Did the mother/caregiver give the baby medication on the night/day of death? If yes, name of medication:			Yes	No
Section E **About the baby**				
1. Where was the baby born?	Hospital	Clinic	Home	Other
Name of hospital/clinic/other				
2. How was the baby born?			Normal vaginal delivery	Caesarian section
3. How much did the baby weigh at birth?				
4. Was the baby		Premature	Full term	Post dates (Overdue)
5. If the baby was premature, how premature was it?				
6. Did the baby receive Kangaroo care (KMC)			Yes	No
7. Did the mother carry the baby on her back?			Yes	No
8. Was the baby		Breast fed	Bottle/formula fed	Both breast and bottle fed
If formula, name of the milk –				
9. Was boiling water used to make the bottle?			Yes	No
10. What other food was use to feed the baby?				
11. Does the mother have the clinic card?			Yes	No
If yes – keep the card for the pathologist. If no – ask the mother to bring it to the facility				
12. Was the baby sick before it died?			Yes	No
If yes	<24h	>24h	> 2 weeks	Never
a) Did the baby have a cold/ runny nose?				
b) was the baby coughing?				
c) did the baby have diarrhea (runny tummy)?				

WC_____

d) Was the baby unusually restless / irritable?				
e) Was the baby crying more than usual?				
f) Was there a difference /change in the appetite / feeding?				
g) Was the baby vomiting?				
h) Any fits / seizures?				
i) did the baby have a fever / showed increased sweating?				
j) Was the baby listless? (floppy)				
k) did the baby turn blue?				
13. Was the now deceased baby taken to	Hospital	clinic	doctor	Pharmacy
	Traditional healer	Other		
14. Did the baby come in contact with someone who is sick in the past two weeks?			Yes	No
If yes – who?				
15. Did the baby ever suddenly stopped breathing?		Yes	No	Unknown
16. When was the baby's last vaccination?				
18. Is the baby known to be allergic to anything?		Yes	No	Unknown
If yes, what?				
19. Did the family visit another country prior to the death of the baby?			Yes	No
If yes, give details				
20. Was the baby admitted to hospital in the past week before the death?			Yes	No
a) If yes, for how long and where:				
b) Why?				
c) Discharge date?				
d) Condition of baby after discharge:				
e) Medication after discharge from the hospital (names please)				
21. Was the baby taken to a traditional healer?			Yes	No
a)If yes, date when the baby was taken to the healer:				

6

WC_____

b) What was given?		
c) Ask for the medication to be given to the pathologist.		
d) Condition of the baby after going to the healer?		
21. What did the baby wear when it died? (list clothing)		
Section F **About the mother**		
1. Is the mother	Married	Single
2. Is the mother employed?	Yes	No
3. Age of the mother?		
4. What standard of schooling did she achieve?		
5. Was she on contraception before she fell pregnant?	Yes	No
6. Did she take iron and vitamin tablets during her pregnancy?	Yes	No
7. Did she receive antenatal care?	Yes	No
8. Did the mother have diabetes in pregnancy?	Yes	No
9. Did the mother have high blood pressure in pregnancy?	Yes	No
10. Did the mother gain weight adequately in pregnancy?	Yes	No
11. Was she diagnosed with any illness during the pregnancy eg. HIV?	Yes	No
12. Was the mother on any medication during the pregnancy?	Yes	No
If yes, what medication:		
13. Were there any difficulties during the delivery?	Yes	No
If yes, what?		
14. Were there any problems with the baby after the delivery?	Yes	No
If yes, what?		

