UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Department of Pathology
Division of Forensic Medicine and Toxicology

The contribution of respiratory pathogens to Sudden Unexpected Death in Infancy

by

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ISHELY001

A minor dissertation submitted in the partial fulfilment of the requirements for the degree of

Master of Philosophy (MPhil) in Biomedical Forensic Science

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Preface

This minor dissertation is submitted in the partial fulfilment of requirements for the degree of Master of Philosophy (MPhil) in Biomedical Forensic Science offered by the Division of Forensic Medicine and Toxicology, Department of Pathology, University of Cape Town, South Africa.

According to the recommendation of the academic committee of the MPhil programme in Biomedical Forensic Science, the MPhil minor dissertation accounts for one-third of the MPhil degree and must include the following parts: (i) research protocol approved by the Human Research Committee (HREC) of the University of Cape Town, South Africa (HREC 635/2014), (ii) structured literature review and (iii) ready to publish manuscript. Therefore, some content duplication may occur between the different chapters.

The present research study was funded by the Division of Forensic Medicine and Toxicology and Division of Medical Microbiology (University of Cape Town, South Africa). All work presented henceforth is a collaborative effort between the Salt River Forensic Pathology Laboratory, the Division of Forensic Medicine and Toxicology, and Division of Medical Microbiology, at the University of Cape Town, South Africa.

Dr. Mamadou Kaba, Dr. Marise Heyns, Dr. Yolande van der Heyde and the MPhil candidate participated in the concept formation of the research study. Forensic pathologists at the Division of Forensic Medicine and Toxicology (University of Cape Town, South Africa) assisted during specimen collection at Salt River Forensic Pathology Laboratory, Cape Town, South Africa. Ms Samantha Africa assisted with laboratory work at the Division of Medical Microbiology and Dr. Lemese Ah Tow assisted in quality controls of the real-time PCR study results. Dr. Mamadou Kaba and Dr. Marise Heyns contributed to the manuscript edits. The MPhil candidate was responsible for data collection at Salt River Forensic Pathology Laboratory, Cape Town, South Africa. The MPhil candidate performed nucleic acids extraction as well as real-time polymerase chain reaction at the Division of Medical Microbiology (University of Cape Town, South Africa). In addition, the MPhil candidate analysed the data and wrote the manuscript.

Besides the contribution of the above mentioned individuals as stated by acknowledgement, the research work describe herein is the work of the MPhil candidate.

Acknowledgments

I would like to respectfully thank Prof. Lorna J Martin and Prof. Mark P Nicol for the provision of laboratory facilities at Salt River Forensic Pathology Laboratory and at the Division of Medical Microbiology and for funding this research work.

I am extremely grateful to my supervisors Dr. Mamadou Kaba and Dr. Marise Heyns for their continuous support, patience, motivation and knowledge. Their valuable input and guidance helped me throughout the research work.

My gratitude to all forensic pathologists at the Division of Forensic Medicine and Toxicology, University of Cape Town, South Africa for their assistance in collecting samples, especially Dr Yolande van der Heyde for her guidance in designing the research protocol. I would also like to thank all forensic officers at Salt River Forensic Pathology Laboratory for their collaboration in administering the questionnaires.

My sincere thanks also go to all my colleagues at the Division of Forensic Medicine and Toxicology, and the people I met at both the Division of Medical Microbiology and at Salt River Forensic Pathology Laboratory for their help and friendship. A special thank goes to Ms Samantha Africa for her endless assistance during laboratory work at the Division of Medical Microbiology.

Finally, I take this opportunity to express my gratitude to my family and friends for their unfailing encouragements. Most of all, I thank my parents for their love and support.

Dedication

To all families that lost a child to Sudden Unexpected Death in Infancy

Table of Content

PART 1: **GENERAL INTRODUCTION**

1. Background and rationale:

Sudden unexpected death in infancy (SUDI) is one of the important causes of death in the first year of life [1, 2]. Various respiratory viruses have been identified in SUDI cases [3-6]. Moreover, a significant number of SUDI cases exhibits pathophysiological signs of bacterial infection and inflammation of respiratory origin[7-9]. In addition, pneumocystis infection was identified in cases of infants dying suddenly and unexpectedly [10, 11]. This high frequency of infections with respiratory pathogens reflects the importance of these pathogenic agents in SUDI. However, the number of respiratory pathogens investigated is limited and the contribution of bacterial and fungal respiratory pathogens is still uncertain.

Little is known about the aetiology of infections in SUDI in Africa [3, 12]. The lack of standardized guidelines regarding specimens to be collected, laboratory analysis to be applied as well as interpretive guidelines for post-mortem microbiological examination renders the investigation of infectious cause of SUDI most challenging.

2. Study aim and objectives:

The aim of this dissertation was to determine the incidence of respiratory pathogens in cases of Sudden Unexpected Death in Infancy (SUDI) and to compare the findings from different techniques used in medico-legal investigation of SUDI cases in Cape Town, South Africa.

3. Thesis outline:

This dissertation begins with the description of the research protocol (Part 2). It is followed by a detailed review (Part 3) that focuses on three main topics: the burden of respiratory pathogens in cases of SUDI, the risk factors associated with the occurrence of respiratory infections in SUDI and diagnosis of respiratory infections in SUDI cases. Part 4 include a ready to publish article that summarises all the important results. Part 5 covers the general discussion of the present dissertation, followed by the final part (Part 6) that summarises the general conclusion and perspectives. A list of referenced literature is provided after each part.

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PART 2: **RESEARCH PROTOCOL**

The Contribution of Respiratory Pathogens to Sudden Unexpected Infant Death

By

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Project Proposal

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Abstract

Sudden unexpected death in infancy (SUDI) is the leading cause of post-neonatal mortality. Infections of the respiratory system are considered as the main cause of death in SUDI. However, the number of respiratory pathogens investigated is still limited, which can lead to an underestimation of the contribution of respiratory pathogens in SUDI. This study aims to evaluate the role of respiratory pathogens in SUDI and examine the correlation among findings from different techniques used in medico-legal investigation of SUDI in Cape Town, South Africa. Consecutive cases of SUDI admitted at Salt River Forensic Pathology laboratory from September through November 2015 will be investigated. This study will follow the routine investigation of SUDI cases at Salt River Forensic Pathology Laboratory, Cape Town, South Africa. Risk factors associated with SUDI will be identified using a questionnaire given to the family of the deceased infant. An assessment of pathological signs of infection in the lungs using Lodox® Statscan® X-ray will be performed followed by a full body post-mortem examination by a forensic pathologist. Sections of the brain, heart, lungs, kidneys, spleen, and liver will be collected during autopsy for histopathological examination using Haematoxylin and Eosin staining microscopy. Cerebrospinal fluid, pericardial fluid, heart blood and percutaneous needle lung biopsies will be collected for the detection of 33 respiratory pathogens using a set of eight multiplex real-time polymerase chain reactions. Post-mortem microbiology, autopsy, histopathology results and findings from the questionnaire will be analyzed to identify respiratory pathogens associated with SUDI and associated pathology and risk factors.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as the death of infants in the interval between seven days and one year of age, that occur suddenly and unexpectedly by clinical history [1]. Following in depth investigation comprising full autopsy, biological examinations, review of the clinical records and examination of the death scene, only 15% of SUDI cases is explained, thus referred as "explained SUDI"[2], while 85% of SUDI cases remain uncertain, therefore classified as "unexplained SUDI" or "Sudden Infant Death Syndrome" (SIDS) [2, 3].

In the developed countries, SIDS is the most frequent cause of death among infant less than one year of age [4, 5]. In England and Wales, unexplained infant death encounters 8% of all infant death [6]. A higher SIDS incidence rate of 15% was observed in United States of America (USA) [7]. In South Africa, although limited data is available, SUDI comprises 25% of all medico-legal autopsy cases admitted to forensic mortuaries [8]. Among them, about 9% are classified as SIDS [8]. The highest SIDS incidence of approximately 15 % is observed in Cape Town-Tygerberg area in the Western Cape [8].

Epidemiological studies on SUDI mainly focussed on the assessment of risk factors [9, 10]. The major risk factors identified include prematurity, low birth weight, low income, intrauterine exposure to smoking, prone sleeping, genetic, cardiovascular and infectious diseases [10]. A high incidence of SIDS, a subclass of SUDI, has been observed in populations who exhibit a high incidence of severe infectious disease, in particular those affecting the respiratory system [11]. An increasing incidence of SUDI is observed during cold periods, and more than 70% of SUDI have been linked to infections, predominantly pneumonia [12]. Despite significant success with the rollout of pneumococcal conjugate vaccine in the past decade [13-15], pneumonia continues to be a significant health burden especially in developing countries [16]. In a multi-centre study on forensic investigation procedures and outcomes of SUDI in South Africa, about 26% were attributed to pneumonia regardless of considerable disparity in the ancillary investigation performed including bacteriology, virology and histopathology [8].

Investigation of SUDI requires a collaborative approach; different protocols for investigating SUDI are available, although it differs across countries as well as within countries [1, 5]. Among many others, the most well-known protocol is Kennedy's report, a protocol developed in 2004 by the Royal College of Pathologists for care and investigation of sudden unexpected child death [17]. It includes a broad range of investigation techniques such as examination of the death scene, review of clinical history, a full body autopsy, histology, microbiology (bacteriology, virology), toxicology and biochemistry [17]. It is noteworthy that toxicology is not mandatory in the Kennedy's report and it is only performed in the cases of suspected parental drug use or intentional intoxication [17]. Vitreous chemistry is not performed on a routine basis in many forensic laboratories due to low rate of metabolic diseases and high cost of tandem mass spectrophotometry [18]. Liver and kidney histology is

used to assess pathological patterns of metabolic diseases rather than the expensive tandem mass spectrophotometry [8, 18].

Respiratory pathogens have been shown to be the leading infectious cause of SUDI [8, 12, 19]. However, identifying respiratory pathogens involved in SUDI has encountered difficulties related to the selection of ideal sampling site for post-mortem microbiology [20] as well as difficulties associated to the cultivability of most of the respiratory pathogens [21]. In addition, there is limited data on high-throughput culture-independent techniques for the identification of respiratory pathogens [22, 23]. In South Africa, few respiratory viruses (Cytomegalovirus (CMV), Respiratory syncytial viruses (RSV) and human Adenoviruses (AdV)) are included in the routine medico-legal investigation of SUDI using polymerase chain reaction (PCR)-based methods [23]. Hence the role of respiratory infections in SUDI might be underestimated due to the low number of pathogens routinely investigated in South Africa.

2. Literature review

The cause of death in SUDI is considered to be multi-factorial, played by three related events: critical development stage, vulnerable infants and exogenous triggers, constituting the "fatal triangle hypothesis" [24]. Epidemiological studies have identified many risk factors for explained and non-explained SUDI and they are found to be similar in both cases [25]. The patterns of SIDS rates vary greatly with age [26] and the peak incidence is observed between two to four months of age, which correspond to the period when the maternal antibodies decrease and the immunity of the infant is still immature [27]. Preterm infants exhibit an increased incidence of SUDI of about four times higher than term infants [28], which has been suggested to be due to the amplified obstructive apnea during sleep in preterm babies [29]. In addition, an increased incidence of SIDS was reported in male infants compared to females [26]. This higher incidence of SIDS in male infants can be linked to X-linked genetic mutations [30] and also correlated with the higher colonisation of *Staphylococcus aureus* in males [31].

A disparity in SIDS incidence is also observed among different races [23, 32, 33]. In USA, American-Indian or Alaska-native exhibits a higher mortality rate due to SUDI than other racial groups in this country [32, 33]. In South Africa, more than 60% of all SUDI cases are observed in black population [23]. This is probably due to low socio-economic status [34].

Poetsch et al. demonstrated a correlation between maternal smoking and SIDS incidence [35]. The level of nicotine metabolic products in infants exposed to cigarette smoke was shown to be similar to those of adult smokers [35]. This high level of nicotine increases the risk of infection and developmental disturbances in infants [35]. Prone sleeping position, especially on contaminated surfaces like sofas, also increases the level of bacterial colonisation in the upper respiratory tract [33, 36]. Furthermore, a combined presence of viral pathogens and heat production by heavy wrapping increases the risk of

death in infants [37]. Epidemiological campaigns mainly focusing on the supine sleeping position were initiated in the early 1990s and yielded a significant decrease in SIDS rate in the past two decades [38]

2.1 Respiratory infections and SUDI

The recent interest in investigating the etiological role of infections, more particularly of respiratory origin in SUDI, has increased the existing awareness of its causes [39]. Infections have been identified as one of the major causes of death in explained SUDI as well as a triggering agent in SIDS [9, 22, 40-42]. For instance, in a large study of more than 1500 paediatric cases conducted in UK; 60% of SUDI cases were due to infections, with pneumonia accounting for 20% [9]. In Africa, limited data is available [8, 23, 43]. In Nigeria, 23% of post-neonatal medico-legal cases are attributed to respiratory diseases [43] while in South Africa, about 26% of SUDI cases are linked to pneumonia [8].

Pathological characteristics of infections and inflammation due to bacterial toxins are frequently found in post-mortem examination in SIDS infants [9, 44, 45]. Significant histopathological findings are even observed in macroscopically normal organs [45]. Therefore, histopathological signs of inflammation provide an easy interpretation of an infectious cause of death in SUDI [45]. However, to determine the aetiology of the infection, microbiology examination is required [17].

Post-mortem microbiology is a compulsory ancillary investigation in SUDI [17]. Blood, cerebrospinal fluid and lung biopsy were considered as most significant samples for post-mortem microbiology [46]. Moreover, recent research recommended pericardial fluid and liver as the best post-mortem sampling sites since they were found to be the most sterile site up to 5 days post-mortem [21]. Enormous variation of positive results obtained using the shell vial culture in viral screening technique and the low sensitivity of bacterial culture technique is a limiting factor in post-mortem microbiology [23, 47]. PCR-based techniques increase the sensitivity of detection of pathogens in post-mortem tissue samples, especially in those pathogens that are not routinely cultured [1, 21].

The interpretation of post-mortem microbiology findings has been always a subject of controversy [47]. Process of agonal spread and bacterial translocation are considered as major problems in post-mortem microbiology [47, 48]. However, microbiological findings from post-mortem samples collected at autopsy within 48 hours after death are proven to be constant [46]. Difficulty in the interpretation of microbiological findings was observed in different groups of specialists involved in the routine investigation of SUDI cases, consisting of paediatric pathologists, general histopathologists, microbiologists, and paediatricians [40] . In 2009, Goldwater proposed that the interpretation of microbiological findings should be done by microbiologists [42] . The mere isolation of potential pathogens in normally sterile tissues such as blood and cerebrospinal fluid was considered as a possible cause of death [20, 49]. Weber et al. suggested that the mere detection of a pathogen in a normally sterile site should be considered as a possible cause of death only if it is supported by relevant histopathological findings [1].

An additional feature in the medico-legal investigation in South Africa, particularly in Cape Town is the use of the X-ray imaging machine, the Lodox® Statscan® (Lodox Systems (Pty), Ltd., Johannesburg, South Africa) in routine autopsy [50]. This imaging system was installed in 2007; and is the only digital radiograph machine which provides a non-stretched, single, full body radiographic image [50, 51]. Although it was initially used for identifying fractures and the presence of foreign materials such as bullets, the Lodox® Statscan® was reported to be able to detect signs of pathology on soft tissue consistent with autopsy findings [51]. Additionally, Douglas et al. observed that in 192 SUDI cases investigated using Lodox® Statscan® (Lodox Systems (Pty), Ltd., Johannesburg, South Africa), 80 cases had lung pathology consistent with respiratory infections [51]. No further examination was done to identify the aetiology of the infection detected using this imaging technique.

