Profiling cardiovascular viral pathogens in cases of sudden and unexpected death in infants (SUDI) at the Tygerberg Medico-legal Mortuary and the role of myocarditis as a possible cause of death

by
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Declaration

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ABSTRACT

Introduction: Sudden unexpected death in infancy (SUDI) is a heterogeneous group in which pathological changes, when observed, can either be an adequate or non-conclusive cause of death (COD). A non-conclusive COD is referred to as Sudden Infant Death Syndrome (SIDS), which is postulated to be the result of multiple risk factors including infection. Viral heart infections, resulting in myocarditis, reportedly contribute to SUDI cases. Numerous viruses have been associated with myocarditis, with few ever having been investigated in a South African context.

Aim: The study aimed to investigate the presence of three specific viruses in the heart of SUDI cases admitted to the Tygerberg Medico-legal Mortuary over a one-year period.

Methodology: Swab samples of the left ventricle of the heart were collected from SUDI cases admitted to the Tygerberg Medico-legal Mortuary over the period of one year. Concurrently, swabs and tissue were retrieved for microbiological and histological analysis, respectively. Conventional qualitative polymerase chain reaction assays were used to detect three deoxyribonucleic acid viruses, namely human adenovirus (HAdV), human bocavirus (HBoV) and parvovirus B19 (PVB19), possibly linked to myocarditis. Clinical history, sociodemographic information and the final COD were collected from case files. All viral results were compared to the histology of the tissue. Associations were investigated between sociodemographic information and viral presence through statistical analysis in order to identify significant risk factors.

Results: Heart swabs were collected from 173 SUDI case, consisting of 93 males and 80 females and a mean age of 12.1 ± 9.8 weeks. Over half of the SUDI cases occurred in the cold seasons. The majority of the cases were assigned *Infection* as a COD, with just under half assigned as *SIDS*. Only one virus, HBoV, was detected in the heart tissue with implications of myocarditis histologically observed in one of the viral positive SUDI cases. Bacterial presence was also confirmed in only one case. All viral infections were observed in the cold seasons. Risk factors were highlighted between variable associations. Significant associations were observed between prematurity, room ventilation, birthweight and the COD. Significant associations were also observed between microbiology results, histology and the temperature on the day of death.

Conclusion: The study expanded the knowledge regarding myocardial infections contributing to SUDI in the study population, as well as significant risk factors. Viral detection in the myocardium, supported by histological evidence, provided an improved way of classifying COD as infection.

OPSOMMING

Agtergrond: Die skielike onverwagte dood in babas, of wiegiedood (SUDI), verwys na alle onverwagte dood in babas waar patologiese veranderinge, as dit waargeneem word, 'n voldoende of onvoldoende oorsaak van dood (OVD) kan bied. Indien daar geen OVD gevind kan word nie, word dit geklasifiseer as "Sudden Infant Death Syndrome" (SIDS). Daar word gepostuleer dat wiegiedood / SUDI plaasvind as gevolg van 'n oorvleueling van verskeie risikofaktore, insluitend infeksie. Virale hartinfeksies, wat lei tot miokarditis, dra by tot SUDI-gevalle. Talle virusse word met miokarditis geassosieer, maar min daarvan is al in 'n Suid-Afrikaanse konteks ondersoek.

Doelstelling: Die doel van hierdie studie was om die teenwoordigheid van drie spesifieke virusse te ondersoek wat in die hart van SUDI-gevalle kan voorkom by die Tygerberg Geregtelik-geneeskundige Lykshuis (GGL) oor 'n tydperk van een jaar.

Metodes: Monsters is van die linker hartkamer van alle SUDI-gevalle geneem wat in Tygerberg GGL opgeneem is gedurende Maart 2018 tot Maart 2019. Terselfdertyd is deppers en weefsel vir mikrobiologiese en histologiese ontleding onderskeidelik geneem. Konvensionele kwalitatiewe polimerase-kettingreaksie toetsings is gebruik om drie deoksiribonukleïensuurvirusse (DNS) op te spoor wat tydens miokarditis kan voorkom, naamlik menslike adenovirus (HAdV), menslike bocavirus (HBoV) en parvovirus B19 (PVB19). Kliniese geskiedenis, sosiodemografiese inligting en die finale OVD is ook genoteer. Alle virale resultate is vergelyk met die histologie van die hartweefsel. Assosiasies en verwantskappe tussen sosiodemografiese inligting en die teenwoordigheid van die viruses is deur middel van statistiese ontleding ondersoek om beduidende risikofaktore te identifiseer.

Resultate: Hartmonsters is geneem van 173 SUDI-gevalle wat 93 manlike en 80 vroulike babas ingesluit het met 'n gemiddelde ouderdom van 12.1 ± 9.8 weke. Meer as die helfte van die gevalle het gedurende die koue seisoene voorgekom. 'n Finale OVD van *Infeksie* is in die meerderheid van die gevalle bevestig. HBoV was die enigste virus wat in die hartweefsel van 'n enkele geval opgespoor kon word tydens die studie en hierdie geval het ook histologiese tekens van miokarditis getoon. Bakteriële teenwoordigheid is ook in slegs een geval bevestig. Alle virusinfeksies is gedurende die koue seisoene waargeneem. Beduidende assosiasies tussen prematuriteit, kamerventilasie, geboortegewig en die OVD kon aangetoon word. Verder is assosiasies ook waargeneem tussen mikrobiologiese resultate, histologie en die temperatuur op die dag van die dood.

Gevolgtrekking: Die studie het nuwe kennis opgelewer oor miokardiale infeksies, asook beduidende risikofaktore in SUDI. Histologiese tekens van miokarditis, ondersteun deur die teenwoordigheid van virus(se) in die miokardium, kan bydra om die klassifikasie van die OVS as *Infeksie* te verbeter.

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All glory be to God.

'History will judge us by the difference we make in the everyday lives of children.'

- Nelson Mandela

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LIST OF ABBREVIATIONS

5-HT	5-hydroxy tryptamine
ARD	acute respiratory disease
COD	cause of death
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EV	enteroviruses
FPO	forensic pathology officer
H&E	haematoxyllin and eosin
HAdV	human adenovirus
HAE-ALI	human airway epithelium at an air-liquid interface
HBoV	human bocavirus
HCoV	human coronavirus
HIV	human immunodeficiency virus
HREC	Health Research Ethics Committee
HRV	human rhinovirus
ICD-10	International Statistical Classification of Diseases and Related Health Problems, Tenth Revision
Ig	immunoglobulin
IL	interleukin
IMR	infant mortality rate
K. oxytoca	*Klebsiella oxytoca*
K. pneumoniae	*Klebsiella pneumoniae*
L. amnigena	*Lelliottia amnigena*
LTRI	lower tract respiratory infections
MCAD	medium-chain acyl-CoA dehydrogenase deficiency
NHLS	National Health Laboratory Service
NP	nuclear phosphoprotein
NPA	nasopharyngeal aspirates
NS	non-structural protein
NTR	non-translated regions
ORF	open reading frame
PCF	pharyngoconjunctival fever
PCR	polymerase chain reaction
PIV	parainfluenza virus
PVB19	parvovirus B19
QCMD	quality control for molecular diagnostics
rcf	relative centrifugal force

REM	rapid eye movement
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT	reverse transcription
RTI	respiratory tract infection
SIDS	Sudden Infant Death Syndrome
S. liquefaciens	*Serratia liquefaciens*
S. marcescens	*Serratia marcescens*
Staph	*Staphylococcus*
Strep	*Streptococcus*
SUDI	sudden unexpected death in infancy
URTI	upper respiratory tract infection
UTM	universal transport medium
VP	viral capsid protein
VPg	viral protein genome-linked
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

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1.

INTRODUCTION

Sudden unexpected death in infancy (SUDI) has been reported throughout history and gained notoriety for being ill-defined, with a standard investigation protocol lacking in several countries. SUDI is a common cause of infant mortality in both developed and developing countries, although more investigation is required to ascertain its full impact in developing countries (Nunes et al., 2001; Bartick & Tomori, 2018). SUDI is a descriptive term incorporating all deaths of an unexpected nature and is, however, not a diagnosis, unlike sudden infant death syndrome (SIDS). SIDS, commonly referred to as 'cot deaths', is defined as the unexpected death during sleep of an infant one year of age or younger, where no explanation for death can be obtained after a series of investigations (Beckwith, 2003).

Infant mortality is significantly higher in South Africa than developed countries, with the infant mortality rate (IMR) decreasing only slightly over recent years. In 2017 an IMR of 30 deaths per 1 000 live births was observed. It was reported that 4.2% of infants under one year of age died in 2015, according to the World Health Organisation (WHO) (2017). A previous study conducted from 2000-2004 at the Tygerberg Medico-legal Mortuary observed that the majority of infants were described as SUDI cases (Du Toit-Prinsloo et al., 2011).

Sudden infant death is hypothesised to be a result of multiple risk factors acting together, and not one sole reason. This phenomenon is referred to as the triple risk model, first described by Filiano & Kinney (1994). Risk factors are thought to disrupt homeostasis and are divided into three groups, namely external stressors, a susceptible infant and a critical developmental stage. Risk factors associated with SUDI include gender, season, bed-sharing, prematurity and birthweight to name a few. Infection is postulated to act as external stressor risk factors for susceptible infants.

Both bacterial and viral infections have been suspected to play a role in SUDI, often in combination with other risk factors, such as prematurity. Certain bacteria have been observed to release toxins causing sudden and unexpected death. Viral infections and co-infections in the respiratory tract have been investigated as contributors to infant mortality and SUDI. Far fewer investigations regarding cardiovascular viral infections contributing to SUDI have been carried out. Bajanowski et al. (2003), however, reported that viral myocarditis due to infection is a significant contributor to SUDI deaths. Certain specific viruses have been observed to infect the heart, namely enteroviruses (EV), human adenovirus (HAdV), Epstein-Barr virus (EBV) and parvovirus B19 (PVB19).

SIDS is a diagnosis through exclusion; therefore, it is vital that all SUDI cases undergo thorough post-mortem investigation. Weber et al. (2008a) reported that the majority of SUDI cases investigated in the study were signed out as SIDS. It is therefore imperative that a specific protocol is in place for all SUDI investigations, in order to minimise the amount of cases signed out as SIDS. This is also necessary to make sure that the correct cause of death (COD) is ascertained. No standardised infant death protocol is available in South Africa, although SUDI cases require formal Medico-legal Inquiry according to the Inquests Act (Act 58 of 1959) (Du Toit-Prinsloo et al., 2013). The Tygerberg Medico-legal Mortuary follows a specific protocol for all SUDI cases. An autopsy is conducted by either a forensic pathology officer (FPO) or Forensic Pathologist, ancillary tests are performed, the death scene is investigated, and sociodemographic information and clinical history are recorded. Ancillary testing consists of human immunodeficiency virus (HIV) testing, microbiology culturing and histological analysis. Chemical pathology analysis is seldom performed. No standardised viral testing of either respiratory or cardiovascular systems exists at present.

This study aided in expanding the limited knowledge regarding myocardial infections contributing to SUDI in South Africa. Understanding sudden death in infants is dependent on the results of the post-mortem and supplementary investigations (Koehler, 2010), therefore more research is needed to understand cardiovascular viral pathogens and the association with SUDI in a South African context.

2.

LITERATURE REVIEW

2.1.   Infant mortality

The IMR is often used as an indication of the health level within a country. IMR is defined as the number of infant deaths of under the age of one year within a specific year, per 1 000 live births that occurred in that same year (StatsSA, 2015). According to the WHO (2017), 75% of deaths of under five-year-old infants occurred within the first year of life.

2.1.1.   Global mortality

The most prevalent COD in developed countries has been attributed to SUDI and SIDS (Nunes et al., 2001). SUDI cases are most commonly observed in populations of underprivileged or marginalised people, even in wealthy countries (Bartick & Tomori, 2018). The prevalence of SUDI in developing countries is not yet fully ascertained, however global numbers have decreased substantially due to different awareness campaigns on the risks of sudden infant death. Certain geographic regions in the United States of America (USA) and Australia displayed a higher prevalence of SUDI cases. A study carried out in Pelotas, Brazil ascertained that SUDI was a possible COD in 4% of deaths under the age of one year (Nunes et al., 2001). According to the WHO, in 2016 4.2 million deaths of infants under one year occurred globally, which comprised 75% of all deaths under five years of age. Africa displayed the highest risk of a child dying under the age of one year in 2017, indicating that 52 infants die per 1 000 live births. This is six times the amount of deaths occurring in Europe, which is eight deaths per 1 000 live births (WHO, 2017).

Throughout the world the IMRs have decreased from approximately 64.8 deaths per 1 000 live births in 1990 to 30.5 deaths per 1 000 live births in 2016. In 1990 annual infant deaths amounted to 8.8 million, whereas in 2016 approximately 4.2 million deaths were recorded (WHO, 2017). First world countries, such as Australia, Germany, the United Kingdom (UK) and the USA, displayed a steadily decreasing IMR from 2000 to 2017. The IMR of Australia decreased from just over 5 deaths per 1 000 live births to 3.2 in 19 years. Similarly, Germany displayed a decrease from 4.7 deaths down to approximately 3.2 deaths per 1 000 live births recently observed in 2017 (UNICEF, 2019). The risk of an infant dying under one year of age was highest in Africa, being reportedly over six times higher than observed in Europe (WHO, 2017).

2.1.2. Mortality in South Africa

The South African IMR was constant at around 60 deaths per 1 000 live births between 2000 and 2007. From 2007 to 2008 there was a large decrease from 60 deaths to 45 per 1 000 live births. A very slight decrease in the number of infant deaths occurred between 2008 and 2014. Approximately 30 deaths per 1 000 live births were recorded in 2017. These numbers, however, are still significantly higher than those infant deaths recorded in first world countries, such as the UK and Australia (StatsSA, 2015).

In South Africa 4.2% of infants under the age of one year died in 2015 (WHO, 2017). There is slight discrepancy between the percentage of male and female deaths under one year – the male percentage being slightly higher at approximately 5.2% of deaths compared to the female percentage of 5.1%. According to StatsSA, 4.8% of the infant deaths were as a result of heart diseases, including viral myocarditis. Viral infections in infant deaths comprised 3.5% of deaths. The unnatural deaths in infants under one year comprised 3.9% of total deaths for the age group (StatsSA, 2015).

In Pretoria and Tygerberg Medico-legal Mortuaries, 344 and 469 infants under one year respectively were admitted between 2000 and 2004. SUDI cases comprised 28.8% of cases from Pretoria and 88.1% of cases from Tygerberg, just over 50% of which were males in both mortuaries. Racial demographics displayed majority of Pretoria SUDI cases were black, whereas majority of Tygerberg SUDI cases were coloured individuals. SUDI numbers increased in the winter months, with Tygerberg showing a second peak in December. The second peak has not been observed again in subsequent studies. Of the SUDI cases recorded, 9.2% of Pretoria mortuary's numbers and 40% of the numbers from Tygerberg mortuary were assigned a final COD of *SIDS* (Du Toit-Prinsloo et al., 2011).

2.2. Cause of death

The South African National Department of Health assumed the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10) codes in 1996, as a national standard used for diagnosis. The standard is used to ensure quality of diagnosis and specifies how something may be measured or tested. ICD-10 codes are used to help the clinical coder, and record and track changes. These codes provide a list of possible causes of death, in order to tabulate mortality data (National Center for Health Statistics, 2002).

There are 130 possible causes of infant death listed in the ICD-10 codes (National Center for Health Statistics, 2002). Various disorders and illnesses occurring in the perinatal period are specified with the code P00-P96 (National Task Team for the Implementation of ICD-10, 2009). Perinatal

respiratory and cardiovascular conditions are coded as P20-P29, whereas infections fall under P35-P39. Viral infections acquired in utero are specifically classified as P35. Infections acquired after birth are found under the A00-B99 and J09-J11 codes and viral carditis as a result of infection is recorded as B33.2. Viral infections where the site is unspecified (B34) include the presence of HAdV, EVs and parvoviruses. These specific viruses were also classified as causes for illnesses classified in other chapters of the ICD-10 in B97. Viral myocarditis or inflammation of the heart muscle, linked to viruses mentioned elsewhere, is specified in the code I41.1. Diseases of the respiratory system, including viral pneumonia and other viruses, are classified from codes J0 J99 (WHO, 2016).

SUDI cases include multiple ICD-10 codes, namely R95, R99 and W75. SIDS as a COD is coded as R95, as well as R95.9 in some countries where no autopsy was performed. Ill-defined and unspecified causes of mortality are registered as R99, and accidental suffocation as W75 (National Center for Health Statistics, 2002; Taylor et al., 2015). Inconsistencies have been observed between different countries' use of the coding system, making comparisons difficult over time (Taylor et al., 2015).

2.3.   History of sudden infant death in infants

The term SIDS was introduced by Bruce Beckwith in 1969 (Bajanowski et al., 2006). Sudden unexpected infant death is well known, with the first reported case due to 'overlaying' being traced back to the Bible (Nunes et al., 2001; Byard & Krous, 2003). The concept of 'overlaying', described to be death due to suffocation while sleeping with an adult, was suspected to be the leading unexpected cause of infant death in the 12th century. It can therefore be concluded that the problem of SUDI has been in existence for centuries. The risk was, in fact, so serious that wooden and metal structures were required to prevent infant suffocation during breast feeding (Byard & Krous, 2003).

SUDI deaths were ill-defined and poorly investigated in the past, with post-mortem examinations seldom performed. As a result, past epidemiology of SUDI and SIDS is often unreliable. In more recent years, however, a more standardised approach to SUDI was implanted with many theories evolving regarding the phenomenon (Byard & Krous, 2003).

2.4.   Definition of SIDS and SUDI

Although the literature often uses both SUDI and SIDS to describe the same phenomenon, there is a definite distinction between the two terms. SUDI describes a heterogeneous group displaying pathological changes which can either be adequate CODs, not conclusively explain death, or not displaying any changes at all and thus being classified as SIDS (Grangeot-Keros et al., 1996). SIDS, however, remains a diagnosis by exclusion (Beckwith, 2003; Du Toit-Prinsloo et al., 2011). Although the literature often uses the term SIDS as an all-encompassing term to describe unexpected death in infants, mostly in the absence of investigations, this thesis will use the term SUDI to describe all

infant deaths before any investigations have been conducted and SIDS for those cases where no COD could be found.

SIDS can be divided into three classifications, each containing different specifications as listed below (Krous et al., 2004).

An infant in **Category IA SIDS** displays all of the following characteristics:

- Ages 21 days - 9 months;

- Normal clinical history and gestational period (37 weeks);

- Normal growth and development observed;

- No similar deaths observed in siblings, close genetic relatives, or other infants in the custody of the same caregiver;

- Investigations display no explanation for death, as well as there being an absence of potentially fatal pathogenic findings; and

- Minor respiratory tract inflammatory infiltrates are acceptable; intrathoracic petechial haemorrhage may be associated with death, however not diagnostic; absence of unexplained trauma, abuse, neglect or unintentional injury; negative toxicologic, microbiologic, radiologic, vitreous chemistry and metabolic screening studies obtained.

Category IB SIDS correlates with the definition and the requirements of Category IA SIDS. In contrast to Category IA SIDS however, not all the various death scene investigations were completed and / or one or more analyses were not completed. Analyses include toxicological, microbiological, radiological, vitreous chemistry and metabolic screening studies.

Category II SIDS meets the Category I SIDS criteria, except for one or more of the following:

- Age range outside categories IA and IB SIDS;

- Similar deaths among siblings, close relatives, or other infants in the custody of the same caregiver(s) that are not genetic or a result of infanticide;

- Neonatal / perinatal conditions that have resolved by the time of death;

- Mechanical asphyxia caused by overlaying not certainly determined;

- Abnormal growth not thought to have contributed to death; and

- Obvious inflammatory changes, but insufficient to cause death

Unclassified sudden infant death does not meet the criteria for categories I or II SIDS. Diagnoses of natural or unnatural conditions are found to be ambiguous. This category may represent SIDS, however atypical features (underlying organic diseases) are present and therefore insufficient information is present to make the judgment (Krous et al., 2004).

SIDS, according to the San Diego definition, is the unexpected death during sleep of an infant one year of age or younger. Infant deaths within the first week of life are excluded, as death in the neonatal period often results from respiratory complications and other defects. Similarly, after the age of a year, the child is no longer regarded as an infant and thus no longer relevant to SUDI investigations. Unexpected death is also uncommon after one year, and more common in early infancy. Deaths typically remain unexplained after autopsy, circumstantial and clinical history investigations. Supplementary tests are also often used to subclassify deaths based on any, albeit minor, pathology observed (Nunes et al., 2001; Byard & Krous, 2003; Krous et al., 2004). SIDS is the main COD in infants, with the highest incidence in two to four months, divided into the aforementioned two categories based on the investigative results (Dwyer & Ponsonby, 1995; Krous et al., 2004).

A distinct difference between SIDS and SUDI has been observed, SIDS being described as a category of SUDI (Prtak et al., 2010). SUDI is a general term incorporating all sudden, unexpected deaths of infants whether the COD can be explained or not. SUDI is often linked to death occurring during sleep, characterised by maladaptation, as well as recurrent diseases, causing homeostatic imbalances that increase the probability of death. Maladaptation could result from physiological immaturity and phenotypical oddities. As a result of this, an infant death may be classified as SIDS if no fatal injury and lesions were observed, and a mild acute respiratory infection was observed insufficient for COD (Vorontsov & Kelmanson, 1990; Nunes et al., 2001; Krous, 2010). If a SUDI case remains unexplained after clinical history and death scene investigation, as well as autopsy, it is classified as SIDS (Du Toit-Prinsloo et al., 2011; Prtak et al., 2010). Postnatal development, family psychosocial history, as well as gestational history, are important to consider in combination with the aforementioned investigations in order to classify the case as possible SIDS (Athanasakis et al., 2011).

SUDI cases admitted to the Medico-legal mortuary undergo a series of investigations in order to ascertain COD and rule out SIDS. The Medico-legal investigations include the clinical history, death scene investigation, autopsy and supplementary laboratory tests. The investigations provide information regarding the infant's health before death, the presence of risk factors, the circumstances of death, pathogenic presence, as well as macro- and microscopic presence of pathology. Together the findings can be used to attempt to ascertain the COD. Occasionally the results of the investigations are indicative of a contribution death, which is not substantial for the COD diagnosis. These cases are

referred to as borderline SIDS. Sufficient and insufficient findings are assigned CODs of natural and SIDS, respectively. A natural COD includes infection (Gregersen et al., 1995; Krous, 2010; Du Toit-Prinsloo et al., 2011). The investigation and path to diagnosis is depicted in Figure 2.1.
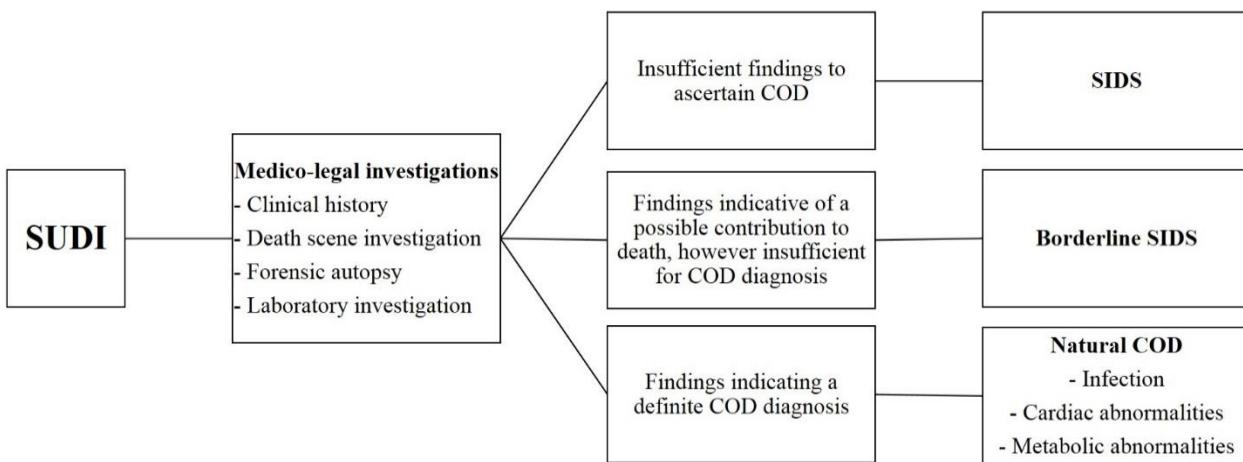
Figure 2.1: Flow chart indicating the constitution of the SUDI groups and subsequent COD (Gregersen et al., 1995; Krous, 2010).

2.5.   Risk factors

Certain similarities have been observed between SUDI cases, such as low economic status, age, gender, season and a young, uneducated mother. These demographics are referred to as risk factors, possibly leading to sudden death (Byard & Krous, 2003). The co-existence or overlap of three individual risk factors, namely a susceptible infant, a critical developmental stage for homeostatic control and an external stressor, is postulated to contribute to SUDI. The phenomenon is known as the triple risk model (Filiano & Kinney, 1994). Infant susceptibility remains dormant until entering a critical developmental stage and the infant is subsequently exposed to an external stressor, resulting in the disruption of homeostasis. The Triple-risk model proposed by Filiano and Kinney (1994) suggests that sudden infant death may be a result of multiple risk factors acting together, and not one sole reason. The risk factors were divided into three main groups: an external stressor, a susceptible infant and a critical developmental stage. An external stressor may include infection, a susceptible infant may include immune deficiency and a critical developmental period includes an infant between 2-4 months (Figure 2.2).

