Infection and immunogenetics in unexplained infant deaths in Australia

Amanda R Hight BSc (Laboratory Medicine) (Hons)

Thesis submitted for the degree of

Doctor of Philosophy

The Discipline of Paediatrics

Faculty of Health Sciences

The University of Adelaide

Australia

December 2009
## Chapter 1. Literature review

Sudden Infant Death Syndrome and unexplained Sudden Unexpected Death in Infancy in Australia

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definition of SIDS in Australia</td>
<td>6</td>
</tr>
<tr>
<td>SIDS Pathology</td>
<td>8</td>
</tr>
<tr>
<td>Organ weight anomalies</td>
<td>9</td>
</tr>
<tr>
<td>Substantiation of an infectious aetiology</td>
<td>9</td>
</tr>
<tr>
<td>Enteric bacteria</td>
<td>14</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>15</td>
</tr>
<tr>
<td>Staphylococcal enterotoxins and the intestinal tract in SIDS</td>
<td>16</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>17</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>19</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>20</td>
</tr>
<tr>
<td><em>Clostridium sordelli</em></td>
<td>22</td>
</tr>
<tr>
<td>Viral infection</td>
<td>25</td>
</tr>
<tr>
<td>Respiratory Viruses</td>
<td>27</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>28</td>
</tr>
<tr>
<td>Fungal infection</td>
<td>29</td>
</tr>
<tr>
<td>Genetic predisposition</td>
<td>30</td>
</tr>
</tbody>
</table>
Chapter 2. Molecular analysis of pathogenicity of *Escherichia coli* from Sudden Infant Death Syndrome, dead control and healthy infants

Abstract

Background

Verotoxicity of *E. coli* isolates from SIDS infants

Cytolysin A and the High Pathogenicity Island in *E. coli* isolates

Methods

Part 1) Evaluation of verotoxicity

Part 2) Testing for Cytolysin A and the High Pathogenicity Island

Results

Part 1) Evaluation of verotoxicity

Part 2) Testing for Cytolysin A and the High Pathogenicity Island

Discussion

References
Chapter 3. Curliated *Escherichia coli*, soluble curlin and the sudden infant death syndrome: expansion of a previous investigation

Abstract

Introduction

Methods

Strains

Evaluation of curli production

Results

Discussion

References

Chapter 4. Staphylococcal enterotoxin genes are common in *Staphylococcus aureus* intestinal flora in Sudden Infant Death Syndrome (SIDS) and live comparison infants.

Introduction

Materials and methods

Ethics approval

Sample collection and preparation

PCR screening of bacterial culture lysates

Single colony analysis and western blot toxin assay

Results

Discussion

References

Chapter 5. *Clostridium sordellii* lethal toxin gene is not detectable by PCR in the intestinal flora of SIDS cases or infants who died of other causes.

Abstract

Background

Methods

Sample material

Demonstration of *C. sordellii* lethal toxin gene presence in simulated (spiked) culture
Chapter 6. Development of a novel hypothesis for unexplained sudden unexpected death in infancy

Abstract

Background

Factor 1: Transient bacteraemia

Factor 2: Pathogen pattern recognition insufficiency

Factor 3: Prenatal infectious event

Mode of Death

Proposed Investigation

Conclusion

References

Chapter 7. T cell receptor BV3 recombination signal sequence allele 2 is not associated with unexplained Sudden Unexpected Death in Infancy (SUDI) in an Australian cohort.

Abstract

Background

Materials and methods

Ethics approval

Sample collection and preparation

DNA extraction from intestinal contents

DNA extraction from dried blood samples

PCR amplification and allele discrimination

Statistics

Results
Chapter 8. Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2

Abstract 143

Introduction 144

Bacteria and their toxins in SUDI and SIDS 144

Interleukin-1 receptor antagonist 144

Materials and Methods 146

Selection criteria and sample preparation 146

PCR amplification 146

Statistics 148

Results 149

Comparison of post-mortem bacteriology findings with IL-1RN genotype 151

Discussion 153

References 156

Chapter 9. IL-1RN allele 2 association with SIDS is not confirmed in a large South Australian cohort.

Abstract 159

Introduction 160

Methods 161

Ethical considerations 161

Selection criteria 161

Sample preparation 164

Statistics 164

Results 165

Discussion 170
Chapter 10. *CD14* (*C-260T*) polymorphism is not associated with SIDS in a large South Australian cohort

Abstract

Introduction

Methods

Selection criteria and sample preparation

PCR amplification and genotype determination

Statistics

Results

Discussion

References

Chapter 11. Toll-like receptor 2 (*R753Q*) polymorphism associated with SIDS in a large South Australian cohort

Abstract

Introduction

Methods

Selection criteria and sample preparation

PCR amplification and genotype determination

Statistics

Results

Discussion

References

Chapter 12. Maternal and perinatal risk factors for SIDS

Abstract

Background

Methods
Chapter 13. Discussion

Bacterial toxins genes are present in the infant intestinal tract
Immunoregulatory gene polymorphisms
Population sampling for genetic studies
Maternal and perinatal risk factors
Study caveats
Future Implications and Directions
Which viruses might be involved?
Use of Neonatal Screening Cards as a source of viral nucleic acids
Limits of detection using NSC
Conclusions

Appendix 1. Oligonucleotides
Appendix 2. Maternal and perinatal risk factors for SIDS. Results and statistical analysis
Appendix 3. Published manuscript based on Chapter 1
Appendix 4. Published manuscript based on Chapter 2
Appendix 5. Published manuscript based on Chapter 4
Appendix 6. Published manuscript based on Chapter 5
Appendix 7. Accepted manuscript based on Chapter 6
Appendix 8. Published manuscript based on Chapter 8
Appendix 9. Published abstract
Addendum

Chapter 1.

<table>
<thead>
<tr>
<th>Year</th>
<th>0-3 m</th>
<th>4-6 m</th>
<th>7-9 m</th>
<th>10-12 m</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 (from 1st July)</td>
<td>31 (66%)</td>
<td>11 (23.4%)</td>
<td>5 (10.6%)</td>
<td>0</td>
<td>47 (100%)</td>
</tr>
<tr>
<td>2001</td>
<td>78 (70.9%)</td>
<td>26 (23.6%)</td>
<td>5 (4.5%)</td>
<td>1 (1%)</td>
<td>110 (100%)</td>
</tr>
<tr>
<td>2002</td>
<td>69 (68.3%)</td>
<td>22 (21.8%)</td>
<td>6 (5.9%)</td>
<td>4 (4%)</td>
<td>101 (100%)</td>
</tr>
<tr>
<td>2003</td>
<td>40 (61.5%)</td>
<td>20 (30.8%)</td>
<td>4 (6.2%)</td>
<td>1 (1.5%)</td>
<td>65 (100%)</td>
</tr>
<tr>
<td>2004</td>
<td>33 (57.9%)</td>
<td>18 (31.6%)</td>
<td>4 (7%)</td>
<td>2 (3.5%)</td>
<td>57 (100%)</td>
</tr>
<tr>
<td>2005</td>
<td>69 (76.6%)</td>
<td>15 (16.7%)</td>
<td>5 (5.6%)</td>
<td>1 (1.1%)</td>
<td>90 (100%)</td>
</tr>
<tr>
<td>2006**</td>
<td>36 (66.7%)</td>
<td>12 (22.2%)</td>
<td>2 (3.7%)</td>
<td>4 (7.4%)</td>
<td>54** (100%)</td>
</tr>
</tbody>
</table>

Page 22, Paragraph 3, Line 2. *Clostridium* should be italicized.

Page 40, Reference 17. The page numbers for this reference are 1242-1254

Page 22, Paragraph 2, Line 2. The table referred to is Table 2.

Page 24. This table is Table 2.
Chapter 2

Page 49, Line 6 should read “Fifty-six *E. coli* strains from 30 SIDS, two dead control and 24 healthy infants were were assayed for verocytotoxicity and for possession of shiga toxin (stx) genes to determine if they remained virulent after retrieval from storage.”

Page 53, Paragraph 2, Line 11. “unviable” should be “non-viable”

Page 54, Line 5 should read “Strains positive for stx-1 and -2, and a C500 control strain negative for stx-1 and -2 were used for comparison.”

Page 70, Line 1. The word “where” has been removed

Page 72, Reference 23. The page numbers for this reference are 1242-1254

Chapter 3

Page 76, Line 5 should read “Dye incorporation into the colonies is achieved via the binding of secreted curli to congo red in the media [2].”

Page 79, Table 1. The title should read “Number of *E. coli* isolates producing curli after 24 and 48 hours incubation at 37°C”

Page 82, Line 1 should read “This study sought to investigate further the role of curli production in *E. coli* from SIDS infants, in relation to a previous investigation in which a higher percentage of *E. coli* isolates from SIDS infants produced curlin compared with isolates from a group of comparison infants.” And Line 11 should read “This study sought to investigate further the role of curli production in *E. coli* from SIDS infants, in relation to a previous investigation in which a higher percentage of *E. coli* isolates from SIDS infants produced curlin compared with isolates from a group of comparison infants.”

Chapter 4

Page 89, Line 8, an additional sentence has been added “We incubated at 30°C for optimum for toxin production by clostridia. The culture supernatant was stored at -80°C for future toxin assays on any samples that tested positive by PCR”

Page 89, Line 1, an additional sentence has been added “A 100μl sample was taken and the cells were pelleted, resuspended in saline and boiled for ten minutes to make a crude lysate of each culture for PCR analysis.”

Page 97, Line 17 should read “Interestingly, the TSST PCR positive control isolate did not demonstrate in-vitro TSST production at 37°C.”
Page 104, Line 5. Pregnancy and childbirth have been suggested to predispose a small number of women to acquire *C. sordellii* in the vaginal tract, where the acidic pH of the vagina enhances the cytopathic effects of its toxins.

**Chapter 5**

Page 104, Line 7. “enhance” should be “enhances”

**Chapter 6 (amendment to Figure 1)**
Figure 1. Proposed interactions between transient bacteraemia (1), PPR gene polymorphisms (2) and prenatal events (3) leading to sudden infant death.
Chapter 7

Page 131, Paragraph 2 Line 2 should read “Testing was done retrospectively on stored material from previous investigations conducted in South Australia, Australia (from the cohort of unexplained sudden unexpected death in infancy (uSUDI) – a broad category of unexplained infant deaths from 1980-1994 that had previously been classified as SIDS according to the 1991 definition [12] - described by Goldwater [13], if stored material was available). There were 18 consecutive cases of SIDS from Victoria, Australia since 2007.”

Chapter 8

Page 153, Paragraph 2, Line 1 should read “This study investigated the relationship between IL-1RN 89bp VNTR genotype and uSUDI, to determine if a particular genotype was associated with the finding of bacteria in a normally sterile site at autopsy. An association was found between the homozygous A2 allele and uSUDI (p=0.007) where carriage of the 2/2 genotype was associated with nearly a five fold increase in relative risk of uSUDI compared with the predominant 1/1 genotype.”

Chapter 9

Page 165, Line 9 and paragraph 2 Line 3. “It was speculated that” has been replaced with “It was suggested that” and Line 7 “compared against carriage of the wild-type this difference” has been replaced with “compared with carriage of the 1/1 genotype, the difference”

Page 171, Line 12. {Zorgani et al., 1999} is citation [6]

Page 175 add the following to the reference list


Chapter 10

Page 177, Line 1 should read “Age-dependent susceptibility to sudden death has been demonstrated in a rat model of endotoxic shock in which the animals displayed gross pathological findings consistent with SIDS in humans [1].”

Page 184, Line 2 should read “Blood-Siegbried et al. reported similarities between a neonatal rat model of endotoxic shock and gross and microscopic pathology observed in SIDS cases [1]. In this investigation, the hypothesis was tested that SIDS infants would have a higher frequency of the CD14 (C-260T) polymorphism compared with non-SIDS controls. This would provide evidence to support, the endotoxic-shock model for SIDS if a higher density of CD14 receptors would render infant more sensitive to LPS. For example, the effect of expression levels of cell differentiation markers on toxin lethality in-vivo has been shown for CD45.”
Chapter 11

Page 190, Line 5 should read “Independent research to date has primarily targeted cytokine gene polymorphisms conferring heightened pro-inflammatory responses.”

Page 191, Paragraph 2, Line 2 should read “The homozygous TLR-2 variant R753Q is a “functional knockout” of LTA stimulation, while the heterozygous carrier type elicits full responses not different from the wild-type [9].”

Chapter 12

Page 211. Add the following to the reference list


Chapter 13

Page 214, Line 4 should read “Over-production of cytolysin A or staphylococcal enterotoxins, whose genes are described here to be present in SIDS infants, could present at lethal levels in the blood, particularly if the intestinal mucosa is damaged as was described by Kamaras and Murell [10].”

Page 216, Paragraph 2, Line 12. “abovementioned” has been deleted

Page 217, Line 9 should read “To prevent erroneous associations the experiments need to be replicated in a cohort larger than this.”
The pathological, epidemiological and genotypic findings in SIDS infants suggest an infectious aetiology possibly being potentiated by immunoregulatory polymorphisms. The objective of this project was to investigate new infectious and genetic risk factors for SIDS which could explain the typical findings and help identify a marker for susceptibility that could be assayed. We conducted a molecular-based investigation into potential candidate bacterial virulence factors of enteric \textit{Escherichia coli}, \textit{Staphylococcus aureus} and \textit{Clostridium sordellii} from SIDS infants. In the case of \textit{E. coli} and \textit{S. aureus}, genes encoding potentially lethal virulence factors were detected in cultures from both SIDS and healthy infants, and \textit{C. sordellii} lethal toxin detected in none. \textit{S. aureus} and its enterotoxins were found significantly more often in intestinal contents in SIDS infants than in comparison babies. The curli-producing phenotype observed to be associated with SIDS in a previous investigation was expanded to cover more serotypes, but in this case failed to demonstrate an association. The investigation then moved on to host factors that influence the outcome of infection by such organisms, in particular the following immunoregulatory gene polymorphisms: 1) Interleukin 1 receptor antagonist gene (IL-1RN) 89bp variable number of tandem repeats polymorphism, which influences the circulating levels of IL-1; 2) T cell receptor Vβ 3.1 recombination signal sequence polymorphism (TCRV3S1), which increases the proportion of T cells responsive to staphylococcal enterotoxin A; 3) CD14 gene promoter C-260T polymorphism which increases monocyte and macrophage responsiveness to endotoxin and 4) Toll-like receptor 2 (TLR-2) R-753Q gene polymorphism where a loss-of-function genotype would compromise pathogen recognition. An association was demonstrated between the homozygous A2 allele of the IL-1RN gene and unexplained sudden unexpected death in infancy (uSUDI) and SIDS.
infants who died prior to 1994. No association was demonstrated between IL-1RN allele 2 and latter SIDS infants (>1994) or between SIDS and TCRBV3S1, CD14 C-260T or TLR-2 R-753Q polymorphism. We constructed a novel hypothesis whereby risk factors for SIDS promote the translocation of bacteria from mucosal surfaces which might explain the finding of potential pathogens in normally sterile body sites, particularly if pathogen pattern recognition is compromised. No association with SIDS could be demonstrated. Prenatal viral infection as a risk factor for SIDS is introduced and a proof of concept study is discussed. Overall, no unique marker of SIDS susceptibility was found, however the higher prevalence of IL-1RN allele 2, predisposing to poor outcomes from infection, in SIDS infants dying before 1994 suggests that the high incidence during this period could point to an infectious aetiology. In the work presented in this thesis we have demonstrated that the intestinal tract contains potentially pathogenic species of bacteria which could contribute to SIDS in a multifactorial hypothesis which involves host predisposition and favourable environmental conditions. We have suggested some immunoregulatory genes that could be involved. The role of IL-1RN is particularly interesting. The work published from this thesis will contribute to the field of infectious disease research in SIDS and hopefully will lead to the identification of the cause of these deaths and future prevention.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Amanda Highet and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the library catalogue, the Australian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

There are no conflicts of interest to declare for myself or my supervisors. This work was supported by a competitive research grant from The Foundation for the Study of Infant Deaths, UK.

Amanda R Highet

December 2009
Acknowledgments

Firstly I would like to thank my supervisors Associate Professor Paul Goldwater, Dr Catherine Gibson and Dr Anne Berry. Without their help I would not have been able to complete so much work and publish much of it within my three years of candidature. I really appreciate the encouragement I received to write manuscripts and present my work at conferences both locally and overseas, and of course for the many hours spent reading thesis drafts. Thankyou also to Ms Gai McMichael who always made time to help me with my lab work, with reading manuscripts and with preparing presentations, and for sharing space with me when I needed to write my thesis. Thankyou to my boyfriend Luke and to my family for supporting me during my many years as a uni student. Thanks also to the staff of the Microbiology and Infectious Diseases Department at the Women’s and Children’s Hospital for taking me in as part of the department, for helping me with lab work and for their friendship.

I would like to thank the people who provided me with assistance in parts of the project, and with materials I needed. Thankyou to Ms Tracey Lumb from the Department of Infectious Diseases at the Institute of Medical and Veterinary Science, North Adelaide for supplying the vero cells, used in Chapter 2, to the Department of Immunology at the Women’s and Children’s Hospital, for use of cell culture facilities, and specifically to Bernadette Boog for patiently teaching me how to split and care for cultures. To Angela Byramji for her assistance with the curli study (Chapter 3). Thankyou also to Dr Karl Bettelheim and the National Escherichia coli Reference Laboratory, Victoria for supplying the E. coli isolates and
supporting data for the resulting publication and to Dr Janice Fletcher, Mr Enzo Ranieri and Ms Rosemarie Gerace from the South Australian Neonatal Screening Laboratory, SA Pathology at the Women’s and Children’s Hospital for helping me to identify and collect Newborn Screening Cards for the gene-association studies. To the Victorian Institute of Forensic Medicine for collecting samples for the Victorian intestinal contents cohort, and lastly to the Foundation for the Study of Infant Death, UK who provided funding for this research with a project grant.
Publications arising from this thesis


2. Highet AR, Berry AM, Bettelheim KA, Goldwater PN. The frequency of molecular detection of virulence genes encoding cytolysin A, high-pathogenicity island and cytolethal distending toxin of *Escherichia coli* in cases of sudden infant death syndrome does not differ from that in other infant deaths and healthy infants. *J Med Microbiol*. 2009;58:285-289. (Chapter 2)

3. Highet AR, Goldwater PN. Staphylococcal enterotoxin genes are common in *Staphylococcus aureus* intestinal flora in Sudden Infant Death Syndrome (SIDS) and live comparison infants. *FEMS Immunol Med Microbiol* (In Press). 2009; (Chapter 4)

4. Highet AR, Gibson CS, Goldwater PN. *Clostridium sordelli* lethal toxin gene is not detectable by PCR in the intestinal flora of Sudden Infant Death Syndrome cases or infants who died of other causes. *J Med Microbiol* (In Press). 2009; (Chapter 5)


6. Highet AR, Berry AM, Goldwater PN. Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2. *Ann Med* (In Press). 2009; (Chapter 8)
Manuscripts in preparation

1. Hight, AR, Gibson, CS and Goldwater, PN. A polymorphism in the staphylococcal enterotoxin A receptor gene (T cell receptor BV3 recombination signal sequence) is not associated with unexplained sudden unexpected death in infancy in an Australian cohort. (Under consideration by *Hum Immunol*) (Chapter 7)
Presentations by candidate arising from this thesis

1. Hight, AR, 2008, „Bacterial Toxins and Genetic Variations Sudden Infant Death Syndrome (SIDS)”. Young Investigator of the Year Semi-Finals Presentation, Adelaide, South Australia

2. Highet, AR, Goldwater, PN 2008, „Sudden Infant Death Syndrome: New research findings”. Children Youth and Women’s Health Service Grand Round Presentation, Adelaide, South Australia

3. Highet, AR 2008 „Toxigenic Escherichia coli and Sudden Infant Death Syndrome: A molecular approach” Paper presented to the Australian Society for Microbiology, South Australia general meeting, Adelaide, South Australia


   Won Best Scientific Presentation by a Young Presenter

6. Highet, AR 2007, „Immune responses to infectious agents in SIDS- Amanda”s research proposal” Presented to the Department of Microbiology and Infectious Diseases, Women’s and Children’s Hospital, Adelaide, South Australia

Contributions made by co-authors

Associate Professor Paul N Goldwater

1. Highet AR, Berry AM, Bettelheim KA, **Goldwater PN**. The frequency of molecular detection of virulence genes encoding cytolysin A, high-pathogenicity island and cytolethal distending toxin of *Escherichia coli* in cases of sudden infant death syndrome does not differ from that in other infant deaths and healthy infants. *J Med Microbiol*. 2009;58:285-289. (Chapter 2)

2. Highet AR, **Goldwater PN**. Staphylococcal enterotoxin genes are common in *Staphylococcus aureus* intestinal flora in Sudden Infant Death Syndrome (SIDS) and live comparison infants. *FEMS Immunol Med Microbiol (In Press)*. 2009; (Chapter 4)

3. Highet AR, Gibson CS, **Goldwater PN**. *Clostridium sordellii* lethal toxin gene is not detectable by PCR in the intestinal flora of Sudden Infant Death Syndrome cases or infants who died of other causes. *J Med Microbiol (In Press)*. 2009; (Chapter 5)


5. Highet AR, Berry AM, **Goldwater PN**. Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2. *Ann Med (In Press)*. 2009; (Chapter 8)

A/Prof Goldwater was my Principal Supervisor during my candidature. As well as co-authoring all of the above manuscripts, Paul had a role in designing the project, collecting of sample material and identities of SIDS cases for collection of Newborn Screening Cards, and of course the reading of many thesis drafts.

**Dr Catherine S Gibson**

1. Highet AR, **Gibson CS**, Goldwater PN. *Clostridium sordellii* lethal toxin gene is not detectable by PCR in the intestinal flora of Sudden Infant Death Syndrome cases or infants who died of other causes. *J Med Microbiol (In Press).* 2009; (Chapter 5)

Dr Gibson was my co-supervisor. She made corrections to many thesis drafts and helped to arrange the chapters in a logical order. She co-authored the manuscripts submitted towards the end of 2009 including the one cited above and another under consideration.

**Dr Anne M Berry**

1. Highet AR, **Berry AM**, Bettelheim KA, Goldwater PN. The frequency of molecular detection of virulence genes encoding cytolysin A, high-pathogenicity island and cytolethal distending toxin of *Escherichia coli* in cases of sudden infant death
syndrome does not differ from that in other infant deaths and healthy infants. *J Med Microbiol*. 2009;58:285-289. (Chapter 2)


3. Hight AR, **Berry AM**, Goldwater PN. Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2. *Ann Med (In Press)*. 2009; (Chapter 8)


Dr Berry was my co-supervisor and provided laboratory advice for the development of PCR assays. Anne co-authored the above manuscripts.

**Dr Karl A Bettelheim**

1. Hight AR, Berry AM, **Bettelheim KA**, Goldwater PN. The frequency of molecular detection of virulence genes encoding cytolysin A, high-pathogenicity island and cytolethal distending toxin of *Escherichia coli* in cases of sudden infant death syndrome does not differ from that in other infant deaths and healthy infants. *J Med Microbiol*. 2009;58:285-289. (Chapter 2)
Dr Bettelheim provided the *E. coli* strains used in the work described in Chapter 2. He also supplied previous results (i.e. verocytotoxicity) obtained for the strains and methods for the verocytotoxicity assays I conducted. Karl also co-authored the manuscript that covered this work (see above), in particular, analysis of the results in terms of serotypes.
Thesis explanation

Format of the Thesis

This thesis is written in a conventional form. Each different investigation forms a chapter presented in a manuscript style, each with a brief introduction, methods, results and discussion. An in-depth literature review (Chapter 1) sets the scene for the work described.

Explanation of the variance in the SIDS cohort throughout the thesis

The work presented in this thesis was conducted both retrospectively on stored material and prospectively on materials collected over a three-year period (2007-2009). Different samples have been used in different chapters, starting with available stored material (E. coli isolates and intestinal contents) and progressing to a large cohort of Newborn Screening Cards that were collected during the third year (2009). Samples from SIDS infants dying in Victoria were collected prospectively and were added to cohorts as they were received. The cohort used in each investigation also varied according to the appropriateness of the sample to an assay and the samples available at the time. For example, intestinal contents were used for bacterial investigations and pilot genetic studies and the genetic studies incorporated the Newborn Screening Cards (which had greater statistical power and appropriately matched controls) in the latter chapters as they became available.

In Chapter 1 “Sudden Infant Death Syndrome and unexplained Sudden Unexpected Death in Infancy in Australia” we introduce the discrepancies in terminology that affect SIDS research.
Over the course of this project we have tried to represent the most popular terminology during that time. The terms used in each chapter are also influenced by the sample type used and the preferred diagnosis at the time of its collection. For example: in the cohort for which we have dried bloodspots we were able to use the definition “SIDS” as they are currently recorded as so in the Pregnancy Outcomes Statistics Unit, SA Health, South Australia. However some of the samples collected in Victoria (included in Chapters 7 and 8) were classified as “unexplained Sudden Unexpected Death in Infancy” or “Unascertained”. In these chapters we have used the term uSUDI to reflect the non-descript cause of death.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>Confidence intervals</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes Simplex Virus 1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus 2</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist protein</td>
</tr>
<tr>
<td>LBW</td>
<td>Low birth-weight</td>
</tr>
<tr>
<td>NBW</td>
<td>Normal birth-weight</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SIDS</td>
<td>Sudden Infant Death Syndrome</td>
</tr>
<tr>
<td>SUDI</td>
<td>Sudden unexpected death in infancy</td>
</tr>
<tr>
<td>-iSUDI</td>
<td>Infectious sudden unexpected death in infancy</td>
</tr>
<tr>
<td>-niSUDI</td>
<td>Non-infectious sudden unexpected death in infancy</td>
</tr>
</tbody>
</table>
-uSUDI  Unexplained sudden unexpected death in infancy

TCRBV3S1 RSS  T-cell receptor V β 3.1 gene recombination signal sequence

TNFα  Tumor necrosis factor alpha

VNTR  Variable number of tandem repeats
Sudden Infant Death Syndrome and unexplained Sudden Unexpected Death in Infancy in Australia

According to the 2003 definition proposed by Beckwith, sudden infant death syndrome (SIDS) is “the sudden and unexpected death of an infant younger than 1 year and usually beyond the immediate perinatal period, which remains unexplained after a thorough case investigation, including performance of a complete autopsy and review of the circumstances of death and of the clinical history. Onset of the lethal episode was presumably during sleep. Minor inflammatory infiltrates or other abnormalities insufficient to explain death are acceptable” [1]. Despite the drop in the number of SIDS cases since 1990 following the Reduce the Risks education program, SIDS has not vanished; it still claims the lives of many Australian infants (Table 1). In the period 2000-2006 there were between 47 and 110 each year deaths due to “Sudden Infant Death Syndrome” (R95 classification of death) (Table 1). Although a genuine decrease in SIDS cases in South Australia, independent of changing terminology, was seen in 1987-1993 [2] the apparent recent decrease in the number of SIDS deaths after the year 2000 is in part due to the increase in the number of deaths due to other ill-defined and unspecified causes. This has been reported by the Australian Bureau of Statistics who quote “In 2006, there were 66 deaths identified as being due to Sudden Infant Death Syndrome (SIDS) (R95). This was fewer than in 2005, when 87 deaths were attributed to SIDS. The decrease in the number of SIDS deaths is in part due to the increase in the number of deaths due to Other ill-defined and unspecified causes (R99). There are 64 deaths in 2006 under the age of 1 year with Other ill-defined and unspecified causes (R99) as the underlying cause of death. In 2005 there were 38 deaths under 1 year of age with the same
underlying cause. In processing Causes of Death, the ABS will only code a death to SIDS if specifically mentioned on the death certificate.” (Australian Bureau of Statistics 3303.0 - Causes of Death, Australia, 2006).
**Table 1.** SIDS deaths in Australia by age range and cases per year (2000-2006)

<table>
<thead>
<tr>
<th>Year</th>
<th>0-3 m</th>
<th>4-6 m</th>
<th>7-9 m</th>
<th>10-12 m</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 (from 1st July)</td>
<td>31 (66%)</td>
<td>11 (23.4%)</td>
<td>5 (10.6%)</td>
<td>0</td>
<td>47 (100%)</td>
</tr>
<tr>
<td>2001</td>
<td>78 (70.9%)</td>
<td>26 (23.6%)</td>
<td>5 (4.5%)</td>
<td>1 (1%)</td>
<td>110 (100%)</td>
</tr>
<tr>
<td>2002</td>
<td>69 (68.3%)</td>
<td>22 (21.8%)</td>
<td>6 (5.9%)</td>
<td>4 (4%)</td>
<td>101 (100%)</td>
</tr>
<tr>
<td>2003</td>
<td>40 (61.5%)</td>
<td>20 (30.8%)</td>
<td>4 (6.2%)</td>
<td>1 (1.5%)</td>
<td>65 (100%)</td>
</tr>
<tr>
<td>2004</td>
<td>33 (57.9%)</td>
<td>18 (31.6%)</td>
<td>4 (7%)</td>
<td>2 (3.5%)</td>
<td>57 (100%)</td>
</tr>
<tr>
<td>2005</td>
<td>69 (76.6%)</td>
<td>15 (16.7%)</td>
<td>5 (5.6%)</td>
<td>1 (1.1%)</td>
<td>90 (100%)</td>
</tr>
<tr>
<td>2006**</td>
<td>36 (66.7%)</td>
<td>12 (22.2%)</td>
<td>2 (3.7%)</td>
<td>4 (7.4%)</td>
<td>54** (100%)</td>
</tr>
</tbody>
</table>

**Possible under-representation due to cases not yet having the medical cause of death field completed at time of data publication (Adapted from Australian National Coroner’s Information System (NCIS) Bulletin: Sudden Unexplained Infant Deaths 1 July 2000-18 May 2007)**
In some Australian states “SIDS” terminology is now used sparingly. For example: In the period 1st July 2000 to 18th May 2007, only 4.4% of South Australian unexplained infant deaths were termed “SIDS” and 39.1% termed “Undetermined (SIDS)” and 54.3% “Undetermined”. According to the Australian Bureau of Statistics the latter two fall into the category of “Other ill-defined and unspecified causes” (R99) and not “SIDS” (R95). Victoria on the other hand prefers use of the term “SIDS” (79.6%) but also use “Unascertained” (19%). (Australian National Coroner’s Information System (NCIS) Bulletin: Sudden Unexplained Infant Deaths 1 July 2000-18 May 2007). Considering that an unknown proportion of “undetermined and unascertained” deaths overlap with SIDS, the true incidence of SIDS is probably higher than the official statistics. While classification is important for statistical and epidemiological purposes and the basis for research, understanding the mechanisms underlying these deaths is of greater importance for development of preventive interventions. Data from England and Wales strongly suggest a similar disparity in defining sudden infant death and similarly indicates an artificial reduction in SIDS cases through terminological reassignment. Figure 1 demonstrates how an increase in unascertained deaths is “mirrored” by a decrease in SIDS, and vice-versa. Similar data has been described in the United States [3]. Use of the term SIDS is often dependant on the preference of the pathologist. A survey of pediatric and forensic pathologists, and general histopathologists in the United Kingdom revealed that 54% of unascertained infant deaths were diagnosed as so because of pathologist’s preference, forensic pathologists more frequently used the term “unascertained”. Criticism of the term SIDS by pathologists exists because SIDS implies a single disease process and prevents further investigation and enquiries, including investigation of the death scene, or is seen as a lazy way of admitting “I don”’t know what happened” [4].
NOTE:
This figure is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.** (Data source: http://www.statistics.gov.uk/STATBASE/)
Due attention has been given to infectious agents and immune responses to infection in SIDS. It has been acknowledged that the pathological, epidemiological and genotypic findings in SIDS infants suggest an infectious aetiology possibly being potentiated by immunoregulatory polymorphisms, however, the cause of SIDS is a mystery and remains open to debate. SIDS investigations frequently ignore key aspects and there seems to be a need to further take into account the inimitable pathology that is generally accepted as being characteristic of SIDS. Similarities exist between these findings and the pathogenesis of toxaemic shock, and the relatively consistent nature of these post mortem findings in SIDS infants suggests that there is a single common factor that contributes to their death. The findings that are consistent with toxaemic shock or sepsis warrant further analysis of the micro-organisms found in SIDS, in particular the roles of *Staphylococcus aureus*, *Escherichia coli*, *Clostridium spp*, and respiratory and enteric viruses. This perspective of SIDS also takes into account genetic predisposition which may influence responses to the above potential microbial triggers.

**Definition of SIDS in Australia**

The following definition published in 2004 by Krous et al. [1] is currently upheld in Australia. SIDS is defined as „the sudden unexpected death of an infant <1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history.”

**Category IA SIDS** cases are those that display the classic features of SIDS, which may include intrathoracic petechial hemorrhage, and are completely documented. The additional criteria required for this classification include: 21 days-9 months of age; unremarkable
clinical history including full term pregnancy; normal growth and development and no similar deaths among siblings, close genetic relatives or other infants in the custody of the same caregiver. The death scene, in particular the sleeping environment, does not provide an explanation for the death and there is an absence of potentially fatal pathologic findings including trauma, neglect, metabolic disorders, or positive toxicology. Minor respiratory system inflammatory infiltrates are acceptable [1].

**Category IB SIDS** exhibit the classic features of SIDS except one or more of the additional criteria for SIDS 1A (i.e. death scene investigation, metabolic screening, toxicology) have not been completed or was not documented [1].

**Category II SIDS** are cases that meet Category I criteria except one or more of the additional criteria are documented to have not been met, i.e. 0-21 days or >9 months of age, similar death among siblings, preterm birth complications that have not resolved by the time of death or marked inflammatory changes insufficient to cause death [1].

**Sudden Unexpected Death in Infancy (SUDI)** is a term used to describe infant deaths between 7 and 365 completed days of life that include: deaths that were unexpected and unexplained at autopsy, deaths during an acute illness that was not recognised as life-threatening, deaths due to an acute illness of less than 24 h duration in a previously healthy infant, deaths from a pre-existing occult condition, and deaths from any form of accident, trauma or poisoning [5]. Unexplained SUDI has been used in research to describe the deaths that were unexpected and unexplained at autopsy where SIDS is not appropriate.
SIDS Pathology

In 1834 SIDS pathology was first identified in the medical literature [6]. 175 years later the there is still no coherent, widely accepted mechanistic explanation, yet a number of major risk factors have been identified [7]. The definition of SIDS has been constructed by exclusion of explained death in infants and gives no identity to SIDS as a disease with a distinct aetiology. Infants that have succumbed to SIDS often have consistent pathological findings, including unclotted blood inside the chambers of the heart, elevated cross-linked fibrin degradation products, small intrathoracic hemorrhages (petechiae) [8], and fluid-laden organs that are reported to be significantly heavier than normal [9]. These findings all display similarities to the pathogenesis of toxaemic shock and/or sepsis. Histological signs of shock including fibrin thrombi in capillaries, megakaryocytes outside the lung, tubular cell necrosis and heart shock were found in 56.4% of SIDS cases studied by Ramboud et al. [10]. The pathology of SIDS often includes an unusual distribution of petechiae which does not present on the conjunctiva, eyelids or any of the soft tissues of the head or neck, but instead involve the intrathoracic organs (thymus, pleura and epicardium). Although this pattern is found in cases of asphyxia, the absence of extrathoracic petechiae point to a non-asphyxial mode of death. The distribution and density of petechiae differs significantly from that found in asphyxia [11, 12]. Moreover, the absence of anatomical airway obstruction is almost universal in SIDS [9]. Infants who died of SIDS whilst on 24 hour memory monitors for recording pulse, respiratory rate and blood pressure showed gasping and cardiac standstill shortly before death [13] indicating the airway was open and functional. Other typical morphologic findings in SIDS include pulmonary congestion and oedema, persistant hepatic erythropoiesis and an empty urinary bladder.
Organ weight anomalies

A hallmark of SIDS pathology is a pattern of organ weights that distinctly deviate from the expected reference ranges. The thymus (p=0·04) and brain (p=0·001) in particular are significantly larger in SIDS victims [14]. Although it was inferred that this was likely due to the reference ranges being based upon infants with illness prior to death (whereas SIDS appear to be healthy immediately before death), the investigators also recognised that the “differences could also reflect disturbances in growth presumably beginning prior to birth”. Other researchers have produced supportive data that suggest that enlarged brains are present at birth of the SIDS infant, rather than a consequence of disproportionate postnatal growth [15]. Extrapolation of post-mortem organ weight data show that disrupted patterns of growth of vital organs appear to originate in the prenatal period [16]. One possibility is “thymic priming” via a massive T-cell proliferative response to a foreign protein or pathogen, possibly prenatally. In this case the enlarged thymus would be consistent with increased thymic activity. A likely cause of this is prenatal viral or bacterial exposure.