2.2 Justification

The implication of respiratory infections is undeniable, as a number of respiratory pathogens have been linked to SUDI [8, 12, 22, 23, 39, 52]. About 80% of SUDI cases caused by infections are linked to bacteria [9] A significant number of SUDI cases exhibits pathophysiological signs of toxins produced by *Staphylococcus aureus; Bordetella pertussis* and *Clostridium perfringens* [11, 53]. In addition, a high prevalence of pneumocystis infection was found in children dying suddenly and unexpectedly [19]. The viruses mainly associated with SUDI consist of RSV, CMV, human AdV and Enteroviruses [22].

In Africa, data on the infectious causes of SUDI are scarce [8, 23, 43]. Only one study conducted in the Western Cape Province, South Africa, assessed the respiratory aetiology of SUDI [23]. The later study focused only on three respiratory viruses, in 82 SUDI cases studied, and found 34, 4and 2 cases positive for CMV, RSV and human AdV, respectively [23]. Although the investigated respiratory viruses represent the most frequent viral pathogen encountered in hospitalized infants in Cape Town [54, 55], it might not be representative of cases where infants die suddenly and unexpectedly before or upon reaching health care facilities, and the number of investigated pathogens is still limited and warranted further investigation, especially for bacterial and fungal respiratory pathogens.

2.3 Aim and Objectives

This study aims to evaluate the role of respiratory SUDI and examine the correlation between findings from different techniques used in medico-legal investigation of SUDI cases in Cape Town, South Africa.

Specific objectives of this study are:

i. to identify risk factors associated with SUDI;
ii. to locate signs of infection in lungs of infants who died suddenly and unexpectedly using the Lodox® Statscan®;

iii. to assess histopathological patterns of infection in the lungs associated with SUDI;
iv. to detect respiratory pathogens in post-mortem specimens from SUDI cases;
v. to evaluate the correlation between radiological, histopathological, microbiological and autopsy findings relevant to this study and their significance in the final diagnosis of SUDI.

3. Methodology

3.1 Study design and setting

This prospective, observational, case-control study will investigate cases of sudden unexpected deaths in infants of less than one year of age at Salt River Forensic Pathology Laboratory (Cape Town, South Africa) from August 2014 through November 2014. The standard procedure routinely used in the forensic investigation of SUDI at this institution will be followed (diagram 1). The standard microbiological investigation will be done by the National Health Laboratory Service at Groote Schuur Hospital, Cape Town, South Africa. Additional samples relevant to this study including percutaneous needle lung biopsies, cerebrospinal fluid, heart blood and pericardial fluid will be collected for microbiological analyses. Laboratory investigations for the "additional" microbiological analysis will be held at the Division of Medical Microbiology, University of Cape Town, South Africa. The laboratory at the Division of Medical Microbiology includes a well-equipped molecular biology unit, with the unidirectional principle respected. Safety practices are strictly enforced following established operating protocols outlined in a Biosafety Manual by the Institute of Infectious Diseases and Molecular Medicine (University of Cape Town, South Africa).

3.2 Study population

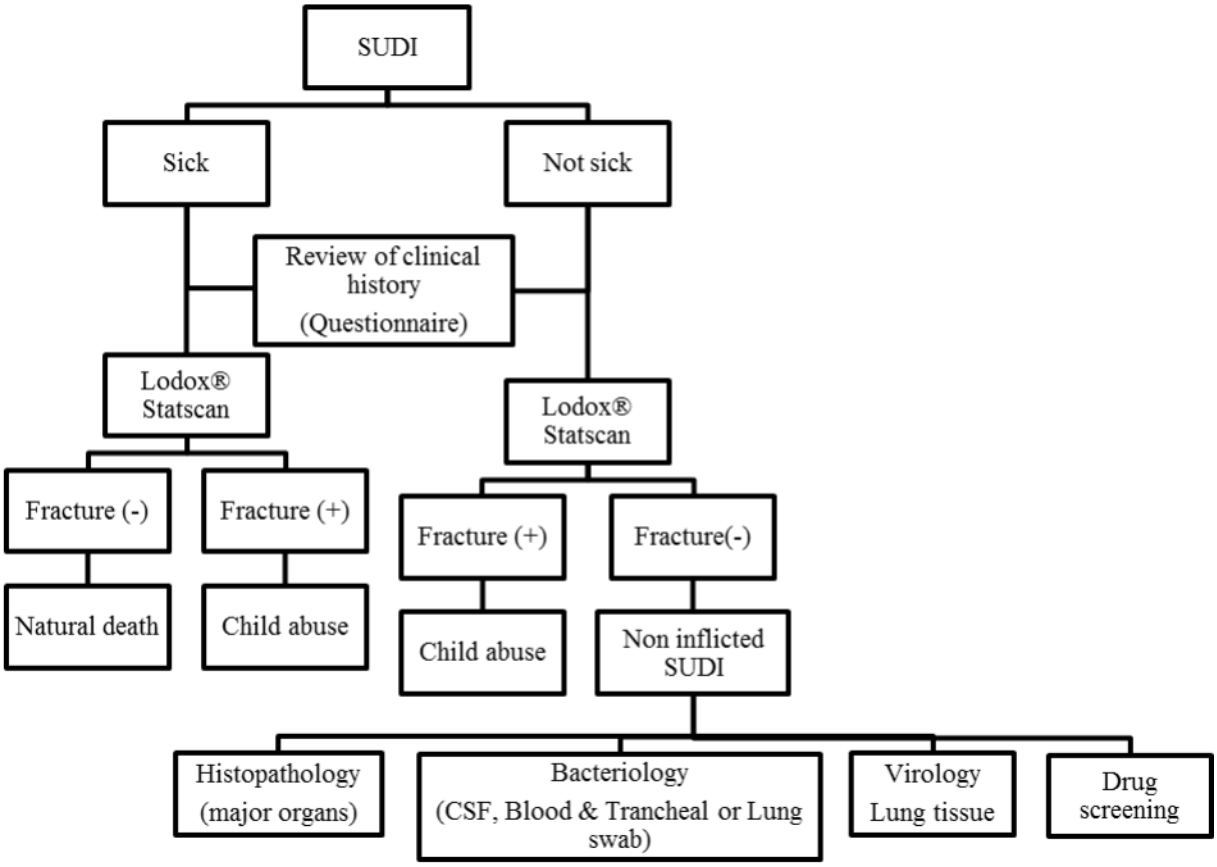
Consecutive cases of non-inflicted SUDI aged from 7 to 366 days admitted at Salt River Forensic Pathology Laboratory from August 2014 through November 2014 will be enrolled in this study. Age and gender matched SUDI cases where the circumstance of death was accidental will be enrolled as controls at a 1:2 ratio.

3.3 Questionnaire

As part of the routine procedure in the investigation of SUDI referred to Salt River Forensic Pathology Laboratory, a questionnaire is used to collect socio-demographic data, details about household environment, events before death and circumstances around death (appendix 1).

3.4 Post-mortem examination and X-ray imaging

First, full body X-ray image will be performed using Lodox® Statscan® digital radiography (Lodox® Systems (Pty), Ltd., Johannesburg, South Africa), and lung pathological patterns assessed by a forensic pathologist. Then a thorough external examination as well as a systematic examination of internal organs will be done by a forensic pathologist as previously recommended [17].



CSF, cerebrospinal fluid

Diagram 1*. Flow chart showing "routine" protocol of SUDI investigation at Salt River Forensic Pathology Laboratory, Cape Town, South Africa.*

3.5 Specimen collection and preservation

3.5.1 Specimen for microbiological analysis: lung biopsy, cerebrospinal fluid, heart blood and pericardial fluid

PrimeStore molecular transport medium (MTM) collection tubes (kept at 0 to - 4°C until use) will be used for sample collection and preservation according to the manufacturer's instructions.

Prior to opening the body, a cerebrospinal fluid (CSF) and lung biopsies will be collected. A CSF sample will be collected using lumbar puncture as described by Gorman et al. [56]. Lung biopsies will be obtained from both lungs using a minimally invasive technique, the percutaneous needle lung biopsy [16]. Furthermore, a pericardial fluid will be obtained via a puncture to the pericardium sac after opening the thorax [21]. A heart blood sample will be collected after proper sterilization of the heart surface following De Jongh et al. procedures [57].

The collected samples in PrimeStore MTM will be immediately (within two hours) transported to the Medical Microbiology Laboratory where they will be kept in the freezer at -80°C prior to analysis.

3.5.2 Tissue samples for histology analysis: brain, heart, lungs, kidneys, spleen, and liver

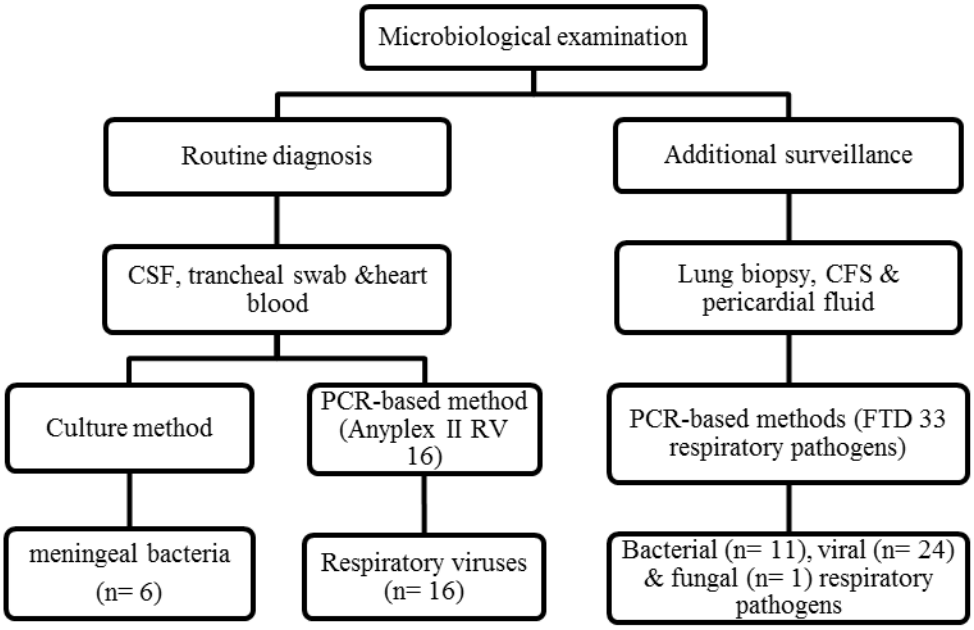
Sufficiently large (≤ 20 mm) sections of tissues from the brain, heart, lungs, kidneys, spleen and liver will be carefully selected by a forensic pathologist from each body. The collected tissues sections will be placed into leak-proof small plastic buckets. A sufficient amount of 10% formal saline, approximately twice the volume of the tissues will be added to fix the collected tissues [58].

3.6 Histopathology examination

The collected sections of tissues will be prepared for microscopic analysis [58]. Briefly, specimens collected from each case will be allowed to be properly fixed and dehydrated in different grades of alcohol, xylol and liquid wax and embedded in wax and cut into fine sections [58]. The resulting fine sections will be stained using Haematoxylin and Eosin and handed to a forensic pathologist for microscopic analysis of histopathological patterns.

3.7 Microbiological examination

"Additional" microbial investigation refers to the use FTD® respiratory pathogens 33 assay (Fast-track Diagnostics, Junglinster, Luxembourg), a real-time PCR-based pathogen detection method. "Routine" diagnosis refers to the microbiological investigation of SUDI cases as it is normally performed at Salt River Forensic Pathology Laboratory using the Anyplex II RV assay (Seegene, South Korea) and bacterial culture.



CSF, cerebrospinal fluid

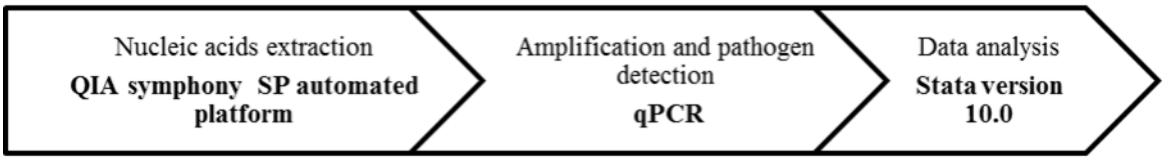
Diagram 2. *Flow chart of the microbiological examination*

3.7.1 "Routine" microbiological diagnosis using: CSF, tracheal or lung swab, and heart blood

From each case of SUDI admitted to Salt River Forensic Pathology Laboratory, a bacteriology and virology investigation is routinely performed on CSF, tracheal or lung swab, and peripheral blood samples collected during autopsy. Blood and CSF are used to isolate the following meningeal pathogens: *Neisseria meningitidis, Staphylococcus aureus, Streptococcus pneumoniae, Listeria monocytogens, Beta haemolytic streptococcus group B and Haemaohilus influenzae*. Tracheal or lung swabs is used to screen a set of sixteen viral respiratory pathogens (Influenza (A+ B), Parainfluenza viruses (1-4), Rhinovirus (A+B+C), Bocavirus (1-4), Human Enterovirus) using a multiplex real-time PCR assay, the Anyplex II RV16 detection kit (Seegene, Seoul, South Korea). CMV testing from tracheal or lung swabs is only performed on request using monoplex conventional PCR.

3.7.2 Microbiological diagnosis using the FTD® respiratory pathogens 33 assay: CSF, pericardial fluid, heart blood and percutaneous needle lung biopsy

Diagram 3 summarizes the workflow for the "additional" microbiological investigation for the detection of respiratory pathogens in samples that will be tested.



qPCR, real-time polymarase chain reaction

Diagram 3. *"Additional" microbiological investigation using FTD® respiratory pahogens 33 assay*

3.7.2.1 Total nucleic acid extraction

Total nucleic acid will be extracted from collected CSF, pericardial fluid, heart blood and percutaneous needle lung biopsies using a commercialized assay, the QIA-symphony® DSP Virus/Pathogen kit in combination of the Complex200 protocol on an automated QIA-symphony SP platform (Qiagen, Hilden, Germany) according to the manufacturer's recommendation with minor modifications. For the lung biopsies, a pre-lysis step will be performed before loading to the QIA-symphony SP instrument. This preliminary step will consist of adding 330 µl of tissue lysis buffer (ATL) and 20 µl of proteinase K to 20 mg of lung tissue and thereafter overnight incubation at 56°C [59]. We will use a protocol (complex off-board lysis protocols) that consists of using a starting volume of 400µl of the target sample and eluting the total nucleic in 110µl. A batch of 13 samples will be extracted. This will include 12 samples of the study population and one negative extraction control.

Before total nucleic acid extraction, 4 µl of an extraction control (Equine arteritis virus) will be added to each sample, including the negative extraction control.

3.7.2.2 Real-time PCR screening for respiratory pathogens detection using the FTD® respiratory pahogens 33 assay

Detection of target respiratory pathogens will be performed on a Bio-Rad CFX96 Touch™ real-Time PCR amplification instrument (Bio-Rad Laboratories, Hercules, CA, USA) using the FTD® respiratory 33 pathogens assay (Fast-track Diagnostics, Junglinster, Luxembourg) together with the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystem®, Ambion, USA) according to the manufacturer's instructions. The FTD® respiratory 33 pathogens (Fast-track Diagnostics, Junglinster, Luxembourg) comprises a set of eight multiplex real-time PCR reactions (table 1). Each set of 12 samples will be performed in two Bio-Rad Hard-Shell 96 microplates (Bio-Rad Laboratories, Hercules, CA, USA), alongside with a negative (nucleic acid extraction) and a positive control (included in the kit provided by Fast-track Diagnostics). Briefly, for each reaction, 10µl of the extracted samples will be added to a PCR mix composed of 1.5µl of primer/probe mix (Fast-track Diagnostics, Junglinster, Luxembourg), 1µl of 25x RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR Kit, Applied Biosystem®, Ambion, USA) and 12.5µl of 2x RT-PCR buffer (AgPath-ID™ One-Step RT-PCR Kit, Applied Biosystem®, Ambion, USA). Cycling will be as follow: initial heat at 50°C for 15 minutes, denaturation at 95°C for 10 minutes and 40 amplification cycles at 95°C for 8 seconds and 60°C for 34 seconds according to the maufacturer's instuctions. Genes target for the detection of different microorganisms included in the FTD® respiratory 33 pathogens assay are listed in Table 1. PCR products within each multiplex will be detected using a dual labelled molecular probe for each pathogen of the multiplex. In order to interpret the real-time PCR results, the negative control should show no amplification and the positive control an exponential amplification curve. Positive results will be indicated by a well defined curve crossing the cycling treshold value according to the manufacture's recommendation. To be considered as valid results, the real-time PCR findings will be agreed by two scientists. Then data will be automatically exported into the Miscrosft Excell (Microsoftcorp, Redmond, WA, USA) file using FTD Resp33 qPCR Analyser version 1.0 software (http://www.gematics.com/analyser.html accesed on the 26th August 2014) for further analysis.