Infants within the first weeks and months of life are vulnerable to certain immune stimuli, as a result of the still developing immune system. The susceptibility of the infant could be aggravated by polymorphisms of the interleukin (IL)-10 gene involved in immunity and mitochondrial deoxyribonucleic acid (DNA), subsequently leading to a susceptible physiological state in situations of stress. Low levels of IL-10 contribute to an increased inflammatory process, which results in hypoxaemia, hypoxia and death. Mitochondrial DNA abnormalities can consequently lead to muscle

weakness, thus preventing the infant from turning its head when in the prone position, which can lead to asphyxiation (Vege & Rognum, 2004). It has been hypothesised that sudden infant death may be due to abnormalities in neurotransmitter regulations, such as serotonin or 5-hydroxytryptamine (5-HT) receptors. Variant genes regulating 5-HT signalling in the brainstem have been associated with ineffective homeostatic responses during sleep, which could result in SUDI. Not enough evidence has been discovered, however, to enforce this theory (Paterson, 2013).
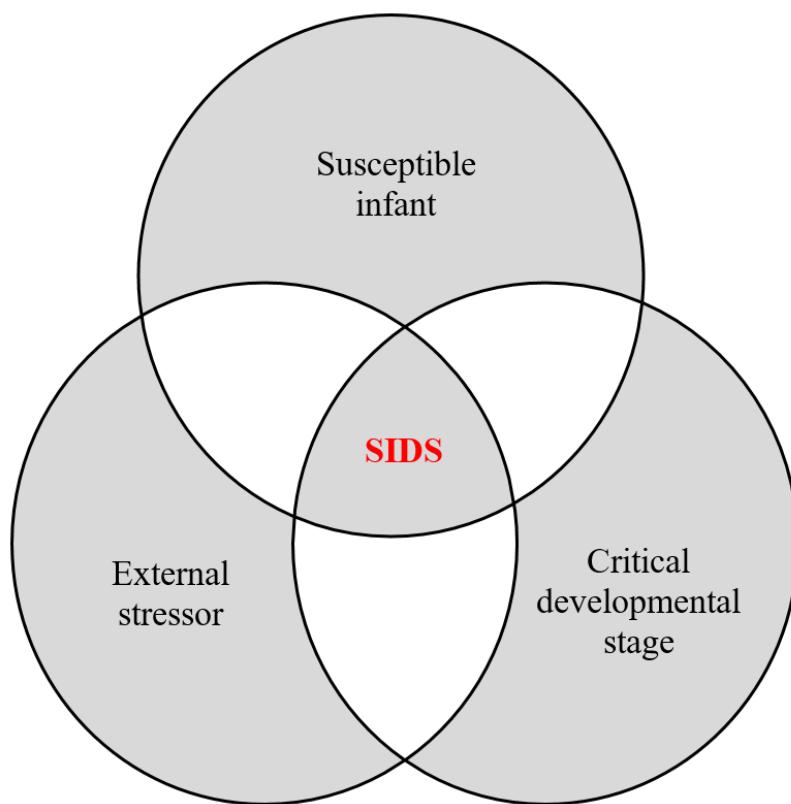
Figure 2.2: Triple-risk model (**Filiano & Kinney, 1994**).

SUDI cases are postulated to result from abnormal sleep arousal, with other contributing risk factors being present (Nunes et al., 2001) and has been hypothesised to be a result of cardiorespiratory events, such as extended apnoea and bradycardia present in a predisposed infant. The hypothesis was related to premature infants, those who suffered idiopathic apparent life-threatening events, as well as SUDI cases (Ramanathan et al., 2001). Apparent life-threatening events are described as cyanotic, apnoeic, limp and choking (Byard & Krous, 2003). It has therefore been postulated that infant death under one year of age is due to maladaptation, resulting in disease and defects, disrupting homeostasis, such as various apnoeas often during the rapid eye movement (REM) sleep cycle (Vorontsov & Kelmanson, 1990).

Subtle neonatal neurological irregularities in the sympathetic / central and autonomic nervous systems have been detected in autopsies of SUDI cases. Post neonatal irregularities in cardiac,

ventilatory and cry patterns also supply evidence for infant susceptibility to SUDI. Pregnancy and maternal factors may also influence the infant's susceptibility to SUDI. Other risk factors include low birthweight and prematurity in the infant, as well as maternal factors, such as age, smoking, anaemia, urinary tract infection, opioid and cocaine usage (Filiano & Kinney, 1994).

SUDI is further postulated to be increased by co-sleeping, thermal imbalances, colder climates, prone position sleeping and parental smoking. It has been reported that male infants have a 60% higher chance of succumbing to SUDI, as well as those that are part of multiple births (Dwyer & Ponsonby, 1995; Nunes et al., 2001). Peaks of SUDI cases have been observed in infants that were born prematurely, as they are often at risk of intraventricular haemorrhages of the heart, which can result in sudden death if the birthweight is lower than 1 500 grams. If the birthweight is less than 2 000 grams and a respiratory infection is present, there is a high risk of sudden death (Vorontsov & Kelmanson, 1990).

Bartick & Tomori (2018) described the aggregation of sequential or concurrent social inequalities affecting patterns of infection and disease. Social stressors for pregnant mothers, linked to low socioeconomic status, have also been associated with low birthweights in infants. Chronic stress due to the effects of racism and poverty causes fluctuation of cortisol levels of the mother, therefore resulting in low birthweight. Both low birthweight and prematurity are significant risk factors for SUDI (Bartick & Tomori, 2018).

According to Vennemann et al. (2007), the same risk factors associated with explained SUDI are often associated with SIDS as well. For example, low socio-economic status, bed-sharing, thermal factors, pacifier use during sleep and maternal smoking during pregnancy all correlated with both SUDI and SIDS groups. The main difference observed between the SUDI and SIDS groups investigated by Vennemann et al. (2007) was the sleeping positions of the infants. Prone position, where the infant is lying face down, has been shown to increase the chances of SUDI. Alcohol abuse and low education of parents were also reported to be associated risk factors for SUDI. Previous SUDI cases were present in 0.7% of families which lost another infant suddenly and unexpectedly (Vorontsov & Kelmanson, 1990). Specific ethnic groups, such as African and Native Americans, have been observed to display higher SUDI rates according to a study by the National Institute of Child Health and Human Development in the USA, however a later study in Brazil displayed that 67% of infant deaths were Caucasian and 33% were of African-American descent (Nunes et al., 2001).

The possible causes for sudden infant death displayed in SUDI (Figure 2.3) can also be caused by generalised infection alone or in combination with aspiration, bronchopneumonia, omphalocele,

meningoencephalitis, dehydration, vitium cordis and medium-chain acyl-CoA dehydrogenase deficiency (MCAD) (Vennemann et al., 2007). In 40% of SUDI cases where a definite COD had been found, diseases such as MCAD, other fatty oxidation defects, heart arrhythmias (long QT syndrome) and neuromuscular disorders were included (Vege & Rognum, 2004). Investigation of the conduction system of the heart has displayed abnormalities in the AV-node, resulting in remodelling of the embryonic heart tissue region that can lead to arrhythmias in infants (James, 1976). Autonomic abnormalities and immaturity of the hypothalamus are linked to an increased risk of SUDI, as well as decreased catecholamine brainstem production and morphological variations of the adrenal glands (Vorontsov & Kelmanson, 1990). Weber et al. (2008b), however, reported that the majority of SUDI occurred due to infection. Respiratory and gastrointestinal viral infections have been observed as predictors to the onset of SUDI cases (Bajanowski et al., 1996).
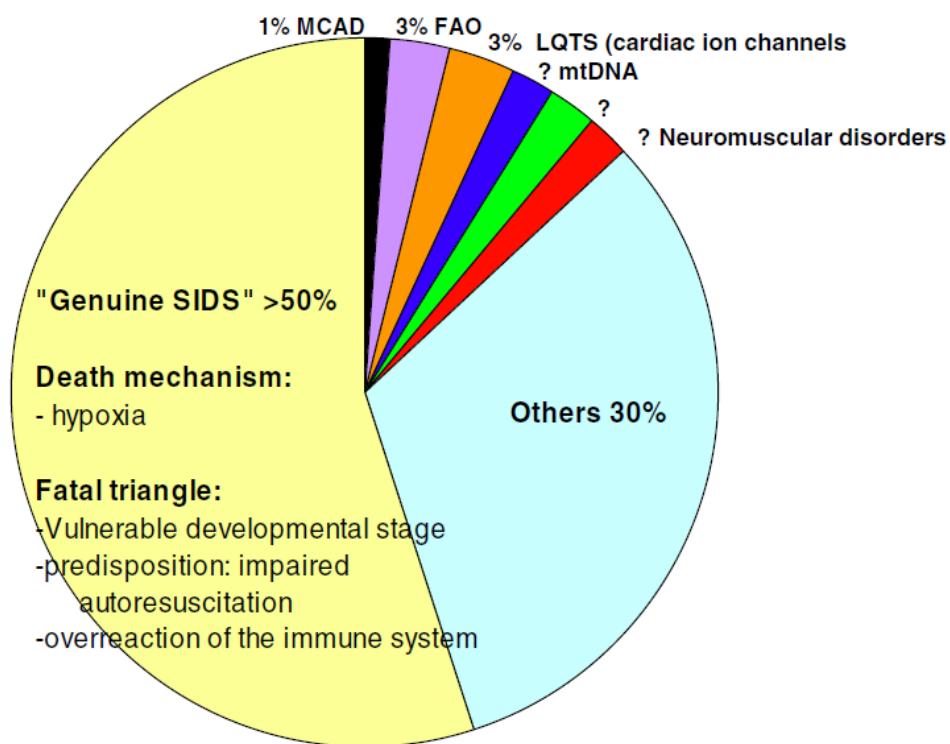
Figure 2.3: Possible causes of death in SIDS **(Vege & Rognum, 2004)**

Typical autopsy findings in SUDI cases include thymus, epicardial and pleural petechiae, laryngeal fibrin foci and necrosis, expanded lungs, pulmonary congestion and oedema, sanguineous exudate in the cardiac cavity, lymphoid stroma and upper respiratory tract inflammation (Nunes et al., 2001). Studies have indicated that both infection and inflammation are linked to SUDI (Alfelali & Khandaker, 2014). It has been postulated that infection, in conjunction with other environmental factors, may act as a trigger for SUDI. For example, seasonality plays a role in infection, as the incidence of SUDI has been observed to be increased during the colder months, furthermore, correlating with the increase in the incidence of epidemic viruses. It is postulated that SUDI occurs

when the viral infection is at an early stage and localised, and thus morphological manifestations may not be clear yet (Grangeot-Keros et al., 1996). Bajanowski et al., (1996) reported 60% of SUDI cases occurred in the autumn and winter months, with exception of those displaying signs of interstitial pneumonitis, where only 44% occurred in the colder months.

2.6.   Infections

Both viral and bacterial infections have been thought to be contributing cofactors to SUDI, specifically infections of the respiratory tract (Blackwell et al., 2001). Fitzgerald et al. (1984) described infection as being the third largest cause of infant death in Australia. Certain risk factors, such as prematurity, maternal smoking and sleeping position have been observed to increase infection incidence in infants. The presence of certain toxigenic bacteria have also been associated with the previously stated risk factors (Blackwell et al., 2001).

In the past a beta-haemolytic *Streptococcus* (*Strep)* bacterium and *Staphylococcus (Staph*) *aureus* were associated with infant mortality and hospital infections. Gram-negative bacteria, however, have become more dangerous to infants. An example of these bacteria would be the *Serratia sp.*, particularly pathogenic in premature infants (Fitzgerald et al., 1984). In a study conducted by Prtak et al. (2010), pathogenic bacterial isolates detected were positively associated with SUDI deaths. The pathogenic bacteria were detected in one or more sites in the SUDI cases. Pathogenic bacteria include *Staph aureus, Strep pyogenes, Escherichia coli (E. coli)* and *Strep pneumoniae* among others (Morris et al., 2006). Toxin producing bacteria have been hypothesised to contribute to SUDI, in the absence of histopathology. These bacteria include *Clostridium botulinum*, *E. coli* and *Staph aureus* (Byard & Krous, 2003; Prtak et al., 2010).

Infection of certain viruses, such as respiratory syncytial virus (RSV), increases chances of infection for bacteria like *Staph aureus* (Blackwell et al., 2001). Viral infections in SUDI cases were observed by An et al. (1993), to occur mainly between 3 weeks and 4 months of age. Histology is referred to as the gold standard for discerning viral infections, however virus specific detection is necessary to confirm the diagnosis (Bajanowski et al., 1996). Viral infections are thought to be a large contributing factor to SUDI, specifically infections of the respiratory tract, gastrointestinal tract and myocardium (Bajanowski et al., 1996; Grangeot-Keros et al., 1996; Blackwell et al., 2001). Respiratory tract infections (RTI) and cardiovascular infections resulting in myocarditis, are discussed further in the following subsections.

2.6.1. Respiratory tract infections

Viral infections are common among children, often resulting in severe RTI. Populations with a high prevalence of HIV display increased morbidity and mortality rates, due to viral RTI (Ghani et al., 2012). Minor inflammatory changes in both the upper and lower respiratory tracts are common among SUDI cases (Bajanowski et al., 2007).

Acute lower RTI (LRTI) is one of the most common causes of mortality in children worldwide (Liu et al., 2015), as it severely compromises the health of infants and children. Monthly changes in the percentage influence on the diagnosed viral LRTI have been observed to range substantially (0%-88%). Clinical symptoms in children frequently present as bronchiolitis due to viruses, such as RSV, human rhinovirus (HRV) and parainfluenza virus (PIV)-3. Similarly, the influenza virus, PIV-1 and human coronavirus (HCoV)-NL63 were discovered to be major agents of croup (Choi et al., 2006).

Ghani et al. (2012) identified HRV-A to be the most common pathogen in children suffering from viral respiratory illness, reaching peak levels in the spring season. RSV was observed to be the second most prevalent, present mainly in the colder seasons, followed by HAdV, which displayed no seasonal trend. Other common viruses, including EVs and human bocavirus (HBoV), have also been detected in respiratory tract samples resulting in disease in young individuals (Oberste et al., 1999; Heim et al., 2003; Gurda et al., 2010). Bajanowski et al. (2003) suggested the viral cause of interstitial pneumonia in SUDI cases was due to HAdV as well as influenza B infection, characterised histologically by oedema, haemorrhage or desquamation of epithelial cells (Bajanowski et al., 1996). Ghani et al. (2012) reported, however, that bacterial and other pathogenic co-infections, have displayed no association with increased child mortality although they are postulated since to be a valid factor for disease by Singer et al. (2017).

A retrospective audit was done on respiratory samples analysed by the National Health Laboratory Service (NHLS) at Tygerberg Hospital, Western Cape, between 2015 and 2017. A total of 1 453 respiratory samples from infants were received in the laboratory during this time. HAdV was positive in 12.3% of cases (118 males and 60 females), while EVs were confirmed in 3.2% (26 males and 21 females) and HBoV in only 2.8% (25 males and 15 females).

2.6.2. Myocarditis

Myocarditis, commonly caused by a virus resulting in inflammatory cardiomyopathy, is often the cause of sudden cardiac death in young individuals (Grangeot-Keros et al., 1996). In keeping with the previous statement, Bajanowski et al. (2003) reported that viral myocarditis is a significant contributor to SUDI. Data derived from animal models indicate that myocarditis can cause dilated

cardiomyopathy probably due to viral persistence or a chronic immune response that was initiated by viral infection (Kuethe et al., 2009).

The condition can be divided into three categories: active, borderline and non-myocarditis. Active myocarditis typically presents with profuse inflammatory cells and myocardial necrosis, whereas borderline myocarditis displays substantially fewer inflammatory cells and myocyte deterioration is not visible with light microscopy (Bohn & Benson, 2002).

Approximately 50% of viral myocarditis conditions are caused by EVs, however Martin et al. (1994) observed that HAdV presence was twice that of the EVs, and thus the former is an integral part in the pathogenesis of myocarditis. The presence of EVs correlated with observed inflammation histologically, whereas HAdV showed no such correlation. It is postulated that the group of EVs, including the coxsackie viruses, promote greater inflammation in comparison to HAdV (Martin et al., 1994). HAdV, EVs and HBoV have all been associated with occurrences of viral myocarditis, attacking the cardiomyocytes or endothelial cells (Nielsen et al., 2014).

A coxsackie-adenoviral-receptor has been observed to be present at birth, and down-regulated thereafter. However, the receptor is again expressed in adults displaying chronic inflammatory myopathies and thus providing a likely molecular explanation of EVs, HAdV, EBV and PVB19 in the heart. The presence of these viruses has also been recorded in affected myocardial samples of SUDI cases, and subsequently indicated in underlying inflammatory processes in SUDI. Inflammation affecting the conduction system throughout the myocardium as a result of viral infection has been postulated to be a valid cause of sudden death, not limited to infants. The detection of viruses does not necessarily coincide with the presentation of histological and immunohistochemical signs of inflammation, implying a very early stage of myocarditis where death can occur before any cellular inflammatory action is observed. This postulation increases the amount of cases of apparent SIDS where the COD can be identified as infection (Dettmeyer et al., 2004).

The Dallas criteria for diagnosing myocarditis indicate visible myocyte necrosis and inflammatory infiltrates, whereas borderline myocarditis only displays inflammatory infiltrates using light microscopy (Baughman, 2006). Dennert et al. (2008), however, stated that interpretation of the Dallas criteria using light microscopy is open to various interpretation errors. Immunostaining is suggested to be used for accurately identifying inflammation, and polymerase chain reaction (PCR) or *in situ* hybridisation for diagnosing viruses. Immunostaining in combination with quantitative PCR are thought to increase accuracy of diagnosis. Immunostaining, however, has been observed to be ineffective in a post-mortem setting (Bültmann et al., 2003; Dennert et al., 2008). Viral myocarditis can be diagnosed with the use of viral cultures and serology; however these methods are not sensitive

or specific. Histology only gives an indication of inflammation (Martin et al., 1994; Dennert et al., 2008). Immune reaction processes are characteristic of the pathophysiology of subacute viral myocarditis, and as a result viral genomic material is often unable to be detected by PCR (Brebion et al., 2014). Studies have shown that molecular methods, such as PCR, can detect viral nucleic acids in up to 70% of endomyocardial samples (Kuethe et al., 2009). Dennert et al. (2008) stated that biopsies are considered to have high sensitivity.

2.7. Viruses

2.7.1. Human adenovirus

2.7.1.1. Structure

HAdV is a medium-sized virus, approximately 80 nm in diameter. The isometric virion consists of 252 capsomeres and is unenveloped (Belshe, 1984). Approximately 240 hexon and 12 penton components are present for each viral particle (Ghebremedhin, 2014). Linear, double-stranded DNA is typical of the HAdV (Belshe, 1984). The genome length is 36 kb, containing 23 – 46 protein coding genes. More than 60 molecular subtypes of HAdV have been discovered and ordered into seven subgroups, namely A, B, C, D, E, F and G (Darville, 1985; Ghebremedhin, 2014; Liu et al., 2015), but only 47 of the antigenically different serotype strains affect humans (Bajanowski et al., 1996). Various HAdV strain recombinants have been observed (Darville, 1985). The structure of HAdV is presented in Figure 2.4.

New HAdV subtypes often form through the homologous recombination between existing subtypes, therefore resulting in different pathogenicities. Varying degrees of oncogenicity have also been observed between HAdV subgroups (Ghebremedhin, 2014).
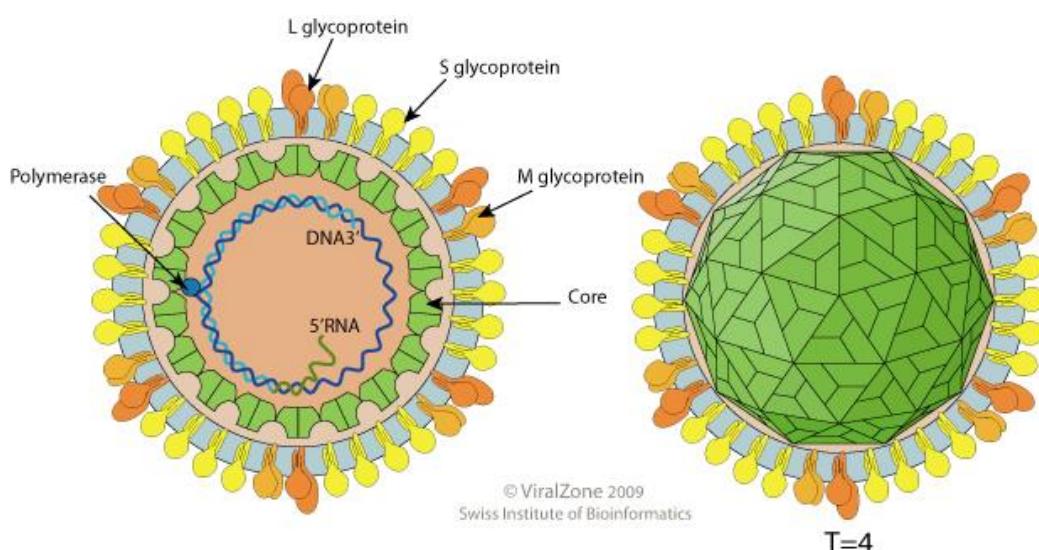

Figure 2.4: Human adenovirus capsid structure **(Swiss Institute of Bioinformatics, 2008).**

2.7.1.2. Pathogenesis, epidemiology and detection

HAdV was first detected in the adenoids by Rowe et al. (1953). Most children display HAdV infection at some point, however, only about 50% are asymptomatic and the symptomatic infections are minor and easily resolved (Gray et al., 2007). In an audit from 2004 to 2007, two out of 116 SUDI cases displayed HAdV infection (Prtak et al., 2010). It has been postulated that the virus could be the most common cause of respiratory infections in infants (Bajanowski et al., 1996). Infection results in acute upper RTI (URTI), however, rarely leads to septic shock or respiratory failure in immunocompetent individuals (Heydari et al., 2011). It has, however, been reported to be severe or even fatal, as observed in various military populations which experienced acute respiratory infections, pneumonia and encephalitis (Gray et al., 2007; Liu et al., 2015). Infection has been observed in conjunction with signs of bronchiolitis, focal peribronchial infiltration and lymphomonocytic infiltration of the alveolar wall. HAdV infection resulting in pneumonia specifically displays necrotising bronchiolitis with desquamation of the epithelium, haemorrhages, atelectasis and intranuclear inclusion bodies (Bajanowski et al., 1996).

The severe epidemics mainly involved strains 4, 7 and 21 (Gray et al., 2007). HAdV infection can cause respiratory illness, pharyngoconjunctival fever (PCF), cystitis, conjunctivitis, gastroenteritis, neurologic and venereal disease (Liu et al., 2015), while it may also result in chronic diseases, such as pulmonary dysplasia, chronic airway obstruction, myocarditis, mononucleosis-like syndrome, sudden infant perinatal death, intussusception and obesity (Guarner et al., 2003; Gray et al., 2007). Severe infection is usually associated with emerging variants of the HAdV (Liu et al., 2015).

Different HAdV serotypes have displayed different tropisms for different tissues (Table 2.1), resulting in different symptoms (Gray et al., 2007). Subtypes 1, 2, 5 and 6 of the subgenus C, as well as subtype 3 of subgenus B, are responsible for viral respiratory infection (Bajanowski et al., 1996). Subgenus B can be genetically separated into two groups, B1 and B2, with B1 being responsible for the development of acute respiratory disease (ARD) and B2 for urinary tract and opportunistic infections in immunocompromised patients (Kajon et al., 2010). Subspecies C1, C2 and C5 result in ARD in children. Pneumonia is a result of B3 and E7, which can also present as PCF, while B3 is associated with PCF, as well as meningitis (Heim et al., 2003).

HAdV strain 7 was reported in conjunction with HBoV, resulting in pneumonia and myocarditis. Focal infiltration of the lungs and arrhythmias were observed in combination with the co-infection (Heydari et al., 2011). HAdV strains 40 and 41 have been classified as enteric viruses (Darville, 1985). The infection persists asymptomatically in the adenoids for months with occasional viral

shedding. HAdV is life-threatening in the case of immunocompromised patients, such as individuals who have undergone haemopoietic transplants (Heim et al., 2003).

Table 2.1: Human adenovirus subgroups associated with infection (adapted from Ghebremedhin, 2014).

Subgroup	Serotype	Infection
A	12, 18, 31	gastrointestinal, respiratory, urinary
B, type 1	3, 7, 16, 21	keratoconjunctivitis, gastrointestinal, respiratory, urinary
B, type 2	11, 14, 34, 35	gastrointestinal, respiratory, urinary
C	1, 2, 5, 6	respiratory, gastrointestinal including hepatitis, urinary
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49	keratoconjunctivitis, gastrointestinal
E	4	keratoconjunctivitis, respiratory
F	40, 41	gastrointestinal
G	52	gastrointestinal

Immunocompromised bone marrow transplant patients have a 3-20% chance for HAdV infections, up to 50% of which are fatal (Wang & Wang, 2003). Severe HAdV infections are reportedly more often present in the winter months. Limited epidemiological investigations have been carried out on HAdV, however it has been observed that certain genetically different strains have varying virulence and are specific to geographical regions. HAdV7 has been associated with serious infections globally. HAdV4, 3 and 21 were the strains most common among military trainees. An increase of HAdV21 and decrease of HAdV4 were observed over time. In infected American civilian children under the age of seven years, the most common genotypes were 1, 2, 3 and 5 (Gray et al., 2007).

HAdV disseminates as a result of viraemia from the lungs to other organs. Viral loads of the pathogenic disseminated disease are recorded as $>1\text{x}10^{7}$ copies/µl and $>1\text{x}10^{11}$ copies/µl in adult and paediatric cases respectively (Ganzenmueller & Heim, 2012). HAdV can be detected through molecular methods, such as conventional PCR and real-time PCR assays, as well as sequencing (Heim et al., 2003; Gray et al., 2007). A case study also described culturing strain 7 of HAdV in order to identify the virus (Heydari et al., 2011). Culturing using human embryonic kidney and human epithelial type 2 cells were used in another study for subtyping the different HAdV strains (Darville, 1985). Viral culture is often referred to as the gold standard for diagnosing viral infection, despite its insensitivity and specificity (Dennert et al., 2008; Ganzenmueller & Heim, 2012). Immunofluorescence microscopy and qualitative PCR performed on serum samples have been used to detect HAdV in pathogenic cases. Qualitative PCR has been described as lacking sensitivity in order to detect low, persistent infections. Quantitative PCR detection for HAdV has been described

as difficult due to the genomic variation between the multitude of viral strains. Designing or finding generic primers is a challenge due to few common conserved regions and sequence homology. Multiple primers and probe reactions have been optimised; however these are costly. Quantitative PCR has higher sensitivity, conveying useful information regarding viral loads (Ganzenmueller & Heim, 2012).

2.7.2. Human bocavirus

2.7.2.1. Structure

HBoV was discovered in 2005, and forms part of *Parvoviridae* family, sub-family *Parvovirinae* which is further divided into eight genera: *Aveparvovirus, Copiparvovirus, Protoparvovirus, Tetraparvovirus, Erythroparvovirus, Dependoparvovirus, Amdoparvovirus* and *Bocaparvovirus* (Guido, et al., 2016). *Erythroparvovirus* and *Dependoparvovirus* are the only known genera to infect humans (Heegaard & Brown, 2002). The name "Bocavirus" is derived from the amalgamation of bovine parvovirus and canine minute virus, due to the close resemblance in genome structure and sequence to these relative viruses. The virus solely infects vertebrates (Schildgen et al., 2012; Guido et al., 2016). The HBoV is the second virus affecting humans in its family, the other being PVB19 (Schildgen et al., 2012). Although HBoV and PVB19 display a close phylogenetic resemblance, differences have been observed in the tissues targeted by each. PVB19 targets bone and heart tissue, whereas HBoV displays tropism for lymphatic tissues, as well as tissues inflicted with chronic sinusitis (Guido et al., 2016).