Substantiation of an infectious aetiology

The major risk factors for SIDS parallel those for increased colonisation and serious bacterial infections. Such examples are prone sleeping position and exposure to cigarette smoke [17]. The risk factors for SIDS may also predispose the infant to the lethal effects of the toxins that are produced by the colonising bacteria, by affecting the baby’s immune competency [18]. The natural variation in the incidence of SIDS cases is typical of an infectious disease (Figure 2), which may rise to high prevalence for a number of decades and then decrease. For example, this phenomenon is seen in B. pertussis infection in New Zealand, where low
incidence was observed between 1873 and 1910 (<2 per 100,000) before a dramatic increase took place from 1910 to 1945 (>12 per 100,000) [19] (Figure 3). The incidence of SIDS follows such a trend: the rate of SIDS was low prior to World War II peaked in the 1980s then decreased again in western countries after 1990 [20]. The seasonality of SIDS appears to be independent of low temperature. In Hawaii, where the temperature remains constant over the seasons, the SIDS incidence still peaks in the winter [21]. This suggests that another factor which varies by season, such as infection, is involved in SIDS in this region.
Figure 2  

a) SIDS in South Australia 1989, 1990 and 2002, b) Influenza in South Australia 2002. Note the number of SIDS and Influenza cases both peak in the winter months. c) SIDS South Australia 1980-2004 shows the consistency of this pattern. Influenza data from Communicable Diseases Australia, SIDS data from Australian Bureau of statistics.
Figure 3 a) SIDS Australia 1980-2004 and b) B. pertussis infection, New Zealand, 1873-2004 Adapted from Somerville et al. [19].
Bacteria isolated from SIDS infants interact synergistically to cause sudden death in gnotobiotic weanling rats, in which the composition of microbial flora present is fully defined, and their toxins interact synergistically to cause death in chick embryos [22, 23]. Furthermore, the addition of nicotine to combined bacterial toxins potentiates their lethal effects [24]. There is considerable evidence that infants exposed to cigarette smoke have similar levels of nicotine by-products in their body to those of active smokers which may add to the lethality of bacterial toxins. Further evidence for the “SIDS toxin hypothesis” lies with the fact that serum from SIDS infants, presumably carrying toxins, is lethal to infant mice and chick embryos, whereas control sera from non-SIDS infants is not [23, 25]. The prone sleeping position is another major risk factor that may also be attributed to increased bacterial colonisation. Infants sleeping prone (particularly on contaminated sleeping surfaces, e.g. sofas) would be more likely to become colonized, and would accumulate more potentially toxigenic nasopharyngeal bacterial flora, than those sleeping supine [26]. Male gender is over-represented in every infection (except pertussis) and in SIDS which suggests there could be an X-linked allele predisposing males to most infections and as a consequence SIDS [27]. The particular vulnerability of males over females to SIDS could also be related to the binding of bacteria. Studies by Harrison et al. demonstrated that males sleeping in the prone sleeping position had significantly higher counts of Gram-positive cocci, including *S. aureus*, than prone females. Interestingly, carriage of *S. aureus* was in parallel to a number of SIDS aspects; it was more common in winter months, greater in males than females and decreased with age [28]. It is important to recognise that there does not necessarily have to be evidence of invasive infection for infection to be implicated; colonization alone would suffice. Deaths from toxic shock syndrome or infant botulism are examples where bacterial toxins cause fatalities without displaying characteristic pathology associated with invasive infection. This
may be significant in SIDS because evidence of invasive infection and cause of death are not usually found [24]. It is noteworthy that a small number of cases of bacterial sepsis (evidenced through sterile site, e.g. cerebrospinal fluid, isolation of pathogens) have been mistaken for SIDS [29]. Examination of many known bacterial toxins from species such as *Bordetella pertussis*, *Haemophilus influenzae*, *Clostridium perfringens*, *Streptococcus pyogenes* and *Helicobacter pylori* has shown that their relationship with SIDS is variable or unsubstantiated. Despite this there are still many bacterial toxins which remain incompletely tested or untested and are worthy of investigation.

**Enteric bacteria**

Most of the organisms suspected of being concerned with SIDS are intestinal bacteria because diarrhoea is a well known associated factor for SIDS [30, 31] and gastrointestinal illness in the last two weeks of life has been shown to be strongly associated with SIDS [32]. The *Results of the National Institute of Child Health and Human Development of SIDS Cooperative Epidemiological Study* report that gastrointestinal illness is strongly associated with SIDS after they found in a cohort of 838 SIDS, each with two controls, that vomiting in the last two weeks before death was significantly more common in SIDS infants (14.6%) than live control infants during the same period (7.7% and 9.7%, the latter being matched for birthweight) \((p=<0.001)\) [32]. Similarly diarrhoea occurring in the last two weeks prior to SIDS death was more common to SIDS infants (19.1%) compared with the controls (12.2 and 12.0%) \((p=<0.001)\).

The potential role of enterotoxigenic bacteria in SIDS has been recognized and it has been found that a significantly higher proportion of toxigenic bacteria are found in the faeces of
SIDS infants than in controls [33]. A study on the effects of enterotoxins by Siarakas et al. demonstrated at critical concentrations, enterotoxins induced hypotension, reduced heart rate, blood pressure and breathing and caused a quiet death in rabbits [34]. This animal model is comparable to SIDS, especially considering that similar signs to the abovementioned were observed in the SIDS deaths recorded on memory monitors. Notably, greater suppressive reactions were observed when the enterotoxins were combined and administered at low doses [34]. Pre-term birth may be explained to be a risk factor for SIDS because pre-term infants have delayed colonisation of the throat and stomach compared with full term infants, and so lack normal flora which is considered to prevent colonisation by toxigenic species [30].

**Staphylococcus aureus**

SIDS infants have notably increased colonisation by *S. aureus* (Harrison et al., 1999), and *E. coli* [35]. Since the introduction of the *common bacterial toxins hypothesis* in 1987 [36], *S. aureus* has been suspected to be implicated in SIDS. Its presence has been demonstrated in 86% of SIDS infants [37] and staphylococcal toxin detected in the tissues [38] and faeces [33] of 53% and 20% respectively. A number of findings characteristic of SIDS can be explained with respect to *S. aureus* involvement. Firstly, it has been demonstrated that the prone sleeping position can increase nasopharyngeal colonisation by bacteria [28], and that approximately 42% of cot mattresses from SIDS infants harbour *S. aureus* [39]. Thus infants sleeping in the prone position are likely to be colonized by *S. aureus*. Secondly, the decline in the expression of the Lewis\(^a\) antigen, a *S. aureus* receptor, parallels the decrease in frequency of isolation of *S. aureus* from infants, and the age when the expression level of Lewis\(^a\) peaks correlates with the peak of SIDS deaths at 2-4 months [17]. Viral infection of human respiratory epithelial cells (a common finding in SIDS) causes changes in cell surface antigens which act as receptors for bacteria, thus significantly enhancing the binding of *S.
*aureus* [17]. *In vitro* experiments have found IgA antibodies to *S. aureus* toxic shock syndrome toxin (TSST), and staphylococcal enterotoxin C (SEC) present in human milk and in higher titers in ethnic groups with lower SIDS incidence, which may explain the protective effect that breast milk has against SIDS as well as variable SIDS frequencies between ethnic groups. Epidemiological studies have shown that immunization with diphtheria, pertussis and tetanus vaccine decreases an infant’s risk of SIDS. Investigating this phenomenon, Essery *et al.* have demonstrated that the protective effect is most likely due to cross-reacting antibodies that neutralize staphylococcal toxins implicated in SIDS [40].

**Staphylococcal enterotoxins and the intestinal tract in SIDS**

Staphylococcal enterotoxins (SEs) are exotoxins secreted by some strains of *S. aureus* and contribute to the pathogenesis of toxic shock. *S. aureus* can be found in many body sites, but of particular interest is the occurrence of *S. aureus* in intestinal contents. Kamaras and Murrell [41] used the rabbit intestinal epithelium to demonstrate the effects of enterotoxigenic bacteria and their toxins on the intestinal mucosa. Both the degree and nature of the damage caused by the toxins were comparable to the mucosal damage that is observed in most SIDS intestinal samples, ranging from damage to villous tips to the removal of epithelial cells and damage to the lamina propria and was more significant when SE was detected in the faeces [41]. When the epithelium has been damaged bacteria gain access to the enterocytes and interstitial space and toxins can be taken up into the blood vessels of the lamina propria and submucosa. Thus there is greater toxin absorption and subsequently a chance of toxemia and death [42]. If a bacterial toxin plays a role in SIDS, a lethal dose of the toxin can be absorbed into the bloodstream through the intestinal walls very rapidly, particularly if there is damage as occurs in a number of cases of SIDS. Anecdotally, mucosal damage observed by pathologists is often
passed off as autolysis and has not been a reason to instigate further investigation (Goldwater, PN, personal communication).

**Escherichia coli**

*E. coli* is of particular interest to SIDS research because early and persistent colonisation of the bowel of neonates renders it sufficiently common to fit the age distribution of SIDS and *E. coli* is often isolated from the intestinal contents of infants who have died of SIDS [35]. Interestingly, the *E. coli* found in the intestinal tract of SIDS infants are typically extra-intestinal serotypes [43]. Several studies to date have identified a link between toxin secreting strains of *E. coli* and SIDS, toxin producing *E. coli* have been found in a high proportion of SIDS infants (39%) but less in comparison healthy babies (1.5%) [35]. The *E. coli* found in these instances are generally of different serotypes from those expected to be isolated from infants [44], and the isolates from SIDS babies, but not healthy babies, are lethal to mice [22]. A study by Bettelheim and Goldwater in 1990 found that 46% of SIDS cases were carrying toxigenic strains of *E. coli*. In the comparison group of live infants, 83% had *E. coli* isolated, but none of them were toxigenic. Also no toxigenic strains could be isolated from the control group of infants who died from other causes [35]. Despite these findings, neither shiga toxin nor heat labile enterotoxin has been attributed to the causation of SIDS, which is accompanied by a lack of compatible pathological evidence that would reflect the effects of these toxins. In 1995 Bettelheim et al. carried out verocytotoxicity assays on *E. coli* isolates from 76 healthy infants, 64 SIDS infants and 20 infants whom had died of other causes [45]. They reported that the *E. coli* from 51.6% of SIDS infants induced vero cell monolayer disruption (affecting ≥50% of cells) compared with only 25% of those from healthy infants and 3.9% of strains from dead controls. The presence of *stx* genes in the “VT”+ isolates could not be confirmed by polymerase chain reaction (PCR) at the time of the study and it remains open that the VT+
strains described in 1995 that induced subtle but distinct changes to vero cell monolayers were non-Stx verotoxigenic. The “verocytotoxin” may indeed be a novel distinct toxin as perceived by the nature of the verotoxicity.

A study investigating the role of extraintestinal *E. coli* in SIDS showed that 15% of SIDS infants had *E. coli* isolated from their blood and 25% had *E. coli* isolated from their lungs. Those with respiratory tract inflammation, a risk factor for SIDS, were more likely to yield *E. coli* in the upper respiratory tract than those without inflammation [30]. Comparison of *E. coli* isolates from sterile sites and lung is difficult to interpret because similar rates of isolation are found in non-SIDS deaths [29, 46].

Human uropathogenic *E. coli* (UPEC) and avian pathogenic *E. coli* (APEC) both belong to the extraintestinal pathogenic *E. coli* subgroup and share overlapping serotypes and phylogenetic and virulence traits. Human UPEC strains have been shown to share pathogenicity-related genes with APEC strains, particularly those located on the High Pathogenicity Island (HPI) which facilitate the uptake of iron and thus survival of the bacterium in extra-intestinal body sites. HPI is found in extraintestinal *E. coli* strains associated with urinary tract infection and sepsis [47]. Interestingly, the predominant *E. coli* found in the intestinal tract of SIDS infants are typically extra-intestinal serotypes and are more consistent with UPEC [45] and thus possibly APEC. APEC produce a high mean lesion score for lethality in one-day-old chicks, perhaps similar to the high lethality of SIDS *E. coli* to mice whereas those isolated from healthy infants were not lethal. Also the recent reporting of *E. coli* in normally sterile sites [48] and coliforms in extraintestinal sites such as the upper
respiratory tract [49] at post-mortem investigation of sudden unexpected death in infancy, including SIDS, may be related to the effects of such islands as the HPI.

Bacteria such as *Staphylococcus aureus* and *Escherichia coli* secrete proteins that act as super-antigens causing uncontrolled overstimulation of the immune system. These species of bacteria are commonly isolated from SIDS infants and thus it remains possible that superantigens could play a role in SIDS pathogenesis. In 2002 a study was undertaken to explore a possible role for curli-producing strains of *E. coli* in the causation of SIDS. All of 92 *E. coli* isolates from SIDS infants produced curli on congo red agar (an indicator of curli protein production). Significantly fewer control isolates from non-SIDS deaths and healthy babies produced curli (76.5% and 80.4% respectively) [50]. Soluble curlin and other bacterial proteins act as potent proinflammatory stimulators and could invoke, with the help of concurrent viral infection, an infant’s immune system to respond abnormally, especially if certain polymorphisms in immunoregulatory genes facilitate a progression to a cytokine storm resulting in circulatory shock [51].

*Clostridium perfringens*

*C. perfringens* has a significant presence in the intestinal tract of SIDS infants. In 1993 Lindsay *et al* demonstrated that high numbers of the organism colonise greater than 80% of SIDS infants compared with less than 2% of non-SIDS control infants, and that *C. perfringens* type A cytotoxic enterotoxin (CTE) was detectable by ELISA coinciding with the presence of the organism. Furthermore, histopathological change to the SIDS infants’ ileal tissue showed remarkable similarity to animal models of CTE damage [52], and in rabbit models CTE has been shown to contribute to intrathoracic petechiae, a pathological hallmark of SIDS [53].
Viral and bacterial infections induce the synthesis of interferons, notably interferon gamma, which can sensitise cells by enhancing permeability, allowing rapid uptake of toxins and reducing the amount required for a lethal dose [54]. Given that a mild viral infection is a risk factor for SIDS, intraintestinal production of *C. perfringens* CTE may contribute to the pathogenesis, particularly where other bacterial toxins are concerned.

*Clostridium botulinum*

Produced by *C. botulinum*, botulinum neurotoxin (BoNT) is the most toxic protein known. BoNT acts by preventing the release of acetylcholine from vesicles at the presynaptic membranes of the motor neurons. Death is a result of flaccid paralysis caused by the toxin’s blockage of neuromuscular signals leading to respiratory failure and/or cardiac arrest [55]. When spores from *C. botulinum* are ingested by an infant they may germinate with vegetative bacterial colonisation and produce BoNT in the intestinal lumen. The resultant disease is infant botulism. The particular susceptibility of infants to *C. botulinum* infection is due to the ideal physiological conditions for spore germination in the infant intestine; the establishment of normal flora usually prevents infection by these organisms but in some young infants this flora may not be established well enough. Infant botulism differs from classical botulism, where the pre-formed food-borne toxin is ingested, because in the latter no colonisation by *C. botulinum* occurs. The fulminant form of infant botulism may lead to sudden and unexpected death which may be deemed to be SIDS. This proposition has been previously demonstrated by Arnon *et al.* [56], whose research concluded that infant botulism can lead to the rapid death of an infant and therefore may account for a small percentage of SIDS cases.

Undetected *C. botulinum* infections have been estimated to cause between 3 and 20% of SIDS deaths, depending upon the prevalence of infant botulism in the region [57]. The curve of age
incidence for infant botulism is almost identical to that of SIDS age incidence [20], thus signifying a relationship between the two.

_Clostridium botulinum_ is not normal flora, and so should not be found in a healthy infant; however, rare occurrences of asymptomatic carriage have been reported. When Bohnel et al. tested 75 tissue samples sourced from autopsy, 57 of which were SIDS cases, free toxin (BoNT) was found in nine samples and typical bacteria detected in another six [58]. The presence of either fits the criteria to assign death to infant botulism. However, these findings are not always consistent. A screening study via culture of small and large bowel contents of 248 infants presenting with SIDS conducted at the Adelaide Children’s Hospital (now the Women’s and Children’s Hospital) produced no isolates of _C. botulinum_ [59].

Due to advances in infant botulism research we now recognize that two other species of _Clostridium_ can produce neurotoxin. BoNT toxigenic _Clostridium butyricum_ and _Clostridium baratii_ have neurotoxin genes that are moderately homologous to those of _C. botulinum_. _C. butyricum_ can produce toxin type E and was first identified as a cause of infant botulism in Italy in 1984 [60]. _C. baratii_ can produce toxin type F and was isolated as the causative agent of a case of infant botulism in 1979 [61]. In the first instance these organisms were determined to be _C. botulinum_ by toxin neutralisation although their cultural and biochemical properties were quite different.

The ability of BoNT to cause sudden unexpected death in infants, and the strong correlation between the SIDS age incidence curve with that of infant botulism, suggest that the two are related. Studies conducted simply by _C. botulinum_ culture may be failing to show this
relationship. The integration of polymerase chain reaction (PCR) into clinical and research laboratories has increased the ability to detect via small amounts of DNA, organisms that are fastidious in culture, have genes for toxins that are atypical of that species, or recently discovered toxins for which there is no immunochemical or biological test. Post-mortem samples may contain other bacteria which produce proteolytic enzymes and destroy free toxin or impede growth of the organisms or their toxin production [58] and so culture of post mortem samples may be misleading. Therefore, we expected that PCR screening for BoNT toxin genes may better characterise the true inhabitants of the intestinal tract of infants dying of SIDS. Since elements of SIDS seem to be attributable to a fast acting and potent neurotoxin such as BoNT, other BoNT detection methods were investigated further before the relationship was ruled out. We screened cultures grown from the intestinal contents of 51 SIDS and 19 infants that had died from other causes for the presence of BoNT A, B, E, F and G encoding genes. We found none to be positive. To investigate the possibility of BoNT toxigenic species being a non-pathogenic inhabitant of the infant intestinal tract we also screened 200 cultures grown from the intestinal contents of healthy infants and yielded no positive results (unpublished data). Here our findings were in line with other studies carried out in Australia, which demonstrate that undetected botulinum infections are not likely to account for SIDS death in this population.

**Clostridium sordellii**

Infection by *Clostridium sordellii* translocating from the gastrointestinal tract has been reported to cause septic shock, often resulting in fatality [62]. The patients shown in Table 2 had underlying medical conditions; however, more recently several cases have been described to occur in previously healthy women who underwent medically-induced abortion [63] or shortly after delivering a baby (vaginal or cesarean section) [64-71]. Pregnancy and childbirth
have been suggested to predispose a small number of women to acquire *C. sordellii* in the vaginal tract, where the acidic pH of the vagina enhance the cytopathic effects of its toxins [63]. The fastidious anaerobic growth, variable staining characteristics and complex biochemical profiles of clostridia probably contribute to a lack of non-lethal *C. sordellii* infection reported in the literature [63]. It is not known whether an infant born to a mother asymptotically colonised by *C. sordellii* acquires the organism at birth through the ingestion of vaginal flora, as has been shown for other organisms such as *Escherichia coli* [72].
Table 2. Reported cases of *Clostridium sordellii* bacteraemia reported in the literature, adapted from [62]

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age (years)/ sex</th>
<th>Presumed portal of entry</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>[73]</td>
<td>18/ male</td>
<td>Gastro-intestinal tract/colon</td>
<td>Fatal</td>
</tr>
<tr>
<td>[74]</td>
<td>61/ male</td>
<td>Oropharynx</td>
<td>Survived</td>
</tr>
<tr>
<td>[75]</td>
<td>54/ male</td>
<td>Gastro-intestinal tract/colon</td>
<td>Fatal</td>
</tr>
<tr>
<td>[75]</td>
<td>40/ female</td>
<td>Unknown</td>
<td>Survived</td>
</tr>
<tr>
<td>[76]</td>
<td>37/ male</td>
<td>?Gastrointestinal tract/colon/?auto-inoculation</td>
<td>Survived</td>
</tr>
<tr>
<td>[77]</td>
<td>48/ female</td>
<td>? Transcutaneous liver biopsy</td>
<td>Fatal</td>
</tr>
<tr>
<td>[78]</td>
<td>55/ male</td>
<td>Gastro-intestinal tract/colon</td>
<td>Fatal</td>
</tr>
<tr>
<td>[64]</td>
<td>29/ female</td>
<td>Gastro-intestinal tract/colon</td>
<td>Fatal</td>
</tr>
<tr>
<td>[79]</td>
<td>37/ male</td>
<td>?Gastrointestinal tract</td>
<td>Fatal</td>
</tr>
<tr>
<td>[80]</td>
<td>73/ male</td>
<td>Gastro-intestinal tract/rectum</td>
<td>Fatal</td>
</tr>
<tr>
<td>[62]</td>
<td>12/ male</td>
<td>Ear</td>
<td>Survived</td>
</tr>
</tbody>
</table>
The major virulence factor of *C. sordellii* is lethal toxin (LT). This toxin belongs to a family of large clostridial cytotoxins [81]. Notable clinical features of *C. sordellii* toxic shock include an absence of fever and rash, refractory tachycardia, hypotension, diarrhea and a relatively short time course (<1 day from hospitalization to death) [63]. These are not unlike the observations made prior to SIDS death, in particular where the event was recorded on memory monitors to include a cardiogenic event preceded by bradycardia [13]. The organism has only been isolated from the blood during one of fourteen cases of fatal toxic shock syndrome reported in the literature [63] and it is recognized that toxin produced from a localized site can cause a shock-like death without bacteraemia [68, 69]. This organism has not yet been investigated in SIDS, in particular the possibility of colonising intestinal *C. sordellii* contributing to sudden unexplained infant death, possibly via toxin absorption into the bloodstream.

**Viral infection**

As mentioned previously, the epidemiology of SIDS strongly suggests that viruses may contribute to an infectious aetiology, either directly or subtly via synergistic interactions with bacterial virulence factors or immunoregulatory polymorphisms. The lethality of bacterial toxins is potentiated by viral infection [28, 82] i.e. sublethal doses of toxin are made lethal through the presence of viral infection. The mechanism by which this occurs is not understood but a cytokine storm is considered most plausible [51]. Considering an infectious hypothesis, viral respiratory infections are the most likely trigger for SIDS.

Where good data exist, SIDS incidence is highest in cooler climates (e.g. New Zealand) and in winter months, which coincides with the increased incidence of several infections under
these conditions [35]. This trend is not likely to be dependant upon temperature since the same seasonal peaks are observed in regions where the climate remains relatively constant, i.e. Hawaii [21]. Since a mild viral infection is a risk factor for SIDS, viral involvement has been considered. The involvement of viruses may be direct, i.e. induction of a cytokine storm upon viral infection, or indirect; colonisation and binding of bacteria including toxigenic species is increased under the conditions of viral infection. Pre-treatment of non-smokers buccal epithelial cells with water-soluble cigarette smoke extract, at serial dilutions down to 1/300, significantly enhanced bacterial binding to the cells. The accumulation of large numbers of bacteria in the respiratory tract due to these increased binding conditions may contribute to SIDS [83]. Alternatively, the contribution of cigarette smoking to the risk of SIDS may relate to its decreased catabolism during infection; activation of systemic host defenses in response to a viral pathogen which causes down-regulation of enzymes involved in toxin metabolism and elimination [84].

Numerous case-control studies have found that viruses are more commonly isolated from SIDS infants than controls [85] with an average isolation rate of 22% and 8% respectively [86]. SIDS infants display similar rates of major respiratory illness to age and sex matched control infants in the two weeks prior to death. However SIDS infants have a significantly higher incidence of minor symptoms, as often indicated in early case notes, during this period. Significantly high concentrations of interferon alpha (IFN-α), an early response molecule to viral infection, are found in nasopharyngeal aspirates from SIDS infants not only when the infant tests positive for virus, but in virus-negative cases also. This suggests that a viral infection was present and has either resolved before death, or the virus was simply undetectable at post mortem or as yet uncharacterized viruses were present.
Respiratory Viruses

A mild viral respiratory infection is commonly seen in up to 80% of SIDS cases [87] in the days preceding death, and evidence of infection is found on post mortem examination. Trachaeitis and/or bronchiolitis are considered classical histopathologic findings in SIDS, yet the degree of inflammation in the trachea and bronchioles is considered inadequate to cause death [88]. Furthermore, SIDS infants have been found to have an increased number of immunoglobulin-producing cells in their salivary glands [89] and tracheal wall [90] which suggest a recent stimulation of the mucosa immune system, i.e. by viral antigen. The consistent presence of the viral infection in SIDS should not be ignored as it may be a clue leading to the true cause of SIDS.

In studies of SIDS virology in Melbourne, Australia, 39% of SIDS infants yielded one or more viruses and 21% of SIDS cases yielded respiratory viruses. Correlation with respiratory virus isolation rates from the Royal Children’s Hospital demonstrated that the monthly isolation rate for respiratory viruses followed the incidence of SIDS during the same time period [91]. In Canterbury, New Zealand, where the SIDS rate is high, Ford et al. reported that a weak association between RSV, influenza A and influenza B with SIDS is most probably due to the effect of independent seasonality in both respiratory viral infection and SIDS [92]. To date no single respiratory virus appears to have been exclusively found in SIDS infants, rather a range of these viruses are found at a higher frequency in this population compared with controls. 24% of SIDS lung parenchyma were found to be positive for respiratory viruses including Respiratory Syncytial Virus (RSV), parainfluenza 2 and adenovirus compared with 3.3% of controls [93]. Influenza B has been found in 6.6% of SIDS but not in controls [94], though in this study the control group, 13 non-natural deaths, may
have been insufficient in number to accurately profile the prevalence of influenza B in the population.

RSV has been considered a candidate virus for involvement in SIDS. Typically RSV infects infants that are over one month old and spares those younger, as does SIDS [95]. Preliminary data suggests RSV is found at similar frequencies in lung specimens from SIDS and control infants [96]. It has been suggested that a window of significant difference opens after three months of age where RSV is detected (by immunofluorescence and isolation methods) in a greater number of SIDS compared with live control infants [97]. It is likely that both SIDS and control infants undergo similar levels of exposure to RSV in the first three months of life as anti-RSV IgG immunoglobulins are found in similar frequencies in both groups [97], and there is a similar age distribution between infants admitted to hospital with RSV bronchiolitis and the age incidence of SIDS [98]. Polymorphisms in toll-like receptor genes (i.e. TLR-4 D259G) are associated with increased severity of RSV infection, causing altered local immune responses and disrupted clearance of bacteria. Furthermore, many viruses produce products with significant structural and functional homology to host cytokines such as interferon and other defense molecules thus escaping recognition [99]. It may be the infant’s own genetic predisposition to the effects of viral infection that determines the risk of SIDS, and not simply the presence of a virus.

**Enteroviruses**

For the fact that diarrhoea is a risk factor for SIDS, enteroviruses are of interest to SIDS research. A study conducted in 1966 isolated five strains of coxsackie B, one of coxsackie A and three strains of echovirus from cases of sudden infant death. Notably all of the coxsackie
B isolates were yielded from spinal cord and lung specimens rather than the faeces [85]. Conflicting studies however suggest that the rates of isolation of enteroviruses and adenoviruses do not differ significantly in SIDS compared to controls [35], and that a viral infection, usually mild, is considered a contributing risk factor for SIDS rather than the causative agent.

Virus isolation in post-mortem samples is rarely positive [94] and success of viral isolation can be hampered by low viral load, degradation of the virus (particularly in the case of RNA viruses which are labile) or the need for fastidious culture conditions. Thus, the actual incidence of viral infection may in fact be a lot higher. Postmortem isolation of respiratory viruses has often given conflicting results with some studies concluding that there is a higher isolation rate from SIDS infants, whilst others observe no difference [100]. Despite these conflicting data it is generally accepted that epidemiological and pathological evidence suggest a role is played by viral infection in the pathogenesis of SIDS, if even simply as a risk factor.

**Fungal infection**

A mycological basis for SIDS was first proposed in 1982. It was thought that the intestinal flora was dominated by *Candida albicans*, which may contribute endogenous alcohol production to play a role in SIDS pathogenesis. Different Candida species were found in various organs, but none appeared to have the degree of growth to cause sepsis [101]. It was concluded that the alcohol produced in the intestines would not exceed the concentration able to be metabolized by the liver. *Pneumocystis (previously carinii)* has been identified in the lungs in a significant number of SIDS cases (14%) in South America and Europe [102]. The
direct involvement of *P. jiroveci/carinii* in SIDS has since been abandoned as it is thought that infants are a natural reservoir for this organism and so the finding of them in SIDS infants is normal [103].

**Genetic Predisposition**

Infections, particularly of the respiratory tract, are very common in the first year of life, more so than the incidence of SIDS [95]. Thus if infection plays a role in the cause of SIDS there must be other contributing cofactors within the process. The mainstream perspective of SIDS is that death may be multifactorial: an amalgam of predisposing host and epidemiological risk factors [26], in particular polymorphisms in immunoregulatory genes that predispose the infant to an exacerbated immune response when challenged by toxigenic bacteria and/or viral infection. Pro-inflammatory cytokines are a crucial defense mechanism; they play a major beneficial role in the host response against microbial invasion. The intensity and duration of an inflammatory response is modulated by T helper cell type 2 cytokines and anti-inflammatory mediators. During sepsis there is a marked inflammatory response typified by high levels of inflammatory cytokines, markers of cellular stress, complement-derived compounds and activated coagulation factors, all of which act synergistically to cause cytokine storm, tissue injury, organ dysfunction and possible death [104]. Such an episode can result in a pathophysiological picture of circulatory collapse or shock which is consistent with SIDS pathology. Exposure to bacterial toxin or viral infection elicits a cytokine storm manifesting as toxic shock, as supported by experimental data [51], and as indicated by the pathological findings in SIDS. Concurrent polymorphisms in immunoregulatory genes which up-regulate or down-regulate circulating levels of cytokines may disrupt the balance between a normal and overstated immune response to toxic stimuli, thus rendering the infant
susceptible to cytokine storm. Research has been conducted into the frequency of single nucleotide polymorphisms (SNPs) that result in an exaggerated pro-inflammatory response in the SIDS population. Some SNPs were found in a higher proportion of SIDS infants compared with controls and will be discussed herein.

**Interleukin 10 (IL-10)**

An underproduction of anti-inflammatory mediators can have a profound effect on responses to toxins. IL-10 is a cytokine capable of inhibiting synthesis of pro-inflammatory cytokines including IL-1, TNF-α and IL-6 [105]. The SEs, SEA through to SEJ, have the immunomodulatory properties of superantigens and many are lethal to rabbits in models of toxic shock syndrome. Therefore, by limiting pro-inflammatory response, IL-10 is protective against staphylococcal enterotoxin induced lethal shock [105]. It has been suggested that between 50% and 75% of variability of IL-10 secretion is explained by genetic determinants. Other factors include those that are associated with SIDS such as cigarette smoking, male gender and ethnicity (risk factors for SIDS). Two SNPs, IL-10 G-1082A and IL-10 C-592A, have been linked to low IL-10 levels *in vitro* and have been suggested to be associated with SIDS [106]. The results of two studies of British infants suggests there is an association between the IL-10 polymorphisms G-1082A and C-592A and SIDS [106, 107].

**Interleukin-1β (IL-1β)**

IL-1β has been implicated in the pathogenesis of SIDS due to its ability to induce vascular shock, hypoglycaemia, deep sleep with prolonged apnea and cardiac irregularities. Clinical [13] and pathological findings in SIDS are similar to endotoxin-induced shock [26, 108] suggesting that high levels of circulating IL-1 may be implicated in their death. The IL-1β C-511T SNP results in over expression of IL-1β and is associated with fatal meningococcal
infections [109]. It has been suggested that individuals homozygous for IL-1β polymorphisms who smoke have much higher IL-1β production in response to bacterial toxins [110]. Thus the involvement of IL-1β polymorphisms may explain the effect smoking has on the risk of SIDS. IL-1β SNPs have been investigated in relation to SIDS, but with only small sample numbers statistically significant frequencies were observed but not strong [110]. Notably, Australian and German SIDS infants had significant difference in the distribution of IL-1RN (T + 2018C) polymorphism, and the frequency of this and the IL-1β (C-511T) SNP was variable among different ethnic groups. Staphylococcal infection gives rise to increased expression of interleukin-1β [111], which may be exaggerated in the presence of a SNP.

**Interleukin 6 (IL-6)**

The most direct evidence for cytokine involvement comes from studies in which half of the SIDS infants investigated had IL-6 concentrations in their cerebrospinal fluid equivalent to those found for infants dying from infectious diseases such as meningitis or septicaemia [112]. This finding may be the result of a polymorphism that causes overexpression of IL-6 in response to a colonising bacterium, as evidence of such invasive infection is seldom found in SIDS [24]. An association has been demonstrated between Australian SIDS infants and IL-6 G-174C SNP [113], but the sample size was insignificant to draw any definitive conclusions. A recent study in the UK has reported similar results with a group of 50 SIDS infants where the frequencies reached statistical significance [114]. Conflicting results have been shared by researchers studying Norwegian SIDS infants where the frequency of the IL-6 G-174C SNP did not differ between SIDS and control infants in that region [115]. High levels of plasma IL-6 in can also be attributed to the CD14 (C-260T) polymorphism [116], where high IL-6 expression is likely to be a downstream consequence of CD14-mediated immune stimulation.
**Vascular Endothelial Growth Factor (VEGF)**

VEGF is an endothelial cell mitogen that stimulates angiogenesis under hypoxic stress to increase tissue capillary density and improve the delivery of oxygen [117]. It is also highly pro-inflammatory, up to 50,000 times more vasoactive than histamine, and induces expression of chemokine genes [114]. Elevated VEGF concentrations were found in 30 of 51 SIDS cerebrospinal fluid samples [117]. The VEGF G-1154*A genotype has been shown to be associated with SIDS infants [114].