7

WC_____

15. Was any specific instruction given about specific health care for the baby?			Yes	No
If yes, what?				
16. Was she depressed after the pregnancy?			Yes	No
17. Did she get any treatment?			Yes	No
18. How many babyren does she have?				
19. How old are they?				
20. Are they healthy?			Yes	No
21. Do any of the babyren have learning disability?			Yes	No
22. Do the living babyren have the same father as the deceased baby?			Yes	No
23. Does she look after the baby?			Yes	No
24. If not, who looks after the baby?				
25. Why is the mother unable to look after the baby?				
26. Did the mother smoke during the pregnancy?			Yes	No
If yes, how many per day?				
27. Did the mother drink during the pregnancy?			Yes	No
a) What did she drink?	Beer	Wine	Spirits	Other
b) how much did she drink?	Every day		Now and again	Weekends
1 glass	Every day		Now and again	Weekends
> 1 glass	Every day		Now and again	Weekends
A bottle of alcohol	Every day		Now and again	Weekends
> 1 bottle	Every day		Now and again	Weekends
28. Does she use drugs?			Yes	No
a) If yes, what drugs does she use?	Tik	Cocaine / Heroin	Mandrax	Other
b) How often does she use drugs?	Every day		Now and again	Weekends
29. Does the mother smoke after the pregnancy?			Yes	No
30. Does the mother know that smoking harms the unborn baby?			Yes	No
31. Does the husband/partner drinks?			Yes	No

8

WC_____

32. Does the mother drink after the pregnancy?	Yes	No
33. Do the parents of the mother drink?	Yes	No
34. Does the mother know that alcohol harms the unborn baby?	Yes	No
35. Did the mother have a previous baby that died suddenly?	Yes	No
a) If yes, how many died?		
b) At what age?		
c) Was a PM done?	Yes	No
If yes, where was it done?		
36. Did the mother have a previous stillbirth?	Yes	No

Section G
Household environment

1. Place where the baby lives	House		Shack	Other
2. Number of bedrooms?				
3. Is the room in which the baby was found well ventilated?			Yes	No
4. Odour(s) present in the room the baby slept in?			Yes	No
5. Peeling paint in the room the baby slept in?			Yes	No
6. Fungal growth (mould) in the room the baby slept in?			Yes	No
7. Are there pets in the house?			Yes	No
If yes, type and number:				
8. Was the following in the room where the baby slept to heat the room?	Electric heater	"Galley"	Fire	Other
Describe other –				
9. Number of adults in the dwelling?				
10. Number of babyren in the dwelling?				
11. Total number of people in the dwelling?				
12. Estimated monthly income?				
13. Number of smokers in the dwelling?				
14. Are there mentally retarded/ challenged people in the dwelling?			Yes	No

9

FPS006(b)

WC_____

COMMENTS TO PATHOLOGIST FROM THE FORENSIC OFFICER WHO ATTENDED THE SCENE AND INTERVIEWED DURING ID PROCESS:

ITEMS RETAINED AT THE SCENE OR FROM THE MOTHER DURING INTERVIEW

Date:

Signature / Thumbprint of deponent

I certify that the above statement was taken down by myself and that the deponent has acknowledged that he / she knows and understands the contents hereof.

Date _____ Time: _____

Place: _____

_____

Department of Health
Forensic Pathology Laboratory

10

Appendix D: Results for PCR assays

PCR and histology results for the SUDI cases

SUDI case	B19	EV	Histology
001	+	+	-
002	+	+	-
003	+	+	-
004	+	-	-
005	+	+	-
006	+	+	-
007	+	+	-
008	-	+	-
009	+	+	-
010	+	-	-
011	+	-	-
012	+	-	-
013	+	+	-
014	+	-	-
015	+	+	+
016	+	+	-
017	+	+	-
018	+	+	-
020	+	+	-
021	+	+	-
022	+	+	-
023	+	+	-
024	+	-	-
025	+	-	-
026	+	-	-
027	+	-	-
028	+	-	-
029	+	-	-
030	+	-	-
031	+	+	-
032	+	-	+
033	+	+	-
034	+	+	-
035	+	+	-
036	+	+	-
037	+	-	-
038	+	-	-
039	+	+	+
040	+	+	+
041	+	+	-

042	+	-	-
043	+	+	-
044	+	+	-
045	+	+	+
046	+	+	-
047	+	+	-
048	+	-	-
049	+	+	-
050	+	+	+
051	+	-	-
052	+	+	-
053	+	-	-
054	+	-	-
055	+	-	-
056	+	-	-
057	+	-	-
058	+	-	-
059	+	-	-
060	+	-	-
061	+	-	-
062	+	-	-
063	+	-	-
064	-	-	-
065	-	-	-
066	+	-	-
067	+	-	-
068	+	-	-
069	+	-	-
070	+	-	-
071	+	-	-
072	+	-	-
073	+	+	-
074	+	-	-
075	+	+	-
076	+	-	-
077	+	-	-
078	+	+	-
079	+	-	-
080	+	-	-
081	+	+	-
082	+	+	-
083	+	+	-
084	+	-	-
085	+	-	-
086	+	-	-