The viruses of family *Parvoviridae* are non-enveloped and contain single stranded linear DNA of a negative or positive polarity with approximately 5 000 bases. The DNA is enclosed in a small icosahedral capsid consisting of six capsule proteins, approximately 18-26 nm in diameter (Gurda et al., 2010; Schildgen, 2013; Nielsen et al., 2014; Guido et al., 2016). Terminal sequences of 32-52 nucleotide bases are involved in the replication of HBoV (Guido et al., 2016).

The HBoV genome (Figure 2.5) encodes three different open reading frames (ORF) (Guido et al., 2016). The large left ORF encodes four non-structural proteins (NS1, NS2, NS3 and NS4), while the middle ORF encodes NS1. The right ORF encodes the viral capsid proteins (VP) 1, 2 and 3 (Schildgen et al., 2012). The non-coding regions consist of palindromic repeats which play a part in replication (Guido et al., 2016). HBoV displayed an absence of the terminal hair-pin structure, present in other parvoviruses (Schildgen et al., 2012).
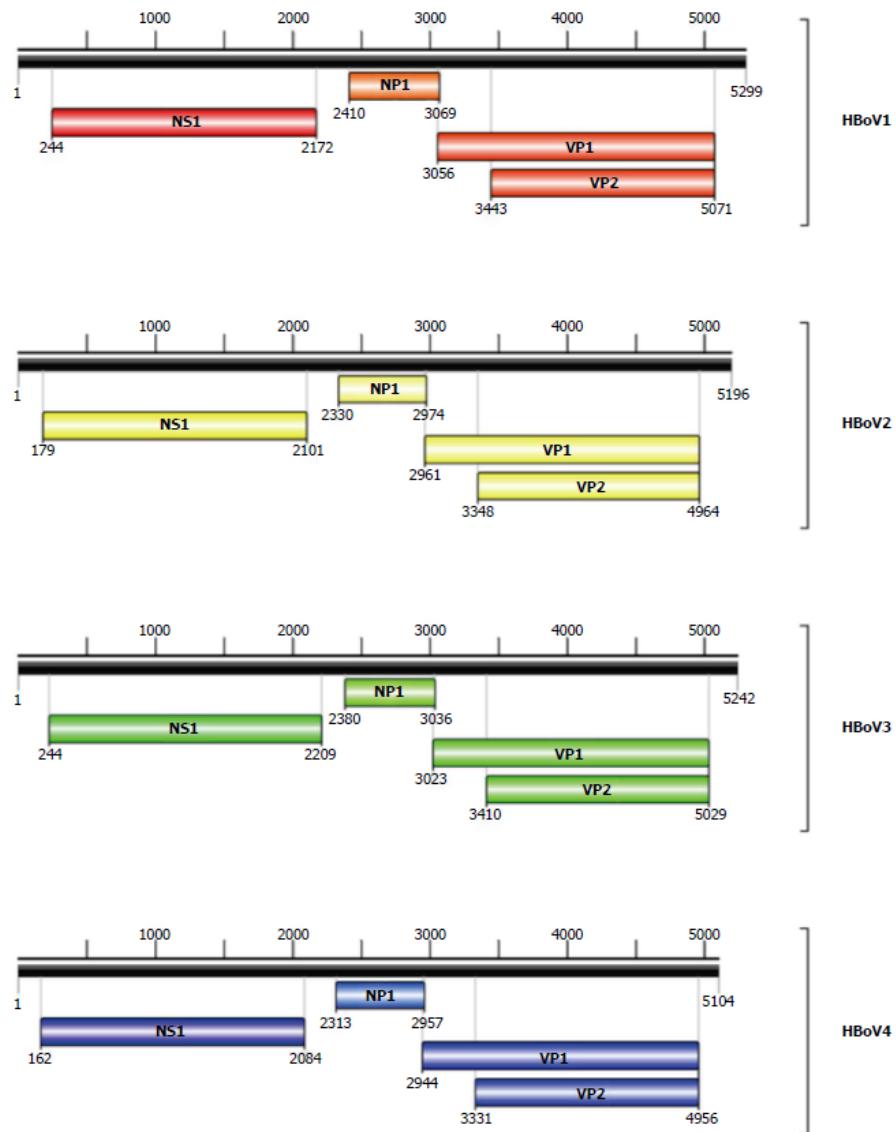
Figure 2.5: The genome structure of the four subtypes of HBoV (adapted from Guido et al., 2016).

Differences between the different strains of HBoV, however, have been observed by Zhao et al. (2012). The HBoV2 strain apparently displays a circular genome (Schildgen, 2013). Two HBoV genome isolate prototypes have been observed, 5 217 and 5 299 nucleotides in lengths respectively (Schildgen et al., 2012).

HBoV3 displays similarities in the sequences of NS1 and NP1 to HBoV1. However, VP1 and VP2 sequences display similarities to HBoV2, thus indicating that HBoV3 is a recombinant of the first and second strains (Guido et al., 2016). Few polymorphisms in HBoV indicate a high rate of recombination and mutations, aiding the rapidly evolving virus. As a result, HBoV has recently been classified into two main groups or species, namely human bocaparvovirus 1 and 2. Human bocaparvovirus 1 consists of the HBoV1 and 3 strains, whereas human bocaparvovirus 2 consists of HBoV strains 2 and 4 (Guido et al., 2016).

The replication of viruses within the *Parvovirinae* family, such as HBoV, is complex and can occur via the rolling-cycle or rolling-hairpin models. The latter, typical to parvovirus, occurs via the creation of concatenameric intermediates. Head-to-head or tail-to-tail structures are typical of this replication model. The head-to-head structures have been observed in HBoV1 respiratory infections, but no such concatenameric intermediates have been discovered in the HBoV2 and HBoV3 subtypes. However, due to recent evidence, HBoV has been postulated to become established within the host cell and forming circular episomes, instead of the previously described concatemers. Such episomes have been discovered in all HBoV genotypes (Guido et al., 2016).

2.7.2.2. Pathogenesis, epidemiology and detection

HBoV was first described by Allander et al. (2005) in children suffering from RTI using random PCR, sequencing and bioinformatics. It has been observed that 3.1% of children displaying unresolved RTI tested positive for HBoV (Guido et al., 2016). There are four subtypes, the most common being HBoV1 observed in the upper and lower respiratory tract, while the others are associated with gastrointestinal tract infections (Schildgen, 2013; Guido et al., 2016). HBoV is also found in the nasopharynx, as well as the myocardial endothelial cells (Gurda et al., 2010; Nielsen et al., 2014). The virus first enters the host via the bloodstream, respiratory tract or direct ingestion (Guido et al., 2016).

The characteristic age of patients suffering from HBoV1 associated RTI ranges between 6 and 24 months of age (Schildgen et al., 2012). HBoV1 infection presents with symptoms of coughing, fever, rhinorrhoea, asthma exacerbation, bronchiolitis, acute wheezing and pneumonia, as well as rare cases of adult nausea, vomiting and diarrhoea. Viral loads of HBoV1 have been observed to be much higher in paediatric respiratory samples than stool samples (Guido et al., 2016). However, only three cases aged between eight months and four years have been reported where HBoV1 LRTI had been life-threatening (Brebion et al., 2014). HBoV1 has also been confirmed in blood samples in cases of systemic infection (Schildgen et al., 2012).

Only three serious HBoV myocardial infections have been reported, two of which were fatal. The age range of these three cases was from 8 weeks to 2 years old (Heydari et al., 2011; Brebion et al., 2014; Sallmon et al., 2017). HBoV2 was detected in one of the cases, a 13 month old female. Symptoms of respiratory grunting as well as tachycardia were observed, with the autopsy revealing no macroscopic cardiac abnormality. Histological examination showed lymphocytic and mononuclear permeation of the cardiac tissue, myocyte degradation in the interventricular septum, oedema and fibrosis. The damaged septum caused the arrhythmias and ultimately heart failure (Brebion et al., 2014). Hospitalised children with acute RTI display HBoV2 as the only species present in nasopharyngeal

aspirates (NPA). HBoV2 and HBoV3 have been associated with gastroenteritis where it was detected in the duodenum, paranasal sinus mucosa, intestinal biopsies and also commonly found in stool samples of children (Schildgen et al., 2012; Guido et al., 2016). HBoV has been observed to persist six months after infection in NPAs (Guido et al., 2016).

The virus is autonomous, meaning that helper viruses are absent (Schildgen et al., 2012), however, infections of increased severity are usually due to co-infections of up to six different pathogens (Schildgen, 2013). Respiratory and gastrointestinal viruses, such as HRV, RSV, HAdV, rotavirus and norovirus, as well as bacteria, show a high rate of co-infection with HBoV. A viral load of greater than 104 copies per millilitre is often correlated to elongated hospital stays and severe symptoms. However, if HBoV is detected in combination with other viruses in asymptomatic individuals, reactivation of a latent virus is often established through super infection. This raises concerns whether HBoV could be a non-pathogenic passenger (Guido et al., 2016). A study performed by Kuethe et al. (2009) investigated whether HBoV DNA persists in human hosts from early childhood infection, much like the closely-related PVB19, possibly a characteristic specific to parvovirus pathobiology (Schenk et al., 2009). However, the study observed no viral persistence in HBoV (Kuethe et al., 2009). According to Koch's modified postulates, it is not possible to ascertain whether HBoV is in fact pathogenic, mainly due to the lack of an animal model, but also as a result of replicating difficulties *in vitro* in cultured cells (Schildgen, 2013; Guido et al., 2016).

An *in vitro* culture for HBoV consists of using pseudostratified human airway epithelium at an air-liquid interface (HAE-ALI). Bronchial epithelium was used to replicate HBoV, as other cell lines proved to be ineffective, possibly due to the absence of certain receptors resulting in the immunity to respiratory viruses. Virions of HBoV are able to infect HAE from basalolateral and apical surfaces, causing damage of the epithelium resulting in the loss of cilia and disruption of the tight junction barrier. Epithelial hypertrophy has also been observed. HAE-ALI cultures showed that HBoV1 can persist in the cells for up to 50 days and can be responsible for DNA damage, which can aid HBoV genome replication (Guido et al., 2016). The viral DNA is postulated to be either included into the human chromosomes, stored as episomes, the virions attached to follicular dendritic cells, or even transmitted into the human body inside macrophage cells (Schildgen et al., 2012).

The incidence of HBoV infection is consistent in both children and adults throughout Europe, Asia, Africa, Australia and the Americas. It persists throughout the year, however it is more common in the spring and winter months. Shortly after its discovery, HBoV was present in cases from different countries in the respiratory and gastrointestinal tracts in 1.0-56.8% and 1.3-63.0% of cases respectively. The prevalence of co-infections with HBoV in RTI ranged between 8% and 100%, while

co-infections in the gastrointestinal tract were 46.7% (Guido et al., 2016). In a study conducted by Kuethe et al., (2009), HBoV antibodies were detected in 96% of patients presenting with no symptoms of myocarditis or cardiomyopathy, however viral DNA could only be detected in 5% of the tissue samples.

Detection is limited to molecular methods, such as conventional qualitative PCR, real-time PCR and Luminex respiratory viral panel assays (Schildgen, 2013). Amplification usually targets the VP1/2 regions, or the NP1/NS1 regions, conserved across genotypes (Manning et al., 2006; Guido et al., 2016). Multiplex assays have been used in order to ascertain HBoV genotypes, including the Luminex. Next generation sequencing in conjunction with sequence independent amplification technologies display much promise for identifying HBoV, among other viruses, simultaneously. Western blotting, ELISA, immunofluorescence and other serological methods have been used to identify HBoV (Guido et al., 2016).

2.7.3   Parvovirus B19

2.7.3.1. Structure

PVB19 was discovered by Cossart et al. (1975) and belongs to the family *Parvoviridae*, known to be pathogenic in humans. The family is known to include some of the smallest DNA containing viruses (Heegaard & Brown, 2002). It belongs to the same family as HBoV. PVB19 was recently reclassified into the genus *Erythroparvovirus*, since the virus infects erythroid progenitor cells. The prior classification in *Tetraparvovirus* genus was due to no helper viruses being needed for infection (Heegaard & Brown, 2002; Guido et al., 2016).

PVB19 consists of a non-enveloped linear single-stranded DNA core and only two capsid proteins (Heegaard & Brown, 2002; Schenk, et al., 2009). Sixty capsomer copies are present. The virions are between 22 to 24 nm in diameter with capsids displaying icosahedral symmetry. PVB19 varies from other viruses in its family, as there are no obvious spikes present on the capsid surface involved in host cell antigenicity identification. The lack of a lipid envelope and the small amount of DNA present make PVB19 resistant to physical inactivation (Heegaard & Brown, 2002).

The linear negative or positive sense DNA consists of 5 596 nucleotide bases, consisting of a 4 830 bp internal coding region (Heegaard & Brown, 2002). The coding region is flanked by palindromic terminal repeats, which assume a hairpin duplex structure, are self-priming and facilitate the synthesis of a complimentary strand (Mori et al., 1987; Heegaard & Brown, 2002). Two main ORFs are present. NS1 is coded in a conserved region on the left side of the genome, while the two capsid proteins VP1 and VP2 are coded by genes subject to variation found on the right (Figure 2.6). Unique to PVB19,

transcripts terminate mid genome with the use of a polyadenylation signal (Heegaard & Brown, 2002). Forty-seven different isolates have been identified, however there is little variation indicating that the PVB19 genome is highly conserved (Mori et al., 1987).
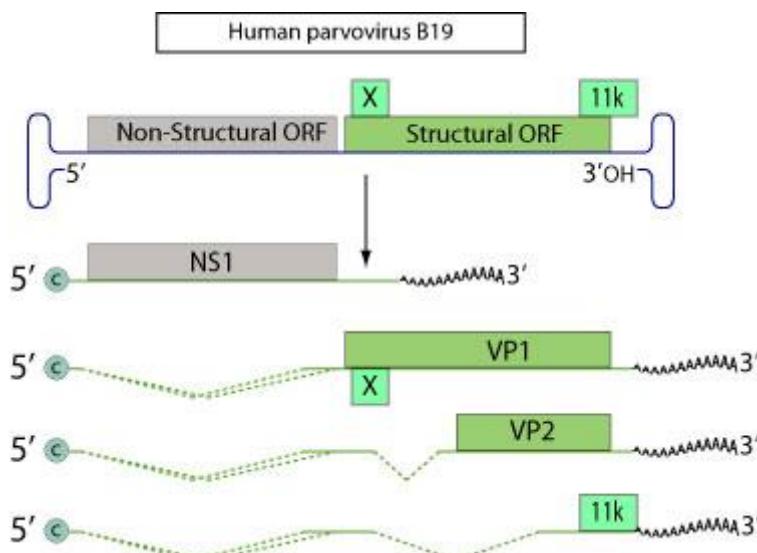

Figure 2.6: The genome structure of human parvovirus B19 (Swiss Institute of Bioinformatics, 2008).

2.7.3.2. Pathogenesis, epidemiology and detection

PVB19, unlike most viruses, targets highly differentiated cell types. This occurs due to a cellular receptor specific to the erythroid lineage of cells in the bone marrow, namely the blood group antigen P (Brown et al., 1993; Kuethe et al., 2009). PVB19 therefore presents as erythema infectiosum, transient aplastic anaemia, arthritis, hepatitis, vasculitis syndromes and hydrops foetalis. The PVB19 DNA has been discovered in the heart tissue of individuals who suffered from dilated cardiomyopathy, peripartum cardiomyopathy and acute myocarditis, as well as in various unaffected tissues of healthy individuals. It is postulated that PVB19 infection resulting in an acute myocarditis, results in vasospasm of the coronary arteries which imitates ischemic heart disease (Kuethe et al., 2009).

Cardiac endothelial are the host cells for PVB19 infection, presuming that the blood group P-antigen is present (Bültmann et al., 2003). The P-antigen acts specifically as a cellular receptor for PVB19. Foetal cardiac myocytes and erythroid progenitor cells also display the presence of the P-antigen, thus making these cells susceptible to PVB19 infection as well (Brown et al., 1993). Kuethe et al. (2009) reported that no there is no distinction between PVB19 persistence in the left atrium and left ventricle. The PVB19 genome has been connected to the development of dilated cardiomyopathy with inflammation as well as chronic myocarditis, and consequently acknowledged as a potential cardiac pathogen (Pankuweit et al., 2003). It displays a permanent persistence in the heart tissue of infected individuals (Guido et al., 2016). Viral loads of 1 000 PVB19 genome equivalents 1×10^6 cells

have been described as normal findings in a subject, and higher copy numbers are indicative of infection as is observed in cases of hydrops foetalis. It is postulated that a higher copy number will result in the onset of acute myocarditis (Kuethe et al., 2009).

Studies have observed genotype 2 of PVB19 was most common in individuals presenting with dilated cardiomyopathy and was the only detected genotype of the virus in patients older than 65 years of age. Younger individuals were more commonly infected with genotype 1 and displayed more intensive left ventricular abnormalities than genotype 2 (Kuethe et al., 2009).

Kuethe et al. (2009) reported that approximately 65% of endomyocardial tissue samples were positive for PVB19 DNA, suggesting it to be a possible major contributor to viral myocarditis. They also found a seroprevalence of 85% in patients with no presenting symptoms of myocarditis or cardiomyopathy, despite the virus being detected in the heart tissue collected from these patients. This raises the question as to whether the virus persists after infection in childhood or adolescence. Over 90% of the German population have displayed seropositivity for both PVB19 and HBoV (Kuethe et al., 2009). In an audit from 2004 to 2007, one out of 116 SUDI cases displayed a PVB19 infection (Prtak et al., 2010).

Diagnosis of PVB19 is often performed using an ELISA to detect certain antibodies, or DNA detection in a PCR assay or dot lot hybridisation (Heegaard & Brown, 2002). DNA detection usually results from both nested qualitative and quantitative PCR, targeting the conserved VP1/VP2 region (Bültmann et al., 2003; Tu et al., 2015).

2.8.　　Post-mortem investigation

In the majority of developed and developing countries SUDI cases must be investigated by Medico-legal Institutions. The COD is determined during extensive investigations, and only thereafter infants with no identifiable COD can be classified as SIDS. A protocol to ascertain the COD of each SUDI victim is necessary, in order to determine risk factors which may differ between regions. No standardised infant death protocol is available in South Africa, although SUDI cases require formal Medico-legal Inquiry according to the Inquests Act (Act 58 of 1959) (Du Toit-Prinsloo, 2013).

Certain international autopsy protocols do exist, but vast differences make comparison difficult. External, internal and X-ray investigations, as well as body measurements are documented to ascertain the development of the infant (Bajanowski et al., 2007). Three important COD enquiries need to be conducted in SUDI cases, namely the review of clinical history; death scene investigation and post-mortem examination consisting of specific laboratory investigations, such as microbiology and virology. Death scene investigation is a crucial part in determining certain risk factors in cases of

unexpected death and perhaps indicate the mechanism of death e.g. suffocation. Bacteriological studies are often unhelpful due to post-mortem flora contamination (Du Toit-Prinsloo et al., 2011). Macroscopic autopsy alone cannot always show morphological changes associated with the COD (Vorontsov & Kelmanson, 1990). Cases in South Africa are recorded as SIDS 1B if certain scene and supplementary investigations are not performed due to reasons such as the lack of funding (Bajanowski et al., 2006; Du Toit-Prinsloo et al., 2013).

In South Africa a natural death of an infant is constituted where a treated disease or condition has been recorded, according to the Births and Deaths Registration Act 51 of 1992. A death notification form is issued by the assisting medical personnel (Du Toit-Prinsloo et al., 2011). According to the Inquests Act (Act 58 of 1959), an unexplained or unexpected death constitutes an unnatural death, and require post-mortem investigations at the relevant medico-legal facility. The following information is usually documented in unexpected deaths: demographic details, date of death, patient history, scene investigations, characteristics of post-mortem and supplementary investigations, COD (if available), and finally the training level of the autopsy physician. Supplementary investigations often include microbiology, virology, histology, toxicology, radiography, genetic and metabolic screening (Du Toit-Prinsloo, 2013). Understanding sudden death in infants is dependent on the results of the post-mortem and supplementary investigations (Koehler, 2010).

2.9. Aims and objectives

2.9.1. Aim

The aim of the study is to prospectively investigate the presence of specific viruses in the heart of SUDI cases admitted to the Tygerberg Medico-legal Mortuary over a 1-year period.

2.9.2. Objectives

- To collect swab samples of the heart from all SUDI cases admitted to the Tygerberg Medico-legal Mortuary in collaboration with the Western Cape Forensic Pathology Services and Division of Forensic Pathology, Stellenbosch University.

- To validate and optimise individual PCR assays for the detection of HAdV, HBoV and PVB19 to establish their prevalence within the SUDI cases at the Tygerberg Medico-legal Mortuary.

- To compare viral results with histopathological assessment of tissue collected from the heart.

- To investigate associations between viruses detected, epidemiology of infection and any clinical signs and symptoms prior to death, using molecular, microbiological and histological techniques.

- To correlate the collected sociodemographic data (e.g. gender, age, season, clinical signs of disease in the days preceding death, etc.) to identify possible trends in relation to infection caused by any of the aforementioned viruses.

3.

METHODOLOGY

3.1.   Introduction

The study prospectively investigated the SUDI cases admitted to the Tygerberg Medico-legal Mortuary, Eastern Metropole referral area of the Western Cape, between March 2018 and March 2019, therefore encompassing all four seasons. Swabs of the heart were collected during autopsy and used specifically for viral analysis. Heart tissue was also excised and processed for routine histological analysis, conducted by the Division of Forensic Medicine. The histology was used in concert with routine diagnostic tests performed by the NHLS to assign a final COD as part of the post-mortem investigation. The viral results obtained from this study were analysed in combination with the histopathological assessment of the tissue, the gold standard in determining evidence of active infection (Weber et al. 2010). All SUDI cases that were included in the study were between the ages of seven and 365 days old. Sample collection was performed under the auspices of the Inquests Act (Act 58 of 1959).

The methodology to be explained can be divided into three main stages: sample collection, laboratory analyses and statistical analysis (Figure 3.1).
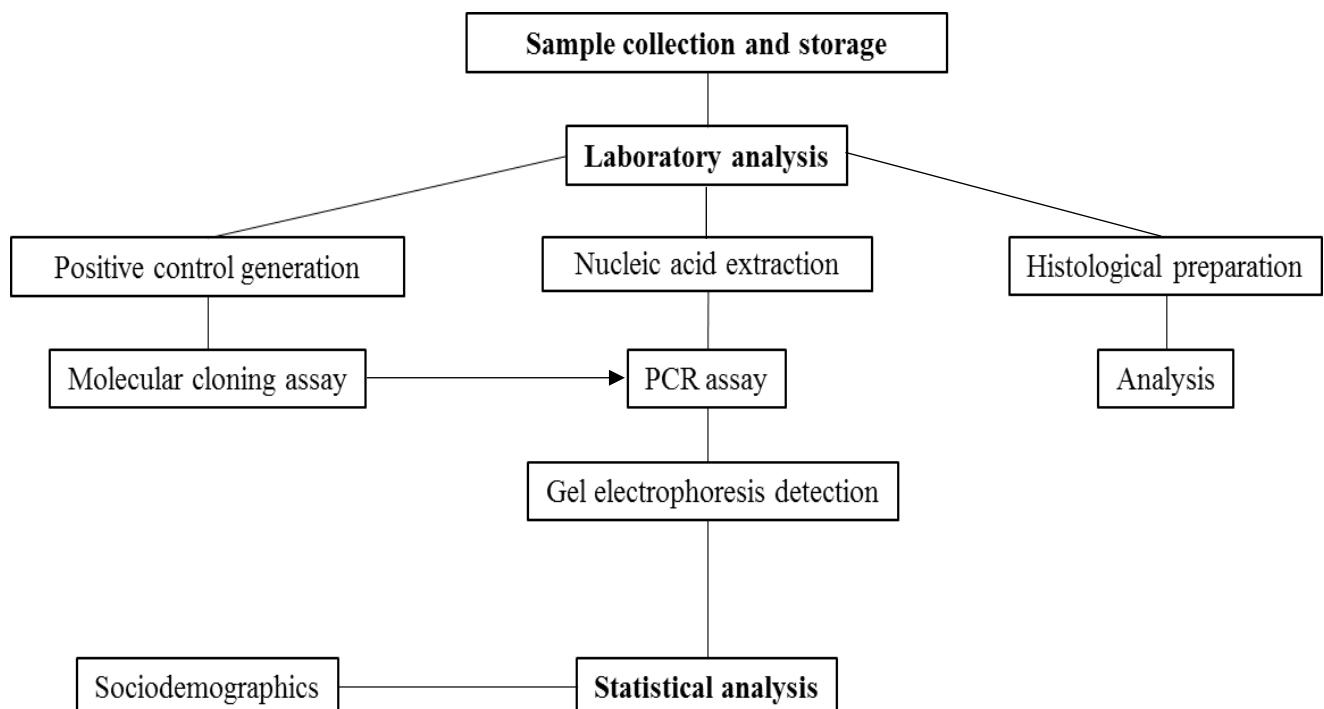

Figure 3.1: Flow chart of the study methodology divided into three main categories; namely sample collection, laboratory analyses and statistical analysis.

3.2. Ethical considerations

The Human Research Ethics Committee (HREC) at the Faculty of Medicine and Health Sciences, Stellenbosch University approved the protocol of the project (reference number: N12/02/007). Ethics approval is renewed annually (Appendix 1). The collection and investigation of samples were classified as part of the autopsy process in order to assign a COD, therefore a waiver of consent was granted providing that the project does not deviate from the routine institutional investigation protocol. As specified in the Inquest Act (58 of 1959) and the Criminal Procedures Act (1977), consent from the next of kin is not required if the investigations are aiding in the determination of the COD.

In order to maintain patient confidentiality, each sample was marked with the death register number only (WC/14/..../2018). Personal information was not used to label the specimens. A liaison officer at the Division of Forensic Medicine protected the identity of the deceased infants, as well as the link to the collected viral swabs and routine microbiology and viral samples.