**Candidate gene polymorphisms for SIDS research**

**Interleukin-1 receptor antagonist**

Interleukin 1 receptor antagonist (IL-1ra) is a competitive inhibitor that binds to IL-1 receptors without inducing intracellular response, by blocking receptors. It is an important endogenous regulator of inflammation and actively limits septic shock and reverses hypotension [118]. Additionally, IL-1ra suppresses the release of IL-1β and TNFα from mononuclear cells [119]. The known genetic variations in the *IL-1β* gene are not major regulators of *in vitro* production of IL-1β, rather the *IL-1RN* (gene encoding IL-1ra) allele type has the decisive role [120]. Therefore to assess the genetic basis of IL-1β production *IL-1RN* polymorphic sites, rather than *IL-1β* polymorphic sites should be considered. In a study by Moscovis *et al.* a single nucleotide polymorphism in *IL-1RN* (T+2018C) was found not to be differently distributed between SIDS infants and controls [110]. A length variation within intron 2 of *IL-1RN* was first reported by Steinkasserer *et al.* [121]. Currently five alleles, A1-A5, are recognised. The A1 allele, and the 1/1 genotype, are predominant [122]. The A2 variant reportedly enhances in vitro IL-1β production almost two-fold [120], and contributes to susceptibility to severe sepsis [123], although at present this remains arguable as conflicting studies have found A2 can increase [124], or have no effect [125], on IL-1ra.
levels. It is generally accepted that homozygous carriers of A2 have a more severe and prolonged pro-inflammatory immune response than do those with other IL-1RN genotypes [126].

*T cell receptor recombination signal*

Host factors, including genetic determinants, influence the outcome of interactions between toxin and host. Accordingly, polymorphisms in genes that influence the expression of toxin receptors could affect the outcome of SE toxaemia, and could therefore play a role in SIDS. SE molecules activate T cells via receptors that express particular Vβ elements. Activation via SE-TCRVβ binding results in overproduction of cytokines which contribute strongly to the onset of toxic shock [127]. Random recombination of TCR segments during T cell maturation ensures a repertoire of diverse TCRs so the proportion that responds to SE is limited. This process is controlled by the *recombination signal sequence (RSS)* gene region.

In transgenic mice with increased Vβ3 (a receptor for SEA) usage and induced staphylococcal sepsis, SEA mortality was greater (85%) than non-transgenic littermates (31%). Fifty percent of Vβ3 transgenic mice died within five days of inoculation and none were accompanied by any change in body weight. Notably, high loads of *S. aureus* were found in the blood, kidneys and spleen [111]. Similar findings concerning true pathogens, *S. aureus* and coliforms, in normally sterile sites have been reported in unexplained SUDI (uSUDI) infants in recent publications [46, 48]. A single nucleotide polymorphism (SNP) in the *RSS* of the *TCRBV3* gene (TCRBV3S1 RSS*2) increases the frequency of expression of the Vβ3.1 chain, resulting...
in large numbers of Vβ3.1 chains expressed on T cells. TCRBV3S1*2 could be involved in a staphylococcal toxic shock hypothesis for SIDS and uSUDI

**Gene polymorphisms affecting pathogen recognition**

Genetic predisposition to inappropriate responses to infection have been the focus of recent SIDS investigations [110, 113, 128], research to date has primarily targeted cytokine gene polymorphisms conferring heightened pro-inflammatory responses. While these are important in determining the outcome of an established inflammatory response, factors influencing susceptibility to invasive infection could also be involved in SIDS. In 2008, two independent retrospective studies reported typically pathogenic bacteria (notably *Staphylococcus aureus*) in normally sterile sites of infants succumbing to sudden unexpected death in infancy (SUDI)[9, 48] (a broad classification of unexplained infant deaths including cases that would have previously constituted SIDS classification). The finding of these potential pathogens in usually sterile sites could reflect insufficient innate immunity permitting the organisms to spread rapidly into normally sterile tissues.

Pathogen Pattern Recognition (PPR) receptors rapidly recognize and bind to conserved molecular patterns on bacteria, viruses and fungi. Upon binding the immune response is triggered. The function of PPR receptors is crucial in the early stages of bacteraemia; activation of the immune response facilitates the clearance of bacteria from the bloodstream to prevent septicemia. It is recognized that susceptibility to sepsis might be due to inherited or acquired mutations of innate immune genes, and that severe sepsis and septic shock are clinical manifestations of a dysregulated immune response to invasive pathogens [129]. Loss-of-function polymorphism among PPR genes could result in impaired recognition of invasive
pathogens, and consequently, impaired induction of an immune response and pathogen clearance. *S. aureus* and coliforms found in normally sterile sites in SIDS and SUDI infants might be a remnant or „footprint“ of a bacteraemic episode during which the pathogen was unrecognised and not cleared from these sites. Reduced pathogen recognition could leave infants particularly susceptible to bacteraemia at 2-4 months of age because the natural diminution in maternal antibodies.

Lipoteichoic acid (LTA) and peptidoglycan from *S. aureus* activate immune cells via Toll-like receptor 2 and CD14 recognition [130, 131]. Activation of the signalling pathways has downstream consequences including pro-inflammatory and chemotactic effects. CD14 is also a receptor for lipopolysaccharide (LPS, or endotoxin). *TLR-2* and *CD14* contain single nucleotide polymorphisms that affect pathogen ligand binding and recognition. The homozygous *TLR-2* variant R753Q is a “functional knockout” of LTA stimulation, while the heterozygous carrier type elicits full responses not indifferent from the wild-type [132]. The TT genotype of the *CD14* C(-260T) polymorphism is associated with a significantly higher density of CD14 receptor expression on monocytes [133] which may make the individual more sensitive to endotoxin.

Given that there are differences in the distribution of these polymorphisms between countries and the ethnic groups within them, all suffering the burden of SIDS it may be the case that there is not a single SIDS genotype, rather there is one of a number of polymorphisms in conjunction with an infectious agent that is common within that population. A single entity may contribute to SIDS pathogenesis in a particular population but conflicting results may
arise from studies in other regions where that genotype or infectious agent is infrequently encountered but another is predominant.

**Conclusion**

Given the extensive evidence for an infectious aetiology for SIDS, it is understandable that the infectious hypothesis proposed in 1966 [85] still persists in research today. Recent advances in molecular biology have equipped us with methods, notably PCR, that may uncover the presence of infectious agents in SIDS that have escaped detection by traditional viral culture methods, when coupled with the analysis of newly discovered immunoregulatory gene SNPs we may gain a new insight into the SIDS enigma and one day know its precise mechanism. The human immune system is extremely complex in nature and is controlled by a vast array of genes. Whilst the list of candidate genes for polymorphism analysis in SIDS appears endless, those involved in immunoregulatory processes deserve attention as they powerfully affect homeostatic balance. Several SNPs within immunoregulatory genes have been associated with dampened or exaggerated immune responses to pathogens which can lead to uncontrolled bacterial infection, or cytokine storm respectively. Thus, these pathways are likely to be the major focus for SIDS research in the field of infectious diseases.
Hypotheses

The work described in this thesis is based upon these initial hypotheses:

1) The SIDS intestinal tract harbors bacteria carrying genes for potentially lethal toxins which contribute to a lethal "toxic shock" response;

2) SIDS infants are genetically predisposed, via immunoregulatory gene polymorphisms, to potentially lethal immune responses when challenged with common bacterial toxins.

Aims

Given the strong evidence for the involvement of bacterial toxins in SIDS this project set out to address the following:

1) To define whether the intestinal tract could be a source of potentially lethal toxins to support a toxic shock hypothesis for SIDS;

2) To compare the frequencies of immunoregulatory gene polymorphisms between SIDS and control infants to determine if SIDS are at increased risk of an inappropriate immune response to bacterial toxins.
References


Chapter 2. Molecular analysis of pathogenicity of *Escherichia coli* from Sudden Infant Death Syndrome, dead control and healthy infants

Abstract

Consistent pathological findings in sudden infant death syndrome (SIDS) are seen which display similarities to the pathogenesis of toxaemic shock and/or sepsis. A key candidate infectious agent that is possibly involved is *Escherichia coli*, given its universal early colonization of the intestinal tract of infants and an increased frequency of toxigenic and mouse-lethal isolates from SIDS compared with comparison infants. An explanation for these findings has yet to be identified. Fifty-six *E. coli* strains from 30 SIDS, two dead control and 24 healthy infants were assayed for verocytotoxicity and for possession of shiga toxin (stx) genes to determine if they remained virulent after retrieval from storage. One of 56 strains possessed shiga toxin genes (stx2), one isolate (non-typable) was found to be non-stx verotoxigenic, and a number of strains elicited subtle but distinct cytopathic effects. Using PCR, we screened *E. coli* isolates from 145 SIDS and 101 dead control and healthy infants for two new candidate pathogenicity-related genes: *clyA* (cytolysin A), *irp2* [high-pathogenicity island (HPI)-specific gene]. The results failed to show a positive correlation with SIDS, instead proving that clyA and irp2 genes were common to the infant intestinal *E. coli*. Interestingly we observed a high rate of carriage of these two potentially pathogenic genes in *E. coli* from healthy infants in the absence of diarrhoeal disease, and we report that in a number of cases, the detection of HPI-specific genes was predictable by serotype. Despite the lack of associations defined so far, there remains the likelihood that genetic determinants influence the interactions between *E. coli* and the host, so these factors may be part of the multi-factorial aspect of SIDS.
Background

Verotoxicity of E. coli isolates from SIDS infants
As explained in Chapter 1, the toxins of E. coli have been extensively researched in isolates from SIDS infants. In 1995 Bettelheim et al. carried out verotoxicity assays on single representative E. coli isolates (i.e. the most predominant and virulent strain from each infant) from 76 healthy infants, 64 SIDS infants and 20 infants who had died of other causes. They reported that the E. coli from 51.6% of SIDS infants induced vero cell monolayer disruption (affecting ≥50% of cells) compared with only 25% of those from healthy infants and 3.9% of strains from dead controls [1]. The presence of stx genes in the “verotoxigenic” isolates could not be confirmed by polymerase chain reaction (PCR) at the time of the study and it remains open that the verotoxigenic strains described in 1995 that induced subtle but distinct changes to vero cell monolayers were non-Stx verotoxigenic.

Cytolysin A and the High Pathogenicity Island in E. coli isolates
Human uropathogenic E. coli (UPEC) and avian pathogenic E. coli (APEC) both belong to the extraintestinal pathogenic E. coli subgroup and share overlapping serotypes and phylogenetic and virulence traits. Human UPEC strains have been shown to share pathogenicity-related genes with APEC strains, particularly those located on the High Pathogenicity Island (HPI) which facilitate the uptake of iron and thus survival of the bacterium in extra-intestinal body sites. HPI is found in extraintestinal E. coli strains associated with urinary tract infection and sepsis [2]. Interestingly, the predominant E. coli found in the intestinal tract of SIDS infants are typically extra-intestinal serotypes and are more consistent with UPEC [1] and thus possibly APEC. APEC produce a high mean lesion
score for lethality in one-day-old chicks, perhaps similar to the high lethality of SIDS *E. coli* to mice whereas those isolated from healthy infants were not lethal. Also the recent reporting of *E. coli* in normally sterile sites [3] and coliforms in extraintestinal sites such as the upper respiratory tract [4] at post-mortem investigation of sudden unexpected death in infancy, including SIDS, may be related to the effects of such islands as the HPI. Alternatively cytolysin A (ClyA) which is cytotoxic and apoptotic to cultured mammalian cells [5-9] may explain cytotoxicity of the strains in the absence of *stx* genes.

This study addressed two questions, 1) would the strains used by Bettelheim *et al.* be amenable to testing for *stx* by PCR after extended periods of storage? Prolonged storage and/or repeated subculture of these isolates may have rendered their *stx*-1 and -2 genes unstable, as has been well documented in other studies [10]. If the *stx* genes are still present and intact, this may explain the verotoxicity of these strains observed by Bettelheim *et al.* Alternatively, if no *stx* is detected, either the genes have been lost over time, or (more importantly) the “verotoxin” may indeed be a novel distinct toxin as perceived by the subtle nature of the verotoxicity, and 2) does the apparent higher frequency of pathogenic *E. coli* genotypes in SIDS infants hold true for the virulence factors HPI and cytolysin A?
Methods

Part 1) Evaluation of verotoxicity

E. coli strains: Enteric E. coli strains isolated from SIDS and control infants were obtained from The National Escherichia coli Reference Laboratory, Microbiological Diagnostic Unit, Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Victoria. Ethical approval had been obtained from the Research Ethics Committee of the Victorian Institute of Forensic Medicine (the institution at which the cases were sampled during autopsy). E. coli isolates from SIDS cases were taken from the intestinal contents of infants who met the 1989 definition of SIDS [11]. Control isolates were from the intestinal contents of infants who died from causes other than SIDS (principally traumatic injury, drowning or congenital defects), and from faecal samples from healthy infants attending a day-care centre in Victoria between 1989 and mid 1990 [1, 12, 13]. These strains were involved in previous investigations by Bettelheim et al. [1, 12-15] and had been assayed at that time for verotoxicity, heat-labile enterotoxin, HeLa cell adherence, haemagglutination, hydrophobicity and cytotoxic necrotising factor (CNF). Selection of isolates and biochemical identification of these strains have been previously described [15]. Principally the single predominant E. coli isolate was taken, and where two isolates of the same serotype were collected, both were tested. On 18 occasions two isolates of different serotypes were taken. Both isolates were taken into account in the analysis of results, but they were both counted as the result for one infant (i.e. results are in terms of number of infants, not number of isolates). All E. coli strains were received in semi-solid medium and were cultured in our laboratory on blood agar plates for 24H at 37°C.

Stx PCR: Fifty-six of the E. coli strains (30 from SIDS, two from dead controls and 24 from healthy infants) were tested for stx genes by PCR. All 56 had been classified as verotoxigenic
by vero cell assay by Bettelheim et al. [1]. Stx-1 and stx-2 positive control strains for PCR testing were sourced from stored clinical isolates from cases of haemolytic uraemic syndrome in the Department of Microbiology and Infectious Diseases, Women’s and Children’s Hospital, North Adelaide, Australia. E. coli K-12 derivative C600 was used as a negative control. An 88bp fragment of the stx-1 gene, and an 121bp fragment of the stx-2 gene were amplified using the primers VS1 and VS2, and VS4 and VS5 respectively [16] (Appendix 1) on a Corbett Research Rotor-gene 3000, with fluorescent stx-1 specific probe VS3 and stx-2 specific probe VS6 under the following cycling conditions: 50°C 2 mins (for uracil DNA glycosylase digestion of carry-over amplicons), 95°C 2 mins initial denaturation, followed by 45 cycles of 95°C 15s and 60°C 45s. The PCR reaction mix contained: 0.05µM MgCl$_2$; 1X PCR Supermix (Invitrogen, Carlsbad CA); 0.02µM each primer (VS1 & VS2 or VS4 & VS5); 0.02 µM of the corresponding probe; 2µl of bacterial lysate and PCR grade water to a total volume of 25µl.

**Verotoxicity assay:** Fourteen isolates (nine SIDS, one dead control and four healthy infant strains) were tested for cytotoxicity on vero cell monolayers. Eight SIDS and four healthy infant strains had been reported by Bettelheim et al. as verotoxigenic, (but were stx-1 and -2 PCR negative in our assay as described in Results). Two isolates, both verotoxin and stx negative, were included for comparison. Toxin production and cell culture assays were performed according to the method described by Konowalchuk et al. [17]. Stx positive and negative control strains for cell culture assays were the same as used for stx-1 and -2 PCR. Vero cell monolayers in 12 well plates were observed by sub-stage/inverted microscopy for four days. Verotoxicity was defined as free-floating vero cells detached from the monolayer or rounding of cells. On the fourth day, eight of the cell cultures underwent a neutral red assay as described by Carbonell et al. [18]. The number of non-viable cells per field was assessed.
by eye in the absence of a suitable plate reader. When the unincorporated dye was removed from viable cells, the solution of removed dye and acetic acid-ethanol mixture was kept and diluted in 1ml PBS. The absorbance of the solution was read in a spectrophotometer at 540nm under the presumption that the quantity of dye removed would be proportionate to the quantity of viable vero cells. Strains positive for stx-1 and -2, and a C600 control strain negative for stx-1 and -2 were used for comparison.
Part 2) Testing for Cytolysin A and the High Pathogenicity Island

**E. coli strains:** PCR testing for *clyA* and *HPI* genes was conducted on 181 of the *E. coli* strains isolated from 145 SIDS infants and 106 *E. coli* strains isolated from 28 dead control and 73 healthy infants (here forth combined as 101 comparison infants). The median age for the SIDS group was 3.5 months with an interquartile range of 3. The median age for dead control infants was four months with an interquartile range of 5.25. The median age for the healthy infant group was three months with an interquartile range of 4. The percentage of males in each of the SIDS, dead control and healthy infant groups were 68%, 57% and 42% respectively.

**ClyA and HPI PCR:** Positive control strains for PCR testing were sourced from stored clinical isolates from cases of haemolytic uraemic syndrome or urinary tract infection in the Department of Microbiology and Infectious Diseases, Women’s and Children’s Hospital, North Adelaide, Australia. Strains were tested by PCR for genes coding for intact *clyA*, without deletion 2, as described by Ludwig *et al* [19] and *irp2* which encodes iron repressible protein 2, a HPI-specific gene. Contents of the PCR mixture contained: 0.1µM MgCl₂; 1X Colourless GoTaq Flexi Buffer; 0.005µM dNTP mix; 0.1µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison WI), 1µl bacterial lysate and water to a total volume of 12.5µl. Primer pairs irp2-F, irp2-R and clyA-F, clyA-R could be duplexed in the same reaction to simultaneously detect *irp2* and *clyA* (*Figure 1*), in which case the primer concentrations were kept at 0.1µM and the volume of water was adjusted accordingly. Genes were amplified on a Hybaid OmniGene Thermal Cycler (Hybaid) programmed as follows: initial denaturation 94°C 2min, 35 cycles of 94°C 30s, 55°C 30s, 72°C 30s, final extension of 2 mins at 72°C. Products, 280bp *irp2* and 204bp *clyAf* were visualised by ethidium bromide stained 2% agarose gel electrophoresis (*Figure 1*).
**Oligonucleotide primers:** Oligonucleotides synthesised by GeneWorks (Thebarton, South Australia) are shown in *Appendix 1*. ClyAF and-R primers amplify clyA when deletion 2 is not present (presumed functional, hereforth assigned the name clyAf). Forward primer clyA-F matches a region of clyA sequence conserved amongst the published sequences for STEC, ETEC and UPEC, GenBank accession numbers AY576656, AY576661 and AY576665 respectively. Reverse primer clyA-R was designed with an 8bp overhang into deletion 2, allowing it to bind to STEC clyAf, but not ETEC or UPEC clyA. Alignment of these primers with three clyA sequences is shown in *Figure 2*. The lack of binding to ETEC and UPEC strains was confirmed by sequencing a larger portion of the clyA gene (Figure 3) amplified from strains that reacted accordingly with these primers and confirming the presence of deletion 2. The primer employed for amplification of the larger product was clyAfull-R (*Appendix 1*).
**Figure 1.** Lanes 1 and 14 pUC19/ *Hpall* DNA MWT marker (Geneworks) Lanes 2-5, 7-8, 12-13 *E. coli* testing positive for irp2 (280bp).

Lanes 4-8, 10-12 *E. coli* testing positive for clyAf (204bp).
<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY576656 (STEC)</td>
<td>CAAACAGTGTATGAATGGTGTGGTGTTGCGACGCAATTGCTCGCAGCGTATATTTTGCTA 540</td>
</tr>
<tr>
<td>AY576661 (UPEC)</td>
<td>--------------------------CGCCCGAGACATCATTCACGAGTATGCGATGCAGAG 600</td>
</tr>
<tr>
<td>AY576665 (ETEC)</td>
<td>CAAACAGTGTATGAATGGTGTGGTGTTGCGACGCAATTGCTCGCAGCGTATATTTTGCTA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY576656 (STEC)</td>
<td>TTTGATGAGTATAATGAGAAGAAAGCATCCGCCCAGAAAGACATTCTCATTAAGGTACTG 660</td>
</tr>
<tr>
<td>AY576661 (UPEC)</td>
<td>GATGACGGCATCACGAAGCTGAATGAAGCGCAAAAATCCCTGCTGGTAAGCTCACAAAGT 720</td>
</tr>
<tr>
<td>AY576665 (ETEC)</td>
<td>GATGACGGCATCACGAAGCTGAATGAAGCGCAAAAATCCCTGCTGGTAAGCTCACAAAGT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY576656 (STEC)</td>
<td>TTCAACAACGCTTCCGGGAAACTGCTGGCGTTAGATAGCCAGTTAACCAATGATTTTTCA 840</td>
</tr>
<tr>
<td>AY576661 (UPEC)</td>
<td>CAAACAGCTATTTCCAGTCAGATGAGTATAAAATCCCTGCTGGTAAGCTCACAAAGT</td>
</tr>
<tr>
<td>AY576665 (ETEC)</td>
<td>TTCAACAACGCTTCCGGGAAACTGCTGGCGTTAGATAGCCAGTTAACCAATGATTTTTCA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY576656 (STEC)</td>
<td>GATGACGGCATCACGAAGCTGAATGAAGCGCAAAAATCCCTGCTGGTAAGCTCACAAAGT 900</td>
</tr>
<tr>
<td>AY576661 (UPEC)</td>
<td>ATTTGAAATTAACCACCGAAATAGCCGCCATCGGGGAGATAAAAACGGAAACTGAAACAAC 960</td>
</tr>
<tr>
<td>AY576665 (ETEC)</td>
<td>ATTTGAAATTAACCACCGAAATAGCCGCCATCGGGGAGATAAAAACGGAAACTGAAACAAC</td>
</tr>
</tbody>
</table>

**Figure 2.** Alignment of STEC, UPEC and ETEC clyA sequences with clyA-F, clyA-R and clyA-fullR primers.
Figure 3. Agarose gel electrophoresis of STEC (414bp), ETEC (402bp) and UPEC (177bp) clyA amplicons using the primer combination clyA-F & clyAfull-R
Results

Part 1) Evaluation of verotoxicity

Stx-1 and -2 PCR: Of the 56 E. coli strains (30 from SIDS, two from dead controls and 24 from healthy infants) that were tested for stx genes by PCR, one O75.H- strain was positive for stx-2. None were positive for stx-1 by PCR.

Verotoxicity assay: Fourteen isolates, nine SIDS, one dead control and four healthy infant strains were tested for verotoxicity. Eight isolates from SIDS and four from healthy infants had been reported by Bettelheim et al. as verotoxigenic, but were stx-1 and -2 PCR negative in our assay. Two isolates, both verotoxin and stx negative, were included as a comparison. Vero cell monolayers treated with SIDS E. coli supernatant exhibited subtle but distinct changes including granulation on the second day. This was consistent with previous observations. One non-typable isolate from a healthy infant disrupted the vero cell monolayer to the same extent of the stx1 control, but was negative for stx-1 and stx-2 genes by PCR

Neutral red viability assay: The absorbance of the unincorporated dye solution was read in a spectrophotometer at 540nm under the presumption that the quantity of dye removed would be proportionate to the quantity of viable vero cells (i.e. the greater the absorbance of the solution the more viable cells). Not surprisingly the E. coli C600 negative control strain gave a high absorbance reading (ABS\(_{540}\) 0.245) compared with the stx-1 and -2 positive control strains (0.027 and 0.043 respectively) and the non-typable isolate which was verotoxic (Ont.Hnt, 0.101). Absorbance values for 14 strains are shown in Table 1 and Figure 4. There was no significant difference between the mean absorbance of assays for isolates described as verotoxin+ (n=5, mean=0.2234) and verotoxin- (n=5, mean=0.2438) in Bettelheim’s study.
We then tested isolates for two *E. coli* virulence factors; Cytolysin A (*clyA*), which may explain cytotoxicity of the non-typable strain in the absence of *stx* genes, and *irp2* from the High Pathogenicity Island, a genetic element common to strains associated with urinary tract infection and sepsis.
Part 2) Testing for Cytolysin A and the High Pathogenicity Island

Resulting data from screening *E. coli* isolates from SIDS and comparison infants for *clyAf* and *irp2* is shown in *Table 2*. SIDS and comparison infants had similar frequencies for both *clyAf* and *irp2*. In total 158 different serotypes were tested. PCR results for a selection of serotypes for which more than one isolate from SIDS was tested are shown in *Table 3*. Detection of *irp2* by PCR was observed to be serotype dependent in most cases; several strains of the same serotype from different infants gave the same result for *irp2*. Although the Ont.Hnt verotoxic isolate was positive for both *irp2* and *clyA*, overall the degree of vero cell viability in the other samples did not appear to be correlated with the presence of either *irp2* or *clyA* (*Table 1*).
**Table 1.** Absorbance at 540nm of unincorporated neutral red dye from viability assay for the tested strains, with irp2 and clyA PCR results.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ABS&lt;sub&gt;540&lt;/sub&gt;</th>
<th>irp2</th>
<th>clyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx-1 positive control</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx-2 positive control</td>
<td>0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C600 negative control</td>
<td>0.245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSB broth only</td>
<td>0.269</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O1.H- SIDS VT+</td>
<td>0.27</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O6.HR SIDS VT+</td>
<td>0.27</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>O145.H21 SIDS VT+</td>
<td>0.268</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>O2.H4 Healthy Baby VT+</td>
<td>0.31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ont.Hnt Healthy Baby VT+</td>
<td>0.101</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O166.H- SIDS VT-</td>
<td>0.209</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>O6.H31 SIDS VT-</td>
<td>0.277</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>O75.H- non-SIDS VT-</td>
<td>0.232</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ont.H- SIDS</td>
<td>0.236</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ont.H- non-SIDS</td>
<td>0.213</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
**Figure 4.** Absorbance at 540nm of unincorporated neutral red dye from viability assay.
Table 2. Results of PCR screening *E. coli* isolates for *clyAf* and *irp2* genes.

<table>
<thead>
<tr>
<th></th>
<th>clyAf (n=145)</th>
<th>irp2 (HPI)</th>
<th>Both clyAf &amp; irp2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SIDS infants</strong></td>
<td>64 (44.1%)</td>
<td>97 (67.0%)</td>
<td>25 (17.2%)</td>
</tr>
<tr>
<td>(n=145)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Comparison</strong></td>
<td>40 (39.6%)</td>
<td>65 (64.4%)</td>
<td>20 (19.8%)</td>
</tr>
<tr>
<td><strong>infants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=101)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. *E. coli* Serotypes commonly isolated from SIDS infants that tested positive for either clyA or irp2 (*Denotes more than one isolate from a single infant*).

<table>
<thead>
<tr>
<th>Serotype</th>
<th>no. strains tested</th>
<th>no. irp2 (HPI) +</th>
<th>no. clyA +</th>
<th>Source infant SIDS/Non-SIDS death/Healthy Baby</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1.H-</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4 SIDS, 1 HB</td>
</tr>
<tr>
<td>O1.H7</td>
<td>15</td>
<td>15</td>
<td>2</td>
<td>13 SIDS*, 1 HB</td>
</tr>
<tr>
<td>O17.H18</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2 SIDS</td>
</tr>
<tr>
<td>O2.H1</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>2 SIDS, 1 NS</td>
</tr>
<tr>
<td>O2.H15</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3 SIDS</td>
</tr>
<tr>
<td>O2.H18</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2 SIDS</td>
</tr>
<tr>
<td>O2.H6</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3 SIDS</td>
</tr>
<tr>
<td>O2.H7</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3 SIDS</td>
</tr>
<tr>
<td>O25.H1</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>3 SIDS, 1 NS, 1 HB</td>
</tr>
<tr>
<td>O25.H1/12</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>6 SIDS</td>
</tr>
<tr>
<td>O25.H2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2 SIDS*</td>
</tr>
<tr>
<td>O4.H5</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>3 SIDS, 1 NS, 3 HB</td>
</tr>
<tr>
<td>O5.H4/17</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2 SIDS</td>
</tr>
<tr>
<td>O6.H-</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>5 SIDS, 5 HB</td>
</tr>
<tr>
<td>O6.H1</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>3 SIDS, 1 HB</td>
</tr>
<tr>
<td>O6.H31</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1 SIDS*, 1 NS</td>
</tr>
<tr>
<td>O75.H-</td>
<td>10</td>
<td>10</td>
<td>1</td>
<td>7 SIDS, 2 NS, 1 HB</td>
</tr>
<tr>
<td>O75.H7</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>2 SIDS, 1 HB</td>
</tr>
<tr>
<td>O77.H18</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>2 NS, 2 HB</td>
</tr>
<tr>
<td>O91.H11/21</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>2 SIDS*</td>
</tr>
<tr>
<td>Ont.H-</td>
<td>17</td>
<td>10</td>
<td>7</td>
<td>10 SIDS*, 3 NS, 1 HB</td>
</tr>
<tr>
<td>Ont.H1</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>3 SIDS, 3 HB</td>
</tr>
<tr>
<td>Ont.H10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2 SIDS</td>
</tr>
<tr>
<td>Ont.H12</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2 SIDS</td>
</tr>
<tr>
<td>Ont.H18</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2 SIDS</td>
</tr>
</tbody>
</table>
Discussion

Only one isolate, of serotype O75.H-, possessed stx2. O75.H- strains have been previously shown to possess stx-1 and stx-2 genes which render them highly cytotoxic to vero cells [20]. The lack of stx in our isolates that were previously reported as verotoxigenic could reflect two possibilities: 1) the strains have lost their stx genes after prolonged storage and subculture, or 2) the verotoxicity described by Bettelheim in 1995 was not due to stx, but to another unidentified mechanism. One isolate (non-typable) was found to be non-stx verotoxigenic, and a number of strains elicited subtle but distinct cytopathic effects. An investigation into other virulence factors was warranted to investigate further possible causes of the toxicity. Cytolysin A is a prime candidate for cytotoxicity as it is cytotoxic and apoptotic to cultured mammalian cells [5-9]; however its effect on vero cells is not well documented. Although the Ont.Hnt verotoxic isolate was positive for both irp2 and clyA, overall the degree of vero cell viability in other samples did not support either HPI or ClyA being responsible for verotoxicity. The cause of this strain’s verotoxicity remains unknown.

Numbers of E. coli isolates containing the genes clyAf and irp2 from the High Pathogenicity Island, were almost identical in both SIDS and comparison infants. This suggests that these genes and their products are not likely to be implicated in the development of SIDS on a purely gene prevalence basis; however, it was interesting to find that of 73 healthy infants, 48 carried irp2 (HPI) positive E. coli, and 26 carried clyAf positive E. coli. Since these infants did not have evident E. coli infection at the time of collection, this suggests that carriage of these genes by intestinal E. coli is normal and there may be other host or environmental factors that contribute to E. coli disease caused by these agents. These findings were unexpected, yet plausible considering Bettelheim et al. in 1993 identified a number of E. coli strains producing CNF in not only SIDS but also in those that died from other causes as well
as healthy infants [15]. Nevertheless, given appropriate conditions that could pertain to a SIDS case, any of these toxins could play a role if they acted in combination with the factors that contribute to a SIDS demise, namely exposure to cigarette smoke [21], viral co-infection [22], and genetic susceptibility [23, 24]. The proposed toxins could act in concert with bacterial super-antigenic products, given that findings indicative of organ shock in SIDS have been observed to be analogous to an *E. coli* endotoxin-induced mouse model of shock [25].

The limitations of this study mainly pertain to the infrequency of non-SIDS infant deaths in Australia. Twenty-eight infants that had died of other causes including drowning or congenital defects were included in the study as controls to represent post-mortem bacteriology of an infant. The random nature of these deaths meant that they did not have the peak incidence of 2-4 months that is characteristic of SIDS and therefore had a slightly higher median age (4 months) and interquartile range (5.25 months) than SIDS infants (3.5 and 3 months). Healthy infants were more suitably matched for median age (3 months) and interquartile range (4 months). Questions can be raised regarding the viability and proportionality of different species between the two sample types. Nevertheless, it has been shown that for *E. coli*, if found in the small intestine and/or colon is without fail also found in the faeces [14]. This would therefore support the use of healthy infant faeces as control material in this investigation. Ideally, healthy control infants should be exposed to the same environmental *E. coli* reservoirs as SIDS infants. However, it has been documented that in most cases the early colonising *E. coli* are acquired from an infant’s mother [26] so perfectly matched case-controls would be particularly difficult in practice, unless the SIDS infant had a twin sibling.
According to Schubert et al. HPI is most prevalent in enteroaggregative *E. coli* and strains isolated from blood cultures (93 and 80% respectively) and rarer in intestinal strains. In a study of 60 *E. coli* from blood cultures they found 50 (83%) to be positive for *irp2*, but only eight of 25 (32%) *E. coli* from stool samples from healthy individuals tested positive for *irp2* PCR [27]. In this study we have found the infant population has a high prevalence of 64-67% carriage of *irp2*. These findings may reflect that the infant intestine normally harbors these strains which then decrease in prevalence after infancy. The isolation of a variety of serotypes between individuals in the healthy infant population negates the idea of one strain of *irp2* positive *E. coli* being transmitted among infants from the childcare centre they were attending. In testing over 150 different serotypes we discovered that detection of *irp2* by PCR appears to be serotype dependent, where several strains of the same serotype from different infants gave the same result for *irp2*. To our knowledge this has not been reported in the literature. ClyAf PCR result on the other hand appeared independent of serotype. This trend is evident in the results shown in Table 3. Serotype seems to play a role in SIDS given that there is a strong association between SIDS and extraintestinal, as well as Avian serotypes of *E. coli* [28]. Therefore there remains the possibility that serotype-associated virulence factors may exist.

In summary, no single *E. coli* entity can be attributed to the causation of SIDS because of either a lack of pathological evidence that would reflect the effects of these toxins, and/or their equal prevalence in non-SIDS infants. One Ont.Hnt strain was found to be verotoxic in the absence of *stx* genes. This was an interesting finding and further investigation is warranted into the mechanism by which this strain elicits cytotoxicity. Two new candidate factors for involvement in SIDS were examined, HPI and ClyA, but failed to show a positive correlation
with SIDS, instead proving to be common to the infant intestinal flora. Interestingly, the finding of HPI was in most cases predictable by serotype, the gene’s presence or absence was consistent in many isolates of the same serotype. Despite the lack of associations defined so far, there remains the likelihood that genetic determinants influence the interactions between E. coli and host and so these factors may be part of the multi-factorial aspect of SIDS.
References


Chapter 3. Curliated *Escherichia coli*, soluble curlin and the sudden infant death syndrome: expansion of a previous investigation

Abstract

In 2002 a study was undertaken by Goldwater and Bettelheim to explore a possible role for curli-producing strains of *E. coli* in the causation of SIDS. All of 92 *E. coli* isolates from SIDS infants produced curli on congo red agar (an indicator of curli protein production). Significantly fewer control isolates from non-SIDS deaths and healthy babies produced curli (76.5% and 80.4% respectively). This chapter describes an expansion of the previous work of Goldwater and Bettelheim over more isolates from the same collection of *E. coli*. The present study of curli production was carried out on 172 *E. coli* strains isolated from 77 SIDS, 10 dead control and 85 healthy infants. An inoculum of each *E. coli* was spread on congo red agar plates and incubated at 37°C for 48 hours. Isolates were scored after 24 and 48 hours according to their incorporation of congo red dye. A positive curli phenotype was observed for 58%, 40% and 73% of isolates from SIDS, dead control and healthy infants respectively after 24 hours, and all except three isolates produced curli after 48 hours. The present investigation did not demonstrate a statistically significant difference between *E. coli* curli fibre production in SIDS and comparison infants, suggesting that the *in vitro* detection of curli production from isolates is not predictive of SIDS. Uncontrolled variables affecting *in vivo* curli production are discussed.
Introduction

Lipopolysaccharides are important mediators of sepsis. However, other bacterial components are also involved in the pathologic changes and pro-inflammatory cytokine induction that occur. Bacterial curli fibers, extracellular structures involved in bacterial adhesion, induce high levels of tumour necrosis factor alpha, interleukin 6 and interleukin 8 and activate the nitric oxide synthase arm of the innate immune system causing a rapid fall in blood pressure [1]. Expression of curli by *Escherichia coli* *in vitro* is temperature regulated with expression occurring in *E. coli K12* at 28°C but not at 37°C. Expression at 37°C is a likely virulence factor for strains causing human sepsis, with reports that greater than 50% of *E. coli* isolated from blood cultures of patients with sepsis are capable of producing curli at 37°C *in vitro*, and patients with Gram negative sepsis often have detectable antibodies to the curlin subunit CsgA [2]. In 2002 a study was undertaken to explore a possible role for curli-producing strains of *E. coli* in the causation of SIDS [3]. The premise that such toxins are present in the SIDS infant bloodstream stems from the fact that SIDS serum is lethal to infant mice and chick embryos, whereas control sera from non-SIDS infants is not [4, 5]. Soluble curlin and other bacterial proteins act as potent pro-inflammatory stimulators and could invoke, with the help of concurrent viral infection, an infant’s immune system to respond abnormally [6] and lead to the shock-like pathology observed in SIDS [7]. In Goldwater and Bettelheim’s study, all of 92 *E. coli* isolates from SIDS infants produced curli on congo red agar (an indicator of curli protein production) at 37°C. Significantly fewer control isolates from non-SIDS deaths and healthy babies produced curli (76.5% and 80.4% respectively) [3]. This chapter describes an expansion of the previous work of Goldwater and Bettelheim [3] with more isolates from the same collection of *E. coli*. 
Methods

Strains
The strains used in this investigation were randomly selected from the cohort described in the previous chapter and their isolation has been described in previous investigations by Bettelheim et al. [8-12]. The present study of curli production was carried out on 172 E. coli strains isolated from 77 SIDS, 10 dead control and 85 healthy infants (one strain from each infant). Twenty-seven of these strains had been tested previously for curli production by Goldwater and Bettelheim [3].