3.8 Statistical analysis

Data will be analysed using Stata version 10.0 software (StataCorp, College Station, TX, USA). We will perform a univariate analysis to describe the socio-demographic and the incidence of respiration infection in the study population. A multiple logistic regression analysis will be performed to identify factors independently associated with SUDI. Mann-Whittney U test will be used to compare differences in continous variables between the SUDI cases and controls. Differences in risk factors exposure between the cases and controls will be compared using chi-square test or Fisher's exact

two-tailed statistics, where appropriate. Kappa statistics will be used to assess the agreement between the investigation methods. The kappa coeficient will be interpreted as proposed by Landis and Koch [60]: 0.81 - 1.00 = excellent agreement, 0.61 - 0.80 = good agreement, 0.41 - 0.60 = moderate agreement, 0.21 - 0.40 = fair agreement, 0 - 0.20 = poor agreement, and <0 = no agreement. For all statistical tests, a p-values of less than 0.05 will be considered significant.

4. Limitations of this project

No upper respiratory specimen will be used in this investigation due to the proliferation of indigenous microorganisms of the upper airways after death [61]. Therefore, the microorganism's colonisation at the time of post-mortem examination does not reflect the ante-mortem situation. In addition, intentional poisoning and metabolic diseases in SUDI are not in the scope of this study; hence, neither toxicology nor clinical chemistry screening will be examined.

5. Ethical considerations and data management

This research protocol will be submitted to the University of Cape Town Human Research Ethics committee for approval. According to the South African Inquests Act 58 of 1959, a medico-legal investigation of all unnatural deaths including SUDI cases is mandatory, hence written consent from the deceased family will not be required for this study. Only the inquest case number assigned to each SUDI will be revealed to the investigators. All post-mortem examination and ancillary investigation data will be kept securely at the Divisions of Forensic Medicine and Medical Microbiology, University of Cape Town, South Africa. This research will follow the routine investigation of SUDI cases at Salt River Forensic Pathology laboratory, Cape Town, South Africa. The findings of this research will not be communicated to the family of the deceased for the reason that the assay that will be used is not validated for diagnostic purpose.

6. Expected output, outcomes and impacts

This research is unique, since it will be able to screen thirty three pathogens. The case-control design will provide a valuable tool in the identification of possible SUDI aetiology regarding the possible disparity in pathogens colonisation in both groups.

In Cape Town particularly, this research has an epidemiological value of identifying respiratory pathogens and associated risk factors contributing to the high incidence of sudden unexpected infant death and could lead to effective approaches to SUDI prevention and control.

Table 1. Target pathogens in the FTD respiratory pathogens 33 multiplex real-time PCR assay

Multiplex no.	Primer/Probe mix	Target pathogen	Target gene sequences
1	FluAB-RH	Influenza A	Matrix gene (post1)
		Influenza B	Segment 8 NS1/NEP
		Rhinovirus	5'untranslated Region (UTR)
2	Para-EAV	Parainfluenza 3	Hemagglutinin-neuraminidase (HN) mRNA
		Parainfluenza 2	Hemagglutinin-neuraminidase (HN) mRNA
		Parainfluenza 4	Fusion protein gene
		Internal control*	
3	Cor	Coronavirus 229	Nucleocapsid protein (N) gene
		Coronavirus 63	Nucleocapsid protein (N) gene
		Coronavirus HKU1	Nucleocapsid protein (N) gene
		Coronavirus 43	Nucleocapsid protein (N) gene
4	BoMpPf1	Parainfluenza 1	Hemagglutinin-neuraminidase (HN) mRNA
		Human Metapneumoviruses A	Fusion glycoprotein (F) gene
		Human Metapneumoviruses B	Fusion glycoprotein (F) gene
		Bocavirus	NP1 gene
		Mycoplasma pneumoniae	Adhesin P1
5	RsEPAcmv	Respiratory syncytial viruses A	Nucleocapsid protein gene
		Respiratory syncytial viruses B	Nucleoprotein mRNA
		Cytomegalovirus	US7+US8 genes
		Enterovirus	Parts of domain IV and V
		Parechovirus	5'untranslated Region (UTR)
		Adenovirus	Hexon gene
6	Bac	*Staphylococcus aureus*	Sensor histidin kinase vick gene
		Chlamydia penumoniae	RNA polymerase beta chain gene
		Haemophilus influenza B	BexA gene
		Streptococcus pneumoniae	LytA gene
7	KLePSa	*Pneumocystis jiroveci*	(mtLSU)r RNA gene
		Legionella spp	16 S rRNA
		Klebsiella pneumoniae	Khe hemolysin gene
		Salmonella spp	Tetrathionate subunit B (ttrB)
8	MoBoCH	*Moraxella catarrhalis*	CopB gene
		Influenza C	Matrix gene
		Bordetella pertussis	IS481
		Haemophilus influenzae spp	ompP2 gene

No., number, *, Equine arteritis virus

7. Research budget

Table 2. Estimated budget for microbial investigation using the Fast-track diagnostic assay.

Item	Description	Amount (ZAR)
QIA-symphony® DSP Virus/Pathogen kit	Nucleic acids extraction kit	6 000
FTD respiratory pathogens33 kit + Fast track Master mix	Multiplex Real Time PCR for detection of respiratory pathogens	33 033.60
PrimeStore Molecular transport medium (MTM)	Molecular transport medium	6 500
Equipment and consumables	Will be provided by the division of Medical Microbiology	-
Total		**45 533.6**

8. Research timeline

Table3. Research timeline

Task	Start	End	Duration	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Planning, Proposal & Ethics	Apr	Jul	16 weeks									
Literature review	Jun	Aug	12 weeks									
Sample collection & analysis	Aug	Oct	12 weeks									
Results & Discussion writing	Oct	Nov	8weeks									
Article writing	Nov	Dec	8 weeks									
Submission of the project and article for publishing	Dec	Dec	4 weeks									

Apr, April; Aug, August; Dec, December; Jul, July; Jun, June; Nov, November; Oct, October; Sept, September

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Supplementary materials

Supplementary material 1. **Research questionnaire**

FORENSIC PATHOLOGY SERVICE

SUDI [Complete If A Baby Should Suddenly And Unexpectedly Die]

FPS laboratory_____ WC _____

Name of baby _____

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

Race:	Sex:

Section B

Person(s) at/called to the scene and relationship

Name/relationship	Date	Time
Name/relationship	Date	Time
Name/relationship	Date	Time
Police response/name	Date	Time
Paramedic response/name	Date	Time
When was the death certified/by whom	Date	Time
If the baby was taken to hospital		
Name of hospital		

Date of arrival:	Time of arrival:
Name of doctor seen / declared death:	
Comment: Get copies of doctors notes	
Was resuscitation done on the baby by the paramedic or the doctors at the hospital?	

Section C

Household environment:

Place where baby lives:	house shack other –

Number of bedrooms Is the room in which the baby is found well ventilated?		
Odour(s) present in the room the baby slept in?	Yes	No
Peeling paint in the room the baby slept in?	Yes	No
Fungal growth (mould) in the room the baby slept in?	Yes	No
Did people smoke cigarettes in the room the baby slept?	Yes	No
Are there pets in the house? If yes – type and number:	Yes	No
Did caregiver use alcohol or drugs on the night baby died?	Yes	No
Was there a heater or open fire or galley blik or other heating device in room where baby slept?	Yes	No
In what position was the baby found lying? Has the baby been moved? Were there any covers/ clothing etc. over the baby's head? Was the baby squashed/wedged between anything (object)?	Yes	No
Was there overlaying (someone lay on top of the baby)?	Yes	No
Comments from forensic officer who attended the scene:		

Section D Circumstances of death / details about events before death

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

7. Did the baby sustain any injuries – eg by falling or being hit: If yes:	Yes	No
a) When did it happen?		
b) How did it happen?		
c) Where did it happen?		
d) What did the caretaker do about it?		

8. a) On what was the baby placed to sleep	Bed with a pillow	Bed without a pillow	Couch with a pillow	Couch without pillow	Cot with pillow
	Cot without pillow	Floor with pillow	Floor without pillow	Other	
b) If placed on a bed/cot, what was the mattress type			Foam rubber	Inner spring	Other
c) Was the mattress covered with a blanket or sheet				Yes	No
d) What position was the baby placed when put to sleep?		Back	Stomach	Side	Other
Other -					
e) what was used to cover the baby: List items					
e) What position was the baby found dead?		Back	Stomach	Side	Other
Other –					

f) Has the baby been moved?		Yes	No
g) Face position when the baby was found dead	To the left	To the right	Face down
	Face up	Unknown	
h) Face and or chest squashed / wedged between any object(s) when the baby was found dead?	Yes	No	Unknown
If yes – details please –			
i) Was the nose and mouth of the baby covered by anything – eg blankets or anything else	Yes	No	Unknown
j) Were there other items in contact with the baby – eg pillow	Yes	No	Unknown
k) Did the baby use a Dummy (pacifier)?		Yes	No
l) Did the baby sleep in the same bed as the mother?		Yes	No
m) Did the baby sleep in her arms?		Yes	No
n) Did the baby sleep on her chest?		Yes	No
o) Did the baby sleep with the mother on a couch?		Yes	No
p) How many other people slept on the same bed as the baby at the time the baby died?			
q) Was anyone found on top of the baby while in the bed (Overlaying)?		Yes	No
r) Was the window where the baby slept on the day /night the baby died		Open	Closed
s) Did the mother or anyone in the house smoke while the baby slept on the night/day of death?			

t) When was the baby last fed?	Date	Time

u) Did the mother/caregiver use alcohol before going to bed with the baby on the night/day the baby was found dead? If yes, how much?	Yes	No
v) Did the mother/caregiver use drugs before going to bed with the baby on the night/day the baby was found dead? If yes, what drugs?	Yes	No
w) Did the mother/caregiver give the baby medication on the night/day of death? If yes, name of medication:	Yes	No

Section E About the baby

1. Where was the baby born?	Hospital	Clinic	Home	Other
Name of hospital/clinic/other				
2. How was the baby born?			Normal vaginal delivery	Caesarian section
3. How much did the baby weigh at birth?				
4. Was the baby		Premature	Full term	Postdates (Overdue)
5. If the baby was premature, how premature was it?				
6. Did the baby receive Kangaroo care (KMC)			Yes	No

7. Did the mother carry the baby on her back?			Yes	No
8. Was the baby	Breast fed		Bottle/formula fed	Both breast and bottle fed
If formula, name of the milk –				
9. Was boiling water used to make the bottle?			Yes	No
10. What other food was use to feed the baby?				
11. Does the mother have the clinic card?			Yes	No
If yes – keep the card for the pathologist. If no – ask the mother to bring it to the facility				
12. Was the baby sick before it died?			Yes	No
If yes	<24h	>24h	> 2 weeks	Never
a) Did the baby have a cold/ runny nose?				
b) Was the baby coughing?				
c) Did the baby have diarrhea (runny tummy)?				
d) Was the baby unusually restless / irritable?				
e) Was the baby crying more than usual?				
f) Was there a difference /change in the appetite / feeding?				
g) Was the baby vomiting?				

h) Any fits / seizures?				
i) Did the baby have a fever / showed increased sweating?				
j) Was the baby listless? (floppy)				
k) Did the baby turn blue?				
13. Was the now deceased baby taken to	Hospital	clinic	doctor	Pharmacy
	Traditional healer	Other		
14. Did the baby come in contact with someone who is sick in the past two weeks?			Yes	No
If yes – who?				
15. Did the baby ever suddenly stopped breathing?		Yes	No	Unknown
16. When was the baby's last vaccination?				
18. Is the baby known to be allergic to anything?		Yes	No	Unknown
If yes, what?				
19. Did the family visit another country prior to the death of the baby?			Yes	No
If yes, give details				
20. Was the baby admitted to hospital in the past week before the death?			Yes	No

a) If yes, for how long and where:		
b) Why?		
c) Discharge date?		
d) Condition of baby after discharge:		
e) Medication after discharge from the hospital (names please)		
21. Was the baby taken to a traditional healer?	Yes	No
a) If yes, date when the baby was taken to the healer:		

b) What was given?
c) Ask for the medication to be given to the pathologist.
d) Condition of the baby after going to the healer?
21. What did the baby wear when it died? (list clothing)

Section F About the mother		
1. Is the mother	Married	Single
2. Is the mother employed?	Yes	No
3. Age of the mother?		
4. What standard of schooling did she achieve?		
5. Was she on contraception before she fell pregnant?	Yes	No
6. Did she take iron and vitamin tablets during her pregnancy?	Yes	No
7. Did she receive antenatal care?	Yes	No
8. Did the mother have diabetes in pregnancy?	Yes	No
9. Did the mother have high blood pressure in pregnancy?	Yes	No
10. Did the mother gain weight adequately in pregnancy?	Yes	No
11. Was she diagnosed with any illness during the pregnancy eg. HIV?	Yes	No
12. Was the mother on any medication during the pregnancy?	Yes	No
If yes, what medication:		

13. Were there any difficulties during the delivery?	Yes	No
If yes, what?		
14. Were there any problems with the baby after the delivery?	Yes	No
If yes, what?		

15. Was any specific instruction given about specific health care for the baby?	Yes	No
If yes, what?		
16. Was she depressed after the pregnancy?	Yes	No
17. Did she get any treatment?	Yes	No
18. How many babyren does she have?		
19. How old are they?		
20. Are they healthy?	Yes	No
21. Do any of the babyren have learning disability?	Yes	No

22. Do the living babyren have the same father as the deceased baby?				Yes	No
23. Does she look after the baby?				Yes	No
24. If not, who looks after the baby?					
25. Why is the mother unable to look after the baby?					
26. Did the mother smoke during the pregnancy?				Yes	No
If yes, how many per day?					
27. Did the mother drink during the pregnancy?				Yes	No
a) What did she drink?	Beer		Wine	Spirits	Other
b) How much did she drink?			Every day	Now and again	Weekends
1 glass			Every day	Now and again	Weekends
> 1 glass			Every day	Now and again	Weekends
A bottle of alcohol			Every day	Now and again	Weekends
> 1 bottle			Every day	Now and again	Weekends
28. Does she use drugs?				Yes	No
a) If yes, what drugs does she use?	Tik	Cocaine	Heroin	Mandrax	Other
b) How often does she use drugs?			Every day	Now and again	Weekends

29. Does the mother smoke after the pregnancy?		Yes	No
30. Does the mother know that smoking harms the unborn baby?		Yes	No
31. Does the husband/partner drinks?		Yes	No

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

Section G

Household environment

1. Place where the baby lives	House	Shack	Other

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

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

ITEMS RETAINED AT THE SCENE OR FROM THE MOTHER DURING INTERVIEW

Date:

Signature / Thumbprint of deponent

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

Date _____ Time: _____

Place: _____

_____

Department of Health

Forensic Pathology Laboratory

Supplementary material 2. **Research participant consent form**

Consent Form

The contribution of respiratory pathogens to Sudden Unexpected Deaths in Infancy

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I have been informed of and understand the purposes of this research, possible benefits and risks.

- □ YES
- □ NO

I have been given opportunities to ask questions

- □ YES
- □ NO

I understand, I can withdraw my permission to use samples from my deceased infant for research purposes at any time without prejudice

- □ YES
- □ NO

I understand that the researches are under an obligation of respect to medical confidentiality; therefore any information which might potentially identify the deceased or any other family member will not be used in published material

- □ YES
- □ NO

I give permission for samples to be collected from my diseased infant during autopsy and used in research to investigate the contribution of respiratory infections in sudden unexpected death in infancy and etiological agents involved.