3.3. Sample collection and processing

3.3.1. Inclusion and exclusion criteria

SIDS remains a diagnosis through exclusion (Du Toit-Prinsloo & Dempers, 2011). Therefore, investigation of the clinical history, death scene and post-mortem examination was performed. Infants were termed as 'SUDI' when death occurred with no apparent reason for death was apparent in the history. SUDI, however, is not the diagnosis. Cases were excluded from the study where intentional injury was observed or suspected (Vennemann, et al., 2007). All SUDI cases that were included in the study were between the ages of 7 and 365 days old. Infants under seven days were not included as described by Vennemann et al. (2007), as death could be due to neonatal causes such congenital abnormalities during the first week.

3.3.2. Autopsy and sample collection

The autopsy dissection was initiated by a FPO, who created a Y-shape incision. The incision began behind each ear of the infant and joined at the manubrium, the start of the sternum. A vertical incision was extended up until the pubic bone. The superficial tissue and muscle layers were dissected from the underlying ribs, which were subsequently cut to facilitate the removal of the anterior aspect of the thoracic framework. The organs of the chest cavity were thus exposed.

Samples of the heart were obtained during the autopsy of the SUDI cases. The heart was released from the pericardial sac, and the left ventricle exposed. In order to create an aseptic surface area to

prevent sample contamination, a small area of the epicardial surface of the left ventricle was seared using a spatula heated by a flame. An incision was created with a sterile scalpel blade in the middle of the burnt area. Subsequently, a flocculated swab (FLOQSwab®, Copan, Murrieta, CA) was inserted into the myocardium and swirled around in order to collect as many heart cells as possible. The swab samples were immediately stored in 3 ml of Universal Transport Medium™ (UTM) (Copan, Murrieta, CA). The samples were transported from the Tygerberg Medico-legal Mortuary to a laboratory at the Division of Forensic Medicine, where they were vortexed vigorously. Aliquots of 1 ml each were subsequently made and stored at -80°C, ready for viral nucleic acid extraction. These samples were stored in the Division of Forensic Medicine and analysed in the Division of Medical Virology, Faculty of Medicine and Health Sciences, Stellenbosch University.

A microbiological swab (Copan, Murrieta, CA) was collected from the same site; however, this sample was collected for routine bacteriological investigation by the NHLS, as part of the routine institutional investigation protocol.

Tissue from the left ventricle of the heart was collected using a sterile scalpel blade and forceps, in order to be processed for histological analysis. The volume of myocardium collected was 2 x 2 x 5 cm. The tissue block was then placed into a Tissue-Tek Uni-Cassette at the Tygerberg Mortuary, the cassette then being stored in a 10% formalin solution until further processing. The samples were transported from the Tygerberg Medico-legal Mortuary to the Forensic Pathology laboratory at Stellenbosch University, Faculty of Medicine and Health Sciences.

3.3.3. Histological analysis

All histological preparation and staining of the tissue were performed by the Chief Forensic Technologist and a Medical Technologist of the Division of Forensic Medicine, Stellenbosch University, Mr André Du Toit and Mr John Segole respectively.

3.3.3.1. Tissue processing

The heart tissue collected for histological analysis was placed in a Tissue-Tek Uni-Cassette and fixed in 10% formalin for up to 24 hours, in order to harden and preserve the tissue. The preserved tissue subsequently underwent routine processing through the steps of a Tissue-Tek® VIP™ 5 Vacuum Infiltrator Processor (Sakura® Finetek, Europe). The process included 12-24 hours of fixation in formalin. This was followed by the dehydration of the tissue in increasing concentrations of ethanol followed by dehydrant clearing as a result of xylene submersion. The xylene was also responsible for the removal of fat within the samples. In a subsequent step, the sample tissue is infiltrated with liquid paraffin wax, which displaced the remaining xylene. These steps were executed using both a vacuum

and pressure source within the processor. The cycling parameters for the clearing process and wax infiltration are detailed in Table 3.1:

Table 3.1: Cycling parameters for the clearing process and wax infiltration uses for tissue processing

10% Formalin	12-24 hours	
70% Ethanol	1 hour	
80% Ethanol	1 hour	
90% Ethanol	1 hour	
100% Ethanol	1 hour	X3
Xylene	1 hour	X2
Paraffin wax	3 minutes	X4

3.3.3.2. Wax block embedding and sectioning

After processing, each heart tissue sample was embedded in a paraffin wax block in order to facilitate the sectioning of the tissue with a microtome. The sample was placed using forceps in a mould containing molten paraffin wax, at the correct orientation. A cassette was subsequently placed over the mould, containing the liquid wax. The paraffin wax was left to harden for approximately 20 minutes, on ice. The wax block was trimmed using a microtome, after which sectioning commenced.

A microtome, Accu-Cut® SRM™ (Sakura® Finetek, Europe), was used to cut 3-5 µm thick sections of the embedded tissue, and subsequently placed in the AWB 210 Water Bath (Amos Scientific) at 60ºC to remove folds. Each section was placed on a microscope slide and placed in an oven (50-60ºC) in order to melt the excess paraffin wax of the sections and ensure adhesion to the slide.

3.3.3.3. Haematoxylin and eosin staining

The microscope slides were stained using autostainer, namely the Tissue-Tek Stainer coverslipper Film machine (Sakura® Finetek, Europe), following a protocol for haematoxylin and eosin (H&E) (Appendix 2). The H&E staining technique enabled the identification of histological structures within the heart tissue, including morphological changes. The unstained sections underwent rehydration prior to the application of the stains. The basic Mayer's haematoxylin dye stains the acid cell structures such as nuclei and ribosomes. The acidic eosin yellowish dye is used to stain basic structures in the cytoplasm of the cell. Cover slips were mounted on the microscope slides using DPX mounting media.

The stained microscope slides were analysed under the Olympus® BX41 light microscope at 40x, 100x and 200x magnification, containing an inbuilt digital camera. Primary histological analysis was

performed by the forensic pathologists and registrars of the Division of Forensic Medicine. Re-evaluation of the myocardial histology, for the sake of this study, was performed by Prof. Johan Dempers, Head of Division and Specialist of Forensic Pathology at the Faculty of Medicine and Health Sciences of Stellenbosch University and Western Cape Forensic Pathology Services. The object of analysis was to identify signs of myocarditis such as lymphocytic and neutrophil infiltrates, as well as necrosis of myocytes.

3.3.4.   Review of case file information

The clinical history of each SUDI case was recorded, as well as the sociodemographic information which is routinely collected by FPOs in the form of a standard questionnaire. All information is stored in hard copy and electronically in case files and the questionnaire is completed by a guardian or family member of the deceased infant at the time of admittance to the Tygerberg Medico-legal Mortuary. The information was then transcribed into a Microsoft® Office Excel spreadsheet. The relevant data was numerically coded in the spreadsheet in order to facilitate the statistical analyses. For example, the assigned COD as *SIDS*, *Infection* and *Other* were coded one, two and three respectively. The gender, age, season of death, race, birthweight, prematurity, sleeping position, bedding, bed-sharing, room ventilation, housing type and COD assigned by the pathologist were all recorded for statistical analysis. The COD was based on all the evidence collected from the post-mortem investigation which included the death scene investigation, autopsy, histological analysis, clinical review, as well as routine laboratory investigations.

3.4.   Laboratory Analyses

In order to generate positive controls and optimise the polymerase chain reaction (PCR) assays, quality control for molecular diagnostics (QCMD) samples for PVB19 and HAdV were acquired from the NHLS. No QCMD sample could be acquired for HBoV however, and a known positive patient sample obtained from the NHLS was used for the purpose of generating a positive control as well as optimising the PCR assay. The QCMD and positive patient control samples were stored at -80°C until the day of analysis. A negative case control was extracted for both DNA and RNA from unused universal transport media (UTM), in which the swab samples were transported. Extractions of both DNA and RNA were performed at room temperature in a Class II Biosafety Laminaire® biosafety cabinet.

3.4.1.   Nucleic acid extraction and quantitation

The DNA of the 173 heart swab samples were extracted using the QIAamp® DNA Mini extraction kit according to the manufacturer's instructions (Qiagen®, Cape Town). DNA was also extracted from QCMD and positive patient control samples for each virus, which were used to test the sensitivity of

the PCR assays. The UTM underwent extraction in order to confirm that no nucleic acid was present that could contaminate further results.

The procedure followed for DNA extraction using the Qiagen DNA mini kit was as follows: 200 µl of fluid virus sample was added to 20 µl protease in order to facilitate the lysis of cellular matter. Subsequently, 200 µl of Buffer AL was added to the mixture, vortexed for approximately 15 seconds and incubated at 56°C for 10 minutes. The mixture was pulse vortexed for approximately 15 seconds after 200 µl absolute ethanol was added. The lysated mixture was added to a QIAamp mini spin column placed in a collection tube and centrifuges at 8 000 rpm (revolutions per minute) for one minute. Exactly 500 µl of AW1 and AW2 wash buffers were added to the spin columns one after the other, separated by centrifugation. After AW2 was added, the spin columns were centrifuged for 4 minutes at 13 200 rpm. The viral DNA was eluted with 60 µl of Buffer AE. Extracted DNA was stored at -80°C until the day of analysis.

The quality and concentration of the DNA extracted from QCMD and positive patient control samples were determined using the NanoDrop ND-1000 spectrophotometer. Both the lower and upper measurement pedestals were wiped clean with paper towel and deionised water. The ND-1000 computer software was opened on the "nucleic acid" option. In order to initialise the microspectrophotometer the sampling arm was opened and a 1 µl drop of nuclease free water was pipetted onto the centre of the lower measurement pedestal. The sampling arm was closed and "initialise" was selected on the software. The microspectrophotometer was blanked using 1 µl of nuclease free water. Subsequently, the measurement surfaces were cleaned. Prior to sample loading, the option for DNA or RNA analysis was selected. Exactly 1 µl of the sample was loaded onto the lower pedestal. The sample arm was lowered and the option to "measure" was selected. The 260/280, 260/230 and ng/µl values were recorded for each sample.

The 260/280 ratio refers to light absorbance at 260 nm and 280 nm in order to ascertain the purity of DNA or RNA. Purity for a DNA sample should indicate a ratio as close to 1.8 as possible. The 260/230 ratio refers to light absorbance at 260 nm and 230 nm and is a secondary measure of nucleic acid purity. A ratio lower than 2-2.2 indicates contaminants. The concentration of DNA present is indicated by ng/µl.

3.4.2. PCR assay

Conventional qualitative PCR assays were used to detect the presence of HAdV, HBoV and PVB19. The protocol established for each virus used published primers from peer-reviewed literature. The BLAST database (https://blast.ncbi.nlm.nih.gov/) was used in order to verify the gene-specific

binding sites of the primers to the viral genomes in question. The nucleotide sequences of the primers were entered into the database, which in turn released a list of possible binding sites to the viruses. The HAdV primers selected targeted the hexon protein gene, similar in all HAdV serotypes (Martin, et al., 1994). As previously stated in section 2.7.1.2, finding generic primers encompassing all strains is a challenge. HBoV primers targeted the conserved NP1 region of all four genotype regions. PVB19 primers targeted the VP1/VP2 coding regions. Primers from HAdV and PVB19 that were previously used to generate inserts in the glycerol stock clones, were also used for this study.

PCR was used to detect the QCMD samples and a positive patient sample in order to generate a positive control via cloning. Colony screening of the transformed bacteria was also performed using the pre-nested protocol. The case samples were analysed with PCR assays, using the primer sequences as displayed in Table 3.2.

One*Taq* Hot Start DNA Polymerase (New England Biolabs®, United States of America.) was used in the PCR assays to detect the viruses. Hot Start *Taq*, a combination of *Taq* DNA polymerase and an aptamer-based inhibitor, allows for greater sensitivity and specificity during amplification of the target DNA sequence. The inhibitor prevents the enzyme from reacting below 45°C. The PCR protocol for each virus was optimised and subsequently tested on QCMD and positive patient samples in order to verify the protocol's diagnostic sensitivity.

A PCR master mix was made up in a sterile 1.5 ml Eppendorf tube with the addition of nuclease free water, standard NEB DNA polymerase buffer, deoxyribonucleotide triphosphates (dNTPs), forward and reverse primers and *Taq* DNA polymerase. The master mix was centrifuged briefly. Equal portions of the PCR master mix were added to sterile 0.2 ml PCR 8-tube strips with strip caps (STARLAB International, Hamburg). The template was added to the PCR tubes and the mixture was subsequently spun down to eliminate any bubbles present. The PCR assay had a final volume of 50 µl (Table 3.3).

The reaction mixture was subsequently placed in a SimpliAmp™ thermal cycler (Life Technologies, Applied Biosystems® by ThermoFisher Scientific™, Randburg) in order to initiate the PCR. The thermocycling conditions used for the pre-nested detection of all viruses are presented in Table 3.4.

During all screening PCRs a positive and negative control were included, as well as a negative case control, as previously mentioned. The positive control PCR was performed using purified plasmid DNA containing the insert of the target virus as a template. The template used for the negative control was 4 µl of nuclease-free water. A template of 4 µl of nucleic acid extracted from UTM was used in

the PCR assay to indicate that nothing from the transport media could affect the results of the DNA/RNA extractions and PCR.

Table 3.2: Primer nucleotide sequences used in PCR assays for HAdV, HBoV and PVB19

Virus	Primer Sequence (5'-3')	Reference	Expected product size	Target sequence	Annealing temperature
HAdV	a-GCC GCA GTG GTC TTA CAT GCA CAT b-CAG CAC GCC GCG GAT GTC AAA GT	Dettmeyer, et al. 2004	300	Hexon protein gene	67.2ºC
HBoV	a-GAG CTC TGT AAG TAC TAT TAC b-CTC GTG TTG ACT GAA TAC AG	Allander, et al. 2005	354	NP1	57.8ºC
PVB19	a-AGC ATG TGG AGT GAG GGG GC b-AAA GCA TCA GGA GCT ATA CTT CC	Bültmann, et al. 2003	290	VP1/VP2	55.7ºC

Table 3.3: Volumes of individual components per reaction used in the PCR assays

Component	Volume
Nuclease-free water	29.75 µl
Buffer	10 µl
dNTPS	1 µl
Primer (forward)	2.5 µl
Primer (reverse)	2.5 µl
Taq DNA polymerase	0.25 µl
Template	4 µl µl
Total	**50 µl**

Table 3.4: Thermocycling conditions for the PCR assays

Stage	Temperature	Time
Initial denaturation	94°C	30 seconds
Denaturation	94°C	30 seconds
Annealing	Specific to virus (see table 2)	1 minute (30 cycles)
Extension	68°C	30 seconds
Final extension	68°C	5 minutes
Hold	4°C	∞

3.4.3. Gel electrophoresis

The presence of HAdV, HBoV and PVB19 were detected on a 2% agarose gel. Two grams of agarose powder was placed in a clean microwavable glass container, thereafter 100 ml of tris acetate EDTA (TAE) buffer (Appendix 2) was added. The mixture was microwaved in 30 second intervals until

transparent. Subsequently, the solution was left to cool to 60ºC before adding 5 µl of EZ Vision staining dye, and subsequently pouring the mixture into the casting plate. The comb was inserted into the casting plate. Fifty mililitres of the liquefied gel was poured and left to polymerise for 15 minutes. Once set, the comb was removed to expose the sample wells. The gel was placed into the Enduro™ Electrophoresis System and TAE buffer was poured in to completely submerge the gel. One microlitre of loading dye was mixed with 5 µl of PCR product, which was subsequently loaded into the gel wells. The GeneRuler 1 kb Plus DNA ladder containing bands of known size was used as a reference in order to confirm the sample DNA fragment size. The PCR product of a cloned positive patient control for each virus was included in the relevant gel electrophoresis reactions, as well as a negative PCR control and the PCR product of the negative case control. The gel tank was connected to the power supply and set to a voltage of 90V, to run for one 50 minutes. The DNA bands were visualised in the UVIprochemi II D-77LS-26M Gel Doc system, using the transilluminator light.

3.4.4. Purification of PCR products and gel extraction

PCR product purification and extraction of sample from a gel were performed using the Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit (ThermoFisher Scientific™, Randburg). All centrifugations were carried out for one minute at 14 000 relative centrifugal forces (rcf).

The manufacturer's instructions for PCR cleanup included adjusting the volume of the PCR product to 200 µl with nuclease-free water. Subsequently, 100 µl of Binding Buffer was added and mixed after which 300 µl of absolute ethanol was added. The mixture was added to the Micro Columns and centrifuged. Exactly 700 µl of Wash Buffer was added, followed by centrifugation. This step was repeated. The empty Micro Columns were subsequently centrifuged. The purified DNA was eluted in 10 µl of Elution Buffer.

DNA extraction from a gel was initiated with excising up to 200 mg of a 1% gel that contained the DNA band and placing the fragment into a 1.5 ml tube. This was performed over a transilluminator. Subsequently 200 µl of Extraction Buffer was added and mixed thoroughly. The mixture was incubated at 58°C for 10 minutes. Exactly 200 µl of absolute ethanol was added, after which the mixture was transferred to a purification Micro Column. Prewash Buffer (200 µl) was added and the mixture was centrifuged. Subsequently, 700 µl of Wash Buffer was added to the mixture which was then centrifuged. This step was repeated. The empty Micro Column was centrifuged, after which the purified DNA was eluted with 10 µl Elution Buffer. Gel extraction worked optimally with a gel percentage of $\leq 1\%$.

The purified DNA was used in a sequencing reaction.

3.4.5. Molecular cloning assay

All positive controls were generated using inserts specific to the selected primer target regions, therefore all positive controls were representative of the strains and genotypes previously mentioned in section 3.4.2.

3.4.5.1. HBoV ligation, transformation and plating

The positive control for HBoV was created using the New England Biolab PCR Cloning Kit. The ligation reaction to join the nucleotide sequence of interest to the pMiniT 2.0 vector had a total volume of 10 µl. The reaction took place in two parts, initially adding the first four components: the vector, insert, amplicon cloning control and nuclease-free water. Subsequently, the mixture of Cloning Mix 1 and Cloning Mix 2 were added. This method ensured that the ligase did not recircularise the vector prior to the presence of the insert. The positive control provided by the PCR Cloning Kit was used in the ligation reaction in order to ascertain its success. The volumes of components used in the ligation reaction are presented in Table 3.5.

The insert added originated from the purified PCR product of a positive patient sample. The amount of insert added was calculated using the DNA concentration of the template (13.9 ng/µl) obtained using the NanoDrop ND-1000 spectrophotometer and the NEBiocalculator web tool (www.NEBioCalculator.neb.com). It is recommended that the linearised vector (2 588 bp) to insert ratio is 3:1. The calculations are summarised below.

a. Relative length calculations:

ng insert to be added   = (3)(25 ng vector)(bp of insert/2 588 bp of vector)

                                     = (3)(25 ng)(354 bp/2 588 bp)

                                     = 10.26 ng

b. Volume (in µl) of insert to add to the ligation reaction:

$$V = \frac{n}{c}$$

10.26 ng/13.9 ng/ µl

= 0.74 µl

~ 1 µl of insert was added, as this was the minimum suggested volume

The mixture was incubated at room temperature (25°C) for 15 minutes to increase transformation levels, as the insert was only 354 bp. Thereafter, the mixture was incubated on ice for 2 minutes. The ligation product was transformed immediately. The remainder of the ligation product was stored at -20°C.

Transformation was completed using the NEB 10-beta Competent *E. coli* cells. Exactly 50 µl of competent cells were thawed on ice for 10 minutes. Subsequently, 2 µl of the ligation reaction was added to the cells and mixed by flicking the tube. Incubation on ice was conducted for 20 minutes, followed by heat shock at 42°C for 30 seconds. The reaction was chilled on ice for 5 minutes. Stable Outgrowth Medium (950 µl) was added to the mixture and incubated at 37°C for 60 minutes with shaking at 250 rpm. The positive control product of the ligation reaction was also transformed using the aforementioned protocol.

The outgrowth and cells were mixed by inversion. Exactly 50 µl of the outgrowth and 50 µl of a 1:10 dilution of the outgrowth was spread plated. The agar plates used contained 100 µg/ml ampicillin and were warmed at 37°C prior to plating. The positive control outgrowth and outgrowth were also plated. The plates were inverted and incubated at 37°C overnight.

Table 3.5: Volumes of individual components used in the ligation reaction

Component	Ligation reaction	Positive control
Linearised pMiniT 2.0 Vector (25 ng/µl)	1 µl	1 µl
Insert	1 µl	
Amplicon Cloning Control (1 kb) (15 ng/µl)		2 µl
Nuclease-free water	3 µl	2 µl
Cloning Mix 1	4 µl	4 µl
Cloning Mix 2	1 µl	1 µl
Total volume	**10 µl**	**10 µl**

3.4.5.2. HAdV and PVB19 plating

HAdV and PVB19 had been cloned using the InsTAclone™ PCR Cloning Kit (Thermo Scientific™, Randburg) and stored as glycerol stocks prior to the start of this study. Due to monetary constraints and the fact that the pTZ57R/T vector plasmid of this kit had been discontinued, the InsTAclone™ kit was not used for cloning HBoV. The glycerol stocks were made up of 500 µl of 50% glycerol solution and 500 µl overnight liquid culture with the vector containing the insert of the virus in question.

The glycerol stock was streaked with a sterile inoculation loop (Figure 3.2). The agar plates were all labelled and placed upside down at 37°C overnight. Thereafter, a colony of bacterial cells was picked up and re-streaked in order to ensure one single colony of transformed *E.coli* cells was used for the PCR positive control. Re-streaking also ensured no contamination was present.
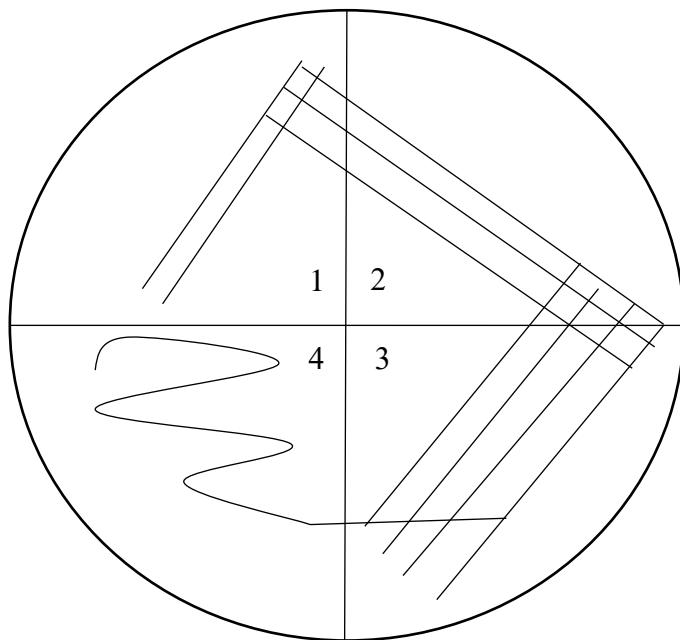
Figure 3.2: Sketch displaying the mechanism of inoculating an agar plate with a glycerol stock solution of transformed bacteria.

3.4.5.3. Screening PCR and bacterial culture

A screening PCR assay was performed on a sampled selected bacterial colony. The same PCR protocol was used as the pre-nested assay, with variation occurring with template addition. A sterile 20 µl pipette tip was used to sample a specific colony, the sample then being added to the mixture of PCR reagents. Four bacterial colonies were selected for screening for each cloning reaction. The PCR reaction was performed and visualised. Once the bands of the appropriate size were observed on gel electrophoresis, the entire selected colony was collected with a sterile pipette tip which was subsequently placed in a sterile falcon tube containing 5 µl of LB broth with 0.05 mg/µl ampicillin concentration. The falcon tubes were placed in the orbital shaker overnight at 37ºC at 250 rpm.

The pipette tip was removed thereafter, and the broth was purified using the GeneJET Plasmid MiniPrep Kit (Thermo Scientific™, Randburg). The bacterial cells of the overnight growth solution were pelleted through centrifugation at 4 000 rpm for 5 minutes at room temperature. The supernatant was discarded thereafter, and the pelleted cells resuspended in 250 µl of the Resuspension Solution. The mixture was vortexed until the pelleted cells were completely dissolved. Subsequently, 250 µl of the Lysis Solution was added and mixed by inverting the tube. Exactly 350 µl of the Neutralisation Solution was added and mixed by inversion, after which the mixture was centrifuged for five minutes. The supernatant was then added to the GeneJET spin column and centrifuged for one minute. All centrifugation from this point was carried out at 8 000 rpm at room temperature. Subsequently, 500 µl of the Wash Solution was added the spin column and centrifuged for one minute. This step was repeated once. The empty spin column was then centrifuged for one minute to ensure residual ethanol

was eliminated. The plasmid was eluted with 50 µl Elution Buffer through centrifugation for two minutes. The purified plasmid was stored at -20°C ready for analyses. Long term storage took place at -80°C. A pre-nested PCR reaction was performed in order to ensure the purification worked.

3.4.6. Sequencing

The purified plasmid product for HAdV and PVB19 was sequenced using the M13 forward (5´d[GTAAAACGACGGCCAG]3´) and reverse (5´d[CAGGAAACAGCTATGAC]3´) primers, in order to target the conserved region of each virus.

HBoV was sequenced using the forward (5´d[ACCTGCCAACCAAAGCGAGAAC]3´) and reverse (5´d[TCAGGGTTATTGTCTCATGAGCG]3´) primers supplied by the NEB® PCR Cloning Kit in order to target the inserted fragment of cloned virus. Sequencing was performed using the BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Scientific™, Randburg) by the Central Analytical Facility (CAF) of Stellenbosch University. After the sequencing reaction was complete, the product clean-up was performed using the BigDye® XTerminator™ Purification Kit (Thermo Scientific™, Randburg). Nucleic acid sequences of the plasmid were then produced from the analysis of the sequence reads. Contigs of the insert were generated using the indicated primers using "De Nova Assemble", which ultimately resembled the insert of the virus.

The nucleic acid sequences of the insert were subsequently sent back by CAF. Once received, the sequences were opened using FinchTV Version 1.4.0 (Geospiza Inc.,) and copied to the online database, BLAST to ascertain the origin of the insert.

Samples in which a band of the correct molecular weight was observed in relation to the DNA ladder and positive control in the gel electrophoresis, were deemed as positive. The PCR product was purified, in order to send the DNA of interest for sequencing. The primers indicated in Table 3.2 were used for the purpose of sequencing the positive samples. The sequence received was copied to the online database, BLAST. This was to ensure the band did represent the nucleotide sequence matched that of the virus in question.