Evaluation of curli production
Congo red agar plates were made by adding 30mg/L Congo red dye to CFA agar base (1% casamino acids, 0.15% yeast extract, 0.4mM MgSO4, 0.4mM MnCl2, 2% agar pH 7.4). The mixture was autoclaved and set in petri dishes to a thickness of approximately 5mm. An inoculum of each E. coli was spread on each plate and the plates were incubated at 37°C for 48 hours. Dye incorporation into the colonies is achieved via the binding of secreted curli to congo red in the media [2]. This was recorded after 24 and 48 hours on a scale of - (colonies remained white) to ++++ (intense red with precipitation) (Figure 1). Strains reported previously by Goldwater and Bettelheim to be representative of each intensity were used as controls.
Figure 1. Congo red staining scale of *E. coli* phenotypes. Image adapted from Bian *et al.* [2].
Results

The proportion of strains positive and negative for curli production after 24 and 48 hours incubation at 37°C are shown in *Table 1* and illustrated in *Figure 2*. All but three strains produced curli (staining intensity greater than or equal to +) after 48 hours. Two of the negative strains were from SIDS infants and one from a healthy baby. No significant difference was observed between SIDS and control isolates after 24 or 48 hours incubation.

Twenty-five strains produced high levels of curli (++++/+++++) after 24 hours. Forty-four percent of these high curli producers were from serotypes associated with extraintestinal infection *E. coli*. The serotypes and source of these strains are shown in *Table 2*. Twenty-seven of the strains had been previously tested for curli production by Goldwater and Bettelheim. The present results were consistent with their findings except that six healthy baby and one dead control strain were recorded as negative in the previous study, while the present study found them to be intermediate curli producers (± or weak +) after 48 hours.
Table 1. Number of *E. coli* isolates producing curli after 24 and 48 hours incubation at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Curli production</th>
<th>No curli production</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SIDS (n=77)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24H</td>
<td>45 (58%)</td>
<td>32 (42%)</td>
</tr>
<tr>
<td>48H</td>
<td>75 (97%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td><strong>Dead control (n=10)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24H</td>
<td>4 (40%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>48H</td>
<td>10 (100%)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Healthy baby (n=85)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24H</td>
<td>62 (73%)</td>
<td>23 (27%)</td>
</tr>
<tr>
<td>48H</td>
<td>84 (99%)</td>
<td>1 (1%)</td>
</tr>
</tbody>
</table>
Figure 2. Percentage of *E. coli* isolates producing curlin after one and two days incubation.
Table 2. Serotypes of the twenty-five strains that produced high levels of curlin (+++/++++) after 24 hours, their source infants and O-serogroup-associated pathogenicity; extraintestinal infection *E. coli* (EIEC); verotoxigenic *E. coli* (VTEC) or enteropathogenic *E. coli* (EPEC)

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Source infant</th>
<th>Serotype pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>O91.H11/2</td>
<td>SIDS</td>
<td>VTEC/EPEC</td>
</tr>
<tr>
<td>O2.H-</td>
<td>SIDS</td>
<td>EIEC</td>
</tr>
<tr>
<td>O6.H31</td>
<td>SIDS</td>
<td>EIEC</td>
</tr>
<tr>
<td>Ont/H11/2</td>
<td>SIDS</td>
<td>-</td>
</tr>
<tr>
<td>O1.H7</td>
<td>SIDS</td>
<td>EIEC</td>
</tr>
<tr>
<td>Ont.H16</td>
<td>SIDS</td>
<td>-</td>
</tr>
<tr>
<td>O25.H6</td>
<td>SIDS</td>
<td>EIEC</td>
</tr>
<tr>
<td>O155.H11/2</td>
<td>SIDS</td>
<td>-</td>
</tr>
<tr>
<td>OR.H1/12</td>
<td>SIDS</td>
<td>-</td>
</tr>
<tr>
<td>O77.H18</td>
<td>Dead control</td>
<td>-</td>
</tr>
<tr>
<td>O2.H1/12</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>Ont.Hnt</td>
<td>Healthy Baby</td>
<td>-</td>
</tr>
<tr>
<td>O2.H4</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>Ont.Hnt</td>
<td>Healthy Baby</td>
<td>-</td>
</tr>
<tr>
<td>O135.H10</td>
<td>Healthy Baby</td>
<td>-</td>
</tr>
<tr>
<td>O6.H-</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>O22.H27</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>O4.H-</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>O62.H48</td>
<td>Healthy Baby</td>
<td>-</td>
</tr>
<tr>
<td>O8.H1/12</td>
<td>SIDS</td>
<td>-</td>
</tr>
<tr>
<td>O18ac.H44</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>Ont.H1</td>
<td>Healthy Baby</td>
<td>-</td>
</tr>
<tr>
<td>O127.H15</td>
<td>Healthy Baby</td>
<td>VTEC/EPEC</td>
</tr>
<tr>
<td>O7.H15</td>
<td>Healthy Baby</td>
<td>EIEC</td>
</tr>
<tr>
<td>O7.H7</td>
<td>SIDS</td>
<td>EIEC</td>
</tr>
</tbody>
</table>
Discussion

This study sought to investigate further the role of curli production in *E. coli* from SIDS infants, in relation to a previous investigation in which a higher percentage of *E. coli* isolates from SIDS infants produced curlin compared with isolates from a group of comparison infants. The present investigation did not demonstrate a statistically significant difference between *E. coli* curli fibre production in SIDS and comparison infants, suggesting that the *in vitro* detection of curli production is not associated with SIDS. The regulation of curli expression is extraordinarily complex and dependent on a number of environmental cues including salt and oxygen concentrations and temperature. Curli can also be produced when a CsgB+A- (phenotypically negative) cell comes into range of a cell secreting unpolymerised CsgA (CsgA+B-). Single nucleotide polymorphisms in the promoter region of *E. coli* csg genes have also been shown to affect curli synthesis [13]. Production of curli on congo red agar is a valuable *in vitro* test; however, the precise role of curli in SIDS cannot be elucidated as the exact *in vivo* conditions for its expression are not known. Analysis of the serogroups and/or serotypes of high curlin producing *E. coli* showed almost half (44%) were obtained from SIDS cases; furthermore, these serogroups/serotypes belonged to *E. coli* with properties of either extraintestinal infection-associated *E. coli* or verotoxigenic *E. coli* or enteropathogenic *E. coli* pathogenicity groups.

The results presented here differ from those published by Goldwater and Bettelheim [3], more specifically, the lack of association between curli production and SIDS. This is likely to be due to the classification of six strains from healthy babies and one strain from a healthy infant being recorded as intermediate curlin producers where they were previously reported as negative. The method of assessing colour intensity by eye is subject to investigator variability which is a major caveat of this study.
Bian et al. have described an almost certain role for curli in nitric-oxide mediated hypotension [1]. Another pathway to increased nitric oxide levels is through polymorphisms in nitric oxide synthase (NOS) genes. An inducible NOS over-producer state can be the result of a single nucleotid polymorphism (-1173CACT) or a CCTTT tandem repeat polymorphism in the iNOS promoter region. Such a polymorphism may predispose the host to production of harmful nitric oxide levels when stimulated by an antigen such as curlin. It would be interesting to investigate whether curli and NOS polymorphisms act synergistically in cases of severe sepsis, or in SIDS. Cigarette smoke, an environmental risk factor for SIDS, is associated with increased arterial iNOS activity and elevated serum nitric oxide [14] and may potentiate the effects of an iNOS SNP.
References


Chapter 4. Staphylococcal enterotoxin genes are common in *Staphylococcus aureus* intestinal flora in Sudden Infant Death Syndrome (SIDS) and live comparison infants.

Abstract

Pathological and epidemiological findings in SIDS suggest an infectious aetiology with indications of involvement of staphylococcal enterotoxins (SEs). While SEA, SEB and SEC have been found in sera and tissues of SIDS cases, little is known about the role of intestinal *S. aureus* or the roles of later described toxins SEE, SEG, SEH, SEI and SEJ in SIDS. We used a molecular-based approach to define whether the intestinal tract could be a source of SEs to support the staphylococcal toxic shock hypothesis for SIDS. Intestinal contents from 57 SIDS infants and faeces from 79 age and gender matched live comparison infants were cultured and tested for *S. aureus* and *sea-b-c-e-g-h-j* and toxic shock syndrome toxin gene by PCR. High proportions of infants in both groups carried toxigenic and non-toxigenic *S. aureus*. Significantly greater proportions of SIDS compared with comparison babies were positive for *S. aureus* (68.4% vs. 40.5%) and for SE genes (43.8% vs 21.5%), suggesting a possible role in SIDS. The results indicate that colonisation by *S. aureus* with SE genes is common in infants; however, their detection is unlikely to be a strong predictive tool for SIDS; other factors (including immune response) might underly a specific susceptibility to SEs in SIDS infants.
**Introduction**

Pathological and epidemiological findings in SIDS suggest an infectious aetiology with involvement of *Staphylococcus aureus* superantigenic enterotoxins [1-6]. Staphylococcal enterotoxins (SEs) have been demonstrated in tissues or sera in over half of SIDS infants from five countries including Australia [6, 7]. Evidence suggests that nasopharyngeal strains could secrete toxin which is absorbed into the bloodstream, but little is known about the role of intestinal *S. aureus*. One Australian study reported that SEs were immunologically detectable in eight of 41 (19.5%) of SIDS infant faecal samples compared with none of 19 healthy babies [5]. Histopathological mucosal damage to the SIDS infant intestine is well documented and has similarities to SEB damage to rabbit ileal lamina propria [8, 9]. A damaged intestinal epithelium would increase accessibility of bacteria and toxins to the bloodstream increasing the chance of toxemia and death [9]. Both diarrhoea and vomiting are recognized risk factors for SIDS [10] which suggests involvement of enteric species.

Previous investigations of SEs in SIDS have principally used immunological methods to detect SEA, SEB, SEC, SED and toxic shock syndrome toxin (TSST) [3, 6]. No studies to date have recognized the potential roles for the other superantigenic SEs in SIDS, in particular SEG and SEI which are capable of initiating toxic shock [11], nor have polymerase chain reaction (PCR) methods been employed to detect SE genes in SIDS bacteria samples. Given the strong evidence for the role of *S. aureus* toxins in SIDS, we used a molecular-based approach to define whether the intestinal tract (also a receptacle for swallowed respiratory secretions) could be a source of SEs to support the staphylococcal toxic shock hypothesis for SIDS.
Materials and methods

Ethics approval

The study was approved by the Research Ethics Committee of the Children, Youth and Women’s Health Service, South Australia, Australia and the Victorian Institute for Forensic Medicine, Victoria, Australia. Samples were non-identifiable for the purpose of this investigation. Gender, date of birth, date of death, date of collection and cause of death, with relevant post-mortem findings, were available for analysis.

Sample collection and preparation

Infants included in the study and the specimen types collected are summarized in Table 1. Testing was done retrospectively on stored material from previous investigations conducted in South Australia, Australia (from the cohort of unexplained sudden unexpected death in infancy (uSUDI) – a broad category of unexplained infant deaths that had previously been classified as SIDS- described by Goldwater [12], where stored material was available), and consecutive cases of SIDS from Victoria, Australia since 2007. Small portions of intestinal contents were collected at autopsy and stored at -80°C. Samples from infants who died in Victoria were collected from the Victorian Institute for Forensic Medicine and transported on dry ice to the Department of Microbiology and Infectious Diseases, Women’s and Children’s Hospital, South Australia where the work was conducted. These infants met the 2004 definition for SIDS and so were further classified into Category 1a (one case) and Category 2 (six cases). These categories are explained by the current definition of SIDS in Chapter 1. The comparison group consisted of anonymous faecal samples (de-identified diagnostic specimens) collected prospectively from live infants visiting the Women’s and Children’s Hospital, North Adelaide, South Australia in 2006. Infants were suspected of having non-chronic enteric disease but were negative for routine microbiological (bacterial and viral)
pathogen testing. None had been treated with antibiotics prior to sample collection. One to two healthy infants were matched by age and gender to each SIDS infant i.e. the samples from comparison infants were collected when they were at roughly the same age (±2 weeks) as the SIDS infants when they died. Seasonal and yearly variation was anticipated to affect the rate of *S. aureus* colonization in the SIDS and comparison infants, the investigation aimed to examine the presence or absence of toxin genes in the *S. aureus* that were in a given sample.

A small portion (<1g) of intestinal contents or faeces was cultured non-selectively in cooked meat broth medium at 30°C for 24 hours. We incubated at 30°C for optimum for toxin production by clostridia. The culture supernatant was stored at -80°C for future toxin assays on any samples that tested positive by PCR. Cells from 500µl of culture were pelleted and resuspended in sterile saline to ABS$_{600}$ 0.1, then heated at 100°C for ten minutes to lyse the cells. Cell debris was pelleted and the lysate stored at -20°C until required. Control *S. aureus* strains were obtained from clinical isolates by replicating the multiplex PCR method described by Monday and Bohach [13] to identify strains carrying toxin genes. Possession of toxin genes by control strains was confirmed by sequencing the amplification products (IMVS Sequencing Centre, Adelaide).
Table 1. Infants and specimens used in this study

<table>
<thead>
<tr>
<th></th>
<th>SIDS (n=57)</th>
<th>Live comparison infant (n=79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (months)</td>
<td>4</td>
<td>4.6</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td>% Male</td>
<td>54</td>
<td>57</td>
</tr>
<tr>
<td>Specimen type</td>
<td>Large and Small intestine contents</td>
<td>faeces</td>
</tr>
<tr>
<td>Source</td>
<td>50 from SA collected between 1989 and 1994 (1991 definition for SIDS [14])</td>
<td>De-identified diagnostic specimens from live infants attending the Women’s and Children’s Hospital, South Australia</td>
</tr>
<tr>
<td></td>
<td>7 from Victoria collected between 2007 and 2008 (2004 definition for SIDS [15])</td>
<td></td>
</tr>
</tbody>
</table>
**PCR screening of bacterial culture lysates**

Detection of *S. aureus* in samples used the published primer pair Sa442-1, Sa442-2 [16]. For *S. aureus* species specific PCR, cycling conditions on a Hybaid OmniGene thermocycler were as follows: initial denaturing 94°C 120s, 35 cycles of 94°C 30s, 54°C 40s, 72°C 70s, then final extension 72°C 120s. Separate PCR reactions were employed for the detection of each SE gene, with the exception of one pair of duplexed reactions (*seg* and *sej*) which could be utilized without losing sensitivity. *seg* and *sei*, and *sed* and *sej* have been shown on numerous occasions to co-exist in *S. aureus* isolates [11, 17, 18]; therefore, for these genes we screened for *seg* and *sej*. Each PCR reaction contained: 0.1µM MgCl$_2$; 1X Colourless GoTaq Flexi Buffer; 0.005µM dNTP mix; 0.1µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison, Wisconsin), 2µl bacterial lysate and water to a total volume of 25µl. The buffer in the reaction for *seg*, *sej* and TSST PCR was decreased to 0.8X to improve the amplification of larger products. *sec*, *seg*, *sej* and TSST genes were amplified using specific oligonucleotide primers (Geneworks, Thebarton, South Australia) *sec*, *seg*, *sej* and TSST combinations as published [13]. *sea* and *seb* genes were amplified using published primer pairs eta-F, eta-R (where „et” stands for enterotoxin and not exfoliative toxin) [19] and GSEBR-1, GSEBR-2 [20] respectively. For toxin gene PCR amplifications cycling conditions were as follows: initial denaturing 94°C 120s; 10 cycles of 94°C 30s; 50°C 30s; 72°C 70s; 35 cycles of 94°C 30s; 54°C 40s; 72°C 70s; then final extension 72°C 120s. All amplification products were visualized on a 2% agarose gel stained with GelRed (Biotium, Hayward, California).

**Single colony analysis and western blot toxin assay**

Samples testing positive by PCR for multiple toxins were inoculated onto blood agar plates and up to 20 colonies per sample were subcultured into 10ml brain heart infusion (BHI) broth
and incubated at 37°C with shaking overnight. A 100µl sample was taken and the cells were pelleted, resuspended in saline and boiled for ten minutes to make a crude lysate of each culture for PCR analysis. Toxin produced by isolates was harvested using the adapted cellophane-over-agar (COA) method described by Jarvis and Lawrence [21] using cellulose membrane retaining proteins of MW >12,000 (dialysis tubing, Sigma, St Louis, Missouri). Samples of COA supernatants (25µl) were denatured in 1x SDS loading buffer and heated at 100°C for ten minutes then separated on 16% PAGE gels at 50mA for one hour and compared with 10 µl Multimark Multi-Coloured Standard (Invitrogen, Carlsbad, California). Proteins were then transferred to a nitrocellulose membrane at a constant 300mA for one hour and probed with rabbit anti-TSST, anti-SEA, anti-SEB or anti-SEC<sub>2/3</sub> immunoglobulin (Toxin Technology, Sarasota, Florida) followed by goat anti-rabbit alkaline phosphatase conjugated immunoglobulin (Bio-Rad, Hercules, California) and developed with 4-nitro blue tetrazolium chloride and X-phosphate/5-Bromo-4-chloro-3-indoyl-phosphate colour precipitate solution.
Results

Significant differences were observed between the two groups for *S. aureus* species detection ($p=0.002; \text{OR } 3.18; \text{95\% CI } 1.47-6.97$) and for infants with one or more *S. aureus* toxin genes ($p=0.0095; \text{OR } 2.85; \text{95\% CI } 1.26-6.47$) (Table 2). No significant difference was observed in the percentage of *S. aureus* positive samples with and without SE genes (toxin positive samples divided by the number of *S. aureus* positive samples) in SIDS compared with controls ($p=0.4673; \text{OR } 1.576; \text{95\% CI } 0.61-4.09$). Ten or more *S. aureus* isolates were purified from each of eight samples where multiple toxin genes were detected, and an attempt was made to demonstrate *in vitro* production of toxins using the cellophane-over-agar toxin harvest and western blot analysis (Figure 1). TSST and SEC were demonstrated in *S. aureus* strains from one SIDS large intestine and one SIDS small intestine respectively. In six other samples (one SIDS small intestine and three comparison infant samples) and four PCR positive control strains, *in vitro* toxin production in PCR positive isolates could not be demonstrated (Table 3). SEG, SEJ, SEH and SEC₁ antitoxins were not available for assay.
Table 2. Results of PCR on crude lysate samples

<table>
<thead>
<tr>
<th></th>
<th>sea</th>
<th>seb</th>
<th>sec</th>
<th>see</th>
<th>seg</th>
<th>seh</th>
<th>sej</th>
<th>TSST</th>
<th>S. aureus species</th>
<th>Total infants with 1 or more toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIDS (n=57)</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>39 (68.4%)</td>
<td>25 (43.8%)</td>
</tr>
<tr>
<td>Comparison infants (n=79)</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>32 (40.5%)</td>
<td>17 (21.5%)</td>
</tr>
</tbody>
</table>

*p = 0.002 OR 3.18; 95% CI 1.47-6.97

*p = 0.0095 OR 2.85; 95% CI 1.26-6.47
Table 3. Toxin PCR results and *in vitro* toxin production by *S. aureus* isolates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crude lysate PCR result</th>
<th>Isolate PCR result</th>
<th>Toxins tested <em>in vitro</em></th>
<th>Toxins detected by western blot analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIDS Large intestine 1</td>
<td>TSST and sec</td>
<td>10</td>
<td>10 TSST and sec</td>
<td>TSST, SEC2/3</td>
</tr>
<tr>
<td>SIDS Small intestine 1</td>
<td>sec, seg and sej</td>
<td>10</td>
<td>10 sec, seg and sej</td>
<td>SEC2/3 SEC2/3</td>
</tr>
<tr>
<td>SIDS Small intestine 2</td>
<td>TSST, seg and sbB</td>
<td>10</td>
<td>10 TSST and seg, none seb</td>
<td>TSST none</td>
</tr>
<tr>
<td>SIDS Small intestine 3</td>
<td>seg and sej</td>
<td>10</td>
<td>10 seg and sej</td>
<td>none</td>
</tr>
<tr>
<td>Healthy infant faeces 1</td>
<td>TSST, sea, seb and seh</td>
<td>20</td>
<td>5 seb only, 2 TSST and sea, 2 TSST, sea and seh, 7 sea and seb, 3 sea, seb, TSST and seh, 1 sea, seb and seh</td>
<td>TSST, SEA, SEB none</td>
</tr>
<tr>
<td>Healthy infant faeces 2</td>
<td>TSST and sej</td>
<td>4</td>
<td>3 TSST, none sej</td>
<td>TSST none</td>
</tr>
<tr>
<td>Healthy infant faeces 3</td>
<td>seb, seg and sej</td>
<td>6</td>
<td>6 seb, seg and sej</td>
<td>SEB none</td>
</tr>
<tr>
<td>Healthy infant faeces 4</td>
<td>TSST and seg</td>
<td>9</td>
<td>9 seG, 8 TSST</td>
<td>TSST none</td>
</tr>
<tr>
<td>Clinical isolates</td>
<td>TSST/ sea/ seb/ sec</td>
<td>4</td>
<td>1 TSST, 1 sea, 1 seb, 1 sec</td>
<td>TSST/ SEA/ SEB/ SEC2/3 none</td>
</tr>
</tbody>
</table>

95
**Figure 1a** Amplicons from screening *S. aureus* isolates for sec: **Lane 1** pUC19/HpaII DNA MWT marker, **2** sec PCR positive control strain (clinical isolate). **3-5** *S. aureus* isolates from SIDS large intestine 1. **6-9** *S. aureus* isolates from SIDS small intestine 1. **10** enterotoxin PCR negative *S. aureus* control strain (clinical isolate). **11** pUC19/HpaII DNA MWT marker

**Figure 1b** Anti-SEC$_{2/3}$ western blot analysis of cellophane-over-agar toxin preparations. **Lane 1** purified SEC$_1$. **2** purified SEC$_2$. **3** purified SEC$_3$. **4** sec PCR positive control strain (clinical isolate) (no toxin detectable). **5-7** *S. aureus* isolates from SIDS large intestine 1. (no toxin detectable) **8-10** *S. aureus* isolates from SIDS small intestine 1. **11** enterotoxin PCR negative *S. aureus* control strain (clinical isolate)
**Discussion**

*S. aureus* possessing SE genes are commonly found in the infant intestinal tract. The proportion of *S. aureus* carrying toxin genes does not differ between SIDS and control infants. These results suggest that colonisation by *S. aureus* with SE genes is normal in infants and their detection is unlikely to be predictive of SIDS. Similarly, in previous work we have demonstrated toxin genes in *Escherichia coli* from SIDS and healthy infants in the absence of disease [22]. The presence of these potentially pathogenic genes in normal flora is intriguing and indicates that host determinants, rather than bacterial virulence alone, determine the outcome of host-pathogen interactions. If given the opportunity, i.e. translocation into the bloodstream or host susceptibility to uncontrolled pro-inflammatory events, these toxins could contribute to disease, and plausibly to an infectious aetiology of SIDS. Gene possession alone does not guarantee that toxin is actually produced *in vivo*. In *S. aureus* the production of toxins is dependent on a number of other factors including temperature, pH and interactions with other bacterial species. We attempted to demonstrate *in vitro* toxin production by a number of strains carrying toxin genes. The value of this assay was limited by the fact that mimicry of the conditions of *in vivo* SE production is inherently difficult to achieve, and *in vitro* immuno-detection was limited to availability of commercially produced anti-toxins, with antibodies to SEE, SEG, SEI or SEJ being unavailable. Interestingly, the TSST PCR positive control isolate did not demonstrate *in-vitro* TSST production at 37°C. This clinical isolate was from a case of wound toxic shock syndrome. The isolate was also positive by PCR for *seg*. Perhaps this was a case of SEG toxic shock syndrome as described by Jarraud [11].

The finding that *S. aureus* species was detected more frequently in SIDS than comparison infant samples is likely to be an artefact of seasonal and yearly variation during the two
periods when the samples were collected rather than a hallmark of SIDS, although the latter cannot be dismissed based on these results alone. Both frequencies were, however, within the expected range for infant samples [23]. The limitations of this study mainly concern the availability of suitable controls. Since SIDS is the most common cause of death in the first year of life, the required number of control infants is unattainable. This is a widely acknowledged problem making statistical analyses difficult [24]. This study utilized faecal samples as a representative source of normal human *S. aureus*. There is no evidence that faecal flora differs from that found in large bowel contents, and for that reason, faeces were considered a valid comparison material in accordance with other studies of this type [25].

It is apparent that SEs are not involved in pathogenesis purely on gene prevalence based on sampling of intestinal contents. They are, however, sufficiently common to fit the *common bacterial toxins hypothesis* for SIDS. The toxins detected in these samples might contribute to SIDS pathogenesis via synergistic interactions with other colonizing species, in particular *Escherichia coli* which has also been shown to have associations with SIDS [26, 27]. The phenomenon of lethal synergy demonstrated with SIDS nasopharyngeal isolates has been previously demonstrated in a gnotobiotic mouse model [2] and in chick embryos [28]. Synergy of bacterial toxins with respiratory viral infection or products of cigarette smoke are other pathogenetic pathways to SIDS described in animal models [29]. SEs have been proposed to facilitate transcytosis of bacteria across the intestinal barrier [30]. Risk factors for SIDS such as diarrhoea and lack of breast feeding also promote the translocation of intestinal bacteria [31]. This raises the possibility of such a mechanism taking place in SIDS, which could also explain the findings of *S. aureus* in normally sterile sites in SIDS infants [12, 32]. Alternatively, the role of *S. aureus* in SIDS may be via an immune response pathway: *in vivo* stimulation with staphylococcal enterotoxin has been shown to condition the innate immune
system to hyper-respond to various pathogen-associated molecular patterns [33]. *S. aureus* toxins are also powerful inducers of interleukin-6 identified in a significant proportion of SIDS infants [34]. A polymorphism in the IL-6 gene associated with high IL-6 responses was over-represented among Australian SIDS infants [35]. A combination of genetic susceptibility with defective pathogen pattern recognition and toxigenic bacterial colonisation in infants has been proposed as an hypothesis to explain SIDS [36].
References


Chapter 5. *Clostridium sordellii* lethal toxin gene is not detectable by PCR in the intestinal flora of SIDS cases or infants who died of other causes.

Abstract

Infection caused by *Clostridium sordellii* translocated from the gastrointestinal tract has been reported to cause septic shock, often resulting in fatality. The organism’s major virulence factor, lethal toxin (LT), is responsible for fatal outcome after *C. sordellii* infection. We designed an experiment to explore the possibility of *C. sordellii* colonising the intestinal tract contributing to Sudden Infant Death Syndrome, possibly via a fatal toxaemia. The feasibility of the methodology was demonstrated using a spiked culture of intestinal contents. Cultures grown from intestinal contents of fifty infants meeting the 1991 definition of SIDS, and thirteen cases of non-SIDS death were tested for the *LT* gene by PCR. None of the SIDS or non-SIDS infant samples tested positive for *LT*. The results of this investigation suggest that intestinal colonization by *LT* toxigenic *C. sordellii* is unlikely to contribute to SIDS. However, based on these results alone we cannot completely exclude the role of *C. sordellii* bacteraemia or toxaemia in SIDS.
Background

Infection by *Clostridium sordellii* translocating from the gastrointestinal tract has been reported to cause septic shock, often resulting in fatality [1]. The cases summarized by [1] had underlying medical conditions; however, more recently several cases have been described to occur in previously healthy women who underwent medically-induced abortion [2] or shortly after delivering a baby (vaginal or cesarean section) [3-10]. Pregnancy and childbirth have been suggested to predispose a small number of women to acquire *C. sordellii* in the vaginal tract, where the acidic pH of the vagina enhances the cytopathic effects of its toxins [2]. The fastidious anaerobic growth, variable staining characteristics and complex biochemical profiles of clostridia probably contribute to low numbers of reports of non-lethal *C. sordellii* infection [2]. It is not known whether an infant born to a mother asymptotically colonised by *C. sordellii* acquires the organism at birth through the ingestion of vaginal flora, as has been shown for other organisms such as *Escherichia coli* [11].

*C. sordellii* produces *lethal toxin* (LT) a highly potent single-chain protein toxin of 250kD molecular mass that acts intracellularly by catalyzing the glycosylation of small GTPases. When LT is injected intraperitoneally into mice, the adherens junctions of lung vascular tissue break down, resulting in the extravasation of blood into the thoracic cavity [12]. Notable clinical features of *C. sordellii* toxic shock include an absence of fever and rash, refractory tachycardia, hypotension, diarrhea and a relatively short timecourse (<1 day from hospitalization to death) [2]. These are not unlike the observations made prior to SIDS death, in particular where the event was recorded on memory monitors to include a cardiogenic event preceded by bradycardia [13]. The organism has only been isolated from the blood during one of fourteen cases of fatal toxic shock syndrome reported in the literature [2] and it
is recognized that toxin produced from a localized site can cause a shock-like death without bacteraemia [7, 8]. We designed an experiment to explore the possibility of colonising intestinal *C. sordellii* contributing to Sudden Infant Death Syndrome, possibly via toxin absorption into the bloodstream by a similar mechanism to infant botulism, which has previously been shown to account for a percentage of SIDS deaths [14, 15].
Methods

Sample material

The study was approved by the Research Ethics Committee of the Children Youth & Women’s Health Service. Testing was done retrospectively on stored material from previous investigations conducted in South Australia, Australia, where stored material was available. Small portions of intestinal contents (both small and large intestine for each infant) from 50 SIDS and 13 non-SIDS comparison infants that died between 1989 and 1994 in South Australia were collected at autopsy into sterile containers and immediately stored at -80 °C until required. SIDS diagnoses retrospectively matched the criteria specified by the 1991 definition for SIDS [16]. The median age of SIDS infants was four months, the interquartile range was 3.65 months and the proportion of male infants was 56%. The comparison group consisted of 13 infants whom had died of non-SIDS causes (nine infectious and four from congenital disease). Their median age was three months, the interquartile range was three months and the proportion of male infants was 46%. All post-mortem investigations were conducted within 48 hours of death. Samples were frozen only once and were not thawed prior to this investigation. Samples had not been stored anaerobically, so a small portion (<1 g) of intestinal contents or faeces was collected from the middle of the sample, i.e. that had not been in contact with air during storage, and cultured in cooked meat broth medium (12.5% cooked meat medium, 2.7% (w/v) Schaedler’s Broth made up to the desired volume in deionised water and autoclaved) containing 0.07 µg/ml neomycin at 30 °C for 72 hours. We incubated at 30°C for optimum for toxin production by clostridia (Hatheway, 1979). The culture supernatant was stored at -80°C for future toxin assays on any samples that tested positive by PCR. Cells from 500 µl of culture were pelleted and resuspended in sterile saline to ABS₆₀₀ 0.1, then heated at 100 °C for ten minutes to lyse the cells. Cell debris was pelleted and the lysate stored at -20 °C until required. American-Type Culture Collection C. sordelli
strain ATCC9714 was used as a control strain as it was shown by Voth et al. to possess LT and express the toxin under appropriate conditions [17]. The suitability of the storage and culture conditions for clostridia has been confirmed previously by testing the crude lysate samples for two common anaerobic flora Clostridium innocuum and Bacteroides thetaiotaomicron. Fifty-four percent of the samples were positive for either species (data not shown). Possession of the LT gene by the control strain was confirmed by sequencing the amplification products (IMVS Sequencing Centre, Adelaide).

Demonstration of C. sordellii lethal toxin gene presence in simulated (spiked) culture

It was necessary to determine whether the organism at low frequency in an intestinal sample could be detected by PCR after 72 hours culture in cooked meat media broth with neomycin. Broths were inoculated with a single infant’s intestinal contents sample (20µl) and 100 µl of an aliquot of a serial dilution of C. sordellii ATCC9714 suspended in sterile saline. Another 100 µl aliquot of the dilution suspension was plated onto Brain-Heart Infusion agar (4.5% (w/v) Brain Heart Infusion, 1.8% (w/v) Yeast extract, 0.0019% (w/v) (autoclaved) Vitamin K, 0.19% Haemin, 4.76% (v/v) defibrinated horse blood) and incubated for 72 hours at 37 °C in an anaerobic chamber. The results indicated that when two organisms were present in the dilution suspension (i.e. two colonies grew on the BHI plate) LT could be detected in the crude lysate made from the spiked cooked meat broth culture (Figure 1). A broth that was not inoculated with C. sordellii suspension (i.e. with intestinal contents only) gave a negative PCR result.
Figure 1. Agarose gel electrophoresis of amplification products.

Lanes 1 & 6: pUC19/HpaII DNA MWT marker (Geneworks). Lane 2: positive control ATCC9714. Lane 3: Spiked culture. Lane 4: unspiked culture. Lane 5: no template control
**PCR screening of bacterial culture lysates**

Detection of *LT* in samples used the published primer pair CLS-F1 and CLS-F2, which are based upon the sequence of *C. sordellii* deposited under GenBank accession number X82638 [2], to screen the crude culture lysate samples. Cycling conditions on a Hybaid OmniGene thermocycler were as follows: initial denaturing 94°C 120 sec, 35 cycles of 94°C 30 sec, 54°C 40 sec, 72°C 70 sec, then final extension 72°C 120 sec. Each PCR reaction contained: 0.1 µM MgCl₂; 1X Colourless GoTaq Flexi Buffer; 0.005 µM dNTP mix; 0.1 µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega), 2 µl crude lysate and water to a total volume of 25 µl. All amplification products were visualized on a 2% agarose gel stained with ethidium bromide. A positive result yielded a 250 base pair product.
Results

None of the SIDS or comparison crude culture lysate samples tested positive for LT by PCR. Anecdotally, a negative result was also obtained from intestinal samples that came from a deceased infant who was excluded from the study on the basis of age (>12 months) who, in their post mortem investigation, reportedly had *C. sordellii* isolated from heart blood. Interestingly, the original cause of death was reported to be SIDS-like with thymic petichial haemorrhages.
Discussion

The results of this investigation suggest that intestinal colonization by LT toxigenic C. sordellii is unlikely to contribute to SIDS. Based on these results we can almost certainly exclude a role for C. sordellii in SIDS. There could be a remote possibility that some babies could have died of C. sordellii bacteraemia, but autopsy bacteriology carried out independently does not support this. Collection of healthy infant faecal samples was anticipated to supplement the low number of dead control samples. However, since no LT positive samples were found this requirement was considered unnecessary.