- □ YES
- □ NO

---------- ---------- ----------

Name of Person Authorizing Consent for collection and use of samples for research purposes. Signature Date

---------- ---------- ----------

Name of Person Obtaining Consent for collection Signature Date

and use of samples for research purposes.

Supplementary material 3. Research study Information sheet

Participant Information Sheet

The contribution of respiratory pathogens to Sudden Unexpected Deaths in Infancy

Good day,

My name is Elyse Sandrine Ishimirwe, and I am currently studying for a Masters degree in Biomedical Forensic Science at the University of Cape Town.

I would like to invite you to take part in a research study. Before you decide, you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully.

Aim of the research

Sudden Unexpected Death in Infancy (SUDI) is the death of infants before their first birthday that occur suddenly and unexpectedly by medical history. The cause of these tragic infant deaths is still unknown. Infections, especially of the respiratory system have been found to have a high implication. However, the number of investigated pathogen is still low which could underestimate the role of these infections. This study aims to identify the pathogens involved in these deaths and to determine the role of microbiological investigation in the final diagnosis.

Procedure

You are being asked to allow the following samples to be taken from your beloved one: 10 ml of pericardial fluid, 5 mg of lung biopsy, 10 ml of cerebrospinal fluid and 10 ml of blood sample from peripheral vessels. These will be collected using minimally invasive techniques during the post-mortem examination procedure. An identification number will be assigned to individual's samples. All samples will be transported to the laboratory of Medical Microbiology Division at the University of Cape Town where they will be stored and further analysed. After research, the samples collected will be discarded of appropriately, and within 10 year-period. All personal information collected during this research will be treated as anonymous and confidential, and will be only used in the context of this research.

Risks

They are no risks to the deceased or the family member consenting to the use of the above named samples for research.

Benefits

I cannot promise the study will help you but the information we get from the study will help to increase the understanding of the implication of respiratory infections in sudden unexpected infant deaths, and possibly contribute to the improvement of diagnostic methods.

Costs/compensation

There will be no charge for participating in this research and also no payment for allowing samples to be collected. No samples will be used for any purpose other than the aim of this study.

Please note that:

1. Your participation is entirely voluntary and you may withdraw form this research at any time and for any reason, without having to give an explanation.
2. Your participation or non-participation will not affect the normal process of post-mortem examination.
3. The data collected will be treated securely and confidentially and stored as required by the University. All investigators involved in this study have signed a confidentiality agreement.
4. Results of this study will not be communicated to you, and, if published, it will be treated in an anonymous manner. No one will be identifiable in any data produced from this study.
5. If you have a concern about any aspect of this study, you can contact Dr. Marise Heyns (Tel.: 021 406 66 04; E-mail: marise.heyns@uct.ac.za) or Ms Elyse Sandrine Ishimirwe (Tel.: 021 406 62 24; E-mail: ishely001@myuct.ac.za) who will do their best to answer any questions.
6. If you have any questions or concern about the safety, right and welfare of participants in human research study, please contact Prof. Marc Blockman, the Chairperson of the Faculty of Health Sciences Human Research Ethics Committee (HREC), on 021 406 63 38.

Supplementary material 4. Research Confidentiality agreement form

Confidentiality agreement

The contribution of respiratory pathogens in Sudden Unexpected Death in Infancy

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I, the undersigned, acknowledge, understand and agree to adhere to the following conditions:

- I will maintain the privacy and confidentiality of all accessible project data and understand that unauthorized disclosure of personal/confidential data is an invasion of privacy and may result in disciplinary, civil, and/or criminal actions against me.

- I will only discuss personal data or confidential information obtained in this research with the researches.

- I will use the data collected only for the purposes for which I am authorized explicitly. On no occasion will allow third parties to access any personal and confidential information.

- I will comply at all times with the practice's data security policies and confidentiality code of conduct.

- I will delete all files containing personal and confidential information from my computer after the completion of the research.

Name of the researcher: ----------

Signature: ----------

Date: ----------

Supplementary material 5. Sample collection form

STORE AT - 80°C

SUDI CASE

INFANT CASE NUMBER: ----------

SAMPLE COLLECTED BY: ----------

FORM COMPLETED BY: ----------

Samples collected		**Time collected**	**Sent to Med Micro Lab**	
a. CSF	☐ Yes ☐ No	_ __h__ __min	☐ Yes ☐ No	__ __h__ __min
b. PCF	☐ Yes ☐ No	__ __h__ __min	☐ Yes ☐ No	__ __h__ __min
c. L-PNLB	☐ Yes ☐ No	__ __h__ __min	☐ Yes ☐ No	__ __h__ __min
d. R-PNLB	☐ Yes ☐ No	__ __h__ __min	☐ Yes ☐ No	__ __h__ __min
d. PB	☐ Yes ☐ No	__ __h__ __min	☐ Yes ☐ No	__ __h__ __min

CSF: Cerebrospinal fluid

L-PNLB: left lung biopsy

PCF: Pericardial fluid

PNLB: Right lung biopsy

Supplementary material 6. **Human research ethics approval letter**

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • Facsimile [021] 406 6411
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

28 November 2014

HREC REF: 653/2014

Dr M Kaba
Public Health & Family Medicine
Falmouth Building
Room 5.26

Dear Dr Kaba

PROJECT TITLE: THE CONTRIBUTION OF RESPIRATORY PATHOGENS TO SUDDEN UNEXPECTED INFANTS DEATH (MPhil-candidate- ES Ishmirwe)

Thank you for your letter responding to the issues raised by the Faculty of Health Sciences Human Research Ethics Committee dated 20th November 2014

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th November 2015.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/research/humanethics/forms)

We acknowledge that the MPhil student Ms E Ishmirwe is also involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC reference no in all your correspondence.

Yours sincerely

Signed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN ETHICS
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

Title page

Respiratory infections and sudden unexpected death in infancy: An update

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Abstract

Sudden unexpected death in infancy (SUDI) is among the most frequent cause of infant mortality worldwide. Respiratory pathogens and bacterial toxins secreted in the upper respiratory tract are considered as the most frequent causal agents involved in these infants' deaths. This review explored the epidemiology and diagnosis of respiratory pathogens in SUDI and the implication of associated risk factors. Some risk factors associated with increased susceptibility to respiratory infection are similar to risk factors associated with increased incidence of SUDI cases. These shared risk factors are associated with increased colonisation by potential pathogens or increased bacterial virulence in the respiratory tract, or imbalance in immunological responses to respiratory infections. Despite the significant role of post-mortem microbiology in the investigation of the aetiological role of various respiratory pathogens involved in SUDI, the diagnosis of respiratory infection in SUDI encounters various drawbacks associated with post-mortem microbiology and also with the lack of standardized protocol for selection of appropriate samples, microbiological diagnosis to be undertaken and the interpretation of the results.

Keywords: sudden unexpected death in infancy, sudden infant death syndrome, respiratory infections, SUDI diagnosis, post-mortem microbiology, SUDI risk factors.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as a non-violent unexpected death of infants between one week and one year of age that is assessed by the absence of clinical history of diseases [1, 2]. Following in depth medico-legal investigations, only a quarter of SUDI cases is explained, thus referred as "explained SUDI" [1, 3], while the larger proportion (≥75%) remain uncertain, therefore classified as "unexplained SUDI", also known as "Sudden Infant Death Syndrome" (SIDS) [1, 3, 4].

The diagnosis of sudden infant deaths is solely exclusive, therefore the cause of death is considered to be multi-factorial rather than being a result of a single cause [2, 5-7], supporting the "triple risk hypothesis" mechanism of death proposed by Morris et al. in 1987 [8, 9]. Epidemiological studies identified a broad range of risk factors (Table 1), including intrinsic factors, originated from the development of an infant [1], and extrinsic factors, related to the environment [10].

Table 1. Risk factors associated with sudden unexpected death in infancy

Intrinsic factors	References	Extrinsic factors	References
Age (2-4 months)*	10-12	Prone sleeping*	13, 14
Prematurity	15	Heavy wrapping	16
Low birth weight	9, 15, 17	Seasonality	18, 19
Small for gestational age	17	Bacterial toxins	20, 21
Genetic disorders*	8, 22-24	Viral Infections*	25, 26
Intra uterine growth restriction	2, 27	Breastfeeding*	28-30
Male gender*	8, 12	Bed sharing/ co-sleeping*	13, 31, 32
Ethnicity	9, 19, 24, 33	Cigarette smoke exposure*	6, 34-37
Inflammatory responses*	23, 38	Low socio-economic status	9, 24, 33
		High parity	2, 27

*, shared risk factors between respiratory infection and Sudden Unexpected Death in Infancy (SUDI)

A number of studies have previously proposed respiratory pathogens as potential cause of death or acting as comorbidity factors by increasing the susceptibility to SUDI death [19, 39, 40]. A high number of risk factors associated with SUDI are shared with risks of increased bacterial colonisation and occurrence of respiratory tract infections in infants and young children [5, 37, 41]. Moreover, the seasonal patterns of most viral pathogens including Respiratory syncytial virus (RSV) [42, 43], Influenza virus [42, 43] and Human Metapneumovirus [43] exhibits a similar seasonality with incidence of cases of SUDI [12, 39, 44], suggesting a high implication of pathogens colonizing the respiratory system in the pathogenesis of SUDI.

Investigation of infectious causes of SUDI have been the focus of forensic microbiology in the past years; however it presents with several drawbacks related to post-mortem body changes and the environment from which post-mortem examinations take place [45-47].

This review discussed the epidemiology of respiratory pathogens in SUDI and the role of specific risk factors associated with increased susceptibility to respiratory infections. It also addresses the relevance of post-mortem microbiological investigation in the diagnosis of respiratory infections in SUDI.

2. Burden of respiratory infections in sudden unexpected death in infancy

Respiratory infections constitute a global health threat accounting for the highest mortality and morbidity in children under the age of five years [48, 49]. In relation to the triple risk hypothesis, respiratory infections are considered to be the main extrinsic triggering agents in SUDI death [2, 19, 34, 50]. An investigation of a SUDI outbreak that occurred in Iowa (US) during winter 2008, revealed that 63% of the cases was associated with respiratory infection [51]. In a retrospective study on SUDI cases in United Kingdom, of the 60% of explained SUDI, 20% were attributed to respiratory infection [27]. In South Africa, about 26% of SUDI are attributed to respiratory infection regardless of the considerable disparity in the ancillary investigation within the country [50].

Furthermore, signs of inflammation in the respiratory tract are frequent findings in SUDI cases [2, 52, 53]. An autopsy study on SUDI conducted in Germany evidenced 60% signs of the upper respiratory tract infections [34]. However, a limited number of respiratory pathogens that might be involved in these fatal infections have been investigated. Table 2 includes respiratory pathogens that have been detected in SUDI cases.

2.1 Bacterial pathogens

Bacteria are the most important causes of SUDI (Table 2) [1, 54, 55]. About 80% of infectious SUDI cases are linked to bacteria [6, 27]. The role of bacterial infection was investigated in 546 SUDI cases in South England (UK) [56]. Of these, 39 cases were excluded due to viral or pneumocystis infections. Among the remaining 507 SUDI cases included in the final analysis, 72 cases were due to bacterial infection with the high prevalence of *Staphylococcus aureus* (16%) [56]. In Norway, a microbiological investigation of 305 SUDI cases was conducted using microbial culture technique. Thirty eight cases were due to infections. Of 17 cases of pneumonia, 13 had a bacterial aetiology [55]. Moreover, histopathological assessment of organs shows signs of bacterial infection in SUDI cases compared to non-SUDI infant deaths [6]. Furthermore, there is a correlation between epidemics of bacterial infection and incidence of unexplained SUDI. For instance, epidemics of *Bordetella pertussis* infection corresponds with the period of increased incidence of unexplained SUDI [57].

The patterns of nasopharyngeal bacterial carriage differ in SUDI and non-SUDI cases. Results from bacterial culture of nasopharyngeal specimens indicate a higher nasopharyngeal colonisation with *S. aureus* in SUDI infants than their age matched healthy controls, especially in the first three months of life [21]. In a study conducted in Australia, 52 SUDI cases and 102 controls were investigated. *S. aureus* was found in 67% of SUDI cases and in 42% controls [40]. This was also evidenced in a case-control study where 46 SIDS cases and 46 age-matched healthy infant controls were investigated for nasopharyngeal bacteria: about 41.3 % SIDS cases carried *S. aureus* compared to 28.3% healthy infants [58]. A significant difference ($P < 0.0001$) in *Streptococci* carriage was observed 78.3% in SIDS and 32.6% in controls [58]. However, the difference in nasopharyngeal and tracheal bacterial colonisation is not always significant as evidenced in a case-control study where about 5.1% of SIDS cases are colonised with *B. pertussis* compared to 5.3% in controls [59]. Hence, the disparity in nasopharyngeal bacterial colonisation between SUDI cases and healthy infants requires further investigation to determine concomitant factors that may be involved.

2.2 Bacterial toxins

Other than inducing infections, some bacteria isolated from SUDI cases are known to produce toxins which can be lethal [20, 60]. A significantly higher prevalence of toxigenic strains of *S. aureus* was found in both unexplained and infectious SUDI deaths than in SUDI deaths due to non-infectious causes [61]. About 78% of *S. aureus* isolated from SUDI cases are toxins producers [62]. Furthermore, *B. pertussis* [57, 59] and *Haemophilus influenza* [58, 63] also produces endotoxins which are involved in pathogenesis of their respective respiratory infections. However the role of both and *H. influenzae and B. pertussis* toxins in the pathogenesis of SUDI is poorly studied.

An important feature in the lethality of bacterial toxins in SUDI deaths is their interaction with viral infections and sleeping conditions such as prone sleeping and overheating. Blackwell *et al.* 2015 proposed that mild viral infections enhance the nasopharyngeal colonisation of toxigenic staphylococcal strains [41]. Furthermore, it was suggested that the toxicity of bacterial toxins is potentiated by increased body temperature in case of mild viral infections and prone sleeping [41, 64, 65]. It is also noteworthy to mention that bacterial toxins isolated from the infant's nasopharyngeal have the ability to potentiate the toxigenicity of one another [66].

The well-known hypothesis that explains the role of bacterial toxins in the occurrence of SUDI is called "common bacterial hypothesis", proposed for the first time by Morris and colleagues in 1987 [26]. According to the "common bacterial hypothesis", SUDI deaths are induced by bacteria producing toxins that inhabits the upper respiratory tract [67]. However, a number of toxigenic bacteria that colonise mainly the gastro-intestinal tract have been also associated with SUDI [5, 68]. Therefore, nasopharyngeal carriage of toxigenic bacteria should be considered as a potential cause of death in the diagnosis of SUDI cases.

2.3 Viral pathogens

Respiratory viral pathogens have been implicated in the occurrence of SUDI [2, 5, 69]. It has been shown that infants with a mild viral infection are at higher risk of SUDI [26]. The incidence of SUDI exhibits a close correlation with the increased prevalence of viral pathogens of the airways, such as respiratory syncytial viruses (RSV) and influenza viruses [1, 2, 12].

A number of viruses have been associated with SUDI, mainly RSV, influenza viruses, herpes simplex virus (HSV), cytomegalovirus (CMV) and adenovirus (AdV) [2, 11, 19, 51, 70]. Using Immunofluorescence and cell culture techniques, 18 cases of SUDI attributed to viral infection were identified in post-mortem lung tissue samples from 490 SUDI cases in a study conducted in UK [2]. The detected viruses in the latter study included: enterovirus (n= 4), RSV (n= 4), HSV (n= 3), CMV (n= 3), AdV (n= 1), Influenza virus (n= 1) and human immunodeficiency virus (n= 1) [2]. A virological surveillance of lung tissues conducted as part of medico-legal investigation of SUDI cases in Western Cape, South Africa, demonstrated a high burden of CMV (29/82,35%) colonisation compared to the low detection rate of AdV (2/82, 2,44%) and RSV (4/82,4.88%) [19]. This low detection rate of AdV and RSV is in contrast with the high incidence rate of both viruses in cases of SUDI worldwide. CMV was detected using all the three detection methods under study, including shell vial culture, real-time PCR and immunohistochemistry. Yet, only real-time PCR detected positive cases of AdV and only immunohistochemistry yield positive results for RSV [19]. The disparity in virological results from different diagnostic techniques evidenced in the study of Burger *et al.*[19] demonstrated also the impact of viral detection methods in the diagnosis of SUDI.