3.4.7. Viral DNA/RNA copy number

The purified plasmid DNA, resulting from the molecular cloning assays of HAdV, HBoV and PVB19 was quantified using the Qubit® DS DNA High Sensitivity Assay Kit (Life Technologies™, Johannesburg). Both Qubit® Assay Kits were used in concert with the Qubit® Fluorometer (Life Technologies™, Johannesburg). For each assay two standard solutions were set up, each consisting of 190 µl working solution and 10 µl of standard one or standard two samples. The working solution

consists of Qubit® DNA diluted in 199 µl of the DNA Buffer. Samples were prepared for analysis by diluting 1 µl of the sample DNA/RNA in 199 µl of the working solution previously made up.

Positive control samples for each virus were analysed in triplicate and the average concentration used in calculating the copy number. The Qubit® concentration obtained for both DNA and RNA viruses can be converted to viral copy number using online software (endmemo.com/bio/dnacopynum.php). Genome structure, sequence size in base pairs and mass per microlitre were required in order to generate the necessary copy numbers per microlitre of nucleic acid.

3.4.8. Optimisation of the PCR assays for viral detection

Serial dilutions of purified cloned positive controls were made in order to ascertain the limit of detection and analytical sensitivity of a PCR assay for each virus. A 1:10 dilution of the DNA of the positive control for each virus was performed, in order to dilute the ascertained viral copy number to one. The tenfold dilutions were made from the highest copy number to the lowest (Figure 3.3). Five microlitres of the stock template DNA was added into 45 µl nuclease free water and stored at -80°C. The dilutions were incorporated in the pre-nested PCR reactions following the previously stated protocols in Section 3.6. The results of the PCR reactions were displayed using gel electrophoresis.
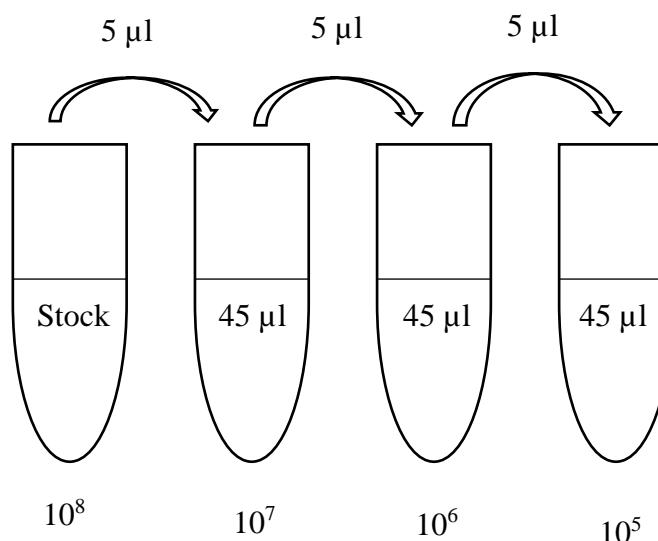
Figure 3.3: Tenfold serial dilution

3.5. Statistical analysis

Statistical analysis was performed by Prof. Martin Kidd at the Centre for Statistical Consultation, Department of Actuarial Sciences, Stellenbosch University. The software used to perform analyses was Statistica® version 13.5.0.17 Statistical Software (TIBCO Software, Inc.).

Associations were calculated using the Fisher Exact test for categorical data and an F-test for numerical data. A least significant difference (LSD) test was also performed for the associations with numerical data. Statistical significance was observed at $p < 0.05$ at a 95% confidence interval, with a strong significance observed at $p < 0.01$. The dataset was stratified according to gender, season (warm and cold) and COD (*SIDS*, *Infection* or *Other*) as major risk factors according to the literature. Descriptive statistics were performed on the recorded age, gender and birthweight of deceased infants. The detection of HAdV, HBoV and PVB19 was specified as categorical variables. Numerical data included age, birthweight and temperature.

4.

RESULTS

4.1.   Introduction

The study aimed to prospectively investigate the presence of HAdV, HBoV and PVB19 in the heart of SUDI cases admitted to the Tygerberg Medico-legal Mortuary over a 1-year period (March 2018 to March 2019). Heart swab samples were collected from all infants that died suddenly and unexpectedly between the ages of 7 and 365 days. Sociodemographic and clinical data, such as birthweight, age, post-mortem interval (PMI), gender, season, prematurity, sleeping position, bed-sharing and ventilation, were recorded for each case in order to identify risk factors that could have contributed to the sudden and unexpected death. Routine microbiology and histology were performed on the heart tissue. PCR assays were designed for HAdV, HBoV and PVB19 DNA detection in the collected heart swab samples and compared to histological or microbiological signs of infection.

4.2.   Study population

A total of 173 SUDI cases were sampled over the one-year period, incorporating all four seasons.

4.2.1.   Cause of death

At the time of completing this thesis, a COD was assigned to only 161 of the 173 cases by the respective forensic pathologists of the Division of Forensic Medicine at Stellenbosch University. The COD was assigned on the basis of an autopsy investigation, review of clinical factors and death scene investigation where the necessary information was available (Figure 4.1). A COD of *SIDS* was assigned in 51 (31.7%) and *Infection* in 98 (60.9%) of the 161 SUDI cases. Twelve (7.5%) SUDI cases were signed out as a COD of *Other*, that included an anatomical defect, cardiac causes, pulmonary causes, an interrupted aortic arch anomaly, congenital brain abnormality and asphyxia.

4.2.2.   Sociodemographic data

The male to female ratio was 1:0.86, with 93 (53.8%) and 80 (46.2%) cases sampled respectively. The majority of deaths, i.e. 107 (61.9%), occurred in the colder months with 50 (28.9%) and 57 (33.0%) cases observed in winter and autumn respectively. The warmer months displayed fewer deaths, i.e. 66 (38.2%), with 43 (24.9%) and 23 (13.3%) cases observed in spring and summer respectively (Figure 4.2).

Accurate ages at the time of death were recorded for the 161 SUDI cases (Figure 4.3). The mean age for the group was 12.1 ± 9.8 weeks. The age interval where the highest number of deaths occurred

was between 4 and 8 weeks, comprising of 25.5% of cases, while the lowest number was reported between 32 and 36 weeks, as well as 44 to 48 weeks, where no infant deaths were recorded.

The mean PMI for the 161 cases included in the analysis was 6.5 ± 2.8 days.

Other information that was recorded included birthweight, prematurity, sleeping position, bed-sharing and room ventilation.

The mean birthweight was $2\,522.5 \pm 817.1$ g. No birthweight was available for 5 (3.1%) out of the 161 cases. The majority of cases (34.2%) displayed a birthweight between 2 600 g and 3 250 g, with only 3.1% of cases having birthweights higher than 3 900 g (Figure 4.4). Prematurity was reported in 68 (42.2%) cases, while 87 (54.0%) were carried to full term. No data was available for the remaining 6 cases.

Fifty (31.1%) of the SUDI cases slept on their stomachs, 20 (12.4%) on their backs and 87 (54.0%) on their sides. Four cases (2.5%) had no data recorded for sleeping position. Bed-sharing was confirmed for 143 (88.8%) cases, denied for 12 (7.5%) and not recorded for 6 (3.7%) cases. No ventilation in the infants' sleeping environments was reported in 76 (47.2%) cases, while open windows of some sort were recorded in 66 (41.0%). No information regarding the ventilation was recorded for the remaining 19 (11.8%) cases. Figure 4.5 summarises the incidence of prematurity and sleeping environment of the 161 cases.
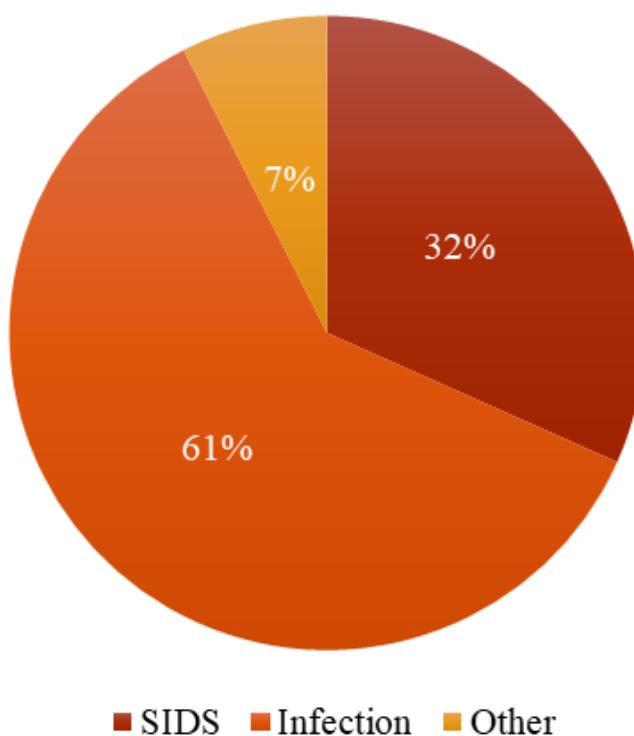
Figure 4.1: Distribution in the COD of SUDI cases (n=161)
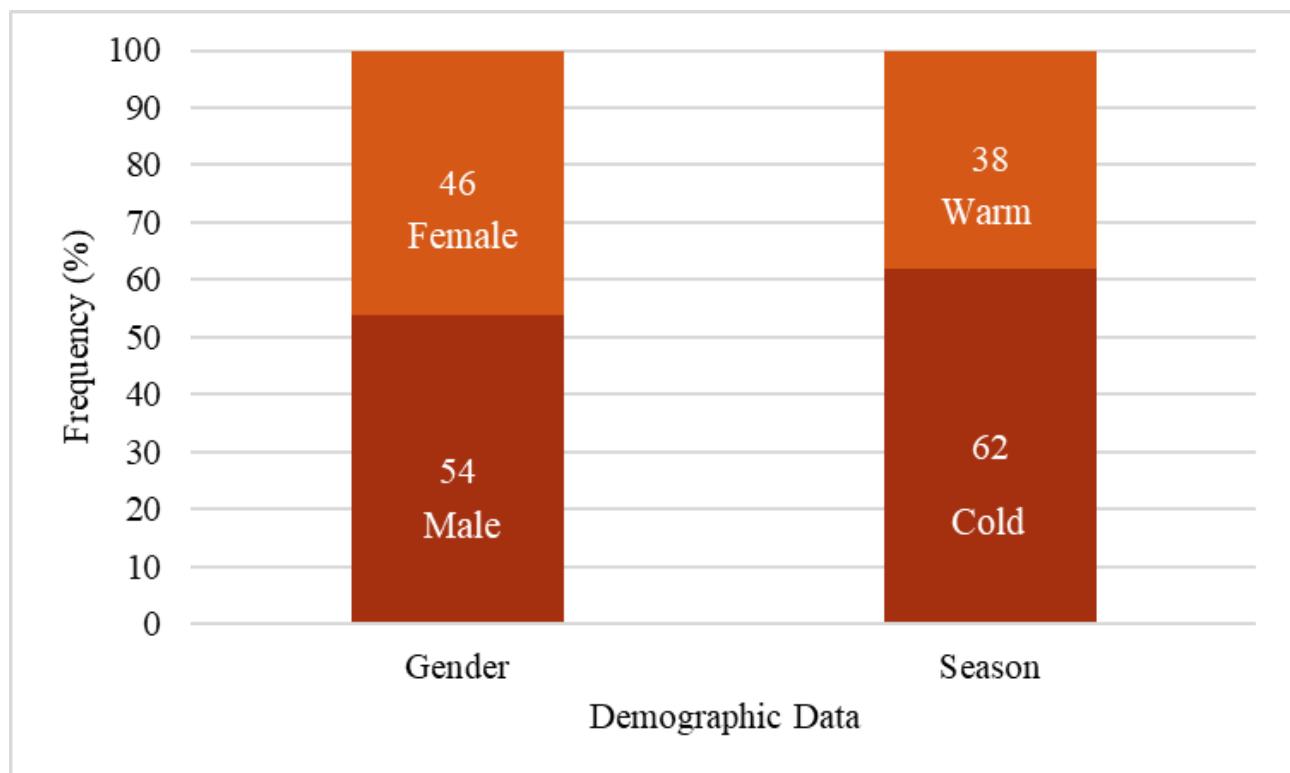
Figure 4.2: Gender and season distribution for SUDI cases (n=161)
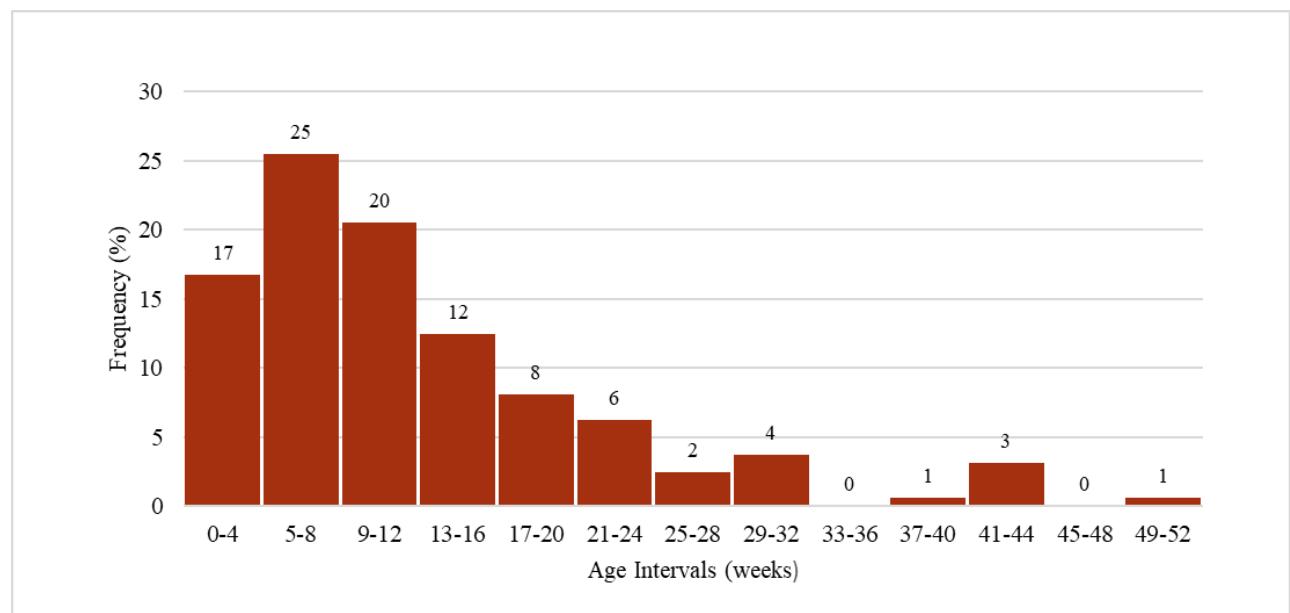
Figure 4.3: Age distribution in SUDI cases (n=161)

44
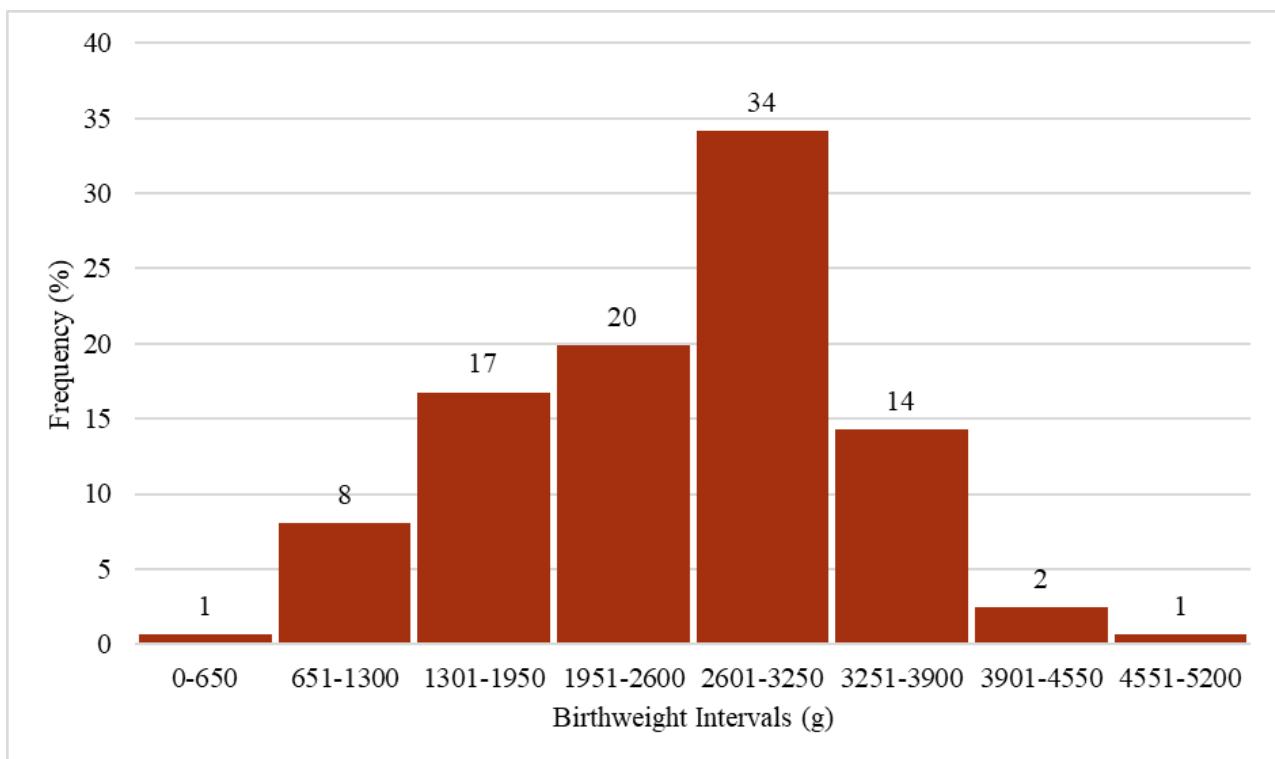
Figure 4.4: Distribution of birthweight in SUDI cases (n=161)
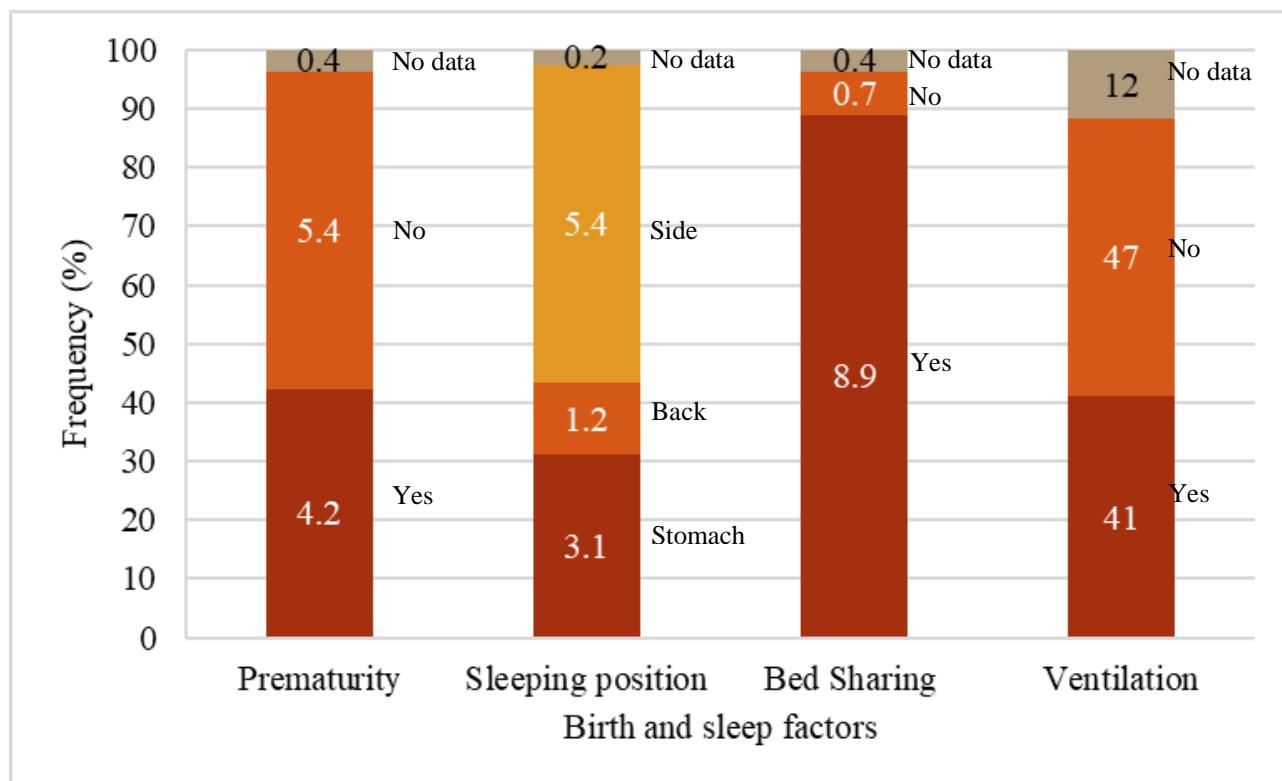
Figure 4.5: Prematurity and sleeping environment in SUDI cases (n=161)

4.2.3.   Daily temperatures

Due to the fact that specific dates of death could only be ascertained for the 161 cases with signed out CODs, the minimum and maximum temperatures on the date of death are only reported for these cases. The mean maximum temperature for these dates was $22.8 \pm 5°C$. The highest number of infant deaths (36.6%) occurred when the maximum temperatures reached 16°C to 20°C, with a steady decrease in the number of deaths as the maximum temperature increased (Figure 4.6).

The corresponding mean minimum temperature was $11.3 \pm 3.8°C$. The highest number of infant deaths (30.4%) occurred when the minimum temperatures were between 12°C and 15°C (Figure 4.7).

The mean difference in daily temperature was $11.5 \pm 4.9°C$. The interval in the difference in daily temperature where the highest number of cases were reported (27.3%) was 6° to 9°C, with a steady decline in the intervals thereafter. The least number of deaths (0.6%) occurred in the 21° to 24°C temperature difference interval (Figure 4.8).
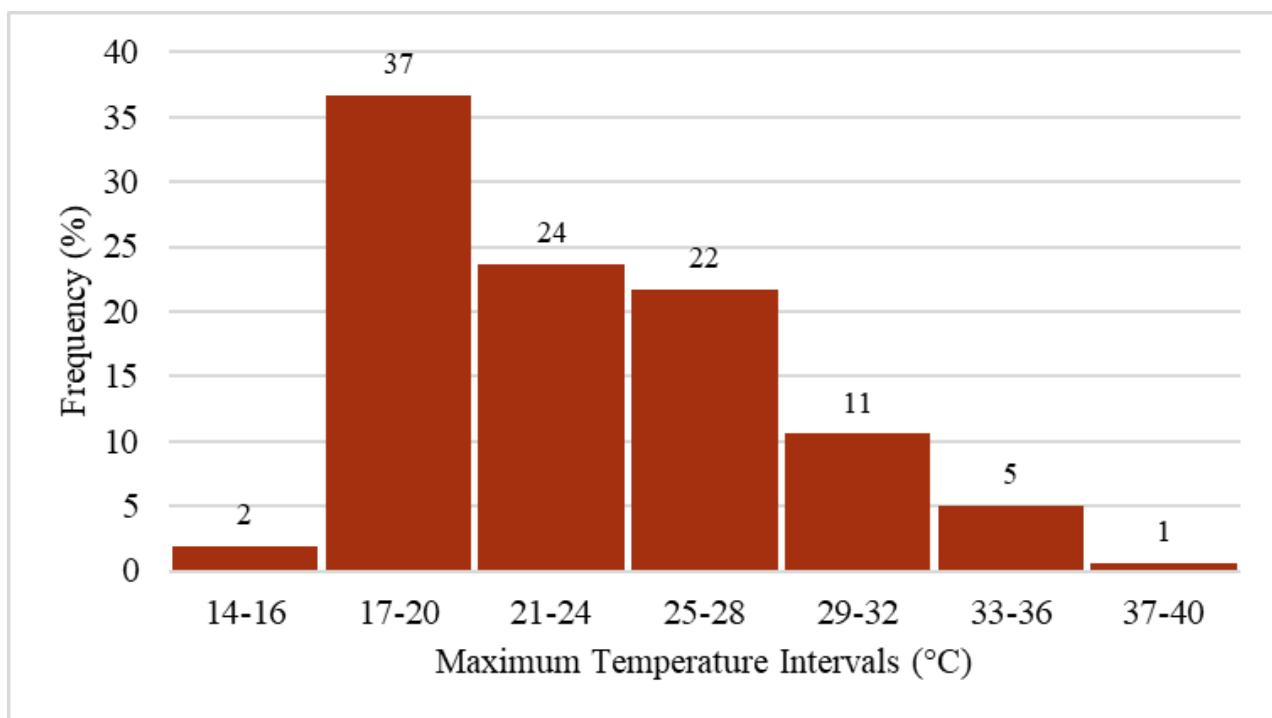
Figure 4.6: Maximum temperature intervals for the dates of SUDI deaths (n=161)
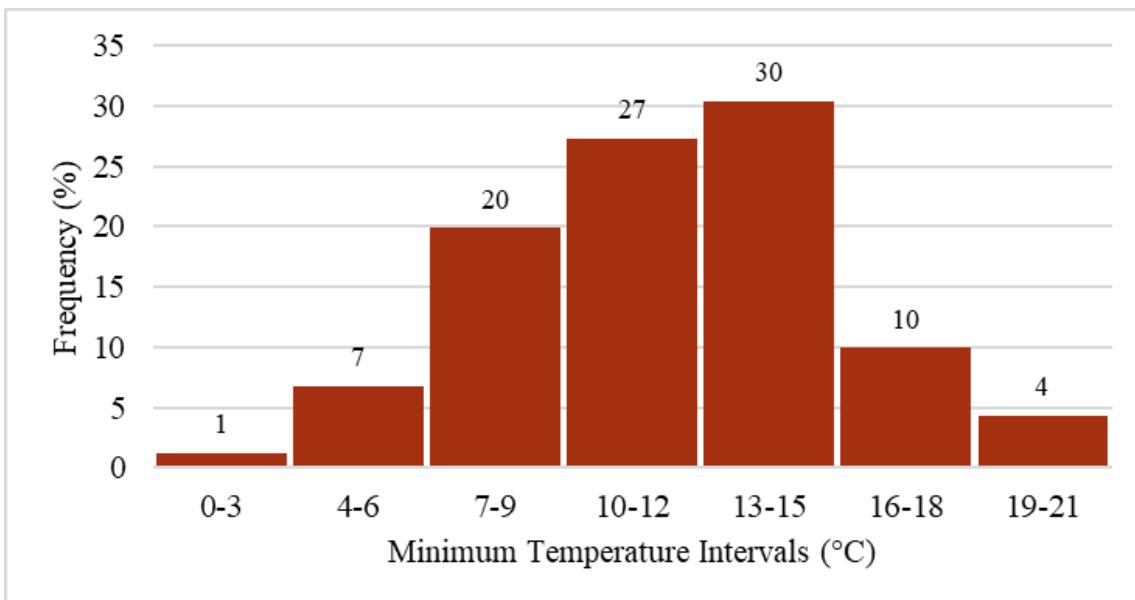
Figure 4.7: Minimum temperature intervals for the dates of SUDI deaths (n=161)
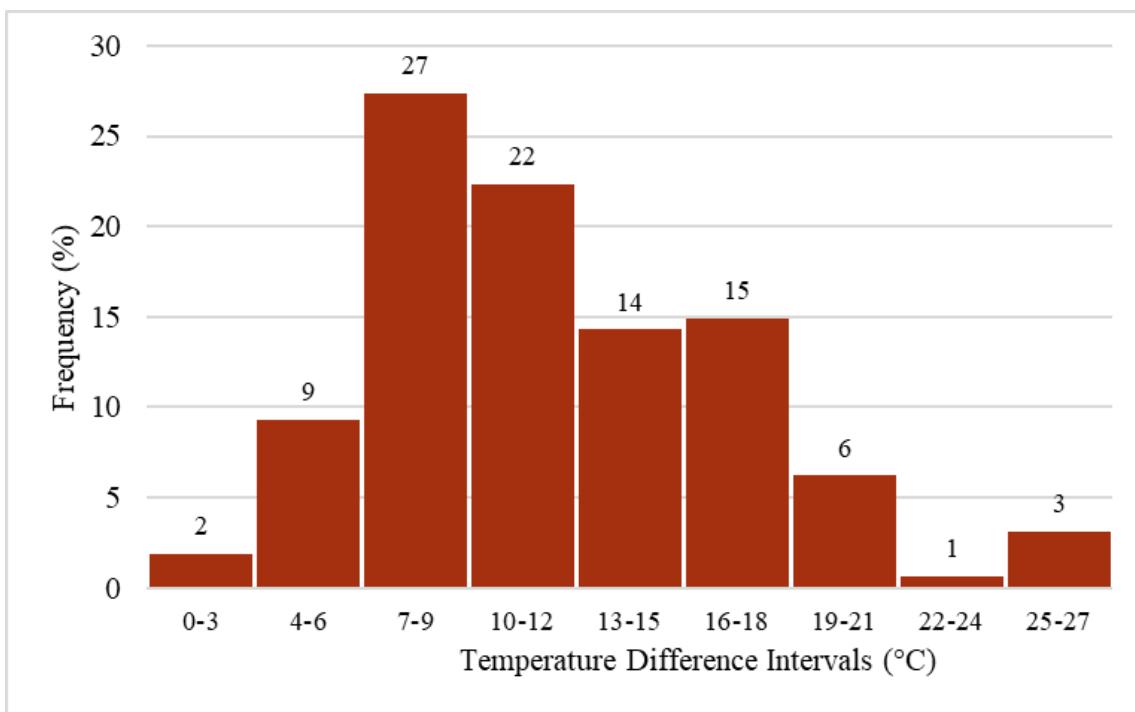
Figure 4.8: Daily temperature differences for the dates of SUDI deaths (n=161)