Toxins of clostridia have on numerous occasions been proposed to be involved in SIDS. The literature covers investigations into the roles of C. perfringens type A cytotoxic enterotoxin [18, 19], and C. botulinum neurotoxin (BoNT) [20, 21] which was shown to be responsible for 4%-24% of SIDS cases [14, 15]. One could speculate that C. sordelli could account for a proportion of SIDS deaths in some populations via a similar mechanism to infant botulism, where the vegetative bacteria colonise and multiply due to the ideal physiological conditions for spore germination in the infant intestine and produce toxin in the intestinal lumen. BoNT producing organisms have been found to cause a number of SIDS deaths in countries in Europe and North America [22] but it does not appear that this is the case the Australian SIDS infant population. Infant botulism is not as prevalent in Australia as in Europe and the United States, and generally is not considered a major differential diagnosis to SIDS. In a prospective culture-based study conducted in 1992 BoNT was found not to be involved in SIDS in South Australia [23]. In a similar study conducted in 2006 (Unpublished data) we tested the 50 SIDS and 13 controls from the present study for the most common C. botulinum toxin types (A and B) as well as the rarer types E, F, G botulinum toxin and C. butyricum neurotoxin by PCR
using degenerate primers described by Fach et al. [24]. We found no positive samples, which was consistent with the results of Byard’s culture-based study. This is to our knowledge the first investigation regarding the role of *C. sordellii* toxin in SIDS. LT was an interesting candidate toxin due to the sudden onset of toxic shock and similarity to events observed leading up to SIDS deaths. It is plausible that LT could play a role in SIDS in regions where perhaps *C. sordellii* is more prevalent than in Australia.
References


Chapter 6. Development of a novel hypothesis for unexplained sudden unexpected death in infancy

Abstract

Two recent retrospective studies independently reported typically pathogenic bacteria in normally sterile sites of infants succumbing to sudden unexpected death in infancy (SUDI). These findings suggested a proportion of unexplained SUDI might be triggered by bacteraemia. The objective was to assess these observations in the context of the pathology and epidemiology of SIDS in relation to the role of infection and inflammation as triggers of these deaths. The literature was reviewed to identify potential risk factors for unexplained infant deaths and a theoretical model for SUDI was constructed. Pathologic and epidemiological evidence led to an hypothesis based on three factors: bacterial translocation; pathogen pattern recognition insufficiency; prenatal exposure to infection. We propose that sterile site infections in which common toxigenic bacteria are identified indicate a brief bacteraemic episode prior to death. This might reflect a defective innate response to invasive pathogens that result in reduced clearance of the bacteria. Thymomegaly observed consistently among infants diagnosed under the category of Sudden Infant Death Syndrome might have its origins in prenatal life, perhaps generated via in utero infection/exposure to microbial antigens which results in thymocyte priming. There is consistent evidence for an infectious aetiology in many unexplained SUDI. Future directions for research are suggested.
Background

Genetic predisposition to inappropriate responses to infection has been the focus of recent investigations into Sudden Infant Death Syndrome (SIDS). Research to date has primarily targeted cytokine gene polymorphisms conferring heightened pro-inflammatory responses [1-3]. While these are important in determining the outcome of an established inflammatory response, factors influencing susceptibility to invasive infection could also be involved in SIDS. This genetic predisposition could be X-linked given the consistent male gender predisposition observed in SIDS [4]. Two independent retrospective studies reported typically pathogenic bacteria (notably *Staphylococcus aureus*) in normally sterile sites of infants succumbing to sudden unexpected death in infancy (SUDI) [5, 6] (a broad classification of unexplained infant deaths including cases that would have previously constituted SIDS classification). The finding of potential pathogens in usually sterile sites could reflect three possibilities: 1) the bacteraemia is missed because of the small volumes of blood often available for testing; 2) bacterial presence might be dismissed as post-mortem contamination; 3) because of the developmental stage of the infant, low antibody levels and/or inefficient innate immunity permit the organisms to spread rapidly into normally sterile tissues. The hypothesis proposed in this chapter is based on the pathologic and epidemiological evidence from unexplained SUDI. In reference to the literature, “SIDS” terminology is used, as was appropriate at the time of the original research publication. The SIDS cases upon which the hypothesis is based met the 1989 [7] or 2004 [8] definition. It is not possible to determine whether or not evidence of bacteraemia/sterile site infection was taken into account for these cases because many pathologists might have regarded the findings as “contamination.” On the other hand, for the diagnosis of SIDS, bacteraemia findings would be excluded as an explainable cause of death.
The literature was reviewed to identify potential risk factors for unexplained infant deaths and construct a theoretical model for SUDI. Three common factors were identified as potential contributors to SIDS: 1) evidence of bacteraemia and/or toxaemia; 2) genetic predisposition to insufficient pathogen pattern recognition; 3) prenatal infectious events that affect organ growth and/or the developing immune system. Their potential roles in unexplained SUDI are developed in this section and interaction among these factors is illustrated in Figure 1.
Figure 1. Proposed interactions between transient bacteraemia (1), PPR gene polymorphisms (2) and prenatal events (3) leading to sudden infant death.
Factor 1: Transient bacteraemia

With the exception of a mild respiratory viral infection and/or diarrhoeal disease there is no significant illness reported prior to SUDI [13]. An episode of transient bacteraemia would logically explain post mortem bacteriological findings of sterile site infection in the absence of bacteraemic symptoms and signs, and an absence of pathogens in the small blood volumes available at autopsy. Transient bacteraemic episodes have been identified as normal infant events and asymptomatic bacteraemia in newborns has been documented [12, 14]. A recent study has identified pyrogenic toxins of *Staphylococcus aureus* in the urine (a possible marker of bacteraemia) in nine percent of a healthy infant cohort, which often coincided with a mild respiratory infection [15]. High levels of these toxins have been identified in tissues or sera in over half of SIDS infants from five countries [16, 17]. Infection or toxins have been proposed to cause sudden life threatening events in infancy, especially when maternal antibody levels decline leaving the infant temporarily hypo-immune, during the 2-4 month age range in which the peak of SIDS occurs [11]. In most cases transient bacteraemia is rapidly cleared by the immune system. This is dependent upon recognition of the pathogen and initiation of an appropriate immune response.

The mild respiratory viral infection that often precedes SUDI can disrupt the mucosal barrier allowing translocation of colonising bacteria into the bloodstream. Virus infections also enhance density of colonization as does prone sleeping [18]. Similarly, damage to the intestinal epithelium during diarrhoeal disease could allow translocation from this site. Other risk factors for SIDS, preterm birth and not breast feeding, are known to increase the ability of enteric bacteria to translocate from the intestine [12]. *S. aureus* is a key candidate for both extra-nasopharyngeal and extra-intestinal translocation. The synergistic events between *S.*
*aureus* and respiratory viral infection has been well documented [18, 19], and staphylococcal enterotoxins are produced in the intestinal tract of infants [20] and genes encoding these toxins have been detected in our recent work on the intestinal contents of SIDS infants [21]. It has been proposed that enterotoxigenic activity by *S. aureus* could facilitate transcytosis [22], which would also explain the findings of *S. aureus* and coliforms in normally sterile sites in SIDS infants at post mortem.

Factor 2: Pathogen pattern recognition insufficiency

Rapid recognition and response to microbial invasion is the function of the innate immune system. Specifically, Toll-like receptor (TLR) 2, Nucleotide-binding oligomerization domain containing 2 (NOD2), and Tumor necrosis factor alpha (TNF-α) receptor 1 are necessary for full responses to *S. aureus* [23]. Similarly, TLRs 2, 4 and 5, MD-2, and CD14 facilitate the recognition and response to *Escherichia coli*. Loss-of-function polymorphisms among genes regulating the above immune components can result in impaired recognition of invasive pathogens and thus impaired induction of an immune response. *S. aureus* and coliforms found in normally sterile sites may be a remnant or „footprint” of transient bacteraemia during which the pathogen was unrecognised and thus not cleared from these sites. Alternatively, the rapidity of death, in some cases, might not allow sufficient time for the immune system (whether compromised or not) to clear the bacteria from the normally sterile sites. Ineffective pathogen recognition may leave infants particularly susceptible to bacteraemia at 2-4 months of age because the natural diminution in maternal antibodies could not be compensated for by the innate immune system.
Factor 3: Prenatal infectious event

A hallmark of SIDS pathology is a pattern of organ weights that distinctly deviate from the expected reference ranges. The thymus (p=0·04) and brain (p=0·001) in particular are significantly larger in SIDS victims [24]. Although it was inferred that this was likely due to the reference ranges being based upon infants with illness prior to death (whereas SIDS appear to be healthy immediately before death), the investigators also recognised that the “differences could also reflect disturbances in growth presumably beginning prior to birth”. Other researchers have produced supportive data that suggest that enlarged brains are present at birth of the SIDS infant, rather than a consequence of disproportionate postnatal growth [25]. Extrapolation of post-mortem organ weight data show that disrupted patterns of growth of vital organs appear to originate in the prenatal period [26] (Figure 2). One possibility is “thymic priming” via a massive T-cell proliferative response to a foreign protein or pathogen, possibly prenatally. In this case the enlarged thymus would be consistent with increased thymic activity. Fetal exposure to virus causes significant increase in T-cell production [27], which may lead to increase in thymus weight as has been demonstrated in other T-cell driven inflammatory conditions [28]. Prenatal infectious events could stand alone as a risk factor for SIDS (through effects of thymic “priming” on pro-inflammatory events). Placental morphology could also be indicative of prenatal virus transmission. Current research by Ansari and colleagues indicate that total trophoblast volume in normal birth weight SIDS is higher due to a significant increase in both cytотrophoblast and syncytiotrophoblast volume (Ansari, TI, Personal communication, 8th January 2009). As these cells are the first-line defense of the fetus against viruses, such anomalies suggest viral involvement. Postnatal exposure (or re-exposure to an antigen possibly via bacteraemia) could also be potentiated by immunoregulatory gene polymorphisms conferring inappropriately high cytokine production, for example those associated with poor outcomes during sepsis. An alternative outcome resulting from prenatal thymic “priming” and subsequent re-exposure to the causal antigen
could include anaphylaxis for which there is some evidence [29]. CMV infection in particular could explain the research findings of Rubens et al. [30] who claim that SIDS infants perform poorly in newborn oto-acoustic emission hearing screening tests. Hearing loss develops in 7.2% of infants born with asymptomatic congenital CMV infection [31] and a similar mechanism may be at work in SIDS.
Figure 2. Simulated a) thymus weight and b) brain weight of SIDS vs. non-SIDS infants. Adapted from Goldwater and Little [26].
Mode of Death

Based on the evidence of Poets et al. [32] speculation as to the mode of death is necessarily narrowed to a non-respiratory, cardiogenic event under conditions of toxic/septic shock or anaphylaxis. Cytokines produced in response to infection can induce physiological responses likely to be involved in SUDI. These include vascular shock, hypoglycaemia, deep sleep with prolonged apnoea, cardiac irregularities and fever [33]. Polymorphisms in genes involved in QT interval control may increase the sensitivity of the heart to exposure to toxin. Because such polymorphisms are not universal in SUDI their presence would not be seen as an „essential“ route to cardiac standstill.

Proposed Investigation

Bacteraemia preceding SUDI might be detected more frequently if molecular methods, as opposed to culture, are employed for the detection of bacteria and their products in blood and normally sterile sites. As bacteria have been identified in normally sterile sites of infants suffering unexplained death, focusing on immunoregulatory gene polymorphisms that specifically affect recognition of bacterial species found to be implicated (i.e. S. aureus and E. coli) will most certainly prove worthwhile. For example, polymorphisms: TLR-2(G2408A) which has been suggested to increase risk of staphyloccocal infection [34], NOD2(302insC) which results in a loss of bacterial sensing and decreased activation of inflammatory pathway [35], or MD-2(G56R) variant which exhibits very low LPS binding [36]. Four TNF-α receptor 1 polymorphisms, TNFRSF1A(A+36T), TNFRSF1A(G-609T), TNFRSF1B(T+196G), and TNFRSF1B(A+1466G) have been identified, but as yet their effect on receptor function is unclear [37]. Prenatal infectious events could be predicted by assaying for a shift from CD45RA+ to CD45RO+ cells in the T lymphocyte population (consistent with previous
exposure to microbial products or other antigens) in peripheral blood collected shortly after birth, should it be available for SUDI infants. In our case these samples would need to be collected prospectively from a large number of infants.

Conclusion

This novel hypothesis takes into account new findings in relation to unexplained SUDI. Ineffective innate responses to invasive pathogens through specific gene polymorphisms would logically increase the likelihood of failure to clear bacteria from the blood and other sterile sites. These sterile site infections involving common toxigenic bacteria could be a “footprint” of a brief bacteraemic episode happening just prior to death. It is plausible that thymomegaly (evidenced by comparative organ weight and simulated organ growth analysis) seen consistently in SIDS has its origins in prenatal life and is probably generated via in utero infection/exposure to bacterial/viral antigen with consequential thymocyte “priming.” Overall, bacteriological and pathological observations appear to be consistent with an infectious aetiology for unexplained SUDI. If an X-linked gene mutation were found, it would provide an explanation of the male preponderance observed in SIDS and also contribute to our understanding of the underlying mechanisms in SIDS.
References


Chapter 7. T cell receptor BV3 recombination signal sequence allele 2 is not associated with unexplained Sudden Unexpected Death in Infancy (SUDI) in an Australian cohort.

Abstract

Polymorphisms in genes that influence the expression of toxin receptors could contribute to Sudden Infant Death Syndrome (SIDS) and unexplained Sudden Unexpected Death in Infancy (uSUDI) for which there is evidence of toxin involvement. We aimed to determine whether TCRBV3S1 allele 2 could be involved in a staphylococcal toxic shock hypothesis for uSUDI. Compared with 96 live comparison infants there was no association observed between TCRBV3S1*2 and 48 uSUDI in this Australian population. The possibility remains that toxin receptors other than TCRBV3 contain similar polymorphisms that affect staphylococcal enterotoxin binding which might play a role in the staphylococcal toxic shock hypothesis for SIDS and uSUDI.
**Background**

Pathological and epidemiological findings in SIDS infants suggest an infectious aetiology with involvement of *Staphylococcus aureus* superantigenic toxins [1-6]. Staphylococcal enterotoxins (SEs) have been demonstrated in tissues or sera in over half of SIDS infants from five countries [6, 7] and pathological findings in SIDS are consistent with toxic shock [8, 9]. Host factors, including genetic determinants, influence the outcome of interactions between toxin and host. Accordingly, polymorphisms in genes that influence the expression of toxin receptors could affect the outcome of SE toxaemia, and could therefore play a role in SIDS and Sudden Unexpected Death in Infancy (SUDI- a broad classification of infant deaths that includes those that would previously have been termed SIDS).

SE molecules activate T cells via receptors that express particular Vβ elements. Activation via SE-TCRVβ binding results in overproduction of cytokines which contribute strongly to the onset of toxic shock [10]. Random recombination of TCR segments during T cell maturation ensures a repertoire of diverse TCRs so the proportion of cells that respond to SE is limited. This process is controlled by the *recombination signal sequence* (RSS) gene region. A C→T single nucleotide polymorphism (SNP) in the *RSS* of the *TCRBV3S1* gene (TCRBV3S1 RSS*2) increases the frequency of expression of the BV3 chain on T cells, individuals homozygous for the 2 allele have a mean of 8.1% BV3+ cells while individuals homozygous for the 1 allele have a mean of only 1.2% BV3+ cells. BV3 chains are receptors for staphylococcal enterotoxins B and C (SEB and SEC) in humans [11]. This investigation was designed to determine whether *TCRBV3S1*<sup>2</sup> could be involved in a staphylococcal toxic shock hypothesis for SIDS and uSUDI.
Materials and methods

Ethics approval

The study was approved by the Research Ethics Committee of the Children, Youth and Women’s Health Service, South Australia, Australia and the Victorian Institute for Forensic Medicine, Victoria, Australia. Samples were non-identifiable for the purpose of this investigation. Gender, date of birth, date of death, date of collection and cause of death, with relevant post-mortem findings, were available for analysis.

Sample collection and preparation

Infants included in the study and the specimen types collected are summarized in Table 1. Testing was done retrospectively on stored material from previous investigations conducted in South Australia, Australia (from the cohort of unexplained sudden unexpected death in infancy (uSUDI) – a broad category of unexplained infant deaths from 1980-1994 that had previously been classified as SIDS according to the 1991 definition [12] - described by Goldwater [13], if stored material was available). There were 18 consecutive cases of SIDS from Victoria, Australia since 2007. Small portions of intestinal contents were collected at autopsy and stored at -80°C at autopsy. Samples from infants who died in Victoria were collected from the Victorian Institute for Forensic Medicine and transported on dry ice to the Women’s and Children’s Hospital, South Australia, where the work was undertaken. Meeting the 2004 definition for SIDS they were classified: one was Category 1a and six Category 2. These categories are explained with the current definition of SIDS in Chapter 1. As a comparison group for TCRBV3S1 genotype frequencies, 103 whole blood samples (in EDTA tubes, surplus to diagnostic requirements) were prospectively retrieved from infants who were not suspected of, or diagnosed with, infectious disease. This group primarily consisted of
infants with specific organ system disease (renal failure, heart disease) haematological
disturbances and infants undergoing surgical procedures. The age at which the blood
sample was taken was less than one year to represent the age of the SIDS infants when
they died. Fifty microlitre samples of blood were absorbed onto filter card and stored at
room temperature until required.
Table 1. Infants and specimens used in this study

<table>
<thead>
<tr>
<th></th>
<th>iSUDI</th>
<th>niSUDI</th>
<th>uSUDI</th>
<th>Live comparison infant</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>13</td>
<td>10</td>
<td>49</td>
<td>103</td>
</tr>
<tr>
<td>Median age (months)</td>
<td>2.5</td>
<td>3</td>
<td>4</td>
<td>5.5</td>
</tr>
<tr>
<td>Interquartile range (months)</td>
<td>5.7</td>
<td>8.6</td>
<td>3.5</td>
<td>6.3</td>
</tr>
<tr>
<td>% Male</td>
<td>62</td>
<td>40</td>
<td>54</td>
<td>62</td>
</tr>
<tr>
<td>Specimen type</td>
<td>intestinal contents</td>
<td>intestinal contents</td>
<td>intestinal contents</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>Source</td>
<td>9 SA, 4 Victoria</td>
<td>6 SA, 4 Victoria</td>
<td>39 SA, 10 Victoria</td>
<td>103 SA</td>
</tr>
</tbody>
</table>
**DNA extraction from intestinal contents**

A small portion (<1g) of intestinal contents or faeces was suspended in 1ml glycerol broth (3% w/v tryptone soya broth and 20% glycerol) and stored at -80°C until required. To extract DNA, 90µl of the suspension was aliquoted into a centrifuge tube with 10µl 10X chelex-lysis solution (50% w/v Chelex-100 (Biorad, Hercules California), 2% w/v SDS, 0.1M Tris, 5mM EDTA) and 4mg Proteinase K (Roche, Mannheim Germany) and vortexed briefly. The mixture was incubated at 55°C for one hour, then 95°C for 30 minutes and centrifuged for one minute. The supernatant was diluted tenfold before use in PCR reactions.

**DNA extraction from dried blood samples**

A single 1.2mm punch biopsy was taken from each dried blood sample with 20 punches of blank card in between to prevent carry-over contamination. Nucleic acids were extracted from the punches by treating with a lysis solution with 50% w/v Chelex-100 (Biorad, Hercules California), 1mg Proteinase K (Roche, Mannheim Germany), and incubation at 60°C for 30 minutes then 95°C 30 minutes. The Chelex was pelleted by centrifugation, the supernatant diluted tenfold and 2µl used in each PCR reaction.

**PCR amplification and allele discrimination**

TCRBV3S1 RSS was amplified using the published primers BV3-intron(sense) and BV3-3” of RSS(antisense) [14]. Thermocycler conditions were as follows: 94°C for 2 mins, 30 cycles of 95°C for 30s, 72°C decreasing 0.5°C each cycle (touchdown) for 30 sec, 72°C for 1 min, 15 cycles of 95°C for 30s, 62°C for 30s, 72°C for 1 min, followed by a final extension of 72°C for 5 min on a Bio-Rad IQ5 cycler. Contents of the PCR mixture contained: 0.05µM MgCl₂, 1X Colourless GoTaq Flexi Buffer, 0.0025µM dNTP mix; 0.05µM each of the primer pair.
and 0.5 units GoTaq DNA Polymerase (Promega, Madison Wisconsin), 2µl DNA extraction and water to a total volume of 12.5µl. The genotype was determined by restriction endonuclease \textit{Pvu}II digestion [14] as illustrated in Figure 1. Amplification and digest products were visualized on a 2% agarose gel stained with GelRed (BioTium, Hayward) alongside a pUC19/\textit{Hpa}II DNA molecular weight marker (Geneworks, Thebarton). Controls for each genotype were sourced from the study cohort and confirmed by sequencing the amplification products (IMVS Sequencing Centre, Adelaide).

\textbf{Statistics}

Unexplained SUDI genotypes were analysed against live control infants, iSUDI and niSUDI by comparing frequencies of the 2/2 with the 1/1 and 1/2 genotypes. Yates Corrected Probability Test using 2x2 contingency table software (Statcalc, Epiinfo v6) was used to calculate \( p \) values, odds ratios and 95% confidence intervals.
Figure 1. Alignment of BV3-intron (sense) and BV3-3' of RSS (antisense) with TCRBV1 gene. C→T SNP is shown in red, PvuII restriction site is highlighted in yellow.
**Results**

Not all samples gave a valid result due to the presence of PCR inhibitors in blood and intestinal contents. Forty-eight uSUDI and 96 live comparison infant samples yielded a TCRBV3S1 RSS PCR amplification product. No significant difference was observed between \textit{TCRBV3S1 RSS} allele or genotype frequencies in uSUDI and live comparison infants (Table 2). Allele frequencies of the live control infants were consistent with those in the published literature [15] and were consistent with Hardy-Weinberg equilibrium. Nine uSUDI infants who tested positive for the presence of staphylococcal enterotoxins B and/or C in their intestinal flora (Chapter 4) were included in the present study. Of these nine infants, three had the 2/2 TCRBV3S1 RSS genotype, three had the 1/1 genotype, two had the 1/2 genotype and four had the 2/2 genotype. Four iSUDI who were positive for \textit{seb} and/or \textit{sec} all had the 1/2 genotype.
Table 2. Distribution of TCRBV3S1 RSS genotypes and alleles among infant deaths and live control infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>iSUDI</th>
<th>niSUDI</th>
<th>uSUDI</th>
<th>Healthy Baby</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>1 (7.7%)</td>
<td>0</td>
<td>11 (22.9%)</td>
<td>21 (21.9%)</td>
</tr>
<tr>
<td>1/2</td>
<td>10 (76.9%)</td>
<td>8 (80%)</td>
<td>20 (41.7%)</td>
<td>39 (40.6%)</td>
</tr>
<tr>
<td>2/2</td>
<td>2 (15.4%)</td>
<td>2 (20%)</td>
<td>17 (35.4%)</td>
<td>36 (37.5%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>13 (100%)</strong></td>
<td><strong>10 (100%)</strong></td>
<td><strong>48 (100%)</strong></td>
<td><strong>96 (100%)</strong></td>
</tr>
<tr>
<td>Allele 1</td>
<td>12 (46.2%)</td>
<td>8 (40%)</td>
<td>42 (43.8%)</td>
<td>81 (42.2%)</td>
</tr>
<tr>
<td>Allele 2</td>
<td>14 (53.8%)</td>
<td>12 (60%)</td>
<td>54 (56.2%)</td>
<td>111 (57.8%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>26 (100%)</strong></td>
<td><strong>20 (100%)</strong></td>
<td><strong>96 (100%)</strong></td>
<td><strong>192 (100%)</strong></td>
</tr>
</tbody>
</table>
Discussion

The results of this investigation suggest that TCRBV3SI RSS polymorphism is not associated with uSUDI in this Australian population. Infectious SUDI showed similar frequencies of alleles and genotypes to niSUDI, although the small number of samples in both these groups does not permit any conclusions to be made. It would be interesting to see if this lack of association holds true in those SIDS infants which have been shown previously to have SEs deposited in their tissues. The number of infants that had SEB and SEC detected in their intestinal flora in Chapter 4 was too small to draw any significant conclusions regarding an association between SE presence, and TCRBV3SI*2.

In mice BV3 is a receptor for SEA, and in humans BV3 is a receptor for SEB and SEC [11]. In an animal model of increased BV3 usage (similar to that which results from TCRBV3SI*2 homozygosity) and induced staphylococcal sepsis, SEA mortality was greater in transgenic mice with more BV3 receptors (85%) than non-transgenic littermates (31%). Fifty percent of BV3 transgenic mice died within five days of inoculation and none were accompanied by any change in body weight. Notably, high loads of S. aureus were found in the blood, kidneys and spleen [16]. Similar findings concerning true pathogens, S. aureus and coliforms, in normally sterile sites have been reported in unexplained SUDI (uSUDI) infants in recent publications [17, 18]. TCRBV3SI polymorphism has been investigated in systemic sclerosis and rheumatoid arthritis, but to date has not shown association with any such inflammatory diseases [19, 20].

Despite the lack of association demonstrated in this investigation, the possibility remains that toxin receptors other than TCRBV3 contain similar polymorphisms that affect SE binding
which might play a role in the staphylococcal toxic shock hypothesis for SIDS and uSUDI. Toxic shock syndrome toxin selectively stimulates T-cells bearing BV2 (Vβ2) chains, for which five variant alleles have been identified [21]. These polymorphisms should be included in any further studies of this hypothesis.
References


Chapter 8. Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2

Abstract

This investigation was designed to explore the role of Interleukin-1 receptor antagonist gene (IL-1RN) genotype in unexplained infant deaths (including SIDS), non-infectious infant deaths and infectious infant deaths, and to investigate whether IL-1RN genotype is related to the finding of organisms in normally sterile sites in infant deaths. IL-1RN 89bp variable number of tandem repeat polymorphism genotype was determined using PCR for 49 cases of unexplained sudden unexpected death in infancy (uSUDI), 13 cases of infectious sudden unexpected death in infancy, 10 cases of non-infectious sudden unexpected death in infancy and 103 live control infants. IL-1RN genotype was then compared with the presence of bacteria in normally sterile sites in infant deaths. An association was found between the homozygous A2 allele and uSUDI (p=0.007 95%CI 1.41-17.67) where carriage of the 2/2 genotype was 4.85 times more likely to increase risk of uSUDI compared with the predominant 1/1 genotype. The role of infection in uSUDI and SIDS may be via an immune response pathway where IL-1RN A2 affects IL-1 regulation. These results are consistent with previous research where polymorphic genotypes conferring more severe pro-inflammatory responses are found more frequently in uSUDI/SIDS infants than controls.
Introduction

**Bacteria and their toxins in SUDI and SIDS**

Unexplained SUDI (uSUDI) is a cause of death defined by exclusion of other recognized causes [1]. Infant deaths that would previously have been termed SIDS constitute the majority of uSUDI cases. Pathological, epidemiological and genotypic findings in SIDS infants suggest an infectious aetiology with involvement of bacteria [2-7]. Staphylococcal enterotoxins have been demonstrated in tissues or sera in over half of SIDS infants from five countries including Australia [7] and *Staphylococcus aureus* and coliforms have been found in normally sterile sites in 10.8 and 17.7% respectively of uSUDI cases [8]. Host factors, including genetic determinants, influence the outcome of interactions between toxin and host, which have been the focus of many recent SIDS investigations [9]. The role of bacteria in SIDS pathogenesis may be via an immune response pathway. Interleukin-1 is a cytokine responsible for causing rapid vasodilation in septic shock [10]. Given that *S. aureus* toxins are powerful inducers of interleukin-1 [10] an investigation of this aspect of the immune response pathway was considered worthwhile.

**Interleukin-1 receptor antagonist**

Clinical and pathological findings in SIDS are similar to endotoxin-induced shock [11-13] suggesting that high levels of circulating cytokines such as IL-1 may be implicated in their death. IL-1β can induce vascular shock, hypoglycaemia, deep sleep with prolonged apnea and cardiac irregularities. The known genetic variations in the *IL-1β* gene are not major regulators of *in vitro* production of IL-1β; rather the *IL-1RN* (gene
encoding IL-1ra) allele type has the decisive role [14]. Interleukin-1 receptor antagonist (IL-1ra) is a competitive inhibitor that binds to IL-1 receptors without inducing an intracellular response by blocking receptors. It is an important endogenous regulator of inflammation and actively limits septic shock and reverses hypotension [15]. Additionally, IL-1ra suppresses the release of IL-1β and TNFα from mononuclear cells [16]. Therefore to assess the genetic basis of IL-1β production IL-1RN polymorphic sites, rather than IL-1β polymorphic sites, was considered apposite. In a study by Moscovis et al., a single nucleotide polymorphism in IL-1RN (T+2018C) was found not to be differently distributed between SIDS infants and controls [17]. A length variation within intron 2 of IL-1RN was first reported by Steinkasserer et al to be due to a variable number of 89bp tandem repeat sequences [18]. Currently five alleles, A1-A5, are recognised corresponding to 4, 2, 5, 3, 6 repeats, respectively. The A1 allele, and the 1/1 genotype, are predominant [19]. The A2 variant reportedly enhances in vitro IL-1β production almost two-fold [14], and contributes to susceptibility to severe sepsis [20], although at present this remains arguable as conflicting studies have found A2 can increase [21], or have no effect [22], on IL-1ra levels. It is generally accepted that homozygous carriers of A2 have a more severe and prolonged pro-inflammatory immune response than do those with other IL-1RN genotypes [23]. This investigation was designed as a pilot study to 1) explore the role of IL-1RN genotype in unexplained infant deaths (uSUDI) including SIDS, non-infectious infant deaths (niSUDI) and infectious infant deaths (iSUDI), and 2) to investigate whether IL-1RN genotype is related to the finding of organisms in normally sterile sites (cerebrospinal fluid, heart blood and/or spleen) in infant deaths which might trigger an uncontrolled pro-inflammatory response.
Materials and Methods

Selection criteria and sample preparation
The cohorts of uSUDI and comparison infants and nucleic acid extraction from dried bloodspots and intestinal contents have been described in Chapter 7.

PCR amplification
IL-1RN was amplified using the primer pair published by Tarlow et al. [19] using the following cycling conditions: 95°C 2 mins, 38 cycles of 95°C 30s, 59°C 30s, 72°C 1 min and a final extension of 72°C 3 mins on a Bio-Rad IQ5 cycler. IL-1RN genotype was determined by the amplicon size (variable number of tandem repeats) as described by Tarlow et al. [19]. Figure 1 shows the two most common alleles IL-1RN*1 and *2. Each PCR reaction contained: 0.05µM MgCl₂; 1X Colourless GoTaq Flexi Buffer; 0.0025µM dNTP mix; 0.05µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison Wisconsin); 2µl of extracted DNA and PCR-grade water to a total volume of 12.5µl. Amplification products were visualized on a 2% agarose gel stained with GelRed (BioTium, Hayward California) alongside a pUC19/HpaII DNA molecular weight marker (Geneworks, Thebarton, South Australia). Homozygous 1/1 and 2/2 controls for each genotype were sourced from the study cohort and confirmed by sequencing the amplification products (IMVS Sequencing Centre, Adelaide, South Australia).
Figure 1. Agarose gel electrophoresis of *IL-1RN* amplification products. Lanes 1 & 14: pUC19/*Hpa*I DNA molecular weight marker. Lanes 2, 4, 5, 10, 11, 12 & 13: *IL-1RN* 1/1 genotype. Lanes 3 & 7: *IL-1RN* 2/2 genotype. Lanes 8 & 9: *IL-1RN* 1/2 genotype. Primer sites are shown in yellow, repeat 1 in pink, repeat 2 in green, repeat 3 in blue, repeat 4 in aqua.
Statistics

Unexplained SUDI genotypes were analysed against live control infants by comparing homozygous and heterozygous A2 carriage with the 1/1 genotype. Yates Corrected Probability Test using 2x2 contingency table software (Statcalc, Epiinfo v6) was used to calculate $p$ values, odds ratios and 95% confidence intervals. Sterile site bacteriology recorded in post-mortem reports, where available, was compared against $IL-1RN$ genotype for uSUDI, iSUDI and niSUDI.
Results

Not all samples gave a valid result due to the presence of PCR inhibitors in blood. Ninety-four of the 103 live control infant, and all of the uSUDI, iSUDI and niSUDI samples gave a valid \textit{IL-1RN} genotype result (\textit{Table 1}). The allele frequencies of the live control infants was similar to those in the previously published literature [19] (±0.04) and were in Hardy-Weinberg equilibrium. Heterozygous 1/2 and homozygous 2/2 genotypes were analysed against 1/1 genotype for uSUDI compared with live control infants. uSUDI infants had a significantly higher frequency of the 2/2 genotype compared with the live control infants (p=0.007; OR 4.83; 95%CI 1.41-17.67).
Table 1. Distribution of *IL-1RN* genotypes and alleles among infant deaths and live control infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>iSUDI</th>
<th>niSUDI</th>
<th>uSUDI</th>
<th>Live Control Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>6 (46%)</td>
<td>3 (30%)</td>
<td>22 (45%)</td>
<td>58 (62%)</td>
</tr>
<tr>
<td>1/2</td>
<td>3 (23%)</td>
<td>6 (60%)</td>
<td>13 (27%)</td>
<td>26 (28%)</td>
</tr>
<tr>
<td>2/2</td>
<td>2 (15%)</td>
<td>1 (10%)</td>
<td>11 (22%)*</td>
<td>6 (6%)</td>
</tr>
<tr>
<td>1/3</td>
<td>2 (15%)</td>
<td>0</td>
<td>2 (4%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>1/4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>2/3</td>
<td>0</td>
<td>0</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>1/5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>10</td>
<td>49</td>
<td>94</td>
</tr>
</tbody>
</table>

| A 1      | 17 (65%) | 12 (60%) | 59 (60%) | 146 (78%) |
| A 2      | 7 (27%) | 8 (40%) | 36 (37%) | 38 (20%) |
| A 3      | 2 (8%) | 0 | 3 (3%) | 1 (0.5%) |
| A 4      | 0 | 0 | 0 | 2 (1%) |
| A 5      | 0 | 0 | 0 | 1 (0.5%) |
| Total    | 26 | 20 | 98 | 188 |

* p=0.007; OR 4.83; 95%CI 1.41-17.67
Comparison of post-mortem bacteriology findings with IL-1RN genotype

Infants with sterile site infection more frequently carried the 2/2 genotype (23%) than infants without sterile site infection (11%) (Table 2). The numbers in each subgroup were not large enough for statistical analysis.
Table 2. IL-1RN 1/1, 1/2 and 2/2 genotypes in infants with and without normally sterile site infection

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>1/1</th>
<th>1/2</th>
<th>2/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>uSUDI with sterile site infection</td>
<td>20</td>
<td>10</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>uSUDI without sterile site infection</td>
<td>14</td>
<td>6</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>iSUDI with sterile site infection</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>iSUDI without sterile site infection</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>niSUDI with sterile site infection</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>niSUDI without sterile site infection</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total with sterile site infection</td>
<td>30</td>
<td>12 (40%)</td>
<td>7 (23%)</td>
<td>7 (23%)</td>
</tr>
<tr>
<td>Total without sterile site infection</td>
<td>19</td>
<td>9 (47%)</td>
<td>7 (37%)</td>
<td>2 (11%)</td>
</tr>
</tbody>
</table>
Discussion

The role of infection in uSUDI and SIDS may be via an immune response pathway where *IL-1RN* genotype plays a critical role. *S. aureus* toxins are powerful inducers of IL-1 [10], a cytokine responsible for causing rapid vasodilation in septic shock. The frequency of *IL-1β* polymorphic site *C-511T* has been investigated in a small group of Australian (n=18) SIDS infants where a trend (*p*=0.59) was observed towards the *TT* genotype, which confers higher IL-1β levels, compared with Australian adult controls [17]. However, Santtilla et al [14] reported that the known variations in the *IL-1β* gene are not major regulators of *in vitro* production of IL-1β, rather the *IL-1RN* allele has this important role. The availability of IL-1ra for neutralization of high IL-1 levels would influence the outcome of the suspected *S. aureus* toxaemia in uSUDI and SIDS. Moscovis et al. have investigated the single nucleotide polymorphism *IL-1RN* (T+2018C) in SIDS, but found it to be similarly distributed between SIDS infants and controls [17].