087	+	+	-
088	+	-	-
089	+	-	-
090	+	-	-
091	+	-	-
092	+	-	-
093	+	-	-
094	+	-	-
095	+	+	-
096	-	-	-
097	+	-	-
098	+	-	-
099	+	-	-
100	+	-	-
101	+	-	-
102	+	-	-
103	+	-	-
104	+	-	-
105	+	-	-
106	+	-	-
107	+	+	-
108	+	-	-
109	+	-	-
110	+	-	-
111	+	-	-
112	+	-	-
113	+	-	-
114	+	+	-
115	+	-	-
116	+	-	-
117	+	-	-
118	+	-	-
119	+	-	-
120	+	-	-
121	-	-	-
122	+	-	+
123	-	-	+
124	+	-	-
125	+	-	-
126	+	-	-
127	+	-	+
128	+	-	-
129	+	+	-
130	+	-	-
131	+	-	-

132	+	+	-
133	+	+	-
134	+	-	-
135	+	-	-
136	+	-	-
137	+	-	-
138	+	-	-
139	+	-	-
140	+	+	-
141	+	-	-
142	+	-	-
143	+	-	-
144	+	-	+
145	+	-	-
146	+	-	-
147	+	-	-
148	+	-	-
149	+	-	-
150	+	-	-
151	+	-	-
152	+	-	-
153	+	-	-
154	+	-	-
155	+	-	-
156	+	-	-
157	+	-	-
158	+	+	-
159	+	-	-
160	+	-	-
161	+	-	-
163	+	+	-
164	+	-	-
165	+	-	-
166	+	-	-
167	+	-	-
168	+	-	-
169	-	-	-
170	+	+	-

Appendix E: Gel extraction

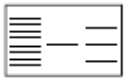
DNA extraction from agarose gel using NucleoSpin® Gel and PCR Clean-up kit () as below:

5.2 DNA extraction from agarose gels

Before starting the preparation:

- Check if Wash Buffer NT3 was prepared according to section 3.

1 Excise DNA fragment / solubilize gel slice



Note: Minimize UV exposure time to avoid damaging the DNA. Refer to section 2.5 for more tips on agarose gel extraction.

Take a clean scalpel to excise the DNA fragment from an agarose gel. Remove all excess agarose.

Determine the weight of the gel slice and transfer it to a clean tube.

+ 200 µL NTI per 100 mg gel

For each **100 mg of agarose gel < 2%** add **200 µL Buffer NTI**.

For gels containing > 2% agarose, double the volume of Buffer NTI.

Incubate sample for **5–10 min** at **50 °C**. Vortex the sample briefly every 2–3 min until the gel slice is **completely** dissolved!

50 °C
5–10 min

2 Bind DNA

Load sample

Place a **NucleoSpin® Gel and PCR Clean-up Column** into a Collection Tube (2 mL) and load up to 700 µL sample.

11,000 x *g*
30 s

Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

Load remaining sample if necessary and repeat the centrifugation step.

3 Wash silica membrane

Add **700 µL Buffer NT3** to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for **30 s** at **11,000 x** *g*. Discard flow-through and place the column back into the collection tube.

+ 700 µL NT3

11,000 x *g*
30 s

Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and low A_{260}/A_{230} (see section 2.7 for detailed information).

+ 700 µL NT3

11,000 x *g*
30 s

4 Dry silica membrane

Centrifuge for **1 min** at **11,000 x** *g* to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.

11,000 x *g*
1 min

5 Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a **new** 1.5 mL microcentrifuge tube (not provided). Add **15–30 µL Buffer NE** and incubate at **room temperature** (18–25 °C) for **1 min**. Centrifuge for **1 min** at 11,000 x *g*.

Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min. See section 2.6 for detailed information.

+ 15–30 µL NE

RT
1 min

11,000 x *g*
1 min