4.3. Routine laboratory results

4.3.1. Microbiology

Routine microbiology of the heart was conducted by the NHLS for 169 of the SUDI cases sampled. Samples were not received for four (2.4%) of the cases. The majority of the bacteriological heart swab cultures (117, 69.2%) displayed no growth. Post-mortem flora was observed in 31 (18.3%) of the cases. Thirteen different bacteria were identified in 21 SUDI cases, which equates to 12.4% of the cases analysed. The most common bacteria detected was *E. coli*, observed in eight (4.73%) cases.

Klebsiella (K.) pneumoniae and *Strep pyogenes* were observed in the heart swabs of three (1.78%) and two (1.18%) cases respectively. *Enterococcus spp., K. oxytoca, Lelliottia (L.) amnigena, Proteus mirabilis, Roaoutella planticola, Salmonella* type D, *Serratia (S.) liquefaciens, S. marcescens, Staph aureus* and *Staph haemolyticus* were each observed in only one (0.59%) case. Two cases (1.18%) had two bacterial species isolated in each; *K. oxytoca* and *E. coli* in the first, and *Staph aureus* and *Strep pyogenes* in the second (Table 4.1).

Table 4.1: Bacteria present in the heart of SUDI cases

Microbiology	Frequency (n)	Percentage (%)
Enterococcus spp.	1	0.6%
E. coli	8	4.7%
K. oxytoca	1	0.6%
K. pneumoniae	3	1.8%
L. amnigena	1	0.6%
Proteus mirabilis	1	0.6%
Raoultella planticola	1	0.6%
Salmonella D	1	0.6%
S. liquifaciens	1	0.6%
S. marcescens	1	0.6%
Staph aureus	1	0.6%
Strep haemolyticus	1	0.6%
Strep pyogenes	2	1.2%
Post-mortem flora	31	18.3%
No growth	117	69.2%

4.3.2. Histology

Only 145 of the 161 cases had histology sections available. Most of these cases (135, 93.1%) were observed to have a histologically normal myocardium, while the remaining 10 (6.9%) cases displayed signs of myocarditis. Myocarditis as a result of infection was confirmed when the histological sections of the heart tissue displayed myocyte destruction and inflammatory cell infiltrates, namely neutrophils and lymphocytes. The cases displaying myocarditis could be described as being overwhelming throughout, underlying or only focally observed, as displayed Figures 4.9-4.13. The histopathological features observed in each case with myocarditis are described in Table 4.2. Case 154 displayed very mild and unremarkable signs of myocytolysis and inflammatory infiltrate, however in combination with the increased heart mass, it was the only case assigned the COD as myocarditis.
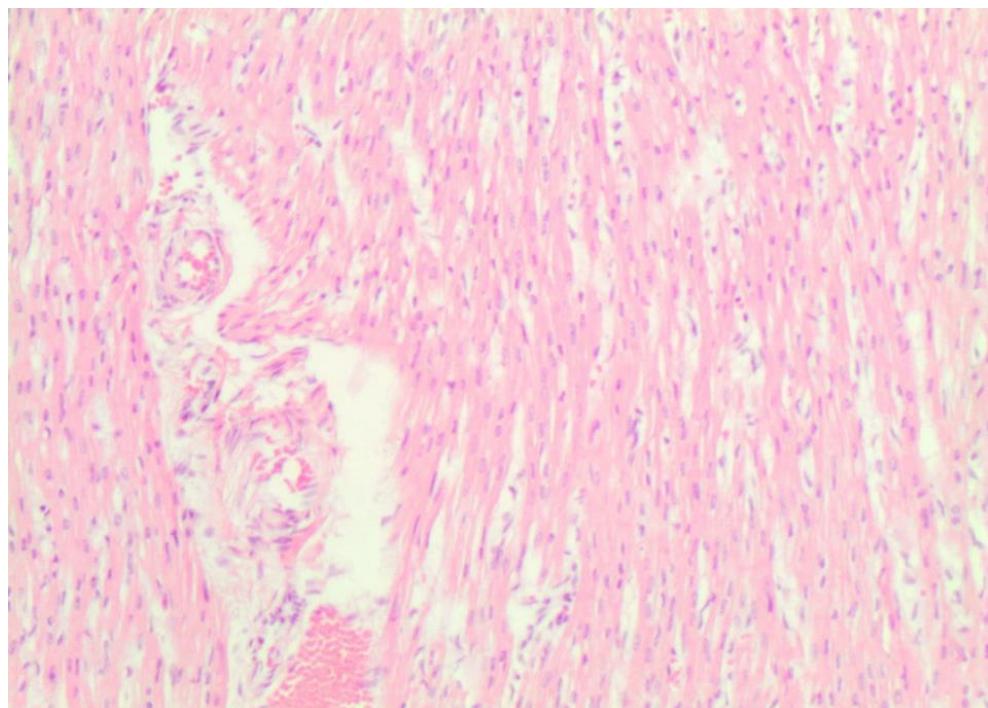
Figure 4.9: Case 128 displayed a histologically normal post-mortem heart section. No visible myocytolysis or inflammatory infiltrates. Continuous, multinucleated fibres are visible. Magnification X10.
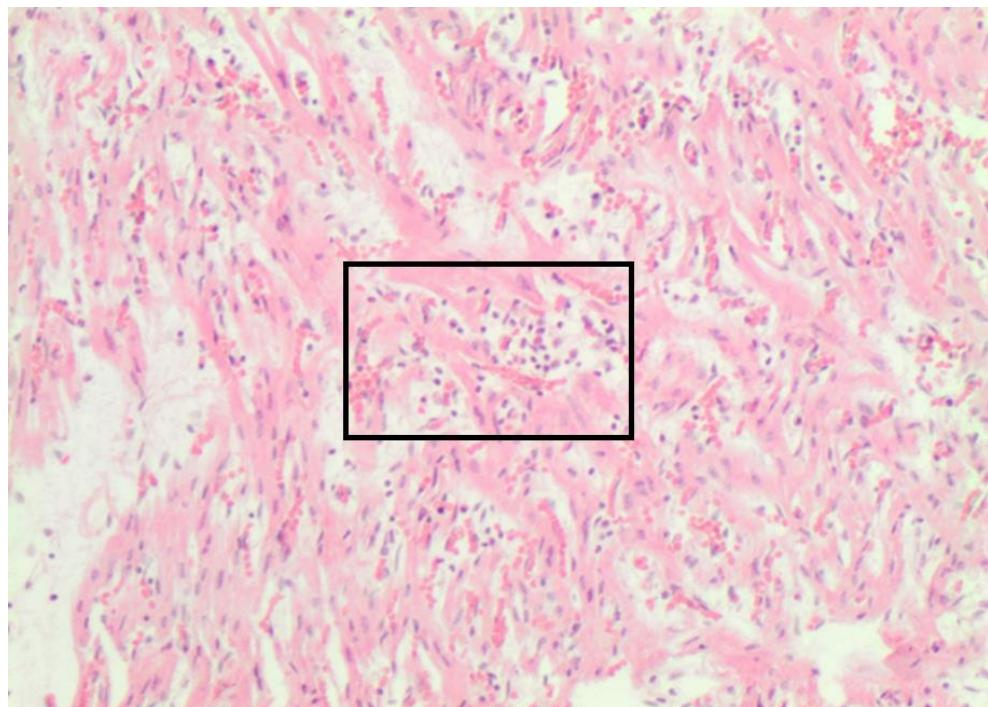

Figure 4.10: Case 76 displaying overwhelming myocarditis. The area in the block displays the infiltration of inflammatory cells into the myocardium. Magnification X10.
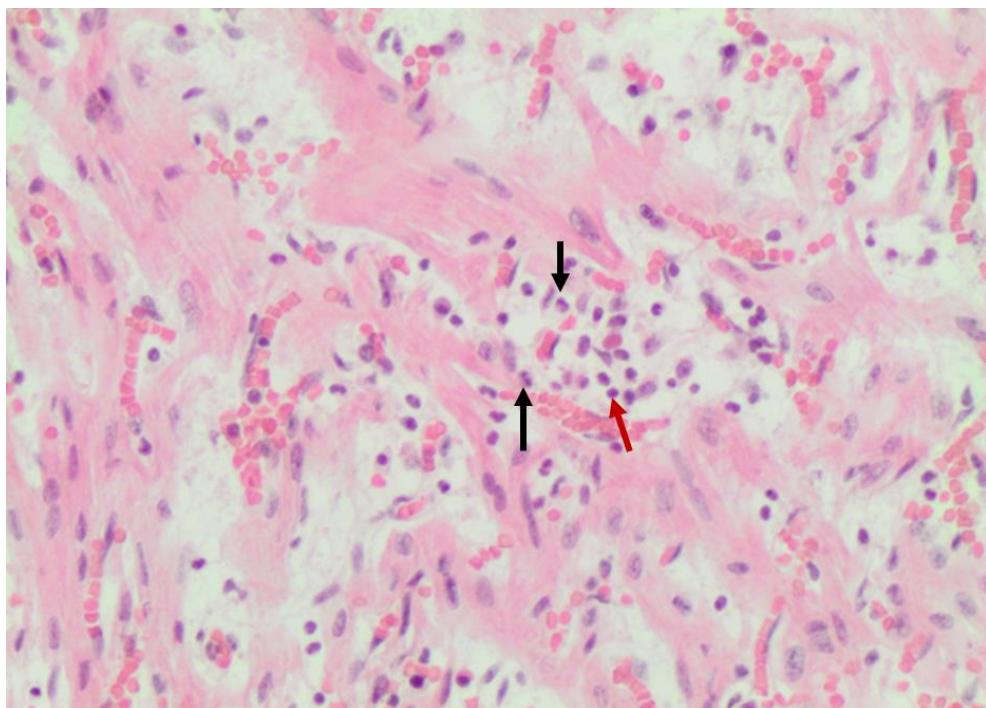
Figure 4.11: Higher magnification of Figure 4.10 shows overwhelming myocarditis. Infiltration of inflammatory cells (i.e. neutrophils), as well as myocyte destruction are visible. Magnification X20. The red arrow indicates chronic inflammatory cell infiltrates such as lymphocytes. The black arrow specifically indicates multinucleated neutrophils.
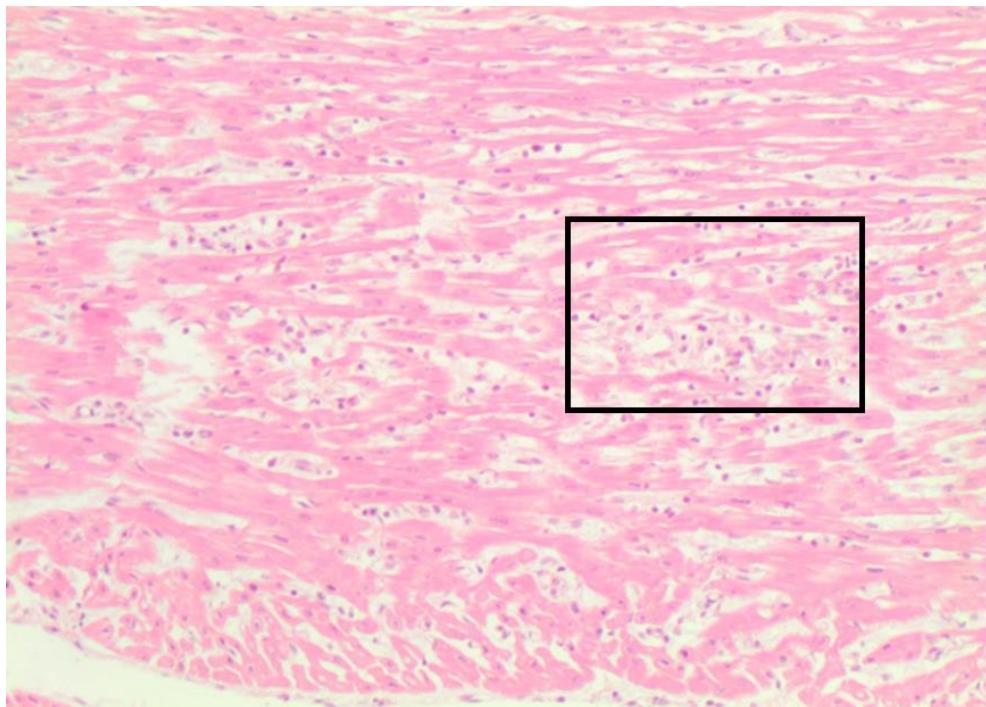
Figure 4.12: Case 82 showing visible focal myocarditis. The blocked area displays the infiltration of inflammatory cells (i.e. neutrophils), as well as myocyte destruction. Magnification X10.
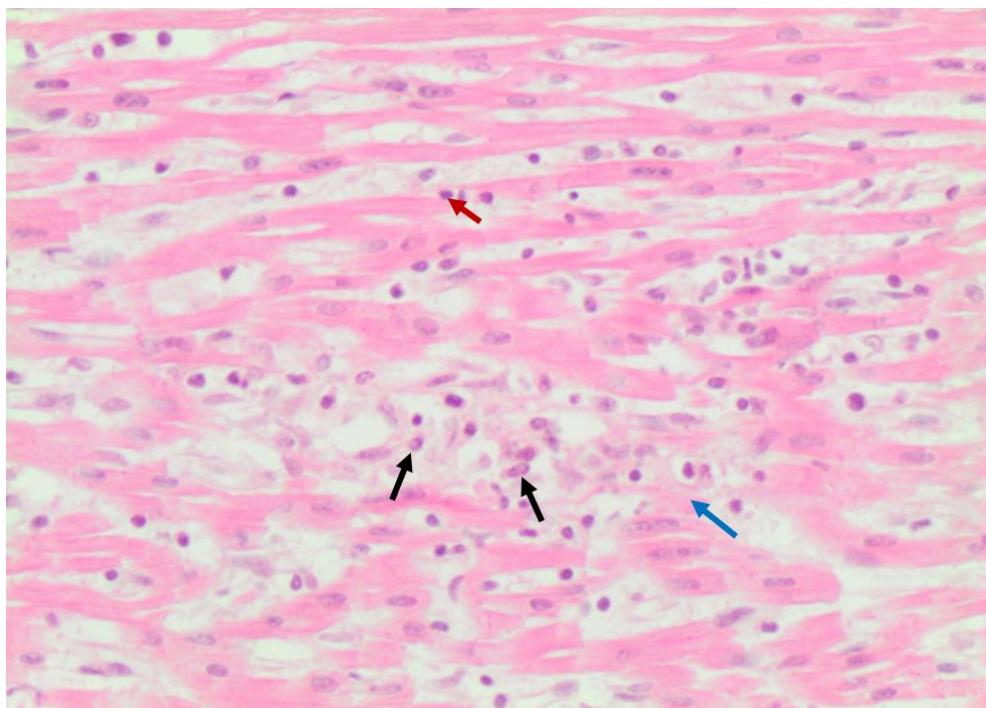
Figure 4.13: Higher magnification from the blocked area in Figure 4.12. Infiltration of inflammatory cells (i.e. neutrophils), as well as myocyte destruction, are visible. Magnification X20. The red arrow indicates chronic inflammatory cell infiltrates such as lymphocytes. The black arrow specifically indicates multinucleated neutrophils. The blue arrow indicates myocyte degradation.

Table 4.2 Histopathological features within the heart tissue of SUDI cases with myocarditis

SUDI case	Histopathology
004	Underlying myocarditis
013	Viral myocarditis, lymphocytic infiltration
039	Myocarditis, not florid
076	Myocarditis, overwhelming
082	Myocarditis, focal
087	Lymphocyte aggregation, myocyte destruction
101	Myocarditis
106	Myocarditis, underwhelming
124	Myocarditis, underwhelming
143	Myocarditis, underwhelming

4.4.   PCR assays

The 173 heart swab samples collected in the Tygerberg Medico Legal Mortuary were all analysed with conventional PCR assays to detect the three viruses selected for this study, i.e. HAdV, HBoV and PVB19.

Positive controls for each virus were created through cloning a viral insert into a suitable vector. Serial dilutions of the positive controls were made: copy numbers for each positive control were recorded, and thus could be calculated for each dilution.

4.4.1. Human adenovirus

The copy number recorded for HAdV was 1.88×10^{10} copies/µl. The lowest dilution that could be detected was 1.88×10^{3} copies/µl. HAdV was not detected in any of the 173 heart samples. A low viral load of $<2x10^{3}$ copies/µl is not associated with disease, with the clinical threshold being officially recorded as $1x10^{4}$ copies/µl. HAdV viral loads in severe disseminated disease cases are $>1x10^{7}$ copies/µl and can be even higher ($>1x10^{11}$ copies/µl) in paediatric cases (Ganzenmueller & Heim, 2012). The minimum detectable copy number was well below the pathogenic viral load observed in the literature, and therefore the assay was theoretically regarded as sensitive enough to detect the virus in a swab or biopsy sample.

In an attempt to address the absence of a suitable comparison group, viral multiplex PCR results from the NHLS Medical Virology laboratory from 760 live infants under one year that were admitted to Tygerberg Hospital between 2015 and 2018 were retrieved. Since collection of samples from the heart of live infants is not possible due to ethical and other constraints, the routine respiratory results were used to ascertain whether the selected viruses were circulating and detected in infants during the same timeframe as the current study. While HAdV was not detected in any of the SUDI cases, it was confirmed in 190 (25.0%) infants from this comparison group of hospitalised infants.

Figure 4.14 shows a gel electrophoresis picture of a positive HAdV band at 300 bp obtained from the positive control.
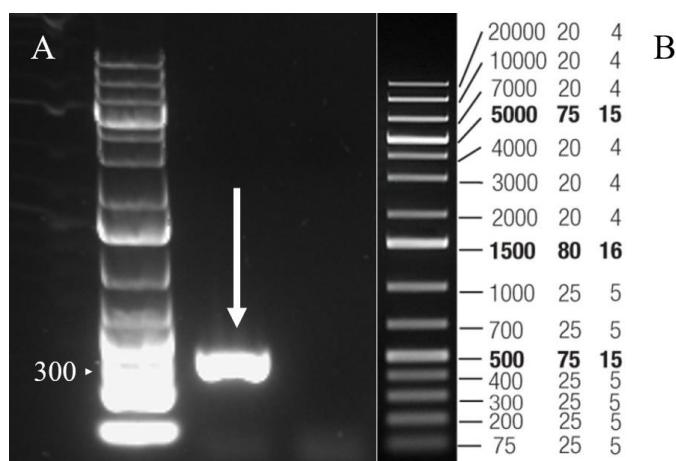

Figure 4.14: Gel electrophoresis showing the detection of HAdV. (A): The cloned insert of the HAdV positive control visible at 300 bp, preceded by a DNA ladder and followed by a negative control. (B): The GeneRuler 1Kb Plus DNA ladder used in the gel picture.

4.4.2. Human bocavirus

The average copy number of the three values recorded for HBoV was 2.42×10^9 copies/µl. The lowest dilution that could be detected was 2.42×10^5 copies/µl. Clinically significant HBoV infections are associated with viral loads $>1.04 \times 10^5$ copies/µl (Guido et al., 2016). The minimum detectable copy number was within range of a clinically significant viral load observed in the literature, and therefore the assay was theoretically regarded as sensitive enough to detect pathogenic levels of virus in a swab or biopsy sample. HBoV was detected in the heart of four (2.3%) cases. Case 109, in which HBoV was detected, also displayed histological evidence of florid myocarditis.

HBoV was detected in 40 infants (5.3%) of the comparison group of hospitalised infants as described above. Figure 4.15 shows a gel electrophoresis picture of a positive HBoV band at 354 bp obtained from the positive control.

All of the HBoV positive PCR products were sequenced as mentioned in subsection 3.4.6. The sequences generated displayed 100% match to HBoV isolates, when entered into the BLAST database.
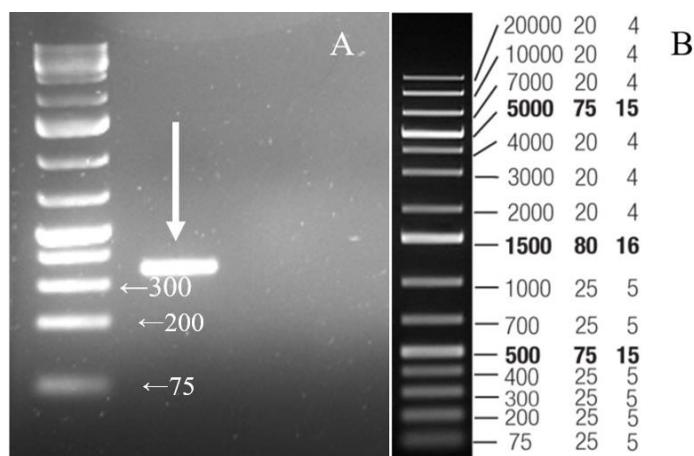
Figure 4.15: Gel electrophoresis showing the detection of HBoV. (A): The cloned insert of the HBoV positive control visible at 354 bp, preceded by a DNA ladder and followed by a negative control. (B): The GeneRuler 1Kb Plus DNA ladder used in the gel picture.

4.4.3. Parvovirus B19

The copy number recorded for PVB19 was 6.94×10^9 copies/µl. The lowest dilution that could be detected was 6.94×10^6 copies/µl. Clinically significant PVB19 infections resulting in acute myocarditis are associated with viral loads of 3.16×10^5 copies/µl (Verdonschot et al., 2016). The minimum detectable copy number was slightly higher than that of a clinically significant viral load observed in the literature, and therefore the assay was theoretically regarded as sensitive enough to detect a pathogenic level of virus in a swab or biopsy sample associated with serious infection. PVB19

was not detected in any of the 173 heart samples and since it does not form part of the routine multiplex PCR assays used for screenings at the NHLS, no comparison data is available on the viral prevalence in past years in Tygerberg Hospital referral area.

Figure 4.16 shows a gel electrophoresis picture of a positive PVB19 band at 290 bp obtained from the positive control.
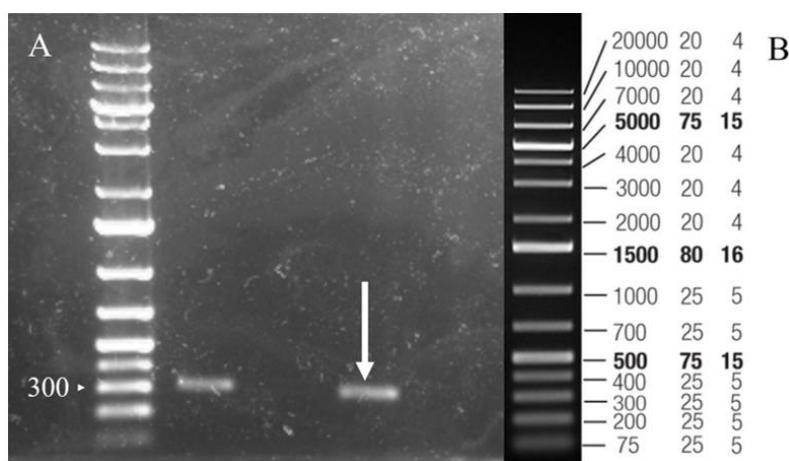

Figure 4.16: Detection of PVB19 positive control. (A): The cloned insert of the PVB19 positive control visible at 290 bp, preceded by a DNA ladder and HAdV detected band (300 bp). Detected PVB19 band is followed by a negative control. (B): The GeneRuler 1Kb Plus DNA ladder used in the gel picture.