This study investigated the relationship between *IL-1RN* 89bp VNTR genotype and uSUDI, to determine if a particular genotype was associated with the finding of bacteria in a normally sterile site at autopsy. An association was found between the homozygous A2 allele and uSUDI (*p*=0.007) where carriage of the 2/2 genotype was associated with nearly a five fold increase in relative risk of uSUDI compared with the predominant 1/1 genotype. These results are consistent with previous research in which polymorphic genotypes conferring more severe pro-inflammatory responses were found more frequently in SIDS infants than controls [17, 26, 27]. Apart from competitively blocking IL-1 receptors, IL-1ra also suppresses the release of IL-1β and TNFα from mononuclear
cells. It could be postulated that unchecked levels of IL-1β and TNFα (through \textit{IL-1RN} polymorphism) could play a role in a sudden cardiogenic event leading to sudden unexpected death in an infant, as these cytokines act synergistically to depress human myocardial function [28]. This mode of death is consistent with a non-respiratory, cardiogenic event as suggested by the observations of Poets \textit{et al.} [13]. The \textit{IL-1RN} 2/2 genotype was more common in infants with bacteria found in normally sterile sites at autopsy than those with no growth from these sites. Although the interpretation of these results is limited by small numbers, the potential relationship is noteworthy and deserves further study with larger numbers of subjects and controls.

Gestational age data were not available for this cohort. Pre-term birth is a risk factor for SIDS and so may be over-represented in our SUDI cohort. However, this is unlikely to introduce bias to the \textit{IL-1RN} genotype as only maternal, not neonatal carriage of A2 demonstrates an association with pre-term birth [29]. The respective infants from the pre-term study showed no difference in A2 carriage, suggesting that in our study, infant A2 carriage is likely to be associated with uSUDI independent of pre-term birth. It has been reported, however, that \textit{IL-1RN} A2 possession by the fetus leads to enhanced intra-amniotic IL-1ß production, which in turn could lead to preterm labour. The live infant control group in the present study had an excess of males (62%) compared with the uSUDI group (54%). This also was unlikely to introduce bias to carriage of \textit{IL-1RN} 2/2 genotype, as a previous study has found the 2/2 genotype to be unaffected by gender [30], although interestingly IL-1ra levels in amniotic fluid and urine of neonates are higher in females than males [31]. Nonetheless, a lack of IL-1ra protection in males might explain why they are at higher risk of SIDS/uSUDI than females.
The possible association between \textit{IL-1RN} A2 and uSUDI needs to be further investigated in a proposed study with a larger cohort, greater statistical power and ideally matched (for date of birth, gestational age, sex and birth weight) controls. This will reveal if the carriage of 2/2 genotype is associated with uSUDI independent of pre-term birth.
References


Chapter 9. \textit{IL-1RN} allele 2 association with SIDS is not confirmed in a large South Australian cohort.

Abstract

In \textit{Chapter 8} we investigated the relationship between \textit{IL-1RN} 89bp VNTR genotype and uSUDI in a pilot study. An association was found between the homozygous A2 allele and uSUDI ($p=0.007$) where carriage of the 2/2 genotype gave a 4.85 times increased risk of uSUDI compared with the predominant 1/1 genotype. The comparison group for the pilot study was not matched for gestational age or gender. This study was designed to investigate if the carriage of 2/2 genotype is associated with uSUDI independent of pre-term birth and gender in a larger cohort of matched infants. The Newborn Screening Cards of 117 SIDS and 232 control infants were assayed for \textit{IL-1RN} genotype. In the present study no association was shown between \textit{IL-1RN} allele 2 and SIDS. Potential reasons for the discrepancy between the two studies are discussed with supporting sub-analyses of data.
Introduction

In Chapter 8 we investigated the relationship between IL-1RN 89bp VNTR genotype and uSUDI in a limited pilot study. An association was found between the homozygous A2 allele and uSUDI ($p=0.007$) where carriage of the 2/2 genotype gave a 4.85 times increased risk of uSUDI compared with the predominant 1/1 genotype. Unfortunately gestational age data were not available for this cohort and as pre-term birth is a risk factor for SIDS it could be over-represented in the SUDI cohort compared with the control group. The live infant control group had an excess of males (62%) compared with the uSUDI group (54%) and was collected prospectively during a different decade to the majority of the uSUDI cases, during which genotypes could be influenced by different selective pressures. The possible association between IL-1RN A2 and uSUDI needed to be further investigated in a larger cohort with greater statistical power and ideally matched (for date of birth, sex and birth weight) controls. This study was designed to investigate if the carriage of 2/2 genotype is associated with uSUDI independent of pre-term birth and gender, and to look for other potential associations with factors recorded in the South Australian Pregnancy Outcomes Statistics Unit Database.
Methods

Ethical considerations

The use of dried bloodspots on Newborn Screening Cards (NSC) as a source of nucleic acids for SIDS research is a novel approach. The use of NSC for the work described in Chapters 9, 10 and 11 required justification of access to this resource and addressing the issues regarding the availability of consent. According to the National Statement on Ethical Conduct in Human Research (The National Health and Medical Research Council, Australian Research Council and the Australian Vice-Chancellor’s Committee, 2007), this study concerned „negligible risk research“ where there is no foreseeable risk of harm or discomfort to participants because they are not actively participating. Obtaining consent was not feasible in this investigation due to the size of the cohort, and time elapsed since sampling, so we were granted Human Research Ethics approval to test the samples in our laboratory as “re-identifiable”, marked with only a laboratory number linked to data held by an external party (The South Australian Pregnancy Outcomes Statistics Unit). The investigators were blind to the identities of the infants, and their SIDS/control status for the duration of this study.

Selection criteria

The South Australian Neonatal Screening Laboratory, and the Pregnancy Outcomes Statistics Unit, SA Health assisted us in identifying the NSC of 120 confirmed SIDS cases and 240 matched healthy infant controls. All SIDS infants met the criteria specific to the definition for SIDS at their time of death (either the 1969 [1] 1989 [2] or 2004 [3] definition) and have been recorded as such in the Pregnancy Outcomes Register. Two control infants were matched to each SIDS infant by date of birth, sex, birthweight (± 500g), gestational age, time lapsed from birth to NSC sampling, and ethnicity (Table 1). They were checked against the pregnancy outcomes database to ensure they were suitably matched, did not suffer any metabolic defects...
and had survived at least one year of life. This method of case-control selection aimed to make SIDS/control status the only major variable in the study. The NSC of 117 SIDS and 233 control infants were able to be located and sampled (changes over time in the accession and storage of NSC rendered some of the older samples inaccessible). Eighty-four percent of NSC were from infants born 1987-1994 and 16% born between 1995-2006. Twenty-nine SIDS were included in both the intestinal contents pilot study (Chapter 8) and the present cohort (i.e. their Newborn screening cards could be identified and located).
**Table 1.** Controlled variables for the SIDS cohort and the control cohorts

<table>
<thead>
<tr>
<th>Race</th>
<th>SIDS (n=118)</th>
<th>Control (n=233)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>113 (95.8%)</td>
<td>213* (93.8%)</td>
</tr>
<tr>
<td>Aboriginal</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Asian</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Males</th>
<th>SIDS (n=118)</th>
<th>Control (n=233)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>68 (57.6%)</td>
<td>131 (56.2%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>SIDS (n=118)</th>
<th>Control (n=233)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>39</td>
<td>39*</td>
</tr>
<tr>
<td>Average</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.8865</td>
<td>2.89</td>
</tr>
<tr>
<td>T-test SIDS vs. controls</td>
<td>p= &gt;0.999</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Birthweight</th>
<th>SIDS (n=118)</th>
<th>Control (n=233)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>3162</td>
<td>3210</td>
</tr>
<tr>
<td>Average</td>
<td>3075.98</td>
<td>3108.09</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>770</td>
<td>685</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>746.66</td>
<td>704.368</td>
</tr>
<tr>
<td>T-test SIDS vs. controls</td>
<td>p= 0.699</td>
<td></td>
</tr>
</tbody>
</table>

* Race and gestational age data available for 227 of the 233 control infants
Sample preparation

Nucleic acid extraction from dried bloodspots and PCR amplification of IL-1RN was carried out as described in Chapter 8.

Statistics

SIDS genotypes were analysed against those of live control infants by comparing homozygous and heterozygous A2 carriage with the 1/1 genotype. Yates Corrected Probability Test using 2x2 contingency table software (Statcalc, Epiinfo v6) was used to calculate $p$ values, odds ratios and 95% confidence intervals.
Results

*IL-1RN* VNTR genotype and allele frequencies are shown in Tables 2 and 3 respectively. Not all samples gave a valid result due to the presence of PCR inhibitors in blood. One hundred and thirteen SIDS and 219 controls gave a valid *IL-1RN* genotype result (Table 2). Eight out of 30 samples from 1987 failed and the remaining nine failed samples were randomly distributed. Heterozygous 1/2 and homozygous 2/2 genotypes were analysed against 1/1 genotype for SIDS compared with live control infants. The genotype frequencies of the control infants was not significantly different from controls in the pilot study (Chapter 8) ($p=0.674$); however, in the present study no association was shown between allele 2 and SIDS. It was suggested that the higher rate of *IL-1RN*2 carriage in uSUDI infants in the previous study might be due to confounding factors. Birthweight and gestational age are closely correlated (Figure 1). Birthweight was chosen for subanalysis because data was available for all infants (six controls had no gestational age recorded). No significant difference in genotype frequencies was observed between males and females, low birthweight (LBW ≤2500g) and normal birthweight (NBW >2500g) infants and infants born before and after 1994 (Table 2).

The uSUDI samples from the pilot study were collected before 1994 and the controls in 2008 (i.e. the controls were collected prospectively in 2008 from infants of the same age as the SIDS age at death), it was suggested that with better matched controls (same DOB, gender and birthweight) the association between *IL-1RN*2 might be dismissed. Ninety-four SIDS (29 from the pilot study) and 185 controls born before 1994 were analysed separately. 18.1% of the SIDS infants carried the 2/2 genotype compared with only 9.1% of the control infants (Table 2). Compared with carriage of the 1/1 genotype, the difference was statistically
significant \((p=0.025, \text{ OR}=2.48, \text{ CI}=1.09-5.59)\). When infants born in or before 1991 were analysed separately (83 SIDS and 164 controls) 20.5\% of the SIDS infants carried the 2/2 genotype compared with only 9.1\% of the control infants \((p=0.014, \text{ OR}=2.80, \text{ CI}=1.19-6.55)\). Five pairs of twins were included in the cohort, where one twin died of SIDS and the other survived to at least one year of age. Four pairs were of the same sex and one mixed. Four pairs of twins both had the 1/1 genotype, and in one pair both had the 2/2 genotype.
Table 2. Distribution of *IL-1RN* genotypes among SIDS and control infants (n, (%))

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SIDS</th>
<th>Control</th>
<th>Male</th>
<th>Female</th>
<th>DOB ≤1994</th>
<th>DOB &gt;1994</th>
<th>SIDS DOB ≤1994</th>
<th>Control DOB ≤1994</th>
<th>Low birthweight (≤2500g)</th>
<th>Normal birthweight (&gt;2500g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>67</td>
<td>148</td>
<td>116</td>
<td>99</td>
<td>180</td>
<td>35</td>
<td>52 (55.3%)</td>
<td>128 (69.6%)</td>
<td>40 (69.0%)</td>
<td>175 (64.1%)</td>
</tr>
<tr>
<td>1/2</td>
<td>23</td>
<td>45 (20.6%)</td>
<td>47</td>
<td>21</td>
<td>56</td>
<td>12</td>
<td>21 (22.3%)</td>
<td>35 (19.1%)</td>
<td>8 (13.8%)</td>
<td>60 (22.0%)</td>
</tr>
<tr>
<td>2/2</td>
<td>18</td>
<td>22</td>
<td>17</td>
<td>34</td>
<td>17 (18.1%)</td>
<td>17 (9.3%)</td>
<td>6 (10.3%)</td>
<td>33 (12.1%)</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>1/3</td>
<td>2 (1.8%)</td>
<td>1 (0.5%)</td>
<td>0</td>
<td>3 (2.1%)</td>
<td>3 (1.1%)</td>
<td>0</td>
<td>2 (2.1%)</td>
<td>1 (0.5%)</td>
<td>2 (3.5%)</td>
<td>1 (0.4%)</td>
</tr>
<tr>
<td>2/3</td>
<td>1 (0.9%)</td>
<td>1 (0.5%)</td>
<td>1 (0.5%)</td>
<td>1 (0.7%)</td>
<td>2 (0.7%)</td>
<td>0</td>
<td>1 (1.1%)</td>
<td>1 (0.5%)</td>
<td>0</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td>3/3</td>
<td>2 (1.8%)</td>
<td>1 (0.5%)</td>
<td>3 (1.6%)</td>
<td>0</td>
<td>2 (0.7%)</td>
<td>1 (1.9%)</td>
<td>1 (1.1%)</td>
<td>1 (0.5%)</td>
<td>1 (1.7%)</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td>4/4</td>
<td>0</td>
<td>1 (0.5%)</td>
<td>0</td>
<td>0</td>
<td>1 (0.4%)</td>
<td>0</td>
<td>0</td>
<td>1 (0.5%)</td>
<td>1 (1.7%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>113</td>
<td>218</td>
<td>190</td>
<td>141</td>
<td>278</td>
<td>53</td>
<td>94</td>
<td>184</td>
<td>58 (100%)</td>
<td>273 (100%)</td>
</tr>
</tbody>
</table>

*p*=0.026, OR 2.46 (1.08-5.55)

**p*=0.039, OR 1.91 (1.04-3.60)
Table 3. Distribution of *IL-1RN* alleles among SIDS and control infants (n, %)

<table>
<thead>
<tr>
<th>Allele</th>
<th>SIDS</th>
<th>Control</th>
<th>Male</th>
<th>Female</th>
<th>DOB ≤1994</th>
<th>DOB &gt;1994</th>
<th>SIDS DOB &lt;1994</th>
<th>Control DOB &lt;1994</th>
<th>Low birthweight (≤2500g)</th>
<th>Normal birthweight (&gt;2500g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>159 (70.4%)</td>
<td>342 (78.5%)</td>
<td>279 (73.4%)</td>
<td>222 (78.7%)</td>
<td>419 (75.3%)</td>
<td>82 (77.3%)</td>
<td>127 (67.5%)</td>
<td>292 (79.3%)</td>
<td>90 (77.6%)</td>
<td>411 (75.3%)</td>
</tr>
<tr>
<td>A 1</td>
<td>60 (26.5%)</td>
<td>88 (20.2%)</td>
<td>92 (24.2%)</td>
<td>56 (19.9%)</td>
<td>126 (22.7%)</td>
<td>22 (20.8%)</td>
<td>56 (29.8%)*</td>
<td>70 (19%)</td>
<td>20 (17.5%)</td>
<td>128 (23.4%)</td>
</tr>
<tr>
<td>A 2</td>
<td>7 (3.1%)**</td>
<td>4 (0.9%)</td>
<td>7 (1.9%)</td>
<td>9 (1.6%)</td>
<td>2 (1.9%)</td>
<td>5 (2.7%)</td>
<td>4 (1.1%)</td>
<td>4 (3.4%)</td>
<td>7 (1.3%)</td>
<td></td>
</tr>
<tr>
<td>A 3</td>
<td>0</td>
<td>2 (0.4%)</td>
<td>2 (0.5%)</td>
<td>0</td>
<td>2 (0.4%)</td>
<td>0</td>
<td>2 (0.6%)</td>
<td>2 (1.7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A 4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>226 (100%)</td>
<td>436 (100%)</td>
<td>380 (100%)</td>
<td>282 (100%)</td>
<td>556 (100%)</td>
<td>106 (100%)</td>
<td>188 (100%)</td>
<td>368 (100%)</td>
<td>116 (100%)</td>
<td>546 (100%)</td>
</tr>
</tbody>
</table>

*p=0.004, OR 1.84 (1.19-2.82)

**p=0.04, OR 3.76 (0.94-17.74) (2-tailed Fisher exact)
Figure 1. Correlation between gestational age and birthweight
Discussion

In adopting a larger cohort of SIDS and matched control infants we have dismissed a significant association between IL-1RN allele 2 and SIDS that was observed in a smaller pilot study. It was speculated that the higher rate of IL-1RN*2 carriage in uSUDI infants in the pilot study might be due to one of three things: 1) a difference in the percentage of males between the uSUDI and controls; 2) a higher percentage of preterm infants in the uSUDI group compared with the controls and/or 3) the higher allele 2 carriage was a kind of “genotype drift” where the allele has become less common due to selective pressures. When the results were broken down into groups, no significant difference in genotype frequencies was observed between low birthweight (≤2500g) and normal birthweight (>2500g) infants, and infants born before and after 1994 (the period when the uSUDI infants from the pilot study were born). The 1/2 genotype was significantly more common in males compared with females. This was unlikely to have accounted for the higher frequency of allele 2 in the SIDS/uSUDI in the pilot study as the live control group had more males than the uSUDI group (54% vs. 62%) and so theoretically should have overrepresented allele 2. By matching the SIDS cases to controls by sex we have limited the effects that gender-associated genotypes might have had on the SIDS vs. control analyses.

When we looked at infants born in or before 1994, a significant association still existed between SIDS and allele 2. These results suggest that the significant results observed in the pilot study were unlikely to be due to the shortcomings of the control group and could possibly be involved in SIDS from this time. The significance increased when infants born in or before 1991 were analysed separately (data not shown), although as the number of cases decreases the results should be interpreted with caution to avoid type I error.
We could speculate that perhaps while the SIDS rate was displaying an infectious-like wave (Figure 2), a major contributing factor to these deaths was infection, giving a higher mortality rate in infants who had the IL-1RN 2/2 allele who were more susceptible to a sustained pro-inflammatory response. In this case selection pressure against the vulnerable genotype could render it less frequent in recent times, or the pathogens present during these time periods might have created different selection pressures thus the vulnerable genotype could have changed. Although it seems unlikely, the significant difference in the 1994 and earlier samples could also be due to a confounding factor that we have not matched for. In Chapter 12 the two cohorts are analysed for differences in maternal and perinatal risk factors. In a study by Moscovis et al. a single nucleotide polymorphism in IL-1RN (T+2018C) was found not to be differently distributed between SIDS infants and controls [5] in SIDS infants who died between 1995 and 1997 [6]. If this experiment was replicated in our cohort of earlier SIDS, born prior to 1994, a higher frequency of IL-1RN (T+2018C) might be observed, in parallel with our findings of higher IL-1RN*2 in these infants. Such a finding would support the involvement of IL-1RN in SIDS.
Figure 2. Number of SIDS deaths per year in Australia. Adapted from SIDS IN AUSTRALIA 1981-2000 A STATISTICAL OVERVIEW Produced by the Australian Bureau of Statistics on behalf of SIDS and Kids.
Allele 3 in the form of both homozygous and heterozygous carriage was found to be significantly more common in SIDS compared with control infants ($p=0.04$). However, as this allele is relatively less common the small numbers observed must be analysed with caution. This possible association could have gone undetected in the smaller cohort examined in the pilot study due to the low statistical power. The biological effects of IL1-RN*3, and whether it alters IL-1β, production is currently unknown [4]. This variant has been reported to lack an inframe coding segment, as compared to variant 2 and the resulting isoform lacks an internal region (NCBI Reference Sequence: NM_000577.3 data). Theoretically, this “less-functional” form of IL-1ra could be inefficient in binding IL-1 receptors and might not exert immune control to the extent of the wild-type. Thus the association of allele 3 with SIDS might reflect unchecked IL-1β levels and progression to a severe pro-inflammatory response and possible death.

Samples that were collected in 1987 had a higher rate of PCR failure; however, almost all samples (53/54) from 1988 gave a valid result. Some possible explanations for this are: 1) an aspect of the method of collection changed during 1987; 2) the DNA on the cards has a “half life” of approximately 22 years or 3) the type of card had changed during 1987 to one more appropriate for computerised record-keeping. We took into consideration that prior to 1987 the cards were overlaid with carbon paper to record the patient details and were somewhat stained with blue ink; however, PCR failure was no more common in cards with carbon paper marks than those without. Visual comparison of cards that failed and passed PCR amplification yielded no obvious differences. To the knowledge of the staff in the Neonatal Screening Lab, the card, material or collection method did not change that year; however due to a change-over to a computerised system, the 1987 cards remained in the laboratory under non-ideal conditions for much longer than usual while data was recorded. This might be a
likely explanation for the high rate of failure. Unfortunately samples from 1986 were not available to test whether it was only 1987 that was tainted. Details of the storage of samples after collection should be considered important for research using dried blood spots.
References


Chapter 10. CD14 (C-260T) polymorphism is not associated with SIDS in a large South Australian cohort

Abstract

Susceptibility to sepsis is influenced by inherited or acquired mutations of innate immune genes, and severe sepsis and septic shock are clinical manifestations of a dysregulated immune response to invasive pathogens. Polymorphisms in genes that influence the expression of toxin receptors could affect the outcome of toxaemia, and could therefore play a role in SIDS. The CD14 gene promoter contains a single nucleotide polymorphism that affects the level of CD14 gene expression. The TT genotype of the CD14 (C-260T) polymorphism causes a significantly higher density of CD14 receptor expression on monocytes which makes the individual more sensitive to endotoxin than those with the wild-type (CC). This investigation was designed to determine whether SIDS infants have a higher frequency of the CD14 (C-260T) polymorphism compared with non-SIDS controls. One hundred and sixteen SIDS and 228 control infants were genotyped using PCR followed by restriction fragment length analysis of amplified product. Carriage of the TT or CT genotypes did not significantly differ between SIDS and control infants ($p=0.218$ and 0.081 respectively). The frequencies observed in the control group were consistent with Hardy-Weinberg equilibrium and did not differ significantly from the published frequencies for a cohort of 443 Caucasian Australians. These results suggest that CD14 (C-260T) polymorphism is unlikely to be implicated in SIDS.
Introduction

Age-dependent susceptibility to sudden death has been demonstrated in a rat model of endotoxic shock in which the animals displayed gross pathological findings consistent with SIDS in humans [1]. As introduced in Chapter 8, host factors, including genetic determinants, influence the outcome of interactions between toxin and host. Accordingly, polymorphisms in genes that influence the expression of toxin receptors could affect the outcome of staphylococcal enterotoxin toxaemia, and could therefore play a role in SIDS. It is recognized that susceptibility to sepsis is influenced by inherited or acquired mutations of innate immune genes, and that severe sepsis and septic shock are clinical manifestations of a dysregulated immune response to invasive pathogens [2]. The CD14 receptor is a cell surface glycoprotein on all myeloid cells, especially monocytes and macrophages, which is stimulated by lipopolysaccharide (LPS, or endotoxin). This stimulation induces overexpression of some cytokines. The CD14 gene promoter contains a single nucleotide polymorphism that affects the level of CD14 gene expression [3]. The TT genotype of the CD14 (C-260T) polymorphism (also known as CD14 C-159T) is associated with a significantly higher density of CD14 receptor expression on monocytes [3] which makes the individual more sensitive to endotoxin than those with the wild-type (CC). High levels of plasma Interleukin-6 has been attributed to CD14 -260*T [4] where high IL-6 expression is a downstream consequence of CD14-mediated immune stimulation [5]. Many SIDS infants have IL-6 concentrations in their cerebrospinal fluid equivalent to those found for infants dying from infectious diseases such as meningitis or septicaemia [6] which gives CD14 (C-260T) the potential for involvement. TT homozygotes also yield significantly higher levels of tumour necrosis factor alpha and significantly lower levels of anti-inflammatory IL-10. This investigation was designed to determine whether SIDS infants have a higher frequency of the CD14 (C-260T) polymorphism compared with non-SIDS controls.
Methods

Selection criteria and sample preparation

The cohort of SIDS and control infants has been described in the previous chapter (Chapter 9), and nucleic acid extraction from dried bloodspots was described in Chapter 7.

PCR amplification and genotype determination

$CD14$ (C-260T) genotype was determined using restriction fragment length analysis of amplified product as illustrated in Figure 1. A 267bp fragment of $CD14$ was amplified using the oligonucleotide primers published by Lichy et al. [7] in a PCR reaction containing: 0.0375µM $MgCl_2$; 0.8X Green GoTaq Flexi Buffer; 0.0025µM dNTP mix; 0.05µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison Wisconsin), 2µl of extracted DNA and PCR-grade water to a total volume of 12.5µl. Amplification was conducted on an Eppendorf Mastercycler EP programmed as follows: initial denaturation at 94°C for 2 minutes, 50 cycles of 94°C 20s, 59.5°C 20s, 72°C 40s, followed by a final extension at 72°C for 2 minutes. Fifty cycles gave a high yield of amplification product for samples that were otherwise weak. Genotypes were distinguished by digesting 1µl of amplification product with five units of $Hae$III (Roche, Mannheim, Germany) as described by Lichy et al. [7] at 37°C overnight, followed by visualisation on 2% agarose gel stained with GelRed (BioTium, Hayward) alongside a pUC19/HpaII DNA molecular weight marker (Geneworks, Thebarton) (Figure 2). Control samples were sourced from the cohort and confirmed by sequencing (IMVS sequencing Centre, SA Pathology, South Australia).
<table>
<thead>
<tr>
<th>Accession</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC_000048</td>
<td>TGGGGGTTGGATAGTGCAGAGTATGGTACTGGCCTAAGGCACTGAGGAT</td>
<td>200*</td>
</tr>
<tr>
<td>AC_000048</td>
<td>CATCCTTTTCCACACCCACCAGAGAAGGCTTAGGCTCCCGAGTCAACAG</td>
<td>250</td>
</tr>
<tr>
<td>AC_000048</td>
<td>GGCATTCACCGCCTGGGGCGCTGAGTCATCAGGACACTGCCAGAGACA</td>
<td>300</td>
</tr>
<tr>
<td>AC_000048</td>
<td>CAGAACCCTAGATGCAGAGTCCTTTCTGTGTTACCTCTCCCCTCCCT</td>
<td>350</td>
</tr>
<tr>
<td>AC_000048</td>
<td>GAAACATCCTTCATTGCCCTATATATTTCCAGGAAAGGAGGGGCTGGTGGG</td>
<td>400</td>
</tr>
<tr>
<td>AC_000048</td>
<td>AGGAAGAGGAGGTGGGAGGTGATCAGGCTTCACAGGAGGGAACTGAAT</td>
<td>450</td>
</tr>
</tbody>
</table>

**Figure 1.** Alignment of CD14-F and –R primers with CD14 gene (GenBank reference no. AC_000048). T→C SNP is shown in red, *Hae*III restriction site GG↓CC is highlighted in yellow.

*Note: Reference sequence AC_000048 nucleotide position 340T=260T in some other sequences*
Figure 2. Agarose gel electrophoresis of CD-14 *Hae*III restriction digest products.

Statistics

SIDS genotypes were analysed against control infants by comparing homozygous and heterozygous CD14 -260*T allele carriage with the wild-type (CC) genotype. Yates Corrected Probability Test using 2x2 contingency table software (Statcalc, Epiinfo v6) was used to calculate p values, odds ratios and 95% confidence intervals.
Results

The results obtained from genotyping 116 SIDS and 228 control infants are shown in Tables 1a and 1b. One sample from a SIDS infant and four from control infants did not yield a CD14 amplification product. Four of these five failed samples were collected in 1987. Carriage of the TT or CT genotypes did not significantly differ between SIDS and control infants \((p=0.218\) and 0.081 respectively). The frequencies observed in the control group were consistent with Hardy-Weinberg equilibrium and did not differ significantly from the published frequencies for a cohort of 443 Caucasian Australians \((p=0.30)\) [8]. The genotype and allele frequencies were sub-analysed by gender, birth year and normal and low birthweight. The T allele was more frequent in infants born in or prior to 1994 (49.0%) compared with those born after 1994 (38.2%) \((p=0.0489)\) and the CT genotype was more common to normal birthweight infants (42.6%) compared with low birthweight infants (24%) \((p=0.0394)\), compared with the C allele and the CC wild-type respectively.

Five pairs of twins were included in the cohort, where one twin died of SIDS and the other was a control who survived to at least one year of age. Four pairs were of the same sex and one pair was mixed. Two pairs of twins all had the CC genotype; one pair had CC in the SIDS and CT in the control case; one pair had CT in the SIDS and CC in the control case and one pair had CC in the SIDS and TT in the control case.
Table 1a. Distribution of *CD14 (C-260T)* genotypes in SIDS and control infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SIDS</th>
<th>Control</th>
<th>Male</th>
<th>Female</th>
<th>DOB ≤1994</th>
<th>DOB &gt;1994</th>
<th>SIDS DOB ≤1994</th>
<th>Control DOB ≤1994</th>
<th>Low birthweight (≤2500g)</th>
<th>Normal birthweight (&gt;2500g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>45 (38.8%)</td>
<td>65 (28.5%)</td>
<td>61 (31.5%)</td>
<td>49 (33.1%)</td>
<td>90 (31.4%)</td>
<td>20 (36.4%)</td>
<td>38 (39.2%)</td>
<td>52 (27.4%)</td>
<td>20 (40%)</td>
<td>70 (29.6%)</td>
</tr>
<tr>
<td>CT</td>
<td>42 (36.2%)</td>
<td>100 (43.9%)</td>
<td>86 (44.3%)</td>
<td>55 (37.2%)</td>
<td>113 (39.4%)</td>
<td>28 (50.9%)</td>
<td>34 (35.1%)</td>
<td>79 (41.6%)</td>
<td>12 (24%)</td>
<td>101 (42.6%)*</td>
</tr>
<tr>
<td>TT</td>
<td>29 (25%)</td>
<td>63 (27.6%)</td>
<td>47 (24.2%)</td>
<td>44 (29.7%)</td>
<td>84 (29.2%)</td>
<td>7 (12.7%)</td>
<td>25 (25.7%)</td>
<td>59 (31.0%)</td>
<td>18 (36%)</td>
<td>66 (27.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>116 (100%)</td>
<td>228 (100%)</td>
<td>194 (100%)</td>
<td>148 (100%)</td>
<td>287 (100%)</td>
<td>55 (100%)</td>
<td>97 (100%)</td>
<td>190 (100%)</td>
<td>50 (100%)</td>
<td>237 (100%)</td>
</tr>
</tbody>
</table>

Table 1b. Distribution of *CD14 (C-260T)* alleles in SIDS and control infants

<table>
<thead>
<tr>
<th>Allele</th>
<th>SIDS</th>
<th>Control</th>
<th>Male</th>
<th>Female</th>
<th>DOB ≤1994</th>
<th>DOB &gt;1994</th>
<th>SIDS DOB ≤1994</th>
<th>Control DOB ≤1994</th>
<th>Low birthweight (≤2500g)</th>
<th>Normal birthweight (&gt;2500g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>132 (56.9%)</td>
<td>230 (50.4%)</td>
<td>208 (53.6%)</td>
<td>153 (51.7%)</td>
<td>293 (51.0%)</td>
<td>68 (61.8%)</td>
<td>110 (48.2%)</td>
<td>183 (48.2%)</td>
<td>52 (52%)</td>
<td>241 (50.8%)</td>
</tr>
<tr>
<td>T</td>
<td>100 (43.1%)</td>
<td>226 (49.5%)</td>
<td>180 (46.4%)</td>
<td>143 (48.3%)</td>
<td>281** (49.0%)</td>
<td>42 (38.2%)</td>
<td>84 (43.3%)</td>
<td>197 (51.8%)</td>
<td>48 (48%)</td>
<td>233 (49.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>232 (100%)</td>
<td>456 (100%)</td>
<td>388 (100%)</td>
<td>296 (100%)</td>
<td>574 (100%)</td>
<td>110 (100%)</td>
<td>380 (100%)</td>
<td>100 (100%)</td>
<td>474 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

*p= 0.0394, OR 2.40 (1.04-5.74)

**p= 0.0489, OR 1.55 (1.00-2.42)
Discussion

Blood-Siegfried et al. reported similarities between a neonatal rat model of endotoxic shock and gross and microscopic pathology observed in SIDS cases [1]. In this investigation, the hypothesis was tested that SIDS infants would have a higher frequency of the CD14 (C-260T) polymorphism compared with non-SIDS controls. This would provide evidence to support, the endotoxic-shock model for SIDS if a higher density of CD14 receptors would render infant more sensitive to LPS. For example, the effect of expression levels of cell differentiation markers on toxin lethality in-vivo has been shown for CD45. Reduced expression of CD45 provides protection against anthrax pathogenesis in transgenic mice whereas normal expression levels resulted in 100% lethality [9]. By genotyping 116 SIDS and 228 control infants we have demonstrated no significant difference in carriage of the CD14 -260*T allele between SIDS and controls. These results suggest that CD14 (C-260T) polymorphism is unlikely to be implicated in SIDS, however, the endotoxic-shock model for SIDS is still viable, as genetic predisposition is potentiating, but not vital to such an event.

The CD14 (C-260T) polymorphism (also known as CD14 C-159T) has been inconsistently associated with a number of conditions, including coronary heart disease and stroke where the inflammatory cytokines released during CD14 stimulation activate the arterial endothelium. Platelet-derived growth factor and tissue factor released from activated monocytes also leads to proliferation of smooth muscle cells and increased coagulability in atherosclerosis [3, 7]. In the cohort tested, the heterozygous CT genotype was more frequent in normal birthweight infants (correlated with term pregnancy) compared with low birthweight infants. In a study by Härtel et al. neither infant nor maternal CD14 C-260T carriage was found to be associated with pre-term birth in a study of 909 pre-term very low birthweight infants and 491 term
Infants [10]. In a latter study the investigators looked at the effects of CD14 (C-260T) on cytokine expression with LPS stimulation in healthy term neonates and found that in vitro stimulation of cord blood increased levels of soluble CD14 and IL-6 in carriers of the CD14 -260*T allele compared with carriers of the wild type. Future studies of the SNP in neonatal infection predict to find T allele carriers at higher risk [11]. A study of the effects of maternal CD14 (C-260T) carriage on soluble CD14 in breast milk found this SNP was not associated with increased CD14 levels, but two other SNPs at positions 1619 and 550 had significant effects on milk sCD14 concentrations [12]. Maternal carriage of these SNPs is interesting in the context of SIDS, as soluble CD14 in breast milk plays a sentinel role in bacterial colonization of the gut and modulation of early immune responses [13]. Perturbation of gut colonization (in particular increased colonization with pathogenic species) has been proposed in SIDS [14, 15] and given the protective role of breast milk [16], CD14 levels in breast milk might be correlated with risk.