Besides the detection of single viruses, it has been shown that SUDI can be also a result of multiple infections [51, 70]. Following PCR-based virological investigation of a SUDI case, a CMV and Varicella-Zoster virus co-infection was detected in heart, lung, small intestine and colonic samples. The highest CMV load was observed in the lungs [70]. Additionally, a RSV and influenza virus co-infection was also detected in nasopharyngeal specimens from a SUDI case [51].

The incidence of respiratory viruses from case-control studies present disparity results [71, 72]. Formalin fixed tissues from 11 SUDI cases and nine controls were analysed for the detection of human herpesvirus-6 (HHV-6), Epsein-Barr virus (EBV) and CMV using real-time PCR. The incidence of CMV, EBV and HHV-6 combined was 72.2% in SIDS infants and 22,2% in previously healthy infants died of non-infectious causes [72]. The viral load for CMV was significantly higher in SIDS infants (45.3 DNA copies/µg) than in controls (20.5 DNA copies/µg) [72]. However, a non-significant difference (p= 0.18) was observed between the prevalence of both AdV and Rotavirus in SUDI (30%) and age-matched controls (20%) [71]. Hence, as respiratory viruses also occur in healthy infants, their role in the pathogenesis of SUDI either as cause of death or contributing factors requires further investigation.

2.4 Fungal pathogens

Pneumocytis jirovecii colonisation is a common finding in children dying suddenly and unexpectedly[73, 74] and the age peak of *Pneumocytis* infection ranging from three to four months is similar to the SUDI critical age period, which ranges from two to four months of life [11, 12]. *P. jirovecii* was detected in 82% of sudden unexpected deaths [73]. However, this organism has been also found in apparently healthy infants of less than one year of age [75], and its role in SUDI deaths is yet to be defined.

3. Risk factors associated with respiratory pathogens in sudden unexpected death in infancy

The recent work by Blackwell *et al.* summarized in details risk factors of SUDI and their role in inflammatory responses to infection [76]. A number of risk factors of SUDI are associated with increased susceptibility to respiratory infections (Table 1) [76]. Following epidemiological surveys, evidence of pathological signs of infection in the respiratory tract or detection of respiratory pathogens have been found to be associated with some genetic, immunological and environmental factors [23, 38, 76].

3.1 Critical developmental period

A decrease in maternal antibody is observed during the critical development period ranging from two to four months of life [10, 11]. During this critical period, infant's immunity is at a developmental stage [11]. Moreover, the immunization process supposed to assist the infant's immunity and therefore reduce risks of SUDI is yet to be completed [77-80]. This immunological imbalance observed during the critical developmental period renders infants more susceptible to new infections.

3.2 Inflammatory responses

The immunological imbalance associated with cytokines levels increase the vulnerability of infants to both infections and unexplained SUDI.[11, 23, 38]. In cases of CMV infection, which is often detected in SUDI cases [2, 19, 70, 72, 81], infants showed a significant reduced level of interferon γ, interleukin II and CD154 production and differentiation [82]. In a mice model of respiratory infection, a late development of CD8+ T cell responses was observed in cases of HSV infection [83], which is also a common virus associated with SUDI [2, 72, 81, 84].

The level of Interleukin (IL) 10, the cytokine involved in protection against staphylococcal enterotoxin induced shock [85] has shared factors with SIDS including cigarette smoking, male gender and ethnicity [5]. Furthermore, IL polymorphisms have been found to influence the effects of cigarette

smoke exposure in infants [5] which indicates that the levels of specific cytokines may constitute a contributing factor to SUDI.

A further support for the theory of inflammatory responses in SUDI cases is the evidence of histological signs of inflammation in respiratory organs [44, 86-88]. A common histological sign in SUDI is intrathoracic petechial haemorrhage suggested to be a result of infant's efforts to overcome hypoxia and respiratory difficulties [44, 89]. About 55% of explained SUDI and 90% of SIDS presents petechial haemorrhages at least in one of intra-thoracic organ [27]. More, inflammatory changes in the laryngeal epithelium have been significantly associated with SIDS suggesting that some SIDS deaths involve preceding inflammation in the respiratory tract [88]. However, since some inflammatory signs are not restricted to respiratory infection in SUDI, further studies on the inflammation of respiratory origin and SUDI is important. The interaction between imbalance in the immunological responses to respiratory infection, histopathological signs of inflammation in the respiratory organs and other environmental risk factors for SIDS may indicate that respiratory infections play a major role in the pathogenesis of SUDI.

3.3 Genetic disorders

Genetic alterations and their subsequent pathogenic role constitute an innate risk factor to unexplained SUDI [8, 22]. A number of genes have been implicated, mainly genes involved in the respiratory regulation or those that have a physiological correlation, causing inherited diseases [8, 34]. The deletion of either complement C4A or C4B genes increases the vulnerability to unexplained SUDI in infants with mild respiratory infections [23, 24]. Gene polymorphisms determine the effectiveness of cytokine responses [23]. IL 10 polymorphisms have been associated with both severity of respiratory infections [90] and unexplained SUDI [91, 92]. The effects of IL 10 polymorphism are more prominent in cases of maternal smoking both during pregnancy and breastfeeding [23].

3.4 Sex

An increased incidence of SUDI is observed in male infants compared to females [8, 15, 38]. During the first six months of life, the levels of testosterone increases in male infants compared to females. This period of increased testosterone levels in male infants corresponds to the period of high incidence of SIDS [12, 93]. Moreover, a positive correlation was observed between testosterone levels and pro-inflammatory responses to lipopolysaccharides in cells previously treated with interferon-Y [12]. Hence, the rise in testosterone levels in male infants may alter immunological responses to respiratory viral infections in male infants.

Table 2. Respiratory pathogens associated with sudden unexpected death in infancy

Microorganisms	Sample types	Detection methods	References
Adenovirus	Lungs	RT-PCR	19
	NPS	NA	51
	Lungs, bronchi and faeces	Culture and PCR	84
	Lungs	PCR	2
	NS and URT aspiration	IF	44
	Lungs	Culture	81
Cytomegalovirus	Lungs	SVC, RT-PCR, IHC	19
	Kidneys and lungs	IHC	70
	All tissues	RT- PCR	70
	Lungs	PCR/DEAFF/ Culture	2
	Lungs, brain, kidney and spleen	RT-PCR	72
	Sera, CSF, tissue samples and body fluids	Complement fixation	81
Influenza virus	NPS	NA	51
	Lungs	IF	2
	Sera, CSF, tissue samples and body fluids	Compliment fixation	81
Parainfluenza virus	Lungs, bronchi and faeces	Culture and PCR	84
	Lungs, bronchi and faeces	Culture and PCR	84
	NS and URT aspiration	IF	44
Respiratory Syncitial Virus	Lungs	IHC	19
	NPS	NA	51
	Lungs, bronchi and faeces	Culture and PCR	84
	Lungs	IF	2
	NS and URT aspiration	IF	44
	All tissues	RT-PCR	70
Herpes simplex virus	Lungs, bronchi and faeces	Culture and PCR	84
	Lungs	PCR	2
	Spleen	Culture	2
	PE lungs, brain, kidney and spleen	RT-PCR	72
	Pharyngeal aspirate and nasal washing	Nested PCR	81

Table 3. Respiratory pathogens associated with sudden unexpected death in infancy (continue)

Microorganisms	Sample types	Detection methods	References
Bordetella pertussis	NPS and TS	Conventional PCR	94
	FFPE tranchea, larynx, bronchi and lungs	IHC	94
Haemaphilus influenzae	NPS	NA	51
	Heart blood, CSF, NPA, swabs from relevant tissues	Microscopy, Culture and PCR	84
	Heart blood, spleen, CSF and lungs	Culture	54
	Heart blood, lungs and kidney	Culture	55
	Blood, liver, lungs, heart, spleen, kidneys, meninges, choroid plexus and bladder	Culture	44
Mycobacterium tuberculosis	Lung and nodes	ZN staining	95
Staphylococcus aureus	NPS	NA	51
	PM samples	Culture	
	Heart blood, CSF, NPA, swabs from relevant tissues	Microscopy, Culture and PCR	84
	Heart blood, CSF and lungs	Culture	54
	Heart blood	Culture	
	Blood, liver, lungs, heart, spleen, kidney, meninges, choroid plexus and bladder	Culture	55
Streptococcus pneumoniae	NPS	NA	51
	Heart blood, CSF, NPA, swabs from relevant tissues	Microscopy, Culture, PCR	84
	Heart blood, spleen, CSF and lungs	Culture	54
	Heart blood	Culture	55
Klebsiella pneumoniae	Heart blood, CSF, lungs, liver, kidneys and spleen	Culture	55
Pneumocytis jiroveci	Lungs	Nested PCR	73
	Lungs	Microscopy	75
	PE lungs	NA	74

CSF: cerebrospinal fluid, DEAFF: detection of early antigen fluorescent foci for CMV, EM: electron microscopy, FFPE: formalin –fixed, paraffin-embedded, IF: immunofluorescence, IHC: immunohistochemistry, NA: not specified, NPA: nasopharyngeal aspirates, NPS: nasopharyngeal swabs, NS: Nasal swabs, PCR: polymerase chain reaction, PM: post-mortem PE: paraffin-embedded, RT-PCR: real-time polymerase chain reaction, SVC: shell vial culture, TS: tracheal swabs, URT: upper respiratory tract, ZN: Ziel-Neelsen.

3.5 Non-safe Sleeping behaviour

Prone sleeping is the most recognised SUDI risk factor [13, 14, 96]. It is suggested that unexplained SUDI results from heat responses especially in the first six months of life [97]. In case of prone sleeping and

mild viral infection, the rise in body temperature leads to increased bacterial colonisation and enhances bacterial toxin production in the upper respiratory tract [6, 98]. The nasopharyngeal colonisation with *S. aureus* is considerably higher in infants who are put down to sleep in a prone position [98]. Non-recommended sleeping behaviour such as prone sleeping, overheating, bed sharing predispose infants to thermal stress and potentiate bacterial toxins production in the upper respiratory tract [10, 13].

3.6 Lack of Breastfeeding

Breast milk, as a core component in the development of infant's immune defence mechanisms, plays an important role in both protection against viral respiratory infections [30, 99] and SUDI [28]. A lower incidence rate of SUDI has been reported in breastfed infants compared to their counterparts [28].

The role of breastfeeding in reducing the severity of respiratory infections is linked to the effect of breast milk in cytokines production [29]. Melendi *et al.* reported high levels of Interferon type 1 associated with breastfeeding in case of viral infections frequently reported in SUDI cases such as Influenza virus infection [29]. At the same time, secretory IgA maternal antibodies and other factors including bactericidal lactoferrin that infants receive from breast milk protects the mucosal membranes infants, therefore protects breastfed infants against respiratory infections [100].

3.7 Exposure to cigarette smoke

Parental smoking highly increases the risk of unexplained SUDI [6, 34]. Prenatal smoke exposure alter the normal development of lungs leading to the impairment of respiratory functions [6, 34]. Moreover, postnatal parental smoking was found to be an important environmental risk factor for SIDS [101].

Adult smokers were found to be more densely and more frequently colonised by bacteria causing respiratory infections including *Streptococcus pneumoniae, H. influenzae, Moraxella catarrhalis* [36] hence infants exposed to cigarette smoking parents are more exposed to infectious agents from their highly and frequently bacterial colonised parents. Other than that, Heininger *et al*. demonstrated that nicotine predisposes infants to respiratory infections [59]. The study by Sayers *et al.* showed that in chick embryos, very low concentrations of nicotine enhances the toxigenicity of bacterial toxins associated with SIDS [35].

3.8 Viral infections

In general, viral infections alter the normal microbial flora which lead to bacterial overgrowth and toxin production [26]. Viral infections increase also body temperature which leads to toxin production [76]. In mice infants, epithelial cells infected with RSV showed a greater susceptibility to *S. aureus* invasion compared to non-infected cells [25]. Further, Zhang *et al.* demonstrated that influenza A infection potentiate staphylococcal enterotoxin B [102]. The administration of staphylococcal enterotoxin

B in transgenic mice infected with a sub-lethal dose of influenza A, resulted in death in all mice [102]. Yet, *S. aureus* is considered as the main bacterial cause of respiratory infection in SUDI [54, 55, 84].

4. Microbiological investigation of respiratory infections in sudden unexpected death in infancy

An aetiological diagnosis of respiratory infection in SUDI cases is based on post-mortem microbiological investigation. However, challenges around the value and procedures in post-mortem microbiology render the interpretation of results less easy.

4.1 Post-mortem microbiology

Post-mortem microbiological investigation aims to determine microbial colonisation prior death [61, 84]. Morentin *et al.* describes the importance of post-mortem microbiology in sudden unexpected death as a valuable tool in the identification of emerging infections [45]. Post-mortem microbiology not only has an epidemiological value but, it also has a huge importance in medico-legal investigations [52, 55, 103].

Post-mortem microbiology is a compulsory ancillary investigation technique in the investigation of SUDI cases [55]. It however presents with several drawbacks [1, 46, 55]:

- Agonal spread [47] defined as the invasion of bacteria through the mucosal surface following ischaemia and /or hypoxia during the process of dying. However, the process of agonal spread does not always occur [46].
- Bacteria migration from the mucosal surface into the blood and body tissues after death, known as post-mortem bacterial translocation [47]. This can be reduced by cooling the bodies below 6°C prior to post-mortem examination [47, 55]. Microbiology findings were proven to be constant in the first 48 hours of death [55, 61].
- Contamination of the specimens [46] is a major artefact in post-mortem microbiological investigation. Specimens are usually collected during autopsy in mortuaries which are highly contaminated environments.

Several methods of improving post-mortem microbiological investigation were suggested, such as a sampling strategy consisting of prioritizing sample collection before open autopsy and starting from body fluid, followed by tissues with rigorous aseptic techniques, all within 48 hours after death to reduce risks of contamination [46, 47, 55].

Detection methods used in microbiological investigation significantly impact the findings and the perceptions on the role of respiratory pathogens in SUDI. An enormous variation is observed in microbial culture-based methods, routinely used in post-mortem microbiology [1, 47, 104]. More

importantly, culture-based methods only detect viable organisms and are known to have a low sensitivity [19, 47, 104]. This is a limiting factor in microbiological investigation of SUDI deaths, where samples are taken several hours to days after death and the corpse have been put into conditions that might be unfavourable for some microorganisms. Alternative molecular diagnostics techniques are known to have a higher sensitivity and specificity hence increase reliability of microbiological findings [1, 72, 73, 104].

Furthermore, because of the lack of published guidelines, the interpretation of post-mortem microbiology findings is difficult [47]. A number of respiratory pathogens involved in SUDI cases are also found in healthy controls (Table 3) [40, 58, 71]. Different specialists involved in the medico-legal investigation of SUDI do not have a consensus as to the interpretation of microbiological findings [61]. Professional microbiologists or a collaborative work between microbiologists and forensic scientists could provide a better interpretation of post-mortem microbiology results [54, 104]. Pryce *et al.* suggested that microbiological findings should be supported by significant histopathological findings for it to be relevant in the final diagnosis [61]. However, SUDI cases might have an infectious aetiology without presenting pathological evidence [61]. Therefore, alternative interpretation guidelines should be elaborated.