4.5.   Frequency data

Four of the 23 cases that had bacteria isolated from the heart also showed signs of myocarditis on histological analysis. Both ventilation present in the room where the infant slept and bacteria isolated from the heart were present in 13 cases. Where there was no ventilation present, six cases displayed bacterial isolates in the heart. Four cases signed out as SIDS displayed bacterial isolates in the heart, namely *E. coli, L. amnigena* and *K. pneumoniae.* Bed-sharing and bacterial presence in the heart were evident in 16 cases. Seven SUDI cases were recorded to have both bacterial isolates detected in the heart, as well as being put to sleep on their stomachs. Ten of the SUDI cases that were put to sleep on their sides had bacteria detected in the heart, while only three cases that slept on their backs had bacteria present in the heart tissue.

Ten of the premature SUDI cases displayed bacterial isolates in the heart tissue. The majority of SUDI cases (7.1%) in which bacteria was present in the heart occurred in autumn. Low birthweight, defined as lower than 2500 g (Fouché et al., 2018), was observed in ten of the cases where bacterial isolates were detected. The vast majority of the 21 cases with positive microbiology results (90.5%) were younger than 6 months.

4.6.   Statistical Analyses

Associations were calculated between categorical and numerical data and COD (*SIDS*, *Infection* or *Other*). The diagnoses in the *Other* category was explained in section 4.3. The Fisher Exact test was used for categorical data (Appendix 4 and 5) and the F-test for numerical data (Appendix 6 and 7). Statistical significance was observed at $p<0.05$ at a 95% confidence interval, with a strong significance observed at $p<0.01$.

4.6.1.   Associations between COD and sociodemographic risk factors

Sociodemographic risk factors from the literature were compared to the COD. The COD of the SUDI cases were divided in three groups, namely *SIDS*, *infection* and *Other*.

4.6.1.1. Categorical data

No statistically significant association between gender and COD was observed ($p=1.00$). However, a trend was evident in all three groups that the number of males was slightly higher than females.

Infection was the most common COD in both seasons, followed by *SIDS* and *Other*. The cold season also showed higher frequencies of cases in the *Infection* and *SIDS* groups, but the differences were not significant ($p=0.43$). As expected, no seasonal difference was found in the *Other* group.

While less than half of the SUDI cases reported prematurity at birth (44.1%), significantly more *Infection* than *SIDS* or *Other* cases were confirmed to have been born prematurely ($p=0.02$) (Figure 4.17). No other significant associations could be demonstrated between COD and prematurity.

While no statistical significance was observed between different sleeping positions and COD ($p=0.20$), certain trends were evident. The majority of deaths occurred in infants that slept on their sides, followed by stomachs and ultimately the least number of SUDI cases were observed to sleep on their backs. This was evident in all three individual groups.

The vast majority (93.2%) of cases in all three groups were reported to be subjected to bed-sharing, but no statistical significance was observed between bed-sharing and COD ($p=0.80$).

Ventilation in the room where the baby slept was significantly associated with COD ($p=0.02$), as observed in Figure 4.18. The highest number of infants that slept in ventilated rooms were found in the *Infection* group, followed by *SIDS*. The same trend, however, with a smaller difference, was observed for infants that were not sleeping in a ventilated room with more cases in the *Infection* group, followed by *SIDS*.
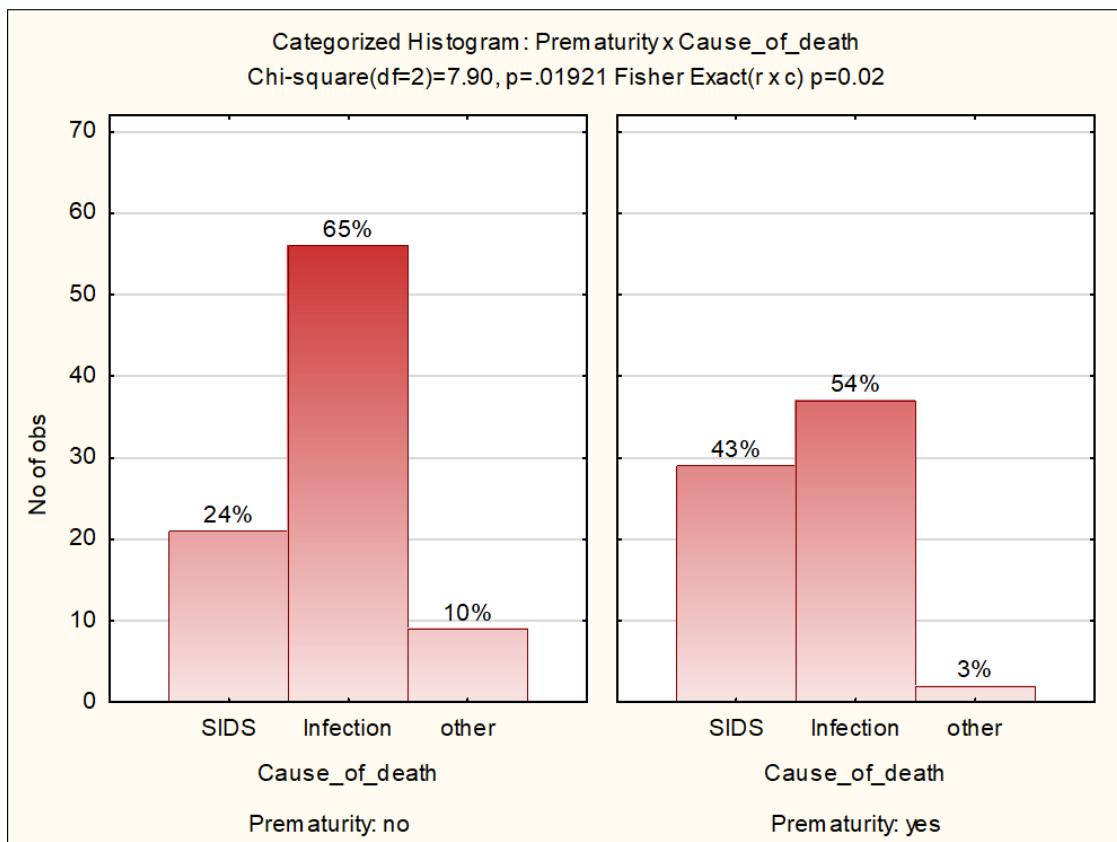
Figure 4.17: Association between prematurity and COD
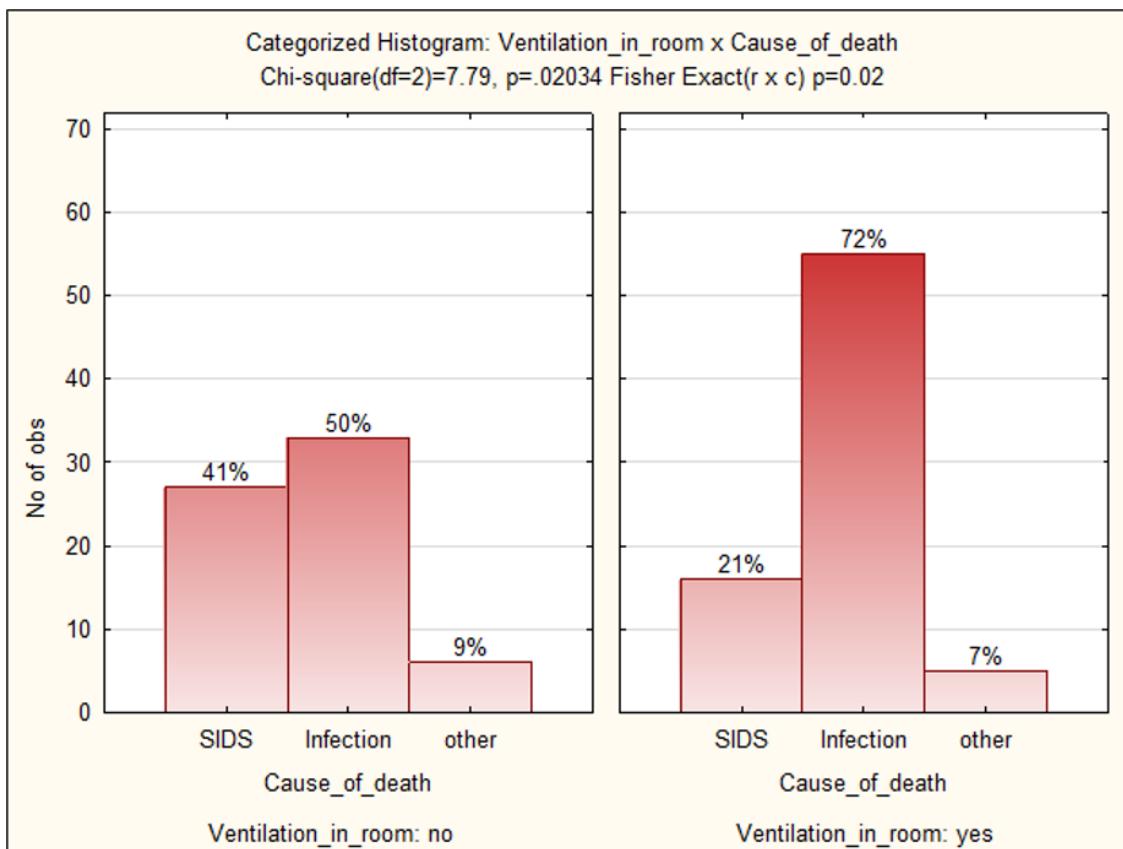
Figure 4.18: Association between ventilation and COD

4.6.1.2. Numerical data

A trend in the age was observed indicating that the *SIDS* cases had the lowest mean age and standard deviation from the three groups (11.0 ± 7.5 weeks). However, the differences were not statistically significant (p=0.77).

The differences in birthweight among the groups were highly significant (p<0.01), with the *SIDS* group producing the lowest values (Figure 4.19). The least significant difference (LSD) test also indicated a p-value of 0.01 between the *SIDS* and *Infection* birthweight, and a p-value of 0.02 between *SIDS* and *Other*.

No statistically significant differences could be demonstrated in the COD for the minimum (p=0.65), maximum (p=0.88) and difference in daily temperature (p=0.88) on the days the infants died. The *SIDS* group had a slightly lower mean minimum temperature and therefore a slightly higher temperature difference than the other groups, but it is unclear if these differences are clinically relevant.
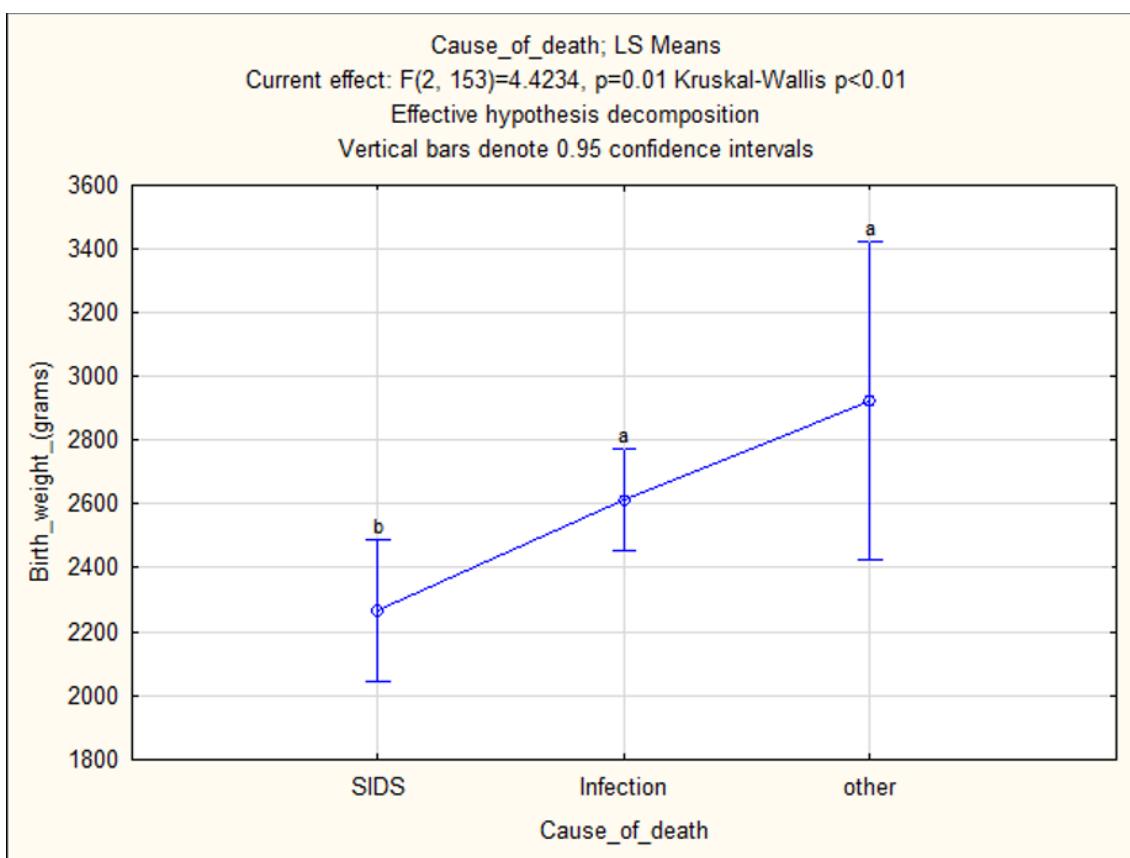
Figure 4.19: Highly significant association between birthweight and COD

4.6.2.   Associations between COD and positive PCR, histology and microbiology results

No statistically significant association could be demonstrated between HBoV and COD (p=1.00). Only one *SIDS* case (0.6%) had a positive HBoV result, while the only other positive results were found in three *Infection* cases (1.9%). Notably, a HBoV positive *SUDI* case was also one of the 10 cases that displayed myocarditis in the histological analysis (p=0.25). Only one HBoV positive case displayed a positive microbiology result in the heart, with no statistical significance being observed (p=0.41).

Histology showed no significant association with COD (p=0.28). However, the vast majority of cases with myocarditis positive histology (55.9%) were observed in the *Infection* group. A statistically significant association (p=0.02) was also found between histology and microbiology results, where the highest frequency of both negative (84.0%) and positive (4.2%) microbiology results were found in the group with normal histology. Case 109 was the only case which had both a positive HBoV PCR result and positive histology for myocarditis. The clinical history and final COD assigned by the forensic pathologist for Case 109 are summarised in Table 4.3.

Table 4.3: Clinical history of Case 109 with both positive PCR results and histology

Case	Histology	Clinical history	PCR result	COD
109	Myocarditis	Runny nose for 3 weeks	HBoV	LRTI

For statistical analysis, post-mortem flora was classified as a 'normal' microbiological result. The majority of microbiology positive heart tissue of SUDI cases were signed out with a COD of *Infection* (10.6%). Four *SIDS* cases displayed microbiology isolates (2.5%). No statistical significance, however, was observed between microbiology positive heart swabs and the COD (p=0.25). Case 29 was the only case which had both a positive PCR result for HBoV and positive microbiology in the heart, displayed in Table 4.4 with the clinical history and final COD assigned by the forensic pathologist.

Table 4.4: Clinical history of Case 29 with both positive PCR results and histology

Case	Microbiology	Clinical history	PCR Result	COD
29	*S. liquefaciens*	Diarrhoea	HBoV	Pneumonia with *E. coli*, gastroenteritis as a contributory cause

4.6.3. Associations between sociodemographic factors, PCR and microbiology results

No statistical significance was observed between HBoV positive SUDI cases and gender (p=0.34). Only one of the four HBoV positive infants was a male. A trend was observed that all four HBoV positive samples (2.3%) were detected in the cold season (p=0.30). Only one (0.7%) HBoV positive SUDI case was premature, therefore no significant association or trend could be observed (p=0.63). All four HBoV positive cases (2.8%) reported sleeping in a ventilated room, indicating a possible trend (p=0.12).

The mean age for HBoV positive samples had a higher mean age (15.0 ± 1.7weeks) than the negative samples (12.1 ± 9.8 weeks). The association, however, was not statistically significant (p=0.55).

The mean birthweight of the four HBoV positive SUDI cases were slightly lower (2 345.0 ± 963.5g) than the HBoV negative cases (2 527.0 ± 816.1g). This trend, however, was not statistically significant (p=0.75). No statistically significant associations between temperature and HBoV results were observed either. The HBoV positive cases displayed on average a 2.6°C lower maximum temperature on the days of death than the negative cases (p=0.23) and similar trends were seen for the minimum (p=0.51) as well as difference in daily temperatures (p=0.26) in the HBoV positive group.

Gender was not significantly associated with positive microbiology results (p=0.35), although slightly more females (7.1%) than males (5.6%) were observed to have positive microbiology results. Even though no statistical significance was observed between positive heart microbiology and season (p=1.00), more microbiology positive cases (7.7%) occurred in the cold season compared to the warm season (4.7%). Exactly the same number of microbiology positives occurred in premature and non-premature SUDI cases, with no observed significance (p=0.54). More cases with positive microbiology results slept in ventilated rooms (9.4%) than non-ventilated rooms (4.3%). However, this difference was not statistically significant (p=0.22).

No significant difference (p=0.57) was observed between age and positive microbiology results, although the microbiology positive group was slightly younger. Similarly, the microbiology positive group also had a slightly lower birthweight, but that was also not significant (p=0.35).

A highly significant association (p<0.01) was found between positive microbiology results and the maximum temperature on the days of death with a difference of almost 4°C (Figure 4.20). A significant association was also found between positive microbiology results and the difference in the minimum and maximum temperatures on the days of death (p=0.02) with the microbiology positive group showing an mean difference of 2.4°C higher than the microbiology negative group (Figure

4.21). No association could however be demonstrated for the minimum daily temperatures between the microbiology negative and positive groups (p=0.58).
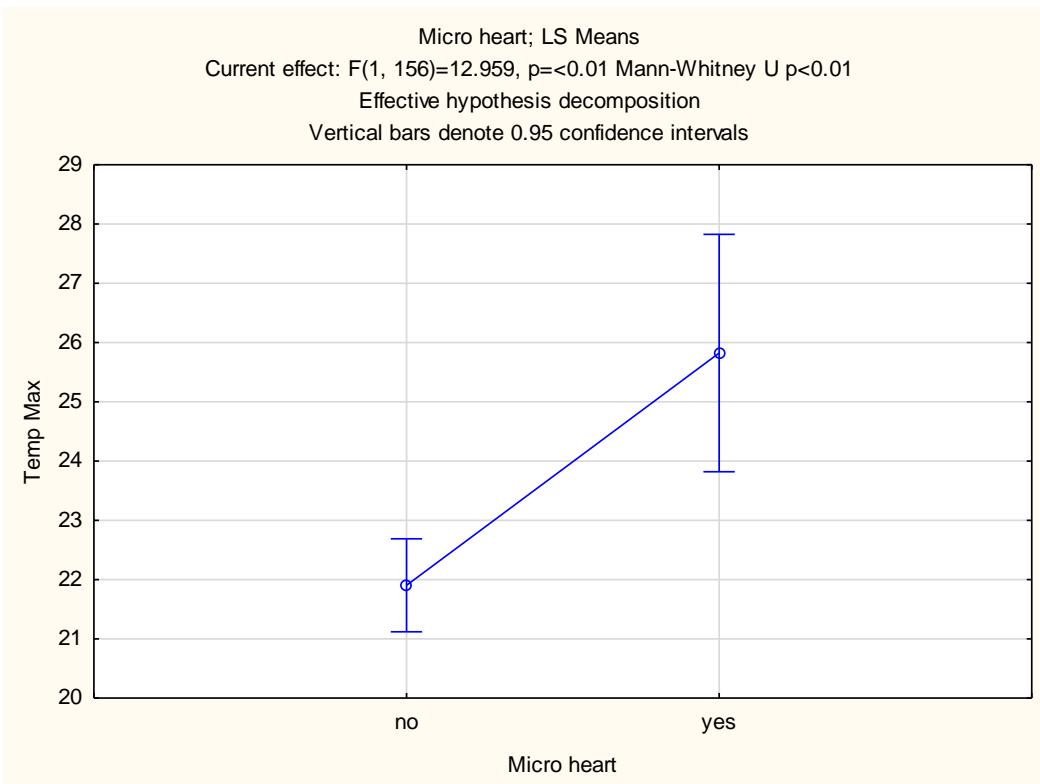

Figure 4.20: A highly significant association was found between the mean maximum temperature recorded on the day of death and the heart microbiology results.
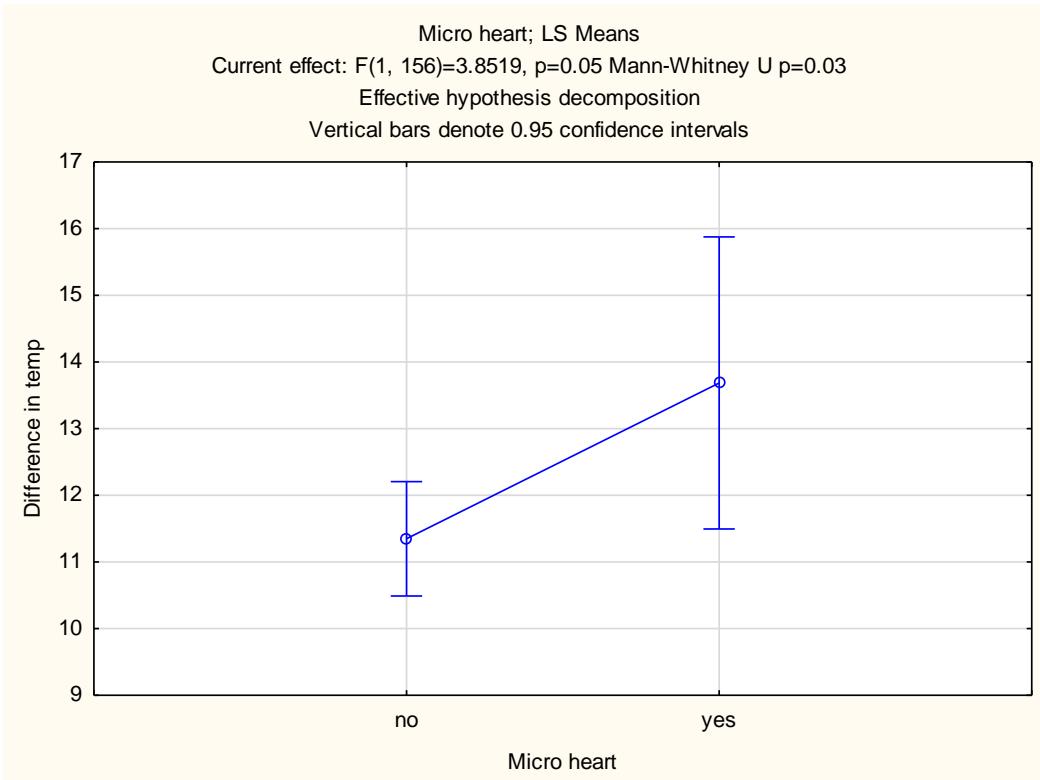

Figure 4.21: A statistically significant association was found between the mean temperature difference recorded on the day of death and the heart microbiology results**.**

5.

DISCUSSION

5.1.   Introduction

Bartick & Tomori (2018) stated that infant mortality can be used as a measure of a health in a population. It is important to take into consideration the demographics of the study population, and not only focus on individual risk factors. SUDI was acknowledged as the second most common COD for infants in 1991 globally, preceded by congenital anomalies. Prematurity, low birthweight and respiratory distress were also common causes of infant death (Goutas et al., 2011). SUDI is reportedly more prevalent in low-income and underprivileged populations, although cases have decreased substantially from 2002 to 2012 (Taylor et al., 2015). According to the Commission on Social Determinants of Health (2008), marginalised populations and poverty play an important role in disease. Social inequalities often lead to increased physiological risk factors for SUDI, such as prematurity and decreased birthweight (Blair et al., 2006; Sosnaud, 2017; Bartick & Tomori, 2018). Decreased rates of breastfeeding, and frequent maternal smoking and alcohol/drug use are all behavioural SUDI risk factors associated with low socio-economic households (Bartick & Tomori, 2018). Infant mortality in recent years has declined, with the exception of regions such as southern African countries, who are experiencing economic restructuring (Goutas, et al., 2011).

The observational study of 173 SUDI cases collected in the Tygerberg Medico-legal Mortuary was conducted in order to ascertain the association between specific viruses detected in the heart tissue and myocarditis observed in the histology. Sociodemographic associations were taken into consideration. Only a few cases displayed signs of myocarditis in the histology, and even fewer had viral positive results.

5.2.   Sociodemographic and sample characteristics

Infant mortality is dependent on various environmental and socioeconomic factors and differences (Goutas et al., 2011). Multiple SUDI risk factors within a population may work to compound one another (Bartick & Tomori, 2018).

5.2.1.   Categorical data

In accordance with the literature regarding the gender distribution of SUDI cases, more males than females were observed. This result, however, was not statistically significant since the totals of both genders only differed marginally. The male to female ratio observed was 1:0.86, similar to the 1:0.82 of the South African population investigated by Du Toit-Prinsloo et al. (2013). There are various immunological theories to why males make up most of the SUDI deaths. Immunological differences

have been observed between males and females, namely that females have higher immunoglobulin (Ig) levels. Female infants also display increased humoral immunity to infection and better antigen response than males. Decreased cytokine inflammatory responses in males compared to females have been observed, the variation possibly due to the sex hormones testosterone and oestradiol (Moscovis, et al., 2013).

Increased numbers of *SIDS* and *Infection* cases were observed in the colder seasons, with *Infection* displaying the highest numbers in both warm and cold seasons. These observed trends were in keeping with the literature where more cases are reported in the autumn and winter months (Guntheroth et al., 1992; Grangerot-Keros et al., 1996), however, the trends in this study were not significant.

It has been recorded that the prone (stomach) sleeping position is a risk factor for SUDI (Bartick & Tomori, 2018). Contrary to the literature, however, the majority of the SUDI cases sampled in this study slept on the side. Prone sleeping position was the next common, followed by the back (supine). Prone position is said to result in SUDI cases, as the position is thought to aid in asphyxia and overheating (Vorontsov & Kelmanson, 1990; Vege & Rognum, 2004; Moon, 2016). According to the literature supine sleeping position poses minimal risk for SUDI. Most *SIDS* cases in this study, however in contrast to the literature, were documented to sleep in supine position.