Infants born in or prior to 1994 had a higher rate of T allele carriage. The reason for the difference in T allele carriage in the two groups is unclear. It does not appear to be related to SIDS during the peak of incidence as was seen in the case of IL-1RN*2 as the frequency was similar in SIDS and controls born in that period. In the five pairs of twins in the cohort, CD14 genotype differed between the sibling infants in three of the pairs. No specific genotype was common to the five SIDS cases. Samples that were collected in 1987 had a higher rate of PCR failure, whereas all samples collected from 1988 onwards gave a valid result. Samples obtained in 1987 which gave a valid result had a visibly lower yield of amplification product compared with more recent samples (data not shown). This was consistent with the previous investigation described in Chapter 8 where samples collected in 1987 also had a higher rate of IL-1RN PCR failure. That this phenomenon was observed in two separate assays suggests that
these older samples contain low recoverable yields of DNA rather than being attributed to laboratory error.

The results obtained from this study suggest that $CD14$ (C-260T) polymorphism is not implicated in SIDS in this South Australian population. Polymorphisms in related genes, such as those in the promoter region of the $IL-6$ gene which is influenced by CD14, should be assayed in this same cohort to see if the carriage of two or more pro-inflammatory gene polymorphisms increases the risk of SIDS. The role of T allele carriage in preterm birth and low birthweight is unclear and requires further investigation.
References


Chapter 11. Toll-like receptor 2 (R753Q) polymorphism associated with SIDS in a large South Australian cohort

Abstract

Factors influencing susceptibility to invasive infection could be involved in SIDS. Loss-of-function polymorphism among pathogen pattern recognition genes could result in impaired recognition of invasive pathogens, and consequently, impaired induction of an immune response and pathogen clearance. The homozygous Toll-like receptor 2 variant TLR-2 (R753Q) is a “functional knockout” of lipoteichoic acid stimulation, which is important in recognition of Staphylococcus aureus, a bacterium suggested to be implicated in SIDS. This investigation was designed to determine whether SIDS infants have a higher frequency of TLR-2 (R753Q) polymorphism compared with non-SIDS controls. TLR-2 (R753Q) genotype was determined for 108 SIDS and 220 control infants using allele-specific PCR. SIDS genotypes were analysed against control infants by comparing homozygous and heterozygous TLR-2*A allele carriage with the wild-type GG genotype. Carriage of the AA or GA genotypes did not significantly differ between SIDS and control infants compared with the GG wild-type (p=0.306 and 0.928 respectively). An association could not be demonstrated between this polymorphism and SIDS, suggesting that it is not involved in SIDS pathogenesis. In this investigation we have addressed one of many functional polymorphisms in the toll-like receptor family. Polymorphisms in other TLRs warrant investigation in SIDS infants.
Genetic predisposition to inappropriate responses to infection have been the focus of recent SIDS investigations [1-3]. Independent research to date has primarily targeted cytokine gene polymorphisms conferring heightened pro-inflammatory responses. While these are important in determining the outcome of an established inflammatory response, factors influencing susceptibility to invasive infection could also be involved in SIDS. Two independent retrospective studies reported typically pathogenic bacteria (notably *Staphylococcus aureus*) in normally sterile sites of infants succumbing to sudden unexpected death in infancy (SUDI) [4, 5] (a broad classification of unexplained infant deaths including cases that would have previously constituted SIDS classification). The finding of these potential pathogens in usually sterile sites could reflect insufficient innate immunity permitting the organisms to spread rapidly into normally sterile tissues.

Pathogen Pattern Recognition (PPR) receptors rapidly recognize and bind to conserved molecular patterns on bacteria, viruses and fungi. Upon binding the immune response is triggered. The function of PPR receptors is crucial in the early stages of bacteraemia; activation of the immune response facilitates the clearance of bacteria from the bloodstream to prevent septicemia. It is recognized that susceptibility to sepsis might be due to inherited or acquired mutations of innate immune genes, and that severe sepsis and septic shock are clinical manifestations of a dysregulated immune response to invasive pathogens [6]. Loss-of-function polymorphism among PPR genes could result in impaired recognition of invasive pathogens, and consequently, impaired induction of an immune response and pathogen clearance. *S. aureus* and coliforms found in normally sterile sites in SIDS and SUDI infants might be a remnant or „footprint” of a bacteraemic episode during which the pathogen was unrecognised and not cleared from these sites. Reduced pathogen recognition could leave
infants particularly susceptible to bacteraemia at 2-4 months of age because the natural diminution in maternal antibodies.

Lipoteichoic acid (LTA) and peptidoglycan from *S. aureus* activate immune cells via Toll-like receptor 2 and CD14 recognition [7, 8]. The homozygous TLR-2 variant R753Q is a “functional knockout” of LTA stimulation, while the heterozygous carrier type elicits full responses not different from the wild-type [9]. This investigation was designed to determine whether SIDS infants have a higher frequency of TLR-2 (R753Q) polymorphism compared with non-SIDS controls.
Methods

Selection criteria and sample preparation
The cohort of SIDS and control infants has been described in Chapter 7, and nucleic acid extraction from dried bloodspots was described in Chapter 9.

PCR amplification and genotype determination

*TLR-2* (R753Q) genotype was determined using allele-specific PCR (Figure 1). A forward oligonucleotide primer was designed to bind the region 2313-2331 of the *TLR-2* gene (GenBank accession no. U88878). Two alternative allele-specific reverse primers TLR-2-A and TLR-2-G (Appendix 1) that bound to the region 2387-2410 were each used with the forward primer in separate reactions and the cycling parameters were optimized to allow binding of only the primer with the complementary 3’ base. Each reaction contained: 0.05µM MgCl$_2$; 0.8X Green GoTaq Flexi Buffer; 0.0025µM dNTP mix; 0.05µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison Wisconsin); 2µl of extracted DNA and PCR-grade water to a total volume of 12.5µl. Amplification was conducted on an Eppendorf Mastercycler EP programmed as follows: initial denaturation at 94°C for 2 minutes, 50 cycles of 94°C 20s, 66.1°C 20s, 72°C 40s, followed by a final extension at 72°C for 2 minutes. The reaction containing the correct complementary reverse primer yielded a 97bp amplification product which was visualized on a 3% agarose gel stained with GelRed (Biotium, Hayward, CA) (Figure 2). Control samples were sourced from the cohort and amplified with a consensus reverse primer (TLR-2-R) that bound at bases 2453-2472 so the amplified region covered the polymorphic site, and were sequenced (IMVS sequencing Centre, SA Pathology, South Australia).
**Figure 1.** Alignment of TLR-2 reference sequence U88878 with TLR-2-F (highlighted yellow), TLR-2-A (blue), TLR-2-G (green) and TLR-2-R (pink) oligonucleotide primers. The polymorphic site is shown in red.
Figure 2. Agarose gel electrophoresis of TLR-2 allele-specific PCR products with pUC19/HpaII DNA molecular weight marker (M).
**Statistics:** SIDS genotypes were analysed against control infants by comparing homozygous and heterozygous TLR-2*A allele carriage with the wild-type G/G genotype. Yates Corrected Probability Test using 2x2 contingency table software (Statcalc, Epiinfo v6) was used to calculate $p$ values, odds ratios and 95% confidence intervals.
Results

The results obtained from genotyping 108 SIDS and 220 control infants are shown in Tables 1a and 1b. Nine sample from SIDS infants and 12 from control infants did not yield a TLR-2 amplification product. Six of the failed samples were collected in 1987. Carriage of the AA or GA genotypes did not significantly differ between SIDS and control infants compared with the GG wild-type ($p=0.306$ and $0.928$ respectively). Hardy-Weinberg analysis was not applicable to the observed frequencies as the number of homozygote A genotypes was less than five, however, the genotype and allele frequencies observed in both the SIDS and the control groups were within the expected ranges as published for this SNP (rs5743708) on the National Centre for Biotechnology Information Single Nucleotide Polymorphism website (http://www.ncbi.nlm.nih.gov/projects/SNP). The results were broken down into male/female, birth date and birthweight categories to determine the presence or absence of any significant findings upon sub-analysis. No significant associations were found for any factor. Five pairs of twins were included in the cohort, where one twin died of SIDS and the other was a control who survived to at least one year of age. All infants in the twin pairs had the GG wild-type, except one which gave an invalid result.
Table 1a. Distribution of TLR-2 (R753Q) genotypes in SIDS and control infants (n, (%))

<table>
<thead>
<tr>
<th>Geno-type</th>
<th>SIDS</th>
<th>Control</th>
<th>Male</th>
<th>Female</th>
<th>DOB ≤1994</th>
<th>DOB &gt;1994</th>
<th>SIDS DOB ≤1994</th>
<th>Control DOB ≤1994</th>
<th>Low birthweight (≤2500g)</th>
<th>Normal birthweight (&gt;2500g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>103 (95.4%)</td>
<td>204 (93.6%)</td>
<td>177 (94.1%)</td>
<td>130 (94.2%)</td>
<td>258 (94.2%)</td>
<td>49 (94.2%)</td>
<td>86 (95.6%)</td>
<td>172 (93.5%)</td>
<td>55 (94.8%)</td>
<td>252 (94.0%)</td>
</tr>
<tr>
<td>GA</td>
<td>5 (4.6%)</td>
<td>10 (4.6%)</td>
<td>9 (4.8%)</td>
<td>6 (4.4%)</td>
<td>12 (4.4%)</td>
<td>3 (5.8%)</td>
<td>4 (4.4%)</td>
<td>8 (4.3%)</td>
<td>2 (3.5%)</td>
<td>13 (4.9%)</td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>4 (1.8%)</td>
<td>2 (1.1%)</td>
<td>2 (1.4%)</td>
<td>4 (1.4%)</td>
<td>0</td>
<td>4 (2.2%)</td>
<td>1 (1.7%)</td>
<td>3 (1.1%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>108 (100%)</td>
<td>218 (100%)</td>
<td>188 (100%)</td>
<td>138 (100%)</td>
<td>274 (100%)</td>
<td>52 (100%)</td>
<td>90 (100%)</td>
<td>184 (100%)</td>
<td>58 (100%)</td>
<td>268 (100%)</td>
</tr>
</tbody>
</table>

Table 1b. Distribution of TLR-2 (R753Q) alleles in SIDS and control infants (n, (%))

<table>
<thead>
<tr>
<th>Allele</th>
<th>SIDS</th>
<th>Control</th>
<th>Male</th>
<th>Female</th>
<th>DOB ≤1994</th>
<th>DOB &gt;1994</th>
<th>SIDS DOB ≤1994</th>
<th>Control DOB ≤1994</th>
<th>Low birthweight (≤2500g)</th>
<th>Normal birthweight (&gt;2500g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>211 (97.7%)</td>
<td>418 (95.9%)</td>
<td>363 (96.5%)</td>
<td>266 (96.4%)</td>
<td>528 (96.4%)</td>
<td>101 (97.1%)</td>
<td>176 (97.8%)</td>
<td>352 (95.6%)</td>
<td>112 (96.6%)</td>
<td>517 (96.5%)</td>
</tr>
<tr>
<td>A</td>
<td>5 (2.3%)</td>
<td>18 (4.1%)</td>
<td>13 (3.5%)</td>
<td>10 (3.6%)</td>
<td>20 (3.6%)</td>
<td>3 (2.9%)</td>
<td>4 (2.2%)</td>
<td>16 (4.4%)</td>
<td>4 (3.4%)</td>
<td>19 (3.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>216 (100%)</td>
<td>436 (100%)</td>
<td>376 (100%)</td>
<td>276 (100%)</td>
<td>548 (100%)</td>
<td>104 (100%)</td>
<td>180 (100%)</td>
<td>368 (100%)</td>
<td>116 (100%)</td>
<td>536 (100%)</td>
</tr>
</tbody>
</table>
Discussion

It has recently become clear that cytokine production in sepsis is under the control of TLR signaling. Loss-of-function polymorphism among pathogen pattern recognition genes can result in impaired recognition of invasive pathogens, and consequently, impaired induction of an immune response and pathogen clearance. In this investigation we looked at the TLR2 (R753Q) polymorphism which impairs recognition of bacterial lipoproteins. An association could not be demonstrated between this SNP and SIDS, suggesting that it might not be involved in SIDS pathogenesis.

To date, only cytokine gene polymorphisms causing heightened pro-inflammatory response have been associated with SIDS [1-3, 10]. It is possible that polymorphisms in pathogen recognition genes are also involved. Interesting interactions have been reported between the two different types of immunoregulatory gene polymorphisms; those impairing pathogen recognition and those exaggerating proinflammatory response. Both can be independent predictors of enhanced mortality in septic shock, and their effects are likely to be additive [11]. In this investigation we have addressed one of many functional polymorphisms in the toll-like receptor family. SNPs in other TLRs have not been investigated in SIDS infants. Two key polymorphisms in the TLR4 gene, TLR4(T399I) and TLR4(D299G), relate to hypo-responsiveness to lipopolysaccharide (LPS or endotoxin) and increased susceptibility to infection [11]. Another SNP of interest is the TLR5 (-392STOP) mutation which abolishes signaling of flagellin, a highly immunogenic component of Escherichia coli [12]. SIDS infants carrying this SNP might be unable to mount a sufficient immune response to E. coli infection, resulting in severe sepsis with remnant coliforms in normally sterile sites at autopsy, such as those reported by Goldwater [13]. Other potential candidate genes are the
frame-shift mutation NOD2 (3020insC) which expresses a truncated protein defective in LPS signaling [14], and MD2 (G56R) which affects the transfer of bacterial LPS from CD-14 to TLR4, resulting in decreased responsiveness to LPS [15]. In future work these other potential candidate gene polymorphisms should be investigated in our large newborn screening card cohort.
References


10. Highe A, Berry A, Goldwater P. Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2. *Ann Med (Accepted for publication)*. 2009;


Abstract

A number of maternal and perinatal factors have been found in past investigations to increase an infant’s risk of SIDS. Data were collected for 118 SIDS cases and 227 controls and the presence of complications or conditions considered being detrimental to the infant’s or mother’s health were analysed as potential risk factors. SIDS was found to be significantly more common in cases in which the infant’s mother was not in a relationship (i.e. divorced, separated or never married) \(p=0.005\), if the infant was not the first born \(p=0.0001\), and when the mother resided in a socioeconomically disadvantaged area \(p=0.03\). Overall, this SIDS cohort appears to display classical SIDS associations, and our findings are consistent with those from other regions.
Background

In 1988 Hoffman *et al.* published the *Results of the National Institute of Child Health and Human Development SIDS Cooperative Epidemiological Study* in which variables “previously neglected in SIDS research” and routinely recorded prenatal, delivery and newborn medical records were statistically analysed for SIDS infants dying between October 1978 and December 1979 with matched controls [1]. Of the maternal factors examined, the authors listed parity greater than two, mother’s age less than 20 years, late onset of or no prenatal care, high crowding index in the home, mother’s education less than 12 years, illicit drug use during pregnancy, lack of breast feeding, cigarette smoking, mother not married and mother’s age less than 20 years at first pregnancy as significant risk factors for SIDS (p=<0.001 for each). Perinatal factors regarding anaemia, low weight gain during pregnancy, urinary tract or venereal infection during pregnancy and APGAR score <7 were also identified to increase risk (p=<0.05). Smith and White published similar findings in 2006 concerning lower maternal age, high parity, maternal smoking and unmarried mother as significant risk factors for SIDS (p=0.001 for each) [2]. They also found that mothers of SIDS infants were likely to have had complications in other pregnancies and concluded from their investigations that there must be a common maternal risk factor for SIDS and obstetric complications [3]. This chapter examines the relationship SIDS has with maternal and perinatal risk factors, and, where possible, re-visits those identified by Hoffman *et al.* (1988) and Smith and White (2006) as significant risk factors.
Methods

We had identified the Newborn Screening Cards of 120 confirmed SIDS cases and 240 matched healthy infant controls. All SIDS infants met the criteria specific to the definition for SIDS at their time of death (either the 1969 [4] 1989 [5] or 2004 [6] definition) and have been recorded as such in the South Australian Pregnancy Outcomes data records. Two control infants were matched to each SIDS infant by date of birth, sex, birthweight (± 500g), gestational age, time lapsed from birth to NSC sampling, hospital of birth, and ethnicity. They were checked against the pregnancy outcomes database to ensure they were suitably matched, did not suffer any metabolic defects and had survived at least one year of life. Human Research Ethics approval was granted for the collection of maternal and perinatal data from the South Australian Pregnancy Outcomes Statistics Unit database for these infants. Data linkage was kindly provided by Associate Professor Annabelle Chan from the Pregnancy Outcome Unit of SA Health and Ms Ann-Marie Twisk of the Health Statistics Unit Epidemiology Branch, SA Health and provided to the investigators with identities removed. Socioeconomic disadvantage values were determined from postcodes by consulting *The Index of Relative Socio-Economic Disadvantage* available on the Australian Bureau of Statistics website. Low index values indicate areas of disadvantage (with many low income earners, low educational attainment, high unemployment, jobs in relatively unskilled occupations) and high index values indicate areas of advantage. Index values have been standardised to have a mean of 1000 and a standard deviation of 100 across all regions in Australia. Thus, lower socioeconomic status in this investigation was defined as an index value <1000.

Data linkage was successful for 118 SIDS and 227 controls. The presence of complications or conditions considered being detrimental to the infant’s or mother’s health were analysed as
potential risk factors. Of the included variables, those with suitable values (values for both groups were >5 or when only one value was <5) were analysed using Yates Corrected Probability Test 2x2 contingency table software (Statcalc, Epiinfo v6) to calculate $p$ values, odds ratios and 95% confidence intervals. Multiple conditions were grouped together into the following for analysis: medical conditions in pregnancy (a group of disorders that might disrupt the *in utero* environment of the infant, including but not limited to anaemia, genito-urinary tract infections, pre-existing hypertension, drug dependence and asthma); obstetric complications (including antepartum haemorrhage, suspected intrauterine growth restriction, pregnancy hypertension, oligohydroamnios and premature rupture of membranes); complications of labour (including fetal distress, prolonged labour >18 hours and anaemia); onset of labour and methods of delivery. Genito-urinary tract infection was also analysed separately as the number of positive cases in each group was more than five and it was identified by Hoffman *et al.* as a risk factor for SIDS [1], as were low one and five minute APGAR scores. Unfortunately maternal smoking was unable to be analysed, as data had only been recorded since 1999 resulting in numbers being too low (19 SIDS and 36 controls) for meaningful statistical analysis. T-test for significant difference between two means using The OpenEpi Collection of Epidemiologic Calculators (Version 2.3 May 2009) was performed for socioeconomic disadvantage value and maternal age to investigate the extent of the difference between SIDS and control cohorts. SIDS and control gender, race, gestational age and birthweight were not analysed in this chapter as these factors were shown in Chapter 9 to be well-matched in the study design and were not significantly different.
Results

Detailed results and statistical analysis are shown in tabular form in Appendix 2. SIDS was found to be significantly more common in cases when the infant’s mother was not in a relationship (i.e. divorced, separated or never married) \( (p=0.005) \), if the infant was not the first born \( (p=0.0001) \), and when the mother resided in a socioeconomically disadvantaged area \( (p=0.03) \). Mothers of SIDS infants had an average age of 24.9 years while mothers of control infants had an average age of 27.7, this difference was highly significant \( (p=0.0000006) \). Plurality >1, any previous stillbirths or miscarriages, maternal medical condition, genito-urinary tract infection, obstetric complications, induced or no labour, labour complications, maternal smoking, assisted delivery, special nursery or neonatal intensive care stay, and APGAR score <7 were all found not to be associated with SIDS \( (p=>0.05) \). Analysis of any nursery care (special nursery, neonatal intensive care or paediatric intensive care) for SIDS vs. controls was also not significant \( (p=0.203) \). Figures 1 and 2 illustrate the odds ratios associated with maternal and perinatal risk factors respectively. Previous neonatal death, presence of congenital abnormalities and paediatric intensive care stay data all had insufficient numbers for statistical analysis.
Figure 1. Odds ratios and 95% confidence intervals of maternal risk factors for SIDS.

* p=0.005

** p=0.0001
Figure 2. Odds ratios and 95% confidence intervals of perinatal risk factors for SIDS.

*p=0.03
Discussion

The aim of this chapter was to examine the relationship between SIDS and maternal and perinatal risk factors, and to see if those risk factors for SIDS identified in 1988 by Hoffman et al. hold true for our cohort. The risk associated with maternal factors was in agreement for most cases with those identified by Hoffman et al. [1]. Those that showed significant associations in both studies were: parity ≥ 2, lower maternal age and mother not in a relationship. Caesarean section delivery was not associated with SIDS in either study. An association between maternal Genito-urinary tract infection and SIDS did not reach significance in our cohort, but did in Hoffman’s study. This may be due to lower numbers in our cohort compared with Hoffman’s cohort of 757 SIDS. We were unable to analyse anaemia in pregnancy or hypertension independently due to low numbers. However, the numbers we observed for anaemia during pregnancy (two SIDS and eight controls) did not reflect the 20.6% observed in the mothers of SIDS cases reported by Hoffman et al. This was possibly due to the different time periods examined in the studies. Hoffman studied infants dying between October 1978 and December 1979, whereas our cohort was more recent (1987-2006) at a time during which anaemia in pregnancy might be more readily prevented. Similarly, Hoffman described urinary tract infection and venereal disease in 15.8% and 4.6% of SIDS infant mothers respectively [1] while we observed a lower frequency of 7.9% of (combined) genito-urinary tract infection. In a Scottish cohort giving birth between 1992 and 2001, Smith and White found that the average age of mothers having infants who died of SIDS was 24 years and the average maternal age for non-SIDS mothers was 28 years [2]. This was similar to the values observed in this cohort, 24.9 and 27.7 respectively. We did not intentionally match for socioeconomic status, however by matching for hospital of birth, where a hospital is most likely to be visited by patients living nearby in suburbs with similar socioeconomic disadvantage values, we could have inadvertently matched for socioeconomic
status. Despite this possible matching, we still observed a significant difference between the proportions of disadvantaged (value less than 1000) cases of SIDS compared with controls.

The fact that SIDS is significantly more common in second and latter born infants is interesting. Mage and Donner propose that the higher risk of SIDS with increasing birth order could be due to greater probability of exposure to respiratory viral infection via contact with a sibling, a low grade respiratory viral infection being a risk factor for SIDS [7]. Perhaps the explanation is as simple as a more relaxed attitude to providing a clean sleeping environment after a first-born child has survived past infancy, in particular the re-use of bedding from the first infant for subsequent siblings. In these situations potentially pathogenic bacteria could thrive in a dirty mattress or sofa and contribute to the death of a prone-sleeping SIDS infant [8].

Overall, this SIDS cohort appears to display classical SIDS associations. This study has demonstrated two things: 1) the infants examined for genetic associations in this thesis are a “typical” SIDS cohort and 2) that the associations observed in the United States [1] and in Scotland [3] hold true for a South Australian SIDS cohort. Statistical analyses were in some cases limited by low numbers and some associations (i.e. with maternal anaemia or genito-urinary tract infection) need to be examined in a larger cohort. This could be achieved practically in the future by combining data from other Australian states.
References


Chapter 13. Discussion

The aim of this project was to examine the aetiology of Sudden Infant Death Syndrome (SIDS) and to investigate the roles played by bacterial toxins, immune regulators and maternal and perinatal risk factors. Over the three year course of the project we accumulated the largest collection of SIDS material in Australia. This enabled us to conduct a genetic study which, to our knowledge, has the most ideal case-control matching for studies of this type in the published literature. The significance of the results presented in the thesis are discussed herein.

Bacterial toxins genes are present in the infant intestinal tract

The first aim of this project was to determine if the SIDS intestinal tract harbored bacteria carrying genes for potentially lethal toxins which could contribute to a lethal "toxic shock" response. This was investigated by examining Escherichia coli isolates, and by non-selectively culturing intestinal contents from SIDS and control infants and using molecular methods to assay for toxin genes. The hypothesis stated that we expected to find a higher frequency of toxin genes present in SIDS infants compared with controls. While this proved true in the case of Staphylococcus aureus enterotoxins, there was a higher frequency of S. aureus species detected in SIDS infants, and so the higher frequency of toxin genes probably reflects this. Indeed the percentage of S. aureus positive samples with and without toxin genes detected did not differ between SIDS and controls. In our studies of E. coli we expected to find a higher frequency of virulence factors in isolates from SIDS infants compared with controls, similar to the higher frequencies of verocytotoxin, heat labile toxin and curli reported in these same isolates previously by Bettelheim et al. [1] and Goldwater [2]. Genes
encoding functional cytolysin A, and the High Pathogenicity Island were equally common in both SIDS and live healthy infants, as was the curli-producing *E. coli* phenotype. No single *E. coli* entity examined in this project can presently be attributed to the causation of SIDS because of their equal prevalence in non-SIDS infants. One Ont.Hnt strain was found to be verotoxic in the absence of stx genes. This was an interesting finding and further investigation is warranted into the mechanism by which this strain elicits cytotoxicity. Future studies should be planned to compare the genetics of isolates from SIDS which are lethal to mice [3] compared with a non-toxic strain of similar phenotype. A subtractive hybridisation method, which isolates pieces of DNA which are not matched between two genomes, could be employed to identify novel virulence mechanisms. Anecdotally, such an experiment was attempted during the course of the *E. coli* work described in Chapter 2 with little success in isolating anything except common *E. coli* housekeeping genes.

We hypothesised that *Clostridium sordellii* lethal toxin (LT) could account for a proportion of SIDS deaths in some populations via a similar mechanism to infant botulism, where the vegetative bacteria colonise and multiply due to the ideal physiological conditions for spore germination in the infant intestine and produce toxin in the intestinal lumen. No LT genes could be demonstrated in cultures grown from intestinal contents of 50 SIDS cases or 13 infants who died of other causes which suggests that LT is not involved in SIDS in South Australia.

Genes encoding cytolysin A, the High Pathogenicity Island and curli fibres in *E. coli*, as well as staphylococcal enterotoxins, are sufficiently common to contribute to a multi-factorial hypothesis for SIDS where other factors, for example inappropriate host response to infection
cigarette smoke exposure or synergistic relationships with other bacterial products [7, 8] could all contribute to death. Evidence for the involvement of toxins in SIDS lies with the fact that serum from SIDS infants, presumably carrying toxins, is lethal to infant mice and chick embryos, whereas control sera from non-SIDS infants is not [3, 9]. Over-production of cytolysin A or staphylococcal enterotoxins, whose genes are described here to be present in SIDS infants, could present at lethal levels in the blood, particularly if the intestinal mucosa is damaged as was described by Kamaras and Murell [10]. This damage could be mediated by the toxins involved (i.e. staphylococcal enterotoxins) or by synergistic actions with other toxins (i.e. *C. perfringens* enterotoxin). It has been proposed, and demonstrated in the case for nasopharyngeal strains, that a particular combination of toxins is required for a lethal event [8]. Potential pathogens colonizing the intestinal tract could also play a role in SIDS pathogenesis if host predisposition permits.

**Immunoregulatory gene polymorphisms**

The human immune system is extremely complex in nature and is controlled by a vast array of genes. Whilst the list of candidate genes for polymorphism analysis in SIDS appears endless, those involved in immunoregulatory processes deserved attention as they powerfully affect homeostatic balance. Our specific hypothesis was that SIDS infants are genetically predisposed, via immunoregulatory gene polymorphisms, to potentially lethal immune responses when challenged with common bacterial toxins. By comparing the frequencies of immunoregulatory gene polymorphisms between SIDS and control infants we expected to find a higher frequency of mutant alleles in SIDS infants, thus putting them at increased risk of an inappropriate immune response to bacterial toxins. The four gene polymorphisms addressed in this project, Interleukin 1 receptor antagonist gene (*IL-1RN*) 89 base pair
variable number of tandem repeats, T cell receptor BV3 chain recombination signal sequence gene \( TCRBV3SI \) RSS C→T single nucleotide polymorphism (SNP), CD14 gene promoter C→T SNP and a toll-like receptor 2 gene \( TLR-2 \) G→A SNP were chosen because the function altered by the polymorphism is important in \( S. \) aureus infection, for which there is some evidence in SIDS [11-13]. \( S. \) aureus toxins are powerful inducers of interleukin-1 [14] making IL-1 receptor antagonist levels important, and \( TCRBV3SI \) RSS, \( CD14 \) (C-260T) and \( TLR-2 \) (R753Q) are receptors for staphylococcal enterotoxin A, lipopolysaccharide and lipoteichoic acid/peptidoglycan respectively.

Overall, no genetic marker of SIDS susceptibility was identified, however the higher prevalence of IL-1RN allele 2, which predisposes to poor outcomes from infection, in SIDS infants dying before 1994 suggests that the high incidence during this period could point to an infectious aetiology. An association with IL-1RN allele 3 with SIDS was also observed, however the significance of this is unknown in the absence of functionality studies for this variant isoform.

**Population sampling for genetic studies**

This work aimed to address the importance of a suitably matched control group in studies of this type. In Chapter 9 we demonstrated how a statistically significant association can be dismissed by employing a larger cohort with more suitably matched controls (i.e. IL-2RN*2 and SIDS in all SIDS infants), whilst other associations might appear only in a larger cohort (i.e. IL-1RN*3 in SIDS). This highlights how possible associations must be interpreted with caution and ideally should be replicated with a large cohort with carefully matched controls to limit the influence of co-variables. It would be interesting to see whether previously reported
gene polymorphism associations (by other research groups in other populations) [4-6] hold true in the large Newborn Screening Card Cohort.

Natural genetic variation between ethnic groups means that associations we have excluded cannot be dismissed from association with SIDS in other populations. Among Indigenous groups and African Americans, who have higher incidences of SIDS and deaths due to infection, there is a higher proportion of single nucleotide polymorphisms (SNPs) among cytokine genes associated with high levels of pro-inflammatory responses [15].

**Maternal and perinatal risk factors**

Maternal and perinatal data for the infant cohort were analysed for potential risk factors. SIDS was found to be significantly more common in cases where the infant’s mother was young (p= 0.0000006) not in a relationship (i.e. divorced, separated or never married) (p=0.005), if the infant was not the first born (p=0.0001), and when the mother resided in a socioeconomically disadvantaged area (p=0.03). We were satisfied that the SIDS cohort appeared to display classical SIDS associations, and our findings were consistent with those from other regions. It is difficult to explain how these risk factors relate to a mechanism of death in SIDS infants. Daltveit *et al.* suggest that the maternal age effect might be related to poorer infant care since young mothers can be less experienced. In the Norwegian population young maternal age and being unmarried relates to low socio-economic status, where the lifestyles of young single mothers are often not compatible with education, and they are more likely to smoke [16]. Thus some of the difficult to explain risk factors (i.e. single mother and socioeconomic disadvantage) could be effects of maternal smoking and less experience in infant care (which could be detrimental to the health of the infant) rather than direct risk
factors for SIDS. If complete data for smoking were available for this cohort it would be interesting to examine whether smoking and low socio-economic status were directly associated. A direct association would pose the question of which is the actual risk factor. Daltveit [16], like Mage and Donner [17], also believe that infection load from elder siblings might explain the birth order effect.

Study caveats

It must be remembered that in performing significance testing of data using an arbitrary $p$ value of 0.05, there is a five percent chance that we will conclude that the two groups differ when they do not (type I error). While the possibility exists that more associations could have been found by conducting multiple comparisons in many sub-analyses (i.e. individual maternal medical conditions), the small numbers of cases could throw doubt on any observed associations. Such “data torturing” is well described by Mills [18]. In splitting data into subgroups (i.e. low and normal birthweight, date of birth categories) was done using pre-determined cut-off values to avoid selectively manipulating the data to present only the strongest association. To prevent erroneous associations the experiments need to be replicated in a cohort larger than this. In our genotype association studies, we were able to obtain and test samples from SIDS and control infants, but not parents. Immunoregulatory genes predisposing to preterm birth and perturbations of the immune system in later life could be determined by the effects of maternal gene polymorphisms during pregnancy. Abnormal alleles might also be required in both maternal and fetal genes to lead to adverse outcomes. In future the effects of maternal immunoregulatory genes is worthy of investigation.
Future Implications and Directions

SIDS research is driven by the need to identify infants at risk in order to prevent deaths. Research to date has identified factors that increase the risk of SIDS (i.e. prone sleeping), many of which are also common to infants who do not succumb to SIDS, and many infants still die of SIDS even when risks are minimised. Home monitoring, the seemingly logical choice for a concerned parent, has been deemed inappropriate either because the risk is not sufficiently enhanced in infants chosen to be monitored or the physiological criteria used to identify infants are not valid. Data to date do not show any advantage for monitored infants [19]. A simple explanation for this is that there is no evidence that SIDS is related to sleep apnoea, and the fatal event appears to be cardiogenic, where gasping is seen after cessation of the heart [20], and documented cases claim that the infants are not able to be revived [19].

Newborn Screening Cards (NSC) have been the standard for screening of metabolic defects for more than 50 years. Chapter 6 introduced some of the gene polymorphisms and viruses that should be investigated in SIDS. If we are able to demonstrate using simple PCR assays on NSC that viruses or gene polymorphisms are predictive of an infant’s risk of SIDS, the assays could be employed as part of the Newborn Screening Program as a panel of genes to identify infants at increased risk. Newborn Screening Cards are fast advancing beyond the use of newborn screening for metabolic disorders to become a valuable genetic research tool. DNA from two 2mm NSC punches (punched-out discs) have been shown to be sufficient to carry out microarray-based whole genome scanning, even after being stored for ten years [21]. RNA extracted from larger samples (eight 3mm punches) of NSC has been demonstrated to be suitable for quantitative gene expression analysis in cards stored for up to nine years [22].
This raises the possibility of testing for expression of inflammatory genes in cards from SIDS infants which might indicate prenatal infection. In Chapter 6 the idea was postulated that prenatal infectious events could be predicted by assaying for a shift from CD45RA+ to CD45RO+ cells in the T lymphocyte population (consistent with previous exposure to microbial products or other antigens) in peripheral blood collected shortly after birth, should it be available for SUDI/SIDS infants. Unfortunately the literature suggests that NSC are unsuitable for protein assay due to the rapid degradation of proteins dried on card [23].

*Which viruses might be involved?*

As explained in Chapter 6, prenatal viral infection could explain 1) the disrupted patterns of growth of vital organs that appear to originate in the prenatal period [24] and 2) the placental morphology that could be indicative of prenatal virus transmission. Current research by Ansari and colleagues indicate that total trophoblast volume in normal birth weight SIDS is higher due to a significant increase in both cytotrophoblast and syncytiotrophoblast volume (Ansari, TI, Personal communication, 8th January 2009). These cells are the first-line defense of the fetus against viruses and become infected during maternal viraemia [25]. Such anomalies suggest viral involvement. Cytomegalovirus (CMV), Epstein - Barr virus, Herpes Simplex Viruses (HSV) 1 & 2, and parvovirus are known to be transmitted in the prenatal period and to cause changes consistent with those seen in SIDS placentae (syncytia formation) [26-28]. Prenatal viral infection may exert a spectrum of biological effects, mouse models of prenatal viral infection results in a sustained decrease in serotonin levels in the brain [29] as well as alterations in a large group of brain genes potentially leading to permanent changes in brain structure [30]. CMV infection in particular can result in immunosuppression [31], decreased clearance of bacterial infection and viral-induced alterations in host resistance to
bacteria and cytokine expression that last well into the postnatal period [32]. Furthermore, poor newborn hearing screening test scores have been reported to be a marker of SIDS susceptibility [33]. Asymptomatic congenital CMV is known to cause such hearing loss [34]. Fetal exposure to parvovirus has been shown to cause a significant increase in T-cell production, which may lead to an increase in thymus weight (characteristic of SIDS) as has been demonstrated in other T-cell driven inflammatory conditions [35]. Ninety-percent of CMV infected infants have no clinical manifestations at birth or at the age when they may have succumbed to SIDS and so CMV infection could silently contribute to their death.

Prenatal viral infection could stand alone as a risk factor for SIDS, or act in combination with the other proposed factors. For example: the consequences of prenatal viral infection could be potentiated by immunoregulatory gene polymorphism conferring inappropriately high cytokine production, or polymorphisms conferring dampened pathogen recognition could increase the likelihood of contracting the infection.

**Use of Neonatal Screening Cards as a source of viral nucleic acids**

Use of NSC for the detection of viral nucleic acids has been well documented. Based upon the published results of Gibson et al. [36, 37] herpesviruses are detectable in NSC of the South Australian population. Barbi et al. have demonstrated 100% sensitivity using NSC samples compared with “Gold-standard” methods (virus isolation from urine &/or saliva) [38]. Recently use of NSC has been employed for diagnosis of congenital viral infections in an Australian laboratory where sensitivities of 100% were achieved for HSV1&2 & 97.4% for parvovirus compared with direct fluorescence antibodies and serology [39]. Cross-contamination of cards during adjacent storage is a major concern and incidence of weak
positives in adjacent cards as high as 16% have been reported in Sweden [40]. Quantitative data showing the contamination rate under Australian storage conditions is not available, but one would expect significant variability in storage conditions between countries, two possible effectors are temperature and humidity. Further research is needed into the suitability of NSC that are more than twenty years old being tested for viral infection. In the PCR assays described in Chapter 9, we found that samples collected in 1987, representing the earliest samples available to us, had the highest failure rate. Accordingly samples from 1987 and earlier may also give false negative viral screen results.

**Limits of detection using NSC**

The average diameter of a bloodspot from 50ul of blood is 12mm ±2mm (unpublished data), although the diameter is dependant on haemoglobin concentration and packed-cell volume (the higher the red cell volume, the smaller the diameter of the bloodspot) [41]. The majority of published methods use between three and nine 3mm diameter punches for viral nucleic acid detection. The area of a 12mm diameter bloodspot is 113.097mm$^2$, and the area of a 3mm diameter punch from that bloodspot is 7.067mm$^2$. Therefore 16 x 3mm punches could be taken from each whole bloodspot, with each punch containing 3.125µl of blood, the total volume of blood to be extracted is 9.375µl. According to the literature, viral load during congenital CMV infection rarely exceeds $10^6$ copies/ml [42] and, in asymptomatic infants with normal hearing, averages $1.1 \times 10^4$ copies/ml [34]. If 9.375µl of blood (in three 3mm punches) goes into each extraction, an average sample will contain 103.125 copies. If the final volume of the extraction solution is 50µl (as is recommended in most commercial kits), each microlitre will contain 2.06 copies. When 5µl of extraction is used in a PCR reaction, theoretically this should contain 10.3125 copies. In summary, it is theoretically possible to
detect viral infection in NSC, assuming that a typical viral load of $10^4$ copies/ml is present and sufficient sample (three 3mm punches) is used. In future our cohort of SIDS and control NSC could be assayed for evidence of prenatal viral infection and ascertain whether or not this is a risk factor for SIDS.

In Chapter 6 we proposed that pathogen pattern recognition could be insufficient in SIDS infants given the findings of bacteria in normally sterile sites and a lack of serious illness before death. If pathogen recognition is believed to be insufficient in these infants, for example if Toll-like receptors are impaired and cannot activate the immune system, other “arms” of the immune system could be strengthened. For example, recent research has identified the vaccine adjuvant *inulin* is capable of inducing cellular and humoral immunity through the alternative complement pathway [43]. If a commonly-acquired virus, such as CMV, is found to increase the risk of SIDS, then immunization of CMV-seronegative potential mothers could be introduced, analogous to rubella immunization of women to protect against congenital rubella. Passive immunization with normal human immunoglobulin (at 1-2 months of age) of infants found to be “at-risk” of SIDS could also be explored.

**Conclusions**

An infectious aetiology of SIDS is strongly supported by experimental evidence. In the work presented in this thesis we have demonstrated that the intestinal tract contains potentially pathogenic species of bacteria which could contribute to SIDS in a multi-factorial hypothesis which involves host predisposition and favourable environmental conditions. We have suggested some immunoregulatory genes that could be involved. The role of *IL-1RN* is particularly interesting. The work published from this thesis will contribute to the field of
infectious disease research in SIDS and hopefully will lead to the identification of the cause of these deaths and future prevention.
References


40. Johansson P, Jonsson M, Ahlfors K, Ivarsson S, Svanberg L, Guthenberg C. Retrospective diagnostics of congenital cytomegalovirus infection performed by


## Appendix 1. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Specificity</th>
<th>Sequence 5’-3’</th>
<th>Nucleotide Position (GenBank accession no.)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS1</td>
<td>Shiga-like toxin 1 (<em>stx-1)</em></td>
<td>CAT AGT GGA ACC TCA CGA CGC AGT</td>
<td>830-854 (M16625)</td>
<td>[1]</td>
</tr>
<tr>
<td>VS2</td>
<td></td>
<td>TTT GCC GAA AAC GTA AAG CTT CA</td>
<td>917-886 (M16625)</td>
<td></td>
</tr>
<tr>
<td>VS3</td>
<td></td>
<td>TGT GGC AAG AGC GAT GTT ACG GTT TG</td>
<td>856-881 (M16625)</td>
<td></td>
</tr>
<tr>
<td>VS4</td>
<td>Shiga-like toxin 2 (<em>stx-2)</em></td>
<td>GGG CAG TTA TTT TGC TGT GGA</td>
<td>442-462 (X07865)</td>
<td>[1]</td>
</tr>
<tr>
<td>VS5</td>
<td></td>
<td>TGT TGC CGT ATT AAC GAA CCC</td>
<td>562-542 (X07865)</td>
<td></td>
</tr>
<tr>
<td>VS6</td>
<td></td>
<td>CTA TCA GGC GCG TTT TGA CCA TCT TCG</td>
<td>481-507 (X07865)</td>
<td></td>
</tr>
<tr>
<td>clyA-F</td>
<td>Cytolysin A (<em>clyA</em>) STEC type</td>
<td>AAG GTA CTG GAT GAC GGC ATC A</td>
<td>592-613 (AY576656, AY576661 and AY576665)</td>
<td>[2]</td>
</tr>
<tr>
<td>clyA-R</td>
<td></td>
<td>ACA CCG GCT GCG GCA C</td>
<td>779-794 (AY576656)</td>
<td></td>
</tr>
<tr>
<td>clyAfull-R</td>
<td>Cytolysin A (<em>clyA</em>) STEC, and ETEC</td>
<td>GTT TTT ATC TCC CCG</td>
<td>979-993 (AY576656)</td>
<td></td>
</tr>
<tr>
<td>irp2-F</td>
<td>Iron repressible protein 2 (<em>irp2</em>) of HPI</td>
<td>AAG GAT TCG CTG TTA CCG GAC</td>
<td>39-59 (NC007946)</td>
<td>[3]</td>
</tr>
<tr>
<td>Protein</td>
<td>Sequence Details</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>-----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>irp2-R</td>
<td>TCG TCG GGC AGC GTT TCT TCT 305-325</td>
<td>[3] (NC007946)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sa442-1</td>
<td>S. aureus specific sequence: AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG</td>
<td>[4] (CP000730)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sa442-2</td>
<td>CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA 1197-1227</td>
<td>[4] (CP000730)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eta-F</td>
<td>Staphylococcal enterotoxin A (sea): TTT GGA AAC GGT TAA AAC GAA TAAG</td>
<td>[5] (M18970)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eta-R</td>
<td>TTT CCT GTA AAT AAC GTC TTG CTT GA</td>
<td>[5] (M18970)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSEBR-2</td>
<td>CCA AAT AGT GAC GAG TTA GG</td>
<td>810-829 [7]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sec-F</td>
<td>Staphylococcal enterotoxin C (sec): CTT GTA TGT ATG GAG GAA TAA CAA</td>
<td>407-430 [X05815]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sec-R</td>
<td>TGC AGG CAT CAT ATC ATA CCA</td>
<td>690-670 [X05815]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>see-F</td>
<td>Staphylococcal enterotoxin E (see): TAC CAA TTA ACT TGT GGA TAG AC</td>
<td>446-468 [M21319]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>see-R</td>
<td>CTC TTT GCA CCT TAC CGC</td>
<td>616-599 [M21319]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seg-F</td>
<td>Staphylococcal enterotoxin G [10]: CGT CTC CAC CTG TTG AAG G</td>
<td>317-335 [AF064773]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seg-R</td>
<td>CCA AGT GAT TGT CTA TTG TCG</td>
<td>644-624 [AF064773]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seh-F</td>
<td>Staphylococcal enterotoxin H (seh): CAA CTG CTG ATT TAG CTC AG</td>
<td>245-264 [U11702]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seh-R</td>
<td>GTC GAA TGA GTA ATC TCT AGG</td>
<td>603-583 [U11702]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Sequence</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>tsst-F</td>
<td>Staphylococcal toxic shock syndrome toxin (TSST-1)</td>
<td>GCT TGC GAC AAC TGC TAC AG</td>
<td>48-67</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGG ATC CGT CAT TCA TTG TTA A</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>tsst-R</td>
<td></td>
<td>ATG AAC TTA GTT AAC AAA GCC CAA</td>
<td>(X82638)</td>
<td></td>
</tr>
<tr>
<td>CLS-F1</td>
<td>C. sordellii lethal toxin (LT)</td>
<td>ATG TTA GAC AAC TGC TAC AG</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGG ATC CGT CAT TCA TTG TTA A</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>IL-1RN-F</td>
<td>Interleukin-1 receptor antagonist (IL-1RN)</td>
<td>CTC AGC AAC ACT CCT AT</td>
<td>28675-28690</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCC TGG TCT GCA GGT AA</td>
<td>(U65590)</td>
<td></td>
</tr>
<tr>
<td>IL-1RN-R</td>
<td></td>
<td>ATG TTA GAC AAC TGC TAC AG</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>BV3-intron(sense)</td>
<td>T cell receptor BV3S1 recombination signal sequence (TCRBV3S1 RSS)</td>
<td>ATG TTA GAC AAC TGC TAC AG</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGG ATC CGT CAT TCA TTG TTA A</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>BV3-3’ of RSS(antisense)</td>
<td></td>
<td>ATG TTA GAC AAC TGC TAC AG</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGG ATC CGT CAT TCA TTG TTA A</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>CD14-F</td>
<td>CD-14 (C-260T)</td>
<td>ATG TTA GAC AAC TGC TAC AG</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGG ATC CGT CAT TCA TTG TTA A</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>CD14-R</td>
<td></td>
<td>ATG TTA GAC AAC TGC TAC AG</td>
<td>(J02615)</td>
<td></td>
</tr>
<tr>
<td>TLR-2-F</td>
<td>Toll-like receptor 2 (TLR2 R753Q) consensus</td>
<td>CAA TGA TGC TGC CAT TCT C</td>
<td>2313-2331</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGT CTT GGT TTG CAT TAT TCT CT</td>
<td>(U88878)</td>
<td></td>
</tr>
<tr>
<td>TLR-2-A</td>
<td>Toll-like receptor 2 (TLR2 R753Q) A allele</td>
<td>GGT CTT GGT TTG CAT TAT TCT CT</td>
<td>2388-2410</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGT CTT GGT TTG CAT TAT TCT CC</td>
<td>(U88878)</td>
<td></td>
</tr>
<tr>
<td>TLR-2-G</td>
<td>Toll-like receptor 2 (TLR2 R753Q) G allele</td>
<td>GGT CTT GGT TTG CAT TAT TCT CC</td>
<td>2387-2410</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGT CTT GGT TTG CAT TAT TCT CC</td>
<td>(U88878)</td>
<td></td>
</tr>
<tr>
<td>TLR-2-R</td>
<td>Toll like receptor 2 (TLR2 R753Q) sequencing reverse</td>
<td>GGT CTT GGT TTG CAT TAT TCT CC</td>
<td>2453-2472</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGT CTT GGT TTG CAT TAT TCT CC</td>
<td>(U88878)</td>
<td></td>
</tr>
</tbody>
</table>
References


Appendix 2. Maternal and perinatal risk factors for SIDS. Results and statistical analysis

<table>
<thead>
<tr>
<th></th>
<th>SIDS</th>
<th>Control</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLURALITY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>singleton</td>
<td>110 (93.2%)</td>
<td>210 (92.5%)</td>
<td><em>Twin vs. singleton</em></td>
</tr>
<tr>
<td>twin</td>
<td>8 (6.8%)</td>
<td>17 (7.5%)</td>
<td><em>p</em>=0.982 OR 0.90 (0.32-2.28)</td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>MARITAL STATUS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>never married</td>
<td>33 (28%)</td>
<td>33 (14.5%)</td>
<td><em>No relationship (divorced, separated, never married) vs. married/defacto</em></td>
</tr>
<tr>
<td>married/defacto</td>
<td>84 (71.2%)</td>
<td>192 (84.6%)</td>
<td><em>p</em>=0.005 OR 2.22 (1.25-3.93)</td>
</tr>
<tr>
<td>divorced</td>
<td>1 (0.8%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>separated</td>
<td>0</td>
<td>2 (0.9%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>PREVIOUS LIVE BIRTHS</strong></td>
<td></td>
<td></td>
<td><em>Parity &gt;1 vs. first baby</em></td>
</tr>
<tr>
<td>0</td>
<td>28 (23.7%)</td>
<td>103 (45.4%)</td>
<td><em>p</em>=0.0001 OR 2.69 (1.60-4.61)</td>
</tr>
<tr>
<td>1</td>
<td>50 (42.4%)</td>
<td>78 (34.4%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>27 (22.9%)</td>
<td>35 (15.4%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11 (9.3%)</td>
<td>9 (4%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2 (1.7%)</td>
<td>1 (0.4%)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>1 (0.4%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>PREVIOUS STILLBIRTHS</strong></td>
<td></td>
<td></td>
<td><em>1 or more vs. none</em></td>
</tr>
<tr>
<td>none</td>
<td>117 (99.2%)</td>
<td>225 (99.1%)</td>
<td><em>p</em>=1 OR 0.96 (0.02-18.65)</td>
</tr>
<tr>
<td>one or more</td>
<td>1 (0.8%)</td>
<td>2 (0.9%)</td>
<td>Fishers exact 2 tailed for values &lt;5</td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>PREVIOUS MISCARRIAGE</strong></td>
<td></td>
<td></td>
<td><em>1 or more vs. none</em></td>
</tr>
<tr>
<td>none</td>
<td>98 (83.1%)</td>
<td>184 (81.1%)</td>
<td><em>p</em>=0.758 OR 0.87 (0.46-1.62)</td>
</tr>
<tr>
<td>one or more</td>
<td>20 (16.9%)</td>
<td>43 (18.9%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>PREVIOUS NEONATAL DEATH</strong></td>
<td></td>
<td></td>
<td><em>Insufficient numbers for analysis</em></td>
</tr>
<tr>
<td>none</td>
<td>118 (100%)</td>
<td>226 (99.6%)</td>
<td></td>
</tr>
<tr>
<td>one or more</td>
<td>0</td>
<td>1 (0.4%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>SIDS</td>
<td>Control</td>
<td>Statistical analysis</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>------------</td>
<td>-------------</td>
<td>----------------------</td>
</tr>
<tr>
<td><strong>MATERNAL MEDICAL CONDITION</strong></td>
<td></td>
<td></td>
<td>Any vs. none</td>
</tr>
<tr>
<td>none</td>
<td>89 (78.1%)</td>
<td>185 (81.9%)</td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>25 (21.9%)</td>
<td>41 (18.1%)</td>
<td>( p=0.491 ) OR 1.27 (0.69-2.29)</td>
</tr>
<tr>
<td>2 or more</td>
<td>4 (3.5%)</td>
<td>7 (3%)</td>
<td></td>
</tr>
<tr>
<td>3 or more</td>
<td>2 (1.8%)</td>
<td>2 (0.9%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>2 (0.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>114 (100%)</td>
<td>226 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>GENITO-URINARY TRACT INFECTION</strong></td>
<td></td>
<td></td>
<td>Any vs. none</td>
</tr>
<tr>
<td>yes</td>
<td>9 (7.9%)</td>
<td>9 (4.0%)</td>
<td>( p=0.206 ) OR 2.07 (0.70-6.05)</td>
</tr>
<tr>
<td>no</td>
<td>105 (92.1%)</td>
<td>217 (96%)</td>
<td></td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>114 (100%)</td>
<td>226 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>OBSTETRIC COMPLICATIONS</strong></td>
<td></td>
<td></td>
<td>Any vs. none</td>
</tr>
<tr>
<td>none</td>
<td>81 (68.6%)</td>
<td>144 (63.4%)</td>
<td>( p=0.398 ) OR 0.79 (0.48-1.30)</td>
</tr>
<tr>
<td>yes</td>
<td>37 (31.4%)</td>
<td>83 (36.6%)</td>
<td></td>
</tr>
<tr>
<td>2 or more</td>
<td>12 (10.2%)</td>
<td>27 (11.9%)</td>
<td></td>
</tr>
<tr>
<td>3 or more</td>
<td>4 (3.4%)</td>
<td>9 (4%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>ONSET OF LABOUR</strong></td>
<td></td>
<td></td>
<td>none/induced vs. spontaneous</td>
</tr>
<tr>
<td>spontaneous</td>
<td>77 (65.3%)</td>
<td>146 (64.3%)</td>
<td>( p=0.957 ) OR 0.94 (0.57-1.56)</td>
</tr>
<tr>
<td>none</td>
<td>18 (15.2%)</td>
<td>23 (10.1%)</td>
<td></td>
</tr>
<tr>
<td>induced</td>
<td>23 (19.5%)</td>
<td>58 (25.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>LABOUR COMPLICATIONS</strong></td>
<td></td>
<td></td>
<td>Any vs. none</td>
</tr>
<tr>
<td>none</td>
<td>81 (68.6%)</td>
<td>153 (67.4%)</td>
<td>( p=0.910 ) OR 0.93 (0.56-1.54)</td>
</tr>
<tr>
<td>yes</td>
<td>37 (31.4%)</td>
<td>74 (32.6%)</td>
<td></td>
</tr>
<tr>
<td>2 or more</td>
<td>10 (8.5%)</td>
<td>15 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>3 or more</td>
<td>1 (0.8%)</td>
<td>3 (1.3%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SIDS</td>
<td>Control</td>
<td>Statistical analysis</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td><strong>MATERNAL SMOKING</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>smoker</td>
<td>10 (52.6%)</td>
<td>9 (25%)</td>
<td>smoking vs non-smoking</td>
</tr>
<tr>
<td>non smoker</td>
<td>9 (47.4%)</td>
<td>25 (69.4%)</td>
<td>p=0.108 OR 3.09 (0.81-11.79)</td>
</tr>
<tr>
<td>unknown</td>
<td>0</td>
<td>2 (5.6%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>19 (100%)</td>
<td>36 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>DELCIVERY METHOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal spontaneous</td>
<td>82 (69.5%)</td>
<td>145 (63.9%)</td>
<td>assisted vs. normal spontaneous</td>
</tr>
<tr>
<td>forceps</td>
<td>8 (6.8%)</td>
<td>27 (11.9%)</td>
<td>p=0.356 OR 0.78 (0.47-1.28)</td>
</tr>
<tr>
<td>assisted breech</td>
<td>0</td>
<td>2 (0.9%)</td>
<td></td>
</tr>
<tr>
<td>elective cesarean section</td>
<td>12 (10.2%)</td>
<td>16 (7%)</td>
<td></td>
</tr>
<tr>
<td>emergency cesarean section</td>
<td>12 (10.2%)</td>
<td>33 (14.5%)</td>
<td></td>
</tr>
<tr>
<td>ventouse</td>
<td>3 (2.5%)</td>
<td>4 (1.8%)</td>
<td></td>
</tr>
<tr>
<td>spontaneous breech</td>
<td>1 (0.8%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>CONGENITAL ABNORMALITIES</strong></td>
<td></td>
<td></td>
<td>Insufficient numbers for analysis</td>
</tr>
<tr>
<td>yes</td>
<td>3 (2.5%)</td>
<td>3 (1.3%)</td>
<td>Any vs. none</td>
</tr>
<tr>
<td>no</td>
<td>115 (97.5%)</td>
<td>224 (98.7%)</td>
<td>p=0.182 OR 1.48 (0.84-2.58)</td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>SPECIAL NURSERY (LEVEL 2) STAY</strong></td>
<td></td>
<td></td>
<td>Any vs. none</td>
</tr>
<tr>
<td>none</td>
<td>87 (73.7%)</td>
<td>183 (80.6%)</td>
<td>p=0.018 OR 1.48 (0.84-2.58)</td>
</tr>
<tr>
<td>yes</td>
<td>31 (26.3%)</td>
<td>44 (19.4%)</td>
<td></td>
</tr>
<tr>
<td>≥ 2 days</td>
<td>24 (20.3%)</td>
<td>38 (16.7%)</td>
<td></td>
</tr>
<tr>
<td>≥ 7 days</td>
<td>16 (13.6%)</td>
<td>29 (12.8%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>NEONATAL INTENSIVE CARE STAY</strong></td>
<td></td>
<td></td>
<td>Any vs. none</td>
</tr>
<tr>
<td>none</td>
<td>108 (91.5%)</td>
<td>215 (94.7%)</td>
<td>p=0.359 OR 1.66 (0.62-4.33)</td>
</tr>
<tr>
<td>yes</td>
<td>10 (8.5%)</td>
<td>12 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>≥ 2 days</td>
<td>9 (7.6%)</td>
<td>11 (4.8%)</td>
<td></td>
</tr>
<tr>
<td>≥ 7 days</td>
<td>6 (5.1%)</td>
<td>10 (4.4%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
<tr>
<td>PAEDIATRIC INTENSIVE CARE STAY</td>
<td>SIDS</td>
<td>Control</td>
<td>Statistical analysis</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------</td>
<td>---------</td>
<td>----------------------</td>
</tr>
<tr>
<td>yes</td>
<td>0</td>
<td>1 (0.4%) 6 days</td>
<td><strong>Insufficient numbers for analysis</strong></td>
</tr>
<tr>
<td>no</td>
<td>118 (118%)</td>
<td>226 (99.6%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SOCIO-ECONOMIC DISADVANTAGE VALUE</th>
<th>SIDS</th>
<th>Control</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>value &lt;1000</td>
<td>80 (67.8%)</td>
<td>124 (55.1%)</td>
<td>T test of 2 means p=0.00003638 value &lt;1000 vs. value &gt;1000</td>
</tr>
<tr>
<td>value &gt;1000</td>
<td>38 (32.2%)</td>
<td>101 (44.9%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>225 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1 MIN APGAR SCORE</th>
<th>SIDS</th>
<th>Control</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min &lt;7</td>
<td>24 (20.3%)</td>
<td>53 (23.3%)</td>
<td>score &lt;7 vs. score ≥7</td>
</tr>
<tr>
<td>1 min ≥7</td>
<td>94 (79.7%)</td>
<td>174 (76.7%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5 MIN APGAR SCORE</th>
<th>SIDS</th>
<th>Control</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min &lt;7</td>
<td>2 (1.7%)</td>
<td>5 (2.2%)</td>
<td>score &lt;7 vs. score ≥7</td>
</tr>
<tr>
<td>5 min ≥7</td>
<td>116 (98.3%)</td>
<td>222 (97.8%)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>118 (100%)</td>
<td>227 (100%)</td>
<td>Fishers exact 2 tailed for values &lt;5</td>
</tr>
</tbody>
</table>

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1111/j.1365-2672.2008.03747.x

**NOTE:** This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1099/jmm.0.005322-0

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1111/j.1574-695X.2009.00592.x](http://dx.doi.org/10.1111/j.1574-695X.2009.00592.x)

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1099/jmm.0.014613-0](http://dx.doi.org/10.1099/jmm.0.014613-0)
Novel hypothesis for unexplained sudden unexpected death in infancy (SUDI)

A R Hightet, A M Berry and P N Goldwater


Updated information and services can be found at:
http://adc.bmj.com/cgi/content/full/94/11/841

These include:

References
This article cites 25 articles, 7 of which can be accessed free at:
http://adc.bmj.com/cgi/content/full/94/11/841#BIBL

Rapid responses
You can respond to this article at:
http://adc.bmj.com/cgi/eletter-submit/94/11/841

Email alerting service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

Topic collections
Articles on similar topics can be found in the following collections

- Immunology (including allergy) (43258 articles)
- Child health (25858 articles)
- Infant health (4363 articles)
- SIDS (333 articles)

Notes

To order reprints of this article go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to Archives of Disease in Childhood go to:
http://journals.bmj.com/subscriptions/
Novel hypothesis for unexplained sudden unexpected death in infancy (SUDI)

A R Highet,1,2 A M Berry,1 P N Goldwater1,2

ABSTRACT
Objective: Two recent retrospective studies independently reported typically pathogenic bacteria in normally sterile sites of infants succumbing to sudden unexpected death in infancy (SUDI). These findings suggested a proportion of unexplained SUDI might be triggered by bacteraemia. The objective was to assess these observations in the context of the pathology and epidemiology of sudden infant death syndrome (SIDS) in relation to the role of infection and inflammation as triggers of these deaths.

Design: A review of the literature to identify potential risk factors for unexplained infant deaths and proposal of a theoretical model for SUDI.

Results: Pathologic and epidemiological evidence suggests a hypothesis based on three factors: bacterial translocation, pathogen pattern recognition insufficiency and prenatal exposure to infection.

Conclusion: We propose that sterile site infections in which common toxigenic bacteria are identified indicate a brief bacteraemic episode prior to death. This might reflect an ineffective innate response to invasive pathogens that results in reduced clearance of the bacteria. Thymomegaly observed consistently among infants diagnosed under the category of SIDS might have its origins in prenatal life, perhaps generated via in utero infection or exposure to microbial antigens which result in thymocyte priming. There is consistent evidence for an infectious aetiology in many unexplained SUDI. Future directions for research are suggested.

It has been almost 175 years since the unexplained pathology of sudden unexpected death in infancy (SUDI) was first identified in the medical literature, yet the cause of these tragic postneonatal deaths remains a mystery. Two independent retrospective studies reported typically pathogenic bacteria in normally sterile sites of infants succumbing to sudden unexpected death. This raised the possibility that undetected bacteraemia is present in a proportion of SUDI cases. There are three possible explanations for this finding of potential pathogens in usually sterile sites: (1) the bacteraemia is normally missed because of the small volumes of blood often available for testing; (2) bacterial presence might have previously been dismissed as post-mortem contamination; (3) because of the developmental stage of the infant, low antibody levels and/or inefficient innate immunity permit the organisms to spread rapidly into normally sterile tissues. This led to the hypothesis proposed in this article based on the pathologic and epidemiological evidence from unexplained SUDI. In reference to the literature, “SIDS” terminology is used, as was appropriate at the time of the original research publication. The SIDS cases upon which the hypothesis is based met the 1989 or 2004 definition. It is not possible to determine whether or not evidence of bacteraemia/sterile site infection was taken into account for these cases because many pathologists may have regarded the findings as “contamination”. On the other hand, for the diagnosis of SIDS, bacteraemia findings would be excluded as an explainable cause of death.

HYPOTHESIS
Three common factors are introduced as potential contributors to SIDS: (1) evidence of bacteraemia and/or toxaemia; (2) genetic predisposition to insufficient pathogen pattern recognition; and (3) prenatal infectious events that affect organ growth and/or the developing immune system. Their potential roles in unexplained SUDI are developed in this section and interaction among these factors is illustrated in fig 1.

Factor 1: Transient bacteraemia
With the exception of a mild respiratory viral infection and/or diarrhoeal disease, no significant illness is reported prior to SUDI. An episode of transient bacteraemia would logically explain post-mortem bacteriological findings of sterile site infection in the absence of bacteraemic symptoms and signs, and an absence of pathogens in the small blood volumes available at autopsy. Transient bacteraemic episodes...
have been identified as normal infant events and asymptomatic bacteraemia in newborns has been documented. A recent study has identified pyrogenic toxins of *Staphylococcus aureus* in the urine (a possible marker of bacteraemia) in 9% of a healthy infant cohort, which often coincided with a mild respiratory infection. High levels of these toxins have been identified in the tissues or sera in more than half of SIDS infants from five countries. Infection or toxins have been proposed to cause sudden life-threatening events in infancy, especially when maternal antibody levels decline leaving the infant temporarily hypo-immune at 2–4 months of age when the peak incidence of SIDS occurs. In most cases transient bacteraemia is rapidly cleared by the immune system. This is dependent upon recognition of the pathogen and initiation of an appropriate immune response.

The mild respiratory viral infection that often precedes SUDI can disrupt the mucosal barrier allowing translocation of colonising bacteria into the bloodstream. Virus infections also enhance the density of colonisation as does prone sleeping. Similarly, damage to the intestinal epithelium during diarrhoeal disease may allow translocation from this site. Other risk factors for SIDS, preterm birth and not being breastfed, are known to increase the ability of enteric bacteria to translocate from the intestine. *S aureus* is a key candidate for both extra-nasopharyngeal and extra-intestinal translocation. The synergistic events between *S aureus* and respiratory viral infection have been well documented and staphylococcal enterotoxins are produced in the intestinal tract of infants and have been detected in our recent work on the intestinal contents of SIDS infants (unpublished data). It has been proposed that enterotoxigenic activity by *S aureus* could facilitate transcytosis, which would also explain the findings of *S aureus* and coliforms in normally sterile sites in SIDS infants at post mortem.

**Factor 2: Pathogen pattern recognition insufficiency**

Rapid recognition and response to microbial invasion is the function of the innate immune system. Specifically, Toll-like receptor (TLR) 2, nucleotide-binding oligomerisation domain containing 2 (NOD2) and tumour necrosis factor alpha (TNF-α) receptor 1 are necessary for full responses to *S aureus*. Similarly, TLRs 2, 4 and 5, MD-2 and CD14 facilitate the recognition and response to *Escherichia coli*. Loss-of-function polymorphisms among genes regulating the above immune components can result in impaired recognition of invasive pathogens and thus impaired induction of an immune response. *S aureus* and coliforms found in normally sterile sites may be a remnant or “footprint” of transient bacteraemia during which the pathogen was unrecognised and thus not cleared from these sites. Alternatively, the rapidity of death, in some cases, might not allow sufficient time for the immune system (whether compromised or not) to clear the bacteria from the normally sterile sites. Ineffective pathogen recognition may leave infants particularly susceptible to bacteraemia at 2–4 months of age because the natural diminution in maternal antibodies could not be compensated for by the innate immune system.

**Factor 3: Prenatal infectious event**

A hallmark of SIDS pathology is a pattern of organ weights that distinctly deviates from the expected reference ranges. The thymus (p = 0.04) and brain (p = 0.001) in particular are significantly larger in SIDS victims. Although it was inferred that this was likely due to the reference ranges being based upon infants with illness prior to death (whereas SIDS infants appear to be healthy immediately before death), the investigators also recognised that the “differences could also reflect disturbances in growth presumably beginning prior to birth”. Extrapolation of post-mortem organ weight data shows that disrupted patterns of growth of vital organs appear to originate in the prenatal period. One possibility is “thymic priming” via a massive T cell proliferative response to a foreign protein or pathogen, possibly prenatally. In this case the enlarged thymus would be consistent with increased thymic activity. A likely cause of this is prenatal

Figure 1  Proposed interactions between transient bacteraemia (1), PPR gene polymorphisms (2) and prenatal (3) events contributing to SIDS.
viral or bacterial exposure. Prenatal infectious events could stand alone as a risk factor for SIDS (through effects of thymic “priming” on pro-inflammatory events). Placental morphology may also be indicative of prenatal virus transmission. Current research by Ansari and colleagues indicates that total trophoblast volume in normal birth weight SIDS infants is higher due to a significant increase in both cytotrophoblast and syncytiotrophoblast volume (TI Ansari, personal communication, 8 January 2009). As these cells are the first-line defence of the fetus against viruses, such anomalies suggest viral involvement. Postnatal exposure (or re-exposure to an antigen possibly via bacteriaemia) could also be potentiated by immunoregulatory gene polymorphisms conferring inappropriately high cytokine production, for example those associated with poor outcomes during sepsis. An alternative outcome resulting from prenatal thymic “priming” and subsequent re-exposure to the causal antigen could include anaphylaxis, for which there is some evidence.\(^{25}\)

**MODE OF DEATH**

Based on the evidence of Poets et al.,\(^\text{21}\) speculation as to the mode of death is necessarily narrowed to a non-respiratory, cardio-geneic event under conditions of toxic/septic shock or anaphylaxis. Cytokines produced in response to infection can induce physiological responses likely to be involved in SUDI. These include vascular shock, hypoglycaemia, deep sleep with prolonged apnoea, cardiac irregularities and fever.\(^\text{22}\) Polymorphisms in genes involved in QT interval control may increase the sensitivity of the heart to exposure to toxin. Because such polymorphisms are not universal in SUDI, their presence would not be seen as an “essential” route to cardiac standstill.

**PROPOSED INVESTIGATION**

Bacteriaemia preceding SUDI might be detected more frequently if molecular methods, as opposed to cultural, are employed for the detection of bacteria and their products in blood and normally sterile sites. As bacteria have been identified in normally sterile sites of infants suffering unexplained death, focusing on immunoregulatory gene polymorphisms that specifically affect recognition of bacterial species found to be implicated (ie, *S. aureus* and *E. coli*) will most certainly prove worthwhile (eg, TLR-2(G2408A) which has been suggested to increase risk of staphylococcal infection,\(^\text{23}\) NOD2(502insC) which results in a loss of bacterial sensing and decreased activation of inflammatory pathway\(^\text{24}\) and MD-2(G56R) variant which exhibits very low LPS binding\(^\text{25}\)). Four TNF-α receptor 1 polymorphisms (TNFRSF1A(A+56T), TNFRSF1A(G-609T), TNFRSF1B(T+196G) and TNFRSF1B(A+1466G)) have been identified, but as yet their effect on receptor function is unknown.\(^\text{26}\) Prenatal infectious events could be predicted by assaying for a shift from CD45RA+ to CD45RO+ cells in the T lymphocyte population (consistent with previous exposure to microbial products or other antigens) in peripheral blood collected shortly after birth, should it be available for SUDI infants.

**CONCLUSION**

This novel hypothesis takes into account new findings in relation to unexplained SUDI. Ineffective innate responses to invasive pathogens through specific gene polymorphisms