Table 4. The prevalence of respiratory pathogens from case-control studies

Countries	Study period	No. of SUDI and controls		Organism	Percentage with respiratory pathogens		P-value	C
		SUDI	Controls		SUDI/SIDS	Controls		
UK	1988	46	46	*S. aureus*	41.3%	28.3%	NA	N
				Streptococci	78.3%	32.6%	<0.0001	
Chile	1991-2000	126	24	Pneumocytis	33%	29%	0.7	1
UK	1993-1996	22	253	*S. aureus*	86.4%	56%	0.02	N
Germany	1995-1997	234	399	*B. pertussis*	5.1%	5.3%	NA	N
Spain	2004-2008	11	9	CMV, EBV, HHV-6	72.7%	22.2%	0.025	9
Australia	2008	38	134	Adv and Rotavirus	30%	20%	0.18	1
Australia	2014	52	102	*S. aureus*	66.6%	41.6%	0.007	2

Adv: Adenovirus, CMV: cytomegalovirus, EBV: Epstein-Barr virus, HHV-6: human herpes virus 6; CI, confidence interva

4.2 Diagnosis of respiratory infection in SUDI

A variety of specimens have been used in the investigation of respiratory pathogens in cases of SUDI, these include but not limited to body fluids (blood, pleural fluid, cerebrospinal fluid), spleen, liver, nasopharyngeal swabs, nasal swabs, bronchi and lung tissues. Lung tissue has been a recommended sample in medico-legal investigation of paediatric cases; and also believed to be the ideal sample in diagnosing respiratory infections [105]. However, lung tissues are susceptible to contamination [47, 55, 105]. About 50% of lung biopsies collected during autopsy are found to be contaminated [105]. The high risk of contamination of lung tissue samples during collection renders more difficult to interpret the results especially in case of multiple pathogen detection, which is common in SUDI cases. Therefore, a broad range of samples and sampling procedures should be investigated before a conclusive identification of ideal sample sites for post-mortem microbiological investigation can be proposed.

Despite major advances in diagnostics techniques, differentiating true pathogens and commensal microorganisms from non-sterile sample site (nasopharyngeal swabs, nasal swabs) have been a major problem in the diagnosis of respiratory infection not only in post-mortem samples but also in living individuals [105, 106]. Alternatively, normally sterile, especially those located at the infection focus (bronchi, lung biopsy and pleural effusion) have a better diagnostic value as the detection of potential pathogen in otherwise normally sterile site is considered as the aetiological cause of infection [54, 104, 107], therefore a potential cause of death.

5. Perspectives

The routine screening for respiratory pathogens in SUDI cases using very sensitive assays would be of great interest because it will not only allow the identification of the known pathogens associated with SUDI but it will also surely allow the identification of new pathogens contributing to SUDI. This approach will be essential in the elaboration of preventive strategies against this fatal illness. Microbiological investigation of SUDI cases should be improved and be standardized at least within countries to enable proper monitoring. This has to include ideal sample site, sample collection methods, diagnostics techniques to be undertaken and also guidelines for results interpretation.

Most of the studies on the aetiology of respiratory infections in SUDI used a pathogen-target approach which does not allow for the diversity of microbial community [2, 19, 70, 72]. More studies are needed to identify pathogens to be included in the routine investigation of SUDI deaths. Since some pathogens detected in SUDI are also found in non-SUDI cases, more case-control studies should be conducted. Advanced diagnostic techniques that allow rapid and accurate detection methods such as MALDI-TOF (matrix-assisted laser desorption ionisation time-of-flight) mass spectrophotometry for fungi and multiplex PCR reactions for viral, bacterial and fungal organisms [108, 109] could be valuable for screening respiratory pathogens in cases of SUDI.

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PART 4: **READY TO PUBLISH RESEARCH ARTICLE**

Title page

Role of respiratory pathogens in sudden unexpected death in infancy, Cape Town, South Africa

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Abstract

Background: Sudden unexpected death in infancy (SUDI) is among the most frequent causes of mortality in infants less than one year of age. Respiratory infections have been identified as the most frequent cause of death in these infants. Yet, the broad range of respiratory pathogen that might be involved in SUDI is poorly studied. This study aimed to investigate the incidence of the respiratory pathogens in SUDI.

Methods: A prospective study was carried out on SUDI cases admitted to Salt River Forensic Pathology Laboratory from February 2015 through May 2015. Cerebrospinal fluid, pericardial fluid and lung biopsy were collected from each study participant during post-mortem examination. Total nucleic acids were extracted on the automated QIAsymphony platform. The microbial diversity was investigated using a commercialized multiplex real-time PCR assay, the "FTD Respiratory pathogens 33" kit. This assay is able to detect 21 viruses, 11 bacteria and one fungus. In each real-time PCR run, a positive and non-template sterile water were included as controls.

Results: Thirty SUDI cases (median age, 3 (interquartile range (IQR): 2 – 8 months) were included in the study. Twenty participants were males. Positive microbiological results from at least one of the three samples were obtained in 28 cases (93%). According to the type of sample, respiratory pathogens were detected in almost all the lung biopsies (93%), while it was only detected in 60% and 50% of the cerebrospinal and the pericardial fluids, respectively. The median cycle threshold value was lower in lung biopsies (30 (IQR: 28 – 35)) compared to both cerebrospinal (34 (IQR: 30– 36)) and pericardial fluids (35 (IQR: 33– 35)) (p= 0.039). In lung biopsies, the most commonly detected bacteria were *K. pneumoniae* (47%, 14/30) and *M. catarrhalis* (20%, 6/30). *H. influenzae* (7%, 2/30) and *M. pneumoniae* (7%, 2/30) were the bacteria often detected in pericardial fluid and cerebrospinal fluid, respectively. Human Metapneumovirus was the most frequently virus detected in all three sample types assessed, accounting for 33% (10/30) in cerebrospinal fluid, 37% (11/30) in pericardial fluid and 57% (17/30) in lung biopsy samples, respectively. A single type of pathogen was detected in seven of the 28 positive cases.

Conclusion: This study highlights the potential implication of respiratory infection in SUDI and it reports one of the highest incidences of respiratory pathogens in SUDI cases. In addition, it is the first to report the high incidence rate of Human Metapneumovirus in SUDI cases. The findings also showed that the majority of SUDI cases are associated with synergetic interaction of multiple respiratory infections. However, data related to histopathology and bacterial culture were not available. A broad range of respiratory pathogens should be included in the routine investigation of SUDI cases with more sensitive diagnostic methods.

Key words: respiratory pathogens, respiratory infection, sudden unexpected death in infancy, real-time PCR, post-mortem microbiology.

1. Introduction

Sudden unexpected death in infancy (SUDI) is the leading cause of post-neonatal mortality in developed countries accounting for over than 50% of all infant death [1-5]. In Africa, all the reported data came from studies conducted in South Africa [6-9], where SUDI comprises 3.3% of all medico-legal autopsy cases admitted to forensic mortuaries [6]. However, the disparity in the investigative procedures renders it difficult to establish national statistics.

Following microbiological investigation, respiratory pathogens and subsequent pathological signs in the respiratory organs have been found to contribute to SUDI [6, 10, 11]. Nasopharyngeal and gastro-intestinal samples from SUDI cases were found to be colonised by more potential bacterial pathogens such as *Staphlylococci* and *Streptococci* than their age matched controls [12-14]. Also, viral pathogens have been identified as a direct cause of death or playing the role of enhancing the invasion of bacterial pathogens or bacterial toxins production [15-18]. In addition, SUDI exhibits a similar seasonality to epidemic viral infections, such as Influenza [19, 20] and Human Metapneumovirus [19]. However, the majority of respiratory pathogens remains undetected due to less sensitive or specific diagnostic techniques or simply is not included in the investigation.

Identification of the pathogen spectrum in infants that die suddenly and unexpectedly would help in the elaboration of preventive methods and would serve as an approach to the identification of microbial pathogens to be included in the standard microbiological investigation in cases of SUDI. This study serves as an initial evaluation of the incidence of a wide range of selected respiratory pathogens.

2. Materials and methods

2.1 Ethical approval

The study protocol was approved by the human research ethics committee of the University of Cape Town, South Africa (HREC 635/2014). Written informed consent was obtained from the parents or the legal guardians of each study participant prior to the sample collection.

2.2 Participants and study design

This prospective study enrolled all the consecutive SUDI (sudden unexpected death in infancy less than one year of age) cases admitted to Salt River Forensic Pathology Laboratory (Cape Town, South Africa) over a four months period, from February 2015 through May 2015. Salt River Forensic Pathology Laboratory is an academic forensic laboratory that serves the Cape Town western metropolitan area (Western Cape, South Africa). Admitted bodies are kept in a refrigerated unit at around 4°C.

2.3 Post-mortem examination

Post-mortem examination was performed by forensic pathologists. It follows the routine protocol of post-mortem examination at the study setting that consisted of an external examination of the body, a full-body imaging and a review of the clinical history. Full-body radiology was performed using Lodox® scanners, the Lodox® Statscan® or Lodox® Xmplar-dr® (Lodox® Systems, Johannesburg, South Africa) according to the manufacturer's instructions. This new digital imaging technique provides a full-body radiological image and detects both soft and hard tissue abnormalities [7, 21].

Characteristics of the study participants were retrospectively obtained from a questionnaire administered to the parents or guardians of the deceased infant during the process of body identification as per standard procedure at Salt River Forensic Pathology Laboratory (Cape Town, South Africa). Data was entered into a SPSS spreadsheet (SPSS Statistics for windows, version 22.0, Chicago, IL, USA).

2.4 Sample collection

Before opening the body, cerebrospinal fluid was obtained by sub-occipital puncture. After opening the thorax, pericardial fluid was obtained by a puncture to the pericardial sac. Thereafter, lung biopsies were obtained from both left and right lungs. All the above mentioned samples were collected under rigorous aseptic conditions consisted of skin surface decontamination by 70% ethanol and changing gloves between each sample type. All the above mentioned samples were collected in sterile 2ml Sarstedt tubes (Qiagen, Hidden, Germany). Collected samples were put into an ice box and transported immediately to the Division of Medical Microbiology at the University of Cape Town (Cape Town, South Africa) where it was kept at - 80°C prior to processing.

2.5 Respiratory pathogens detection by multiplex real-time polymerase chain reaction

The detection of respiratory pathogens was performed at the Division of Medical Microbiology, University of Cape Town, South Africa. The laboratory at the Division of Medical Microbiology includes a well-equipped molecular biology unit, with the unidirectional principle respected. Safety practices are strictly enforced following established operation protocols outlined in a biosafety manual by the Institute of Infectious Diseases and Molecular Medicine (University of Cape Town, South Africa).

2.5.1 Total nucleic acids extraction

Samples to be tested were thawed at room temperature (22°C). Total nucleic acids were extracted using the QIAsymphony® DSP virus/ Pathogen Mini Kit (Qiagen, Hidden, Germany) on the automated QIAsymphony platform (Qiagen, Hidden, Germany) using the complex cell free 200V5 virus application protocol according to the manufacturer's instructions. Total nucleic acids were extracted from a starting volume of 300µl and eluted in a final volume of 60µl. The extracted nucleic acids were stored at -70°C until further analysis.

For lung biopsy, a pre-extraction step was undertaken in a biosafety hood: lung biopsy was crushed with a manual hand crusher in normal saline. After proteinase K and ATL tissue lysis buffer treatment (25mg/25µl, 25mg/375µl; 37°C for 24 hours), the supernatant was used for nucleic acids extraction as described previously.

2. 5.2 Multiplex real-time polymerase chain reaction

Real-time amplification for 33 respiratory microorganisms commonly associated with respiratory infections (Table 1) was performed on the BioRad CFX96^{TM} real-time PCR instrument (Bio-Rad, Hercules, Calfornia, US) using the FTD respiratory pathogens 33 Kit (Fast-Track diagnosis, Junglinster, Luxembourg) as per the manufacturer's recommendations, with minor changes. The real-time PCR reactions were performed in 12.5µl volumes instead of the 25µl as recommended by the manufacturer. The FTD respiratory pathogens 33 real-time PCR protocol has eight multiplexes per sample (Table 1). Each PCR reaction consisted of 5µl of the extracted nucleic acids, 6.25µl of the 2×real-time PCR buffer, 0.75µl of primer/probe mix and 0.5µl of enzyme mix (AgPath-IDTM one-step RT-PCR kit, Applied Biosystems, Carlsbad, CA, USA). The thermal cycling was performed using the following parameters: initial denaturation at 95°C for 10 min, followed by 40 amplification cycles of 94°C for 8 sec and 60°C for 34°C sec. To monitor the assay performance, negative and positive controls were included in each run. An internal control (equine arteritis virus) provided in the kit, was included in all samples to monitor nucleic acids extraction and amplification process. Positive results were indicated by an exponential amplification curve that crossed the threshold cycle within 40 cycles. Amplification data analysis and interpretation were performed using the FTD resp. 33 Analyser software version 1.0 (Cape Town, South Africa) according to the manufacturer's instructions (http://www.gematics.com/analyser.html).

Table 1. Microorganisms targeted in the FTD respiratory pathogens 33 kit

Reaction number	Reaction name	Organism-targeted
1	FluAB-RH	Influenza virus A and B, and Rhinovirus
2	Para.EAV	Parainfluenza 2,3,4 and Internal control (Equine arteritis virus)
3	Cor	Coronavirus NL63, 229E,C433 and HKU1
4	BoMpPf1	Parainfluenza 1, Human metapneumovirus A/B, Bocavirus and *Mycoplasma pneumoniae*
5	RsEPAcmv	Respiratory syncytial viruses A/B, Cytomegalovirus, Adenovirus, Enterovirus and Parechovirus
6	Bac	*Chlamydia pneumoniae, Streptococcus pneumoniae, Haemophilus influenzae type B* and *Staphylococcus aureus*
7	KLePSa	*Klebsiella pneumoniae, legionella spp., Salmonella spp.* and *Pneumocystis jirovecii*
8	MoBoCH	*Moraxella catarrhalis, Bordetella pertussis, Haemophilus influenzae* and Influenza C.

2.6 Statistical analysis

Descriptive analyses are expressed as median and interquartile range for continuous variables and number (percentage) for categorical variables. Logit analysis model was done using Fisher's exact or Likelihood ratio where applicable. Agreement between pathogen detection results and full body radiology results was evaluated with Cohen's kappa (k) statistics. All statistical analyses were done with SPSS for windows, version 22.0 (Chicago, IL, USA). In all statistical analyses, *p* values less than 0.05 were considered statistically significant.

3. Results

3.1 Characteristics of study participants

Thirty SUDI cases were included in this study, 20 (67%) males and 10 (33%) females. The median age was three months (interquartile range (IQR) 2 − 8 months). Most of the cases were of African ancestry (50%, 15/30) and of mixed race ancestry (40%, 12/30). The vaccine coverage pattern among the study participants was 83% (25/30).

Of the study participants, 64% (16/25) died in bed whilst asleep (Table 2). About two-third (61%), 11/18) of infants were exposed to cigarette smoke on a daily basis. The median number of smokers in the dwelling was two (IQR, 1 – 2 persons). The median age of the infant's mothers was 26 years (IQR, 21 – 32 years) and the majority of infant's mothers (83%, 20/24) were unemployed. The only characteristic of the mothers that was statistically associated with unexplained SUDI death was marital status (*p*-value = 0.028) (Table 3).

3.2 Post-mortem examination findings

The median post-mortem interval was three days (IQR, 2 – 4 days). From the 30 SUDI cases examined, 24 (80%) were explained SUDI and six (20%) unexplained. Of the 24 explained SUDI, 14 (58%, 14/24) had apparent lung pathology consistent with lower respiratory tract infection (Figure 1). The main pathological sign of lower respiratory tract infections observed on full-body radiological imaging was bilateral lung opacities (4/18, 22%) (Figure 2).

3.3 Microbiological findings

3.3.1 Real-time PCR data using the Fast-track Diagnostics® kit

Microbiological analysis revealed the presence of a potential pathogen in 93% (28/30) of the SUDI cases. Most of the respiratory pathogens assessed were detected in the lung biopsies (93%, 28/30), followed by the pericardial fluid (63%, 19/30) and the cerebrospinal fluid (47%, 14/30). According to the sample type, the median cycle threshold value was lower in lung biopsy samples (30 (IQR: 28 – 35)) compared to both cerebrospinal fluid (34 (IQR: 30 – 36)) and pericardial fluid (35 (IQR: 33 –

35)) samples. The median cycle threshold value was significantly different among the three samples (*P*= 0.039). There was 60% (18/30) positive SUDI cases that involved at least one bacterial pathogen, 73.3% (22/30) involved at least one viral pathogen, and 50% (15/30) involved *Pneumocystis jirovecii*.

Table 2. Demographic and background characteristics of SUDI cases

Characteristics	Number (percent)		Total	P-value
	explained SUDI	unexplained SUDI		
Age (n=30)				0.093
< 2 months	8 (66.7%)	4 (33.3%)	12	
2 – 4 months	8 (82.4%)	0 (17.6%)	8	
>4 months	8 (85.7%)	2 (14.3%)	10	
Gender (n=30)				0.372
Male	17 (85%)	3 (15%)	20	
Female	7 (70%)	3 (30%)	10	
Prematurity (n=27)				1
Preterm	7 (87.5%)	1 (12.5%)	8	
Born at term	15(78.9%)	4 (21.1%)	19	
Race (n=27)				1
African	12 (80%)	3 (20%)	15	
Mixed race	10 (83.3%)	2 (16.7%)	12	
Feeding practice (n=28)				0.492
Breastfeeding	10 (76.9%)	3 (23.1%)	13	
Formula feeding	10 (83.3%)	2 (16.7%)	12	
Both breast and formula feeding	3 (100%)	0	3	
Birth weight (n=27)				1
Low (< 2500g)	8 (80%)	2 (20%)	10	
Normal (≥ 2500g)	14 (82.4%)	3 (17.6%)	17	
Vaccination status (n= 30)				0.254
Vaccination status up to date	21 (84%)	4 (16%)	25	
No vaccination records	3 (60%)	2 (40%)	5	

Table 2. Demographic and background characteristics of SUDI cases (continue)

Characteristics	Number (percent)		Total	P-value
	explained SUDI	unexplained SUDI		
Place of death (n=25)				0.312
Bed	14 (87.5%)	2 (12.5%)	16	
Couch	1 (100%)	0 (0%)	1	
Mother's arm	1 (33.3%)	2 (66.7%)	3	
Unknown	4 (80%)	1 (20%)	5	
Sleeping position at death (n=23)				0.539
Supine	5 (100%)	0 (0%)	5	
Prone	4 (66.7%)	2 (33.3%)	6	
Side	8 (88.9%)	1 (11.1%)	9	
Unknown	2 (66.7%)	1 (33.3%)	3	
Face position at death (n=23)				1
Face up	2 (100%)	0 (0%)	2	
Face down	1 (100%)	0 (0%)	1	
Face to the side	6 (60%)	4 (40%)	10	
Other (unknown)	5 (100%)	0 (0%)	5	
Bed sharing (n=27)				1
Yes	22 (84.6%)	4 (15.4%)	26	
No	1 (100%)	0 (0%)	1	
Smoking while baby is sleeping (n=21)				1
Yes	6 (85.7%)	1 (14.3%)	7	
No	11 (78.6%)	3 (21.4%)	14	
Presence of smokers in the dwelling (n=17)				
Yes	9 (81.8%)	2 (18.2%)	11	1
No	5 (83.3%)	3 (16.7%)	6	

SUDI, sudden unexplained death in infancy; n, total number tested.

Human Metapneumovirus A/B was the most common pathogen detected, accounting for 33% (10/30) in cerebrospinal fluid, 37% (11/30) in pericardial fluid and 57% (17/30) in lung biopsy. The most frequently detected bacteria was *Klebsiella pneumoniae* (47%, 14/30) in lung biopsy, *Moraxella catarrhalis* (10%, 3/30) and *K. pneumoniae* (10%, 3/30) in pericardial fluid, and *Mycoplasma pneumoniae* (7%, 2/30) in cerebrospinal fluid. *P. jirovecii* was more commonly detected in lung biopsies (47%, 14/30). Adenovirus (AdV), Cytomegalovirus (CMV), Respiratory syncytial virus (RSV), and *Bortdella pertussis* were not detected in this study (Figure 3).

Table 3. Characteristics about the mother of the infants

Characteristics	Number (percent)		Total	P-value
	explained SUDI	unexplained SUDI		
Marital status (n=26)				0.028
Married	1 (25%)	3 (75%)	4	
Single	19 (86.4%)	3 (13.6%)	22	
Employment status (n=24)				0.544
Employed	4 (100%)	0 (0%)	4	
Unemployed	15 (75%)	5 (25%)	20	
Education status (n=20)				0.162
Primary level (grade 0-7)	2 (50%)	2 (50%)	4	
Secondary level (grade 8-12)	14 (87.5%)	2 (12.5%)	16	
Unhealthy behaviour* during pregnancy (n=24)				1
Yes	9 (81.8%)	2 (18.2%)	11	
No	10 (76.9%)	3 (23.1%)	13	
Unhealthy behaviour *after pregnancy (n=23)				1
Yes	6 (85.7%)	1 (14.3%)	7	
No	12 (75%)	4 (25%)	16	
Partner drinking habits (n=20)				0.319
Yes	8 (88.9%)	1 (11.1%)	9	
No	7 (63.6%)	4 (36.4%)	11	
Previous SUDI (n=21)				1
Yes	3 (75%)	1 (25%)	4	
No	13 (76.5%)	4 (23.5%)	17	
Stillbirth (n=22)				1
Yes	7 (87.5%)	1 (12.5%)	8	
No	11 (78.6%)	3 (21.4%)	14	

*Unhealthy behaviour, alcohol/cigarette smoke/drug use; SUDI, sudden unexplained death in infancy

The same type of pathogen was detected in all three samples in nine of 28 positive cases (32%); of the nine cases, eight involved Metapneumovirus A/B and one involved *K. pneumoniae.* Similarly, 50% (14/28) cases had the same type of pathogen detected in two of three samples under study, including four cases infected with Metapneumovirus A/B, three with *M. pneumoniae*, two with *K. pneumoniae*, two with *Streptococcus pneumoniae*, one with *P. jirovecii*, one with Bocavirus and one with *Staphylococcus aureus.*

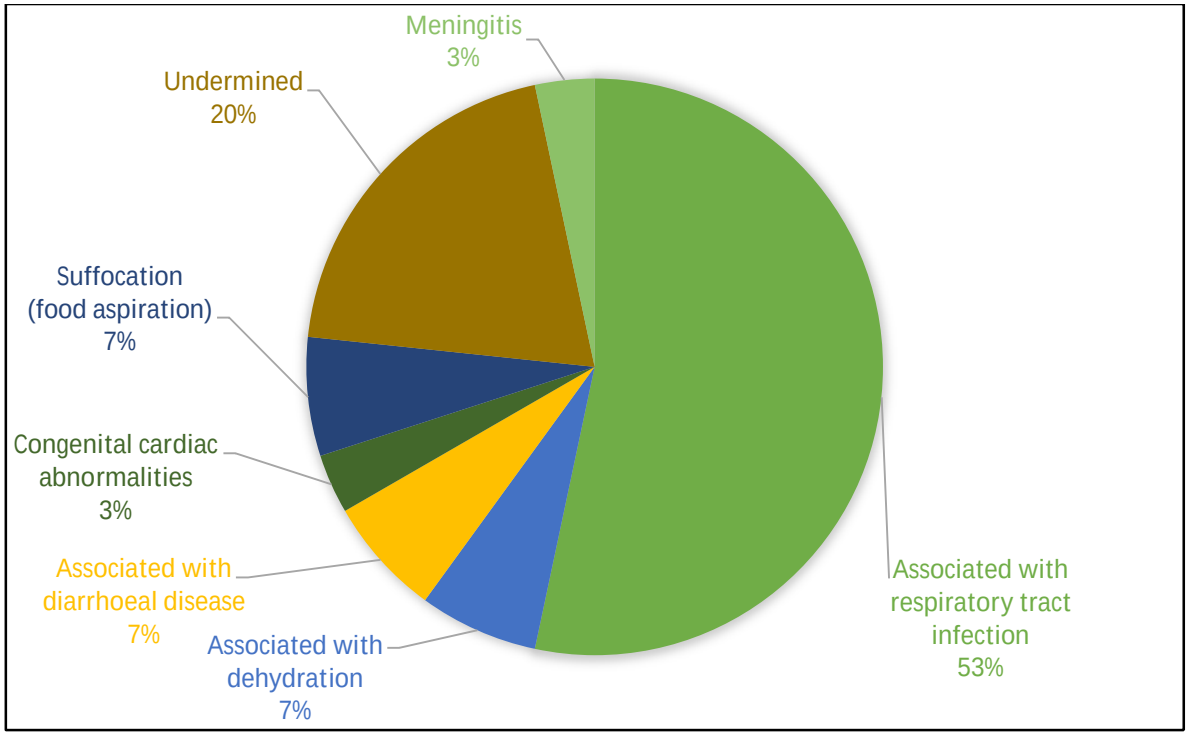

Figure 1. Cause of death (n= 30)

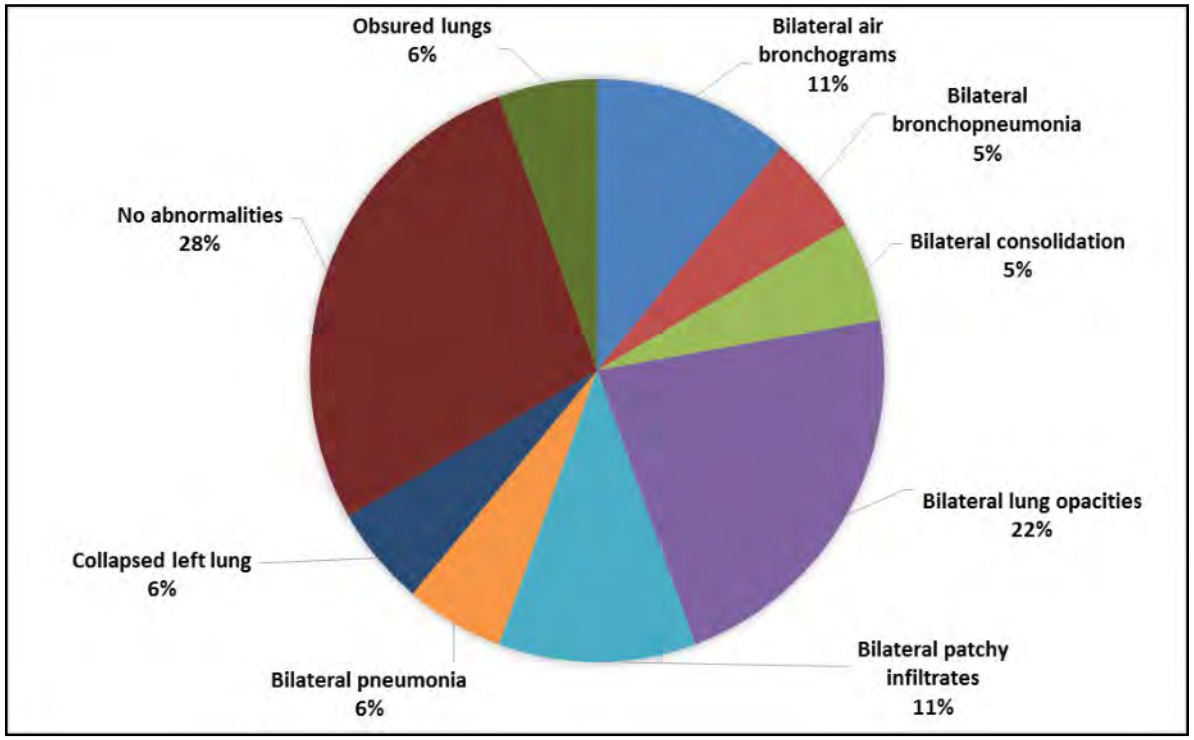

Figure 2. Lodox® scanners full body imaging findings (n= 18)

Among the 28 SUDI cases with positive pathogen detection, seven (25%, 7/28) had a single type of pathogen detected. Table 4, summarized the distribution of a single pathogen detection from all the three samples. Co-infection by at least two respiratory pathogens under study was observed in 75% (21/28). Most of multiple pathogens co-detection was found in lung biopsy (Table 7). Metapneumovirus A/B was the most pathogen involved in pathogen co-detection accounting for 10% (1/10), 45% (5/11) and 71% (12/17) in cerebrospinal fluid, pericardial fluid and lung biopsy, respectively. The combination of pathogens detection in cerebrospinal fluid, pericardial fluid and lung biopsy are described in Tables 5, 6 and 7, respectively.

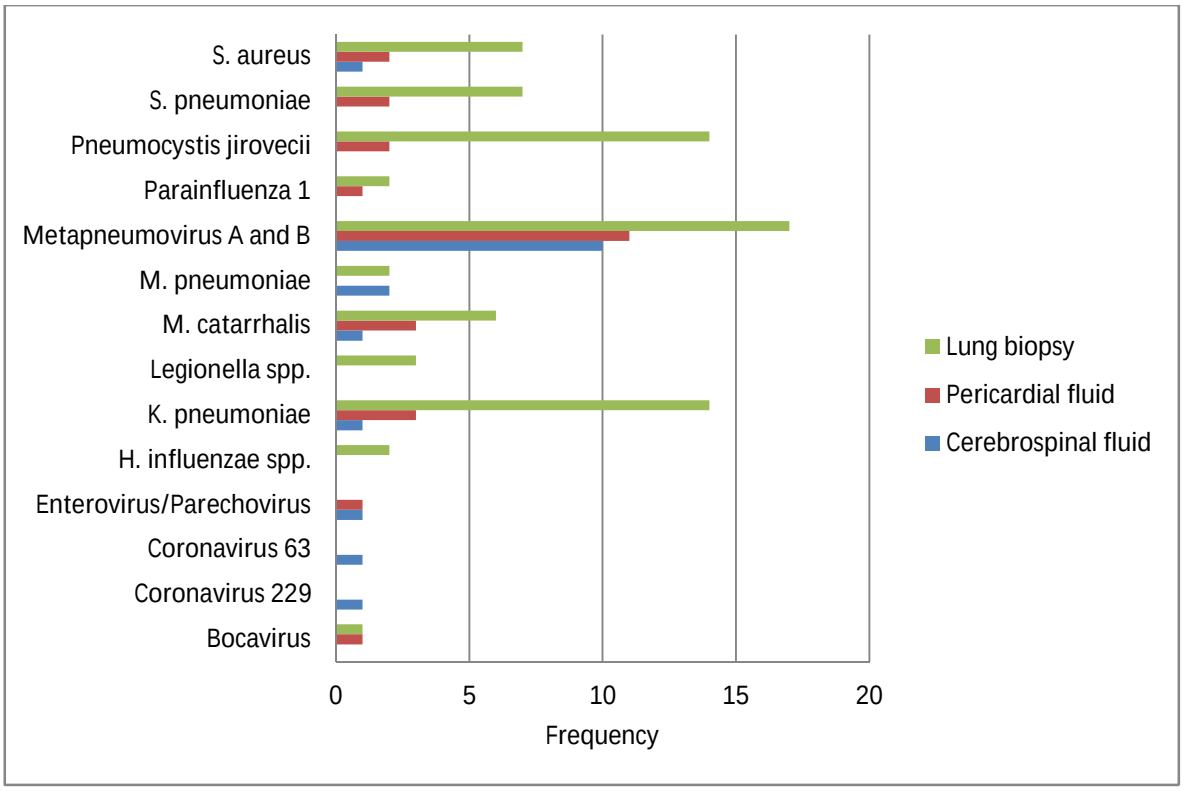

Figure 3.Respiratory pathogens distribution in all the three samples

3.3.2 Microbiological data from routine laboratory investigation

Only two cases had blood culture data available, one of which had a positive detection of *K. pneumoniae* which was also confirmed by real-time PCR with positive detection in both the lung biopsy and pericardial fluid. The other, had *S. aureus* but this was not confirmed by PCR results.

Table 4. Frequency of single detected respiratory pathogens from post-mortem samples

Respiratory pathogen	Cerebrospinal fluid	Pericardial fluid	Lung biopsy
Virus			
Human Metapneumovirus A/B	9	6	5
Parainfluenza 1	–	1	–
Bacteria			
Klebsiella pneumoniae	1	1	–
Mycoplasma pneumoniae	2	3	–
Staphylococcus aureus	1	1	–
Fungi			
Pneumocystis jirovecii	–	–	3
Total	**13**	**10**	**8**

–, not found

Table 5. Distribution of respiratory pathogens co-detections in cerebrospinal fluid

	Hcor229E	HcorNL63	EV/PV	HMPVAB	Morax
Hcor229E		–	–	–	–
HcorNL63	1		–	–	–
EV/PV	0	0		–	–
HMPVAB	0	0	1		–
Morax	1	1	0	0	

Hcor 229, Human coronavirus 229E HcorNL63, Human coronavirus NL63 EV, Enterovirus PV, Parechovirus HMPVAB, Human Metapneumovirus A and B Morax, *Moraxella catarrhalis* –, not found

Table 6. Distribution of respiratory pathogens co-detections in pericardial fluid

	HboV	EV/PV	FluC	HMPVAB	Kpneu	Spneu	Saur	PCP
HboV		–	–	–	–	–	–	–
EV/PV	0		–	–	–	–	–	–
FluC	0	0		–	–	–	–	–
HMPVAB	0	1	0		–	–	–	–
Kpneu	0	0	0	1		–	–	–
Spneu	0	0	0	1	1		–	–
Saur	1	0	0	0	0	0		–
PCP	0	0	0	2	0	0	0	

Hbov, Human bocavirus EV, Enterovirus PV, Parechovirus FluC Influenza virus C HMPVAB, Human Metapneumovirus A and B Kpneu, *Klebsiella pneumoniae* Spneu, *Streptococcus pneumoniae* Saur, Staphylococcus aureus PCP, *Pneumocystis jirovecii* –, not found

Table 7 Distribution of respiratory pathogens co-detections in lung biopsy

	HboV	HMPVAB	Para1	Haeinf	Kpneu	Legio	Morax	Mpneu	Spneu	Saur	PCP
HboV		–	–	–	–	–	–	–	–	–	–
HMPVAB	0		–	–	–	–	–	–	–	–	–
Para1	0	0		–	–	–	–	–	–	–	–
Haeinf	0	1	0		–	–	–	–	–	–	–
Kpneu	1	9	0	2		–	–	–	–	–	–
Legio	0	3	0	0	2		–	–	–	–	–
Morax	1	2	0	2	6	0		–	–	–	–
Mpneu	0	1	0	2	2	0	2		–	–	–
Spneu	0	4	1	2	6	0	3	2		–	–
Saur	1	4	1	0	4	2	2	0	2		–
PCP	0	8	0	1	8	1	2	1	4	3	

Hbov, Human bocavirus HMPVAB, Human Metapneumovirus A and B Para 1, Parainfluenza virus 1 Haeinf, *Haemophilus influenzae* Kpneu, *Klebsiella pneumoniae* Legio, Legionella species Morax, *Moraxella catarrhalis* Mpneu *Mycoplasma pneumoniae* Spneu, *Streptococcus pneumoniae* Saur, Staphylococcus aureus PCP, *Pneumocystis jirovecii* –, not found

3.4 Correlation between pathological and microbiological investigation

The detection of significant pathological signs of infection in the lungs using Lodox® scanners correlate with positive detection of a potential pathogen in the lung biopsy (kappa= 0.73). Human Metapneumovirus A/B detection in the lung biopsy correlate with lower respiratory tract pathology findings on full body imaging using Lodox® scanners (kappa= 0.96). Among the six cases of unexplained SUDI, only two cases have Lodox® scanners results available. One shows bilateral pulmonary patchy infiltrate and the other does not show any abnormality. However four out of the six unexplained SUDI (67%, 4/6) had a positive PCR pathogen detection in at least one of the collected samples.

4. Discussion

A considerable number of SUDI mortalities are caused by respiratory tract infections [8, 22, 23], which are considered as one of the most frequent causes of death in children under one year of age worldwide [24]. However, the association between respiratory infections and SUDI have been less well studied. The detection of specific respiratory pathogens in cases of SUDI provides data relevant to the development of prevention strategies. The aim of this study was to estimate the incidence of respiratory pathogens in SUDI cases in Cape Town (Western Cape, South Africa). This is the first

study to investigate a large panel of respiratory pathogens in SUDI cases, including 21 viruses, 11 bacteria, and one fungus. It also includes recommended samples for post-mortem microbiology such as pericardial fluid that have been recently identified as less affected by post-mortem bacterial translocation [25]. Cerebrospinal fluid is considered as the most sterile post-mortem sample [26] and lung biopsy for being at the focal point of respiratory infection [27]. Hence, detection of a potential pathogen from these samples under study should be considered as potentially causing or contributing to a cause of death.

Human Metapneumovirus is an important cause of respiratory tract infections in previously healthy infants during the first year of life [28, 29]. In the present study, the Human Metapneumovirus was the most predominant detected pathogen, accounting for more than half (57%, 17/30) of all cases. Eight of nine cases (89%) that had the same type of pathogen detected in all the three samples involved Human Metapneumovirus. Also, it was involved in 10% (1/10), 45% (5/11) and 71% (12/17) of respiratory pathogen co-detections in cerebrospinal fluid, pericardial fluid and lung biopsy, respectively. Prior to this study, Human Metapneumovirus has never been reported in SUDI cases. This might be due to the fact that majority of studies include a limited number of pathogens. Therefore, the role of Human Metapneumovirus as a pathogen or co-pathogen in the occurrence of SUDI requires further investigation.

In contrast to the high incidence rate of AdV [23, 30], CMV [22] and Respiratory syncytial virus RSV [10, 23, 31] in SUDI cases worldwide, the present study did not find any case with the above mentioned viruses. A previous virological surveillance of SUDI cases conducted in the same area as the present study showed concordant results, with unexpected low detection rate of both RSV and AdV [8]. However, the peak incidence of RSV and AdV is observed during winter period [19], which was not included in the present study period. In addition, the absence of AdV, CMV and RSV might imply that the spectrum of respiratory pathogens involved in SUDI cases may be different across regions.

S. aureus and *S. pneumoniae* have been implicated in SUDI deaths [10, 23, 26, 32, 33]. In this study, both *S. aureus* and *S. pneumoniae* are detected mainly in lung biopsy samples (7/30, 23%). Similarly, previous studies have isolated *S. aureus* and *S. pneumoniae* from the same sample site using culture based techniques [26], suggesting a high implication of both bacteria in fatal lower respiratory tract infections. *K. pneumoniae* was the most frequently detected bacteria in lung biopsy (47%, 14/30). *K. pneumoniae* has been previously isolated from SUDI cases [32]. Yet, both *K. pneumoniae* and *M. catarrhalis* have been given less consideration in SUDI investigation. An alternative possible explanation of the high incidence rate of *K. pneumoniae* in lung biopsy is the increased susceptibility to contamination in lung biopsy [27, 32, 34], which renders the interpretation of post-mortem microbiological results from lung samples more challenging [35]. Also, the specificity of *khe* assay used to detect *K. pneumoniae* in the FTD® respiratory pathogens 33 Kit was found to detect some species of *Klebsiella* other than *K. pneumoniae* [44].

Haemophilus influenzae has been frequently isolated from lung biopsy and cerebrospinal fluid samples in SUDI cases [10, 26, 32]. However, the significant success of the pneumococcal conjugate vaccine might have reduced the incidence rate of *H. influenzae* infection [36]. Hence the low detection rate of *H. influenzae* (2/30, 7%) in lung biopsy, and the absence of *H. influenzae type B* in the present study could be due to the fact that most of the study participants (83%, 25/30) had up to date immunization records. This high rate of immunization in the study participants would also explain the absence of *Bordetella pertussis*.

A high incidence rate of *P. jirovecii* (50%, 15/30) was observed in lung biopsies and most of *P. jirovecii* positive SUDI cases (37%, 11/30) involved multiple pathogen detection. As *P. jirovecii* is also found in healthy infants [37], the detection of *P. jirovecii* should be considered as a co-morbidity factor rather than the cause of death. Similarly, detected pathogens from lung biopsy should be regarded as contributing to death. However, pathogens that were detected from both cerebrospinal and pericardial fluid should be considered as potential cause of death. Recent studies have shown that respiratory infections in infants less than one year of age are a result of multiple pathogen colonisation [38, 39]. In this study, 73% showed multiple pathogen colonisations. Hence, SUDI deaths might be due to synergetic interaction between multiple pathogens colonisation in the respiratory system.

The use of Lodox® scanners in the investigation of SUDI provided an effective, rapid diagnosis of lung pathology in SUDI cases. This agrees with previous studies in which pulmonary pathology are commonly observed on the radiology of SUDI cases [7, 40]. The detection of significant pathological signs of infection in the lungs correlate with positive detection of a potential pathogen in the lung biopsy (kappa= 0.73), indicating the ability of the Lodox® Statscan® full-body imaging system to detect pathology on soft tissue. However, to determine the aetiology of the infection, further microbiological investigation is required.

In six cases that remained unexplained after routine post-mortem examination, respiratory pathogens were detected in four (67%), suggesting an infectious cause of death rather than unexplained. This suggests that some SUDI cases are miscategorised as unexplained and results from post-mortem microbiology could provide a possible cause of death.

The most common characteristics of SUDI cases were in line with the literature. The male infants were predominant (67%, 20/30), which was also evidenced in several previous studies [8, 41]. Improper sleeping conditions were a common observed characteristic in our study population, which also agrees with previous studies [8, 42, 43]. Only the mother's marital status was statistically significant with unexplained SUDI deaths; however, this may be due to the limited sample size.

This study has several limitations. The present study follows the routine investigation of SUDI cases at Salt River Forensic Pathology Laboratory (Cape Town, South Africa). Data related to toxicology

and histopathology were not available. The mentioned investigations are only investigated in the absence of pathological sign of infection after full-body imaging and post-mortem examination. The cell culture technique, considered as the ultimate diagnostic technique for infections in post-mortem settings. Only two cases had blood culture results available. One was confirmed by PCR results; however, the other one with a positive *S. aureus* did not match with microbiological PCR investigation results. A possible explanation to this finding is that *S. aureus* is part of the normal skin microbial community; therefore, it can be a result of contamination during sample collection or processing.

5. Conclusion

This study provides the incidence of the most frequent respiratory pathogens involved in SUDI in the Cape Town metropolitan, Western Cape, South Africa. The high rate of multiple pathogen detection in otherwise normally sterile site implicates respiratory co-infections in the pathology of SUDI. In addition, this study demonstrates the relevance of post-mortem microbiology as it provided an explanation of death in 67% of otherwise unexplained SUDI cases. The use of large panels of pathogens should be considered in the aetiological investigation of respiratory infection in SUDI. Further research should be considered to determine the seasonal respiratory pathogen distribution in cases of SUDI.

Authors' contributions

ESI MK MH conceived and designed the experiments. ESI performed the experiments and analysed the data. ESI, MK and MH wrote the paper.

Competing interests

The authors declare that they have no competing interests.

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PART 5: **GENERAL DISCUSSION**

The aim of this research project was to study the incidence of respiratory pathogens in SUDI and analyse the correlation between findings from different techniques used in medico-legal investigation of SUDI cases in Cape Town, South Africa.

The aim of the research was achieved by screening all cases of SUDI admitted at Salt River Forensic Pathology Laboratory (Cape Town, South Africa) during the period from February through May 2015. The investigative techniques performed were post-mortem examination, full-body radiology and microbiological investigation. The study period mentioned in the research protocol was postponed due to the delay in obtaining the ethics approval from the Human Research Ethics Committee at the University of Cape Town, South Africa. The lack of control samples rendered the case control sampling approach proposed not achievable.

The sampling procedure for lung biopsy was modified from the intended percutaneous needle lung biopsy to the lung biopsy after opening the body. The percutaneous needle lung biopsy sampling procedure, though it was previously found to be efficient for post-mortem bacteriological examination [1,2] was not able to provide the required sample size (25mg of lung tissue) for the automated nucleic acids extraction intended in this study. Cerebrospinal fluid was collected via sub-occipital puncture instead of lumbar puncture before opening the body. This minor change in cerebrospinal fluid collection was made after sub-occipital puncture was found to be more efficient as standard post-mortem investigation recommend the use of either sub-occipital puncture or lumbar puncture to collect post-mortem cerebrospinal fluid for microbiological examination [3]. Furthermore, sub-occipital puncture was used to collect cerebrospinal fluid in post-mortem virological investigation in Bali [4]. Due to the budget constraints, we did not screen blood samples collected in this study.

The routine microbiological investigation by the National Health Laboratory Service was not performed for all the cases included in the present study. According to the protocol, autopsy and subsequent histopathological and microbiological investigation were performed at Salt River Forensic Pathology Laboratory only in the absence of relevant pathological signs in keeping with natural causes of death after review of the clinical history, external body examination and full-body radiology. Therefore, only two cases (7%, 2/30) had their routine microbiological investigation by National Health Laboratory Service results available. This explains the lack of histopathology findings as was previously intended in the research protocol. These guidelines of SUDI investigation currently followed at the Salt River Pathology Laboratory could lead to missing potential infections and therefore to miss-categorisation of SUDI cases. According to Kennedy's protocol, an international protocol for sudden unexpected child death investigation, a SUDI case undergoes extensive medico-legal investigation including: full body autopsy, histopathology, toxicology, biochemistry, bacteriology and virology examination before it is categorised [5].

The main findings of this study were the high rate of respiratory pathogens (including multiple pathogens) detection in otherwise normally sterile body sites (cerebrospinal fluid and pericardial fluid) in SUDI cases. Pericardial fluid is the most recommended sample type in the diagnosis of post-mortem infection [6]. Human Metapneumovirus A/B was the most common pathogen detected. Moreover, the present study is the first to report Human Metapneumovirus A/B in SUDI cases, and its high implication in SUDI cases warrants further investigation.

In this study, the transitioning from Lodox® Statscan® to Lodox® Xmplar-dr® did not allowed performing the full body radiological imaging for all the SUDI cases. Nevertheless, using Lodox® scan, lung pathology suggestive to lower respiratory infections was found in two-third (12/18) of explained SUDI cases in our study. Of note, the accuracy of post-mortem imaging using Lodox® scan was not in the scope of this study. Therefore, studies investigating the value of Lodox® scan in the diagnostic of respiratory infection are needed.

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PART 6: **GENERAL CONCLUSION AND PERSPECTIVES**

This pilot study provided a baseline for post-mortem microbiological analysis of SUDI cases in our study setting. Although the samples analysed in the present study provided relevant information on the contribution of respiratory pathogens in SUDI cases, a larger sampling strategy with various diagnostic techniques would have eliminated biases associated with limited sample size and therefore add value to the present knowledge. A control group of healthy infants would determine the accuracy of post-mortem imaging in the diagnosis of infections. The use of other recommended samples for post-mortem diagnosis of infections such as heart blood and liver biopsy should also be considered. Furthermore, the present study investigated SUDI cases during a specific period of the year (February to May) and therefore, a study investigating the dynamics of respiratory pathogens involved in SUDI cases over a year period should be envisaged.

The lack of standard procedures in the investigation of respiratory pathogens in cases of SUDI that have been outlined in the research protocol (Part 2) of this dissertation highlighted the need to establish a general protocol at national level. The major respiratory pathogens found in cases of SUDI as detailed in Part 4 (a ready to publish article) of this dissertation provide an initial step in the identification of respiratory pathogens that should be included in the standard procedures. However, if a control group is used, it would facilitate the interpretation of microbiological results.