Bed-sharing is an acknowledged risk factor contributing to SUDI. Antenatal and postnatal smoking has a significant effect on serotonin production in the brain, in turn negatively affecting arousal responses and homeostasis. Both bed-sharing and maternal smoking in concert have been described to increase the risk of SUDI (Bartick & Tomori, 2018). Even though no significance was observed in the association between bed-sharing and COD, the majority of the SUDI cases were reported to share a bed with other individual(s). The literature has reported that bed-sharing may increase the risk of overheating (Moon, 2016). Bed-sharing between parents and infants has been linked to SUDI, in concert with certain other risk factors, such as maternal smoking, alcohol consumption, prematurity and low birthweight. In the absence of the described risk factors, bed-sharing alone had no increased risk on SUDI (National Institute for Health and Care Excellence, 2014; Ball et al., 2016). Routine bed-sharing has been reported to have little contributing risk on SUDI, in contrast to unintentional bed-sharing (Vennemann et al., 2007). Certain studies, however, have observed that bed-sharing contributes no risk to SUDI if no other contributing factors are present (Blair et al., 2014). In contrast, other studies have observed that an absence of bed-sharing can lead to early weaning, often resulting in stress of the infant, increasing the vulnerability of the infant. This increased stress level is

postulated to occur due to increased cortisol levels at night when the mother is absent (Middlemiss et al., 2012; Huang et al., 2013).

Low birthweight is often a result of prematurity in infants, which is observed to be a significant contributing risk factor for SUDI (Ball et al., 2016). Short gestational age is described in the literature as a risk factor for the onset of SUDI, mirrored by the results obtained in this study (Goutas et al., 2011). In correlation to the literature, a significant association was observed between premature SUDI cases and COD. Significantly more *Infection* cases were premature, with just under half of all SUDI cases being born prematurely. This observation is supported by the fact that premature infants are described to have high susceptibility to infections (Strunk et al., 2011). Dwyer and Ponsonby (1995), however, described prematurity increasing the risk of SUDI. In this study, an increase in SIDS numbers was observed in the premature infants, therefore correlating with the observations of Dwyer and Ponsonby (1995).

Significance regarding ventilation and COD was observed, where the majority of SUDI cases found in ventilated rooms were in the *Infection* group. It could be postulated that ventilation could decrease the body temperature of susceptible infants and lead to infection. A higher number of *SIDS* cases were observed where no ventilation was present during sleep, which correlates with the literature. It is stated that overheating during sleep can contribute to SUDI, with a lack of ventilation increasing the chances of this phenomenon (Moon, 2016). McGlashan (1989) discovered that well-ventilated sleeping areas decreased the risk of SUDI.

5.2.2. Numerical data

No significance was observed between age and COD, however an overall trend could be observed in which the *SIDS* group had the lowest overall mean age. All three groups, *SIDS, Infection* and *Other* displayed mean ages between the range of two to four months. This age bracket has been recorded in the literature as the optimal age for SUDI to occur (Filiano & Kinney, 1994).

Low birthweight, defined as below 2 500g, is regarded as an important risk factor for SUDI (Filiano & Kinney, 1994; Fouché et al., 2018). In accordance with the literature, a highly significant association between birthweight and COD was observed in this study. Strong significance was especially observed between birthweight, *SIDS* and *Infection. SIDS* cases displayed the lowest birthweights of the study population, as also observed in Dwyer and Ponsonby (1995). Low birthweight in infants has also been attributed to high maternal stress levels and resultant increased cortisol levels. Chronic stress associated with racism or poverty results in the decrease of diurnal cortisol variation. This chronic stress is particularly relevant between pregnancies and is postulated

to affect the birthweight of the subsequent infant. Increases in infant mortality between 2002 and 2003 were linked to a sudden decrease in birthweights (Bartick & Tomori, 2018).

No statistical significance was observed between minimum, maximum or temperature difference. *SIDS*, however, did display a slightly lower mean minimum temperature resulting in a slightly increased mean temperature difference. The lower minimum temperature correlates to the literature reporting that SUDI cases are more likely to occur in colder weather (Guntheroth et al., 1992). A high difference between maximum and minimum temperatures could possibly result in a difficulty in an infant's ability to maintain a constant body temperature for homeostasis.

5.3. Microbiology

The majority of the SUDI cases that had positive microbiology results were signed out as *Infection*, however this trend showed no significance. Only a few *SIDS* cases displayed positive heart microbiology.

Gender was not significantly associated with microbiology positive results; however, a trend was observed in which more females than males produced positive microbiology isolates. This contrasts with the theory described in Moscovis et al. (2013), whereby female infants were described to have a more developed immune response to microbial infections than males.

Another trend observed was that majority of the microbiology positive SUDI cases occurred in cold seasons, correlating with the microbiological seasonality of URTI (Harrison et al., 1999). This association, however, was not statistically significant possibly due to the small number of microbiology-positive cases. A trend regarding ventilation and microbiology results was observed. Infants that slept in ventilated rooms displayed more positive microbiology results, than infants in rooms with no ventilation. Strong significance was observed in the association between the maximum temperature and positive microbiology results, as well as between the temperature difference and microbiology. Even though microbiological prevalence in colder months and ventilated areas was observed, the maximum temperature was found to be higher in the SUDI cases with positive microbiology results, than the cases with negative microbiology. As a result, however, the difference between maximum and minimum temperatures was increased in the cases which had positive microbiology results.

Infants that had positive microbiology results followed the trend of being slightly younger than the cases that displayed negative results. The association was not statistically significant, however, and contrasted with the study of Weber et al. (2008b), which observed that death due to positive microbiology results usually occurred in slightly older infants.

E. coli was the most commonly detected bacterium which, according to Weber et al. (2008b), is almost always associated with bacteraemia. Weber et al. (2008b) also reported *Strep pyogenes*, which was also detected in two cases of this study, often represents a true infection of the tissue and not resultant of contamination. *Enterococcus sp.,* however, were reported to be due to contamination, while *Staph aureus,* that was detected in one case of this study, is reportedly toxigenic and can contribute detrimentally to SUDI by altering the inflammatory process of the infant. Both *E. coli* and *Strep pyogenes* are also reportedly toxigenic, and could contribute to SUDI (Blackwell et al., 2001).

5.4.   Viral detection

Viral infection is postulated to be an important co-factor in regarding SUDI (An et al., 1993; Blackwell et al., 2001).

All molecular assays were performed on specialised flocculated swabs collected from the heart as described previously. A previous study done at Tygerberg Medico-legal Mortuary compared the DNA/ RNA yield obtained from tissue and flocculated swabs collected from SUDI cases (2012-2014) and found comparable results (unpublished data). In order to preserve the organs for other forensic macroscopic and microscopic investigations, the sample collection protocol at Tygerberg Medico-legal Mortuary no longer includes tissue collection for molecular investigations.

HAdV and PVB19 were not detected in any heart samples of the SUDI cases. This could be due to the sample population and perhaps the seasonality of the viruses. It is also possible that these viruses were in fact present, but in different organs, as the number of HAdV detected in the comparison group of respiratory samples in by the NHLS in Tygerberg hospital, differed substantially to the results of this study. This could be due to the comparison group samples not being myocardium, and therefore not related to myocarditis. The heart tissue of living infants in hospitals is not sampled, due to the invasiveness of obtaining the sample. PVB19 is not part of the routine viral screening conducted by the NHLS, Tygerberg Hospital.

In contrast to the literature, no PVB19 was detected in the myocardium of the SUDI infants. According to Kuethe et al. (2009), 65% of myocardial tissue samples showed PVB19 presence. In another study, however, conducted by Prtak et al. (2010) only one out of 117 cases had PVB19 detected. The German population was observed to have a high PVB19 presence (Kuethe et al., 2009), however the Prtak, et al. (2010) study population was specific to the UK. The stark variation between the two study populations of Kuethe et al. (2009) and Prtak et al. (2010) could be indicative of a similar phenomenon occurring between different populations within South Africa.

HAdV is typically reported in the literature as one of the main causes of respiratory illness Bajanowski, et al. (1996), thus supporting the presence of the virus in the respiratory samples of the comparison group. In contrast to the results obtained in this study, HAdV has been reported to be a common cause of myocarditis (Heydari et al., 2011). In a study conducted by Nielsen et al. (2014), no HAdV was detected in the heart samples of both control cases and cases displaying myocarditis. Certain strains of HAdV have been described to occur in specific geographic areas, perhaps contributing to the reason why HAdV was not detected in this study population (Gray et al., 2007). HAdV and HBoV are associated with coinfection, resulting in myocarditis. This, however, was not observed in this study (Heydari, et al., 2011).

The comparison population previously described displayed a slightly higher prevalence of HBoV in the respiratory samples, in comparison to SUDI cases with HBoV detected in the heart. The low HBoV detection rate contrasted to the high seropositivity for HBoV observed by Kuethe et al. (2009) in a German population. The low HBoV detection in the heart could be due to the fact that the virus could preferentially infect the respiratory system, as it was reported to the fourth most commonly found virus in the respiratory system, detected in 3.1% of children (Allander et al., 2005; Heydari et al., 2011; Guido et al., 2016). Da Silva et al. (2013) described HBoV detection in the study of LRTIs in infants and children in Brazil, a developing country similar to South Africa, a new discovery.

The organ of infection, however, is usually dependent on the HBoV genotype. HBoV1 genotype commonly infects the respiratory tract, whereas HBoV2 and 3 have been most commonly reported in the gastrointestinal tract (Guido et al., 2016). HBoV2, however has been associated with fatal myocarditis (Brebion, et al., 2014). Detection in the myocardium was observed by Kuethe et al. (2009), and again by Sallmon et al. (2017) in an infant younger than one year. Possibly due to the low number of HBoV detected in this study, no significant association was observed between viral detection and COD. One case in which HBoV was detected was attributed to *SIDS*, the other cases were all signed out as *Infection*.

One of the HBoV positive SUDI cases also displayed positive histology for myocarditis. Previous cases in which the COD was resultant from HBoV myocardial infection reported varying findings regarding myocarditis. Sallmon et al. (2017) found evidence of subacute myocarditis in the myocardium where HBoV was detected. No myocyte necrosis was observed in the aforementioned case, similar to the case study of Brebion, et al. (2014), who also reported mononuclear infiltrate, fibrosis as well as myocyte degradation in the heart, where the COD was confirmed to be due to HBoV infection. The histology of Case 109, in which HBoV was detected, displayed neutrophilic and mononuclear infiltrates, consistent with the findings of both Brebion et al. (2014) and Sallmon,

et al. (2017). The mononuclear infiltrates consisted only of lymphocytes, not macrophages, and myocyte degradation could be observed, which was different from the previous two studies mentioned. Sallmon et al. (2017) described the HBoV associated myocarditis as subacute, with no myocytolysis observed.

It was ascertained by Kuethe et al. (2009) that unlike PVB19, a close relative of HBoV, the viral DNA does not persist in the heart tissue after infection. The clinical history of Case 109, with both HBoV and myocarditis present, indicated that the infant had been suffering from a runny nose for three weeks prior to death. HBoV genotype 1 most commonly results in rhinorrhoea (Guido et al., 2016). The COD of the infant was, however, a LRTI due to a *Staph aureus* infection and it is plausible that the simultaneous infection could possibly have been detrimental. Heydari et al. (2011) reported a case in which COD was pneumonia, associated with myocarditis. Both HBoV and HAdV were detected in this case.

HBoV infections alone are rare, with coinfections occurring more often (Guido et al., 2016). One SUDI case displayed both positive microbiology results and HBoV detection in the heart, however a COD of pneumonia and gastroenteritis were assigned, not a heart related infection. The bacteria isolated in the heart was *S. liquifaciens.* Along with *S. marcescens*, these viruses have been described as a potentially fatal threat in premature infants. Both cases in which *S. liquifaciens* and *S. marcescens* were detected were premature, therefore correlating with the literature (Fitzgerald et al., 1984). Multiple pathogens, resulting in coinfection, can be a valid factor in disease (Singer et al., 2017). Simultaneous infection can significantly increase symptom severity, pathogenic load and progression of the disease. Disease load is increased due to coinfection between a pathogenic bacterium and virus, which works synergistically and lead to treatment not working as efficiently or diagnostic difficulties. Coinfections have been described as predominant in underprivileged communities, and thus an example of syndemic interaction (Singer et al., 2017). No viral coinfections were observed between the viruses investigated in this study, in contrast to the literature where 75% of HBoV infections displayed the presence of another virus, most commonly HAdV (Calvo et al., 2016).

No association between gender and viral detection was observed, with only one of the positive cases being a male. This result contrasted with the literature, in which it was reported that the majority of HBoV positive respiratory samples (83%) were detected in males (Dina et al., 2009). This difference, however, could be due to the small number of HBoV positive samples detected in this study.

All HBoV positive samples observed in SUDI cases were collected in the cold seasons, and the infants all slept in a ventilated room. The seasonal distribution coincided with a study performed in the northern hemisphere, in which HBoV detections spiked in the cold months of November and

December (Calvo, et al., 2016). HBoV detected in the southern hemisphere also seemed to peak in the winter months (Da Silva et al., 2013). Therefore, slightly lower mean minimum and maximum temperatures observed in HBoV positive SUDI cases also agreed with the literature. In contrast, however, a study conducted in Korea displayed peak HBoV detection in the spring and summer months (Choi et al., 2011).

Only one case displayed prematurity and HBoV detection. HBoV positive SUDI cases displayed a slightly lower mean birthweight than negative samples. The mean age of HBoV positive samples showed a trend of being higher than that of the negative samples, averaging at just under four months. In contrast, it has been reported that HBoV is most commonly detected in infants 6-24 months (Guido et al., 2016). Choi et al. (2011), however, detected the highest amount of HBoV in infants 3-24 months.

Paraffin embedded myocardial biopsies were used by Dettmeyer et al. (2004) in order to detect HAdV using the same primers as this study in a post-mortem setting, similar to this study. The hexon gene was targeted, the sequence that Martin et al. (1994) stated as similar in all HAdV serotypes. No sensitivity data of the primers was reported by Dettmeyer et al. (2004), however, Martin et al. (1994) did state that endomyocardial biopsies decreased viral sensitivity and suggested nested PCR to be used in clinical settings.

Nested multiplex PCRs are used to detect HBoV in endomyocardial biopsies of live patients (Manning et al., 2006). Allander et al. (2005) used a single pair of primer sequences for DNA extracted from nasopharyngeal aspirates in a diagnostic clinical setting. This study used the same primer sequences to detect HBoV in flocculated swabs and although the samples were different in the two studies, the primers do target the NP1 genome region which is conserved over the genotypes. Sensitivity in the Allander et al. (2005) study was estimated to 10^5 viral copies in 96 clones, similar to what was found in this thesis.

PVB19 is detected using nested quantitative PCR in a clinical setting. The primers required to detect PVB19 used DNA extracted from deparaffinised heart tissue as the template when used in the original study. The original study by Bültmann et al. (2003), similar to this thesis, took place in a post-mortem setting, but they were also able to describe the detection of the VP1/VP2 region of PVB19 in a clinical setting. No primer sensitivity was described and could therefore not be compared to the findings of this thesis.

5.5.   Histology

The vast majority of myocarditis positive histology SUDI cases were signed out as *Infection*, no significant association was observed, however. The inflammatory infiltrate observed in myocarditis histology could vary drastically between different cases. Filtrate intensity and distribution are factors subject to change (Basso et al., 2013). Histology sections positive for myocarditis displayed foci of inflammatory infiltrate, as well as dispersed infiltrate throughout the tissue, as depicted in section 4.3.2. A few of the SUDI cases displayed borderline myocarditis, similar to a case study of an eight-week-old infant with positive HBoV PCR detection. The infant displayed borderline myocarditis, with increased lymphocytic infiltrate, mild fibrosis but no necrosis (Sallmon et al., 2017). This was similar to the results observed in the majority of SUDI cases with abnormal histology. Few cases displayed myocyte necrosis; however, lymphocyte and neutrophil infiltrates were fairly common. In cases of young children with documented viral myocarditis, abnormalities in the septum of the heart was observed (Brebion, et al., 2014; Sallmon, et al., 2017). Septal involvement should therefore be investigated in further histological sampling.

In a study performed in Quebec by Dancea et al. (2002) over 12 years, the second most common cardiac complication in SUDI cases was myocarditis (n=13, 15.9%). The most common cardiac complication observed was due to primary endocardial fibroelastosis. The numbers in the study of Dancea et al. (2002) contrasted quite strongly with the present study. Nearly the same amount myocarditis cases (n=10) were observed in one year in the Tygerberg Mortuary, as opposed to the 13 cases observed in the provence of Quebec in a 12 year period. The COD of 'myocarditis' was only one assigned to one of the histologically confirmed myocarditis.

5.6.   Limitations and recommendations

A limitation of this study was the lack of a comparison control group, or heart samples of non-SUDI infants. This, however, was not possible due to multiple restrictions. Heart sample collection of unnatural infant death is difficult due to ethical reasons, since the samples are not used for ascertaining COD and thus do not fall under the waiver of consent. Due to the invasive nature of obtaining the heart samples, it is not possible to sample the myocardium of living infants. The NHLS database of respiratory infections in infants in the Tygerberg hospital provided a primitive comparison group for HAdV and HBoV frequencies. The literature, with similar study objectives and control groups, was therefore used for further comparisons.

Due to financial constraints conventional qualitative PCR was used instead of quantitative real-time PCR assays. Quantitative PCR could be used in future studies in order to increase sensitivity and specificity of viral detection. Sensitivity and specificity statistical analyses tests could not be

performed due to the low number of viral positive samples detective. The test could, however, be performed in studies with a higher number of viral positive samples. Financial constraints also limited the number of viruses that could be investigated.

A few of the SUDI cases (6.9%) had not had the COD released by the Forensic Pathologists in time for the submission of this thesis. As a result, associations between the data of certain cases could not be included in the analysis. This posed a slight limit on the interpretation of COD associations. Not all the SUDI cases had histology available (16.2%), thus slightly limiting the interpretation of histological analyses to a degree.

Few studies have been conducted regarding viral infection and myocarditis in SUDI cases in South Africa. This study is possibly one of the first South African studies investigating HAdV and HBoV prevalence in the heart samples of SUDI cases. The study therefore brings to light new descriptions regarding the sample population's myocardial infections. This could in turn lead to standardised routine viral screening of the heart in concert with histological analysis for SUDI post-mortem investigations. The results described in this study could possibly lead to further investigations regarding the clinical significance of HAdV, HBoV and PVB19.

Future studies could investigate the genotypes of each virus, as well as the impact on SUDI myocardial infection and inflammation. Further investigations of HAdV, HBoV and PVB19 prevalence in other study populations in South Africa could also be performed. A longitudinal study of HBoV could be beneficial in describing the prevalence over time. Viral detection could ultimately be used in concert with immunological results and histology to characterise infectious threats.

6.

CONCLUSION

The study aimed to prospectively investigate the presence of specific viruses in the heart of SUDI cases admitted to the Tygerberg Medico-legal Mortuary over a 1-year period. Profiling viral pathogens in the heart and the effect on myocarditis in South African SUDI cases is relatively new. Few studies, to the knowledge of the author, have been conducted specifically profiling HAdV, HBoV and PVB19 in the sample population of the Tygerberg Medico-legal Mortuary.

Certain sociodemographic factors investigated have been deemed to affect the incidence of SUDI cases and the assigned COD, according to both the literature and this study. In the study population, premature infants were more significantly linked to death due to *Infection.* Ventilation of the room in which the infant slept was also found to have a significant impact on COD in this study. Low birthweight was also significantly associated to *SIDS* and *Infection,* with *SIDS* cases displaying the lowest mean birthweight. These risk factors were prevalent in the Tygerberg Medico-legal Mortuary referral area, and negatively impacted the homeostasis of the infants leading to SUDI.

Only one virus, HBoV, was detected in the heart tissue with implications of myocarditis histologically observed in one SUDI case. Even though HBoV is reportedly common in coinfections, only one microbiology positive result was obtained, with none of the other viruses investigated present. All viral infections were observed in the cold seasons, with slightly lower mean temperatures than HBoV negative cases. The study provided a platform for describing the ever-evolving viral infections of the Tygerberg referral area, and which possible risk factors were linked to HBoV infection and SUDI cases.

The study aided in expanding the knowledge regarding myocardial infections contributing to SUDI deaths in the study population. Viral detection in the study, supported by histological evidence, provided an improved way of classifying COD as infection, thus aiding in standardising a protocol for the profiling of viral pathogens in the heart during SUDI post-mortem examinations. The study provided a basis for potential studies to come, in the hope of better characterising viral myocardial infections and SUDI in a South African context, ultimately providing specific infection prevention.

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APPENDICES

Appendix 1: Approval and renewal from the Health Research Ethics Committee of Stellenbosch University (2017-2019)

Appendix 2: Haematoxylin and Eosin

Incubation

Incubate samples for ± 1 hour in 60°C

Hydration

Xylene	2 minutes (repeat twice)
100% Ethanol	1 minute (repeat twice)
96% Ethanol	1 minute
70% Ethanol	1 minute
Tap water	2 minutes

Staining

Haematoxylin	4 minutes
Water rinse	3 minutes
Eosin	2.5 minutes
Water rinse	2 minutes

Dehydration

70% Ethanol	5 minutes
96% Ethanol	5 minutes (repeat twice)
100% Ethanol	5 minutes (repeat twice)
Xylene	5 minutes
Xylene	5 minutes

Appendix 3: TAE buffer (1X)

50X TAE buffer

- 242 g Tris base

- 57.1 ml glacial acetic acid

- 100 ml of 500 mM EDTA (pH 8.0)

- Final volume 1 L in milliQ water

20 ml 50X TAE buffer diluted in 980 ml of milliQ water

Appendix 4: Statistical comparison between categorical data and Cause of Death using the Fisher Exact test

Variable		Cause of Death			p-value
		SIDS	*Infection*	*Other*	
Gender	Male	17.4%	32.9%	4.4%	1.00
	Female	14.3%	28.0%	3.1%	
Season	Warm	14.3%	21.1%	3.1%	0.43
	Cold	17.4%	39.8%	4.4%	
Prematurity	No	13.6%	36.4%	5.8%	*0.02**
	Yes	18.8%	24.0%	1.3%	
Sleeping position	Stomach	7.0%	23.6%	1.3%	
	Back	5.2%	6.4%	1.3%	0.20
	Side	19.2%	31.2%	4.5%	
Bed-sharing	No	1.9%	5.2%	0.7%	0.80
	Yes	29.7%	56.1%	6.5%	
Ventilation in room	No	19.0%	23.2%	4.2%	*0.02**
	Yes	11.3%	38.7%	3.5%	
HBoV Positive	No	31.1%	59.0%	7.5%	1.00
	Yes	0.6%	1.9%	0.0%	
Histology Positive	No	30.3%	55.9%	6.9%	0.28
	Yes	0.7%	6.2%	0.0%	
Microbiology Positive	No	29.4%	50.6%	6.3%	0.25
	Yes	2.5%	10.6%	0.6%	

Appendix 5: Statistical comparison between other categorical variables using the Fisher Exact test

Variable 1	Variable 2	Negative	Positive	p-value
Microbiology results	Male gender	49.1%	5.6%	0.35
	Female gender	38.5%	7.1%	
Microbiology results	No ventilation in room	41.0%	4.3%	0.22
	Ventilation in room	45.3%	9.4%	
Microbiology results	Premature	37.1%	6.6%	0.54
	Full-term	49.7%	6.6%	
Microbiology results	Warm season	32.5%	4.7%	1.00
	Cold season	55.0%	7.7%	
Microbiology results	Histology negative	84.0%	9.0%	*0.02**
	Histology positive	4.2%	2.8%	
HBoV results	Male gender	53.2%	0.6%	0.34
	Female gender	44.5%	1.7%	
HBoV results	Full-term	53.9%	2.0%	0.63
	Prematurity	43.5%	0.7%	
HBoV results	No ventilation in room	46.5%	0.0%	0.12
	Ventilation in room	50.7%	2.8%	
HBoV results	Warm season	38.2%	0.0%	0.30
	Cold season	59.5%	2.3%	
HBoV results	Microbiology negative	85.8%	1.8%	0.41
	Microbiology positive	11.8%	0.6%	
HBoV results	Histology negative	91.2%	2.0%	0.25
	Histology positive	6.1%	0.7%	

Appendix 6: Statistical comparison between numerical data and Cause of Death using the F-test

Variable	Cause of Death			p-value
	SIDS	*Infection*	*Other*	
Age (weeks)	11.0 ± 7.5	12.6 ± 10.4	13.0 ± 13.4	0.77
Birthweight (gram)	$2\ 267.3 \pm 684.8$	$2\ 613.7 \pm 851.9$	$2\ 922.5 \pm 820.6$	***<0.01****
Maximum temperature (°C)	22.7 ± 4.8	22.5 ± 5.0	22.3 ± 4.1	0.88
Minimum temperature (°C)	10.9 ± 4.3	11.4 ± 3.8	11.8 ± 3.2	0.65
Temperature difference (°C)	12.7 ± 5.3	11.3 ± 5.1	10.5 ± 4.1	0.33

*Statistical significance indicated between variables ($p < 0.05$)

Appendix 7: Statistical comparison between non-parametric data using the F-test

Variable 1	Variable 2	Negative	Positive	p-value
Microbiology results	Age (weeks)	12.4 ± 10.0	11.2 ± 9.0	0.57
	Birthweight (gram)	2 555.9 ± 776.8	2 400.1 ± 1 042.1	0.35
	Maximum temperature (°C)	22.0 ± 4.7	25.7 ± 5.2	***<0.01****
	Minimum temperature (°C)	11.2 ± 3.9	11.9 ± 4.0	0.58
	Temperature difference (°C)	11.4 ± 5.1	13.8 ± 5.1	***0.02****
HBoV results	Age	12.1 ± 9.8	15.0 ± 11.7	0.55
	Birthweight (gram)	2 527.0 ± 816.1	2 345.0 ± 936.5	0.75
	Maximum temperature (°C)	22.6 ± 4.9	20.0 ± 4.7	0.23
	Minimum temperature (°C)	11.29 ± 3.94	10.3 ± 1.3	0.51
	Temperature difference (°C)	11.7 ± 5.1	9.6 ± 5.9	0.26

*Statistical significance indicated between variables (p <0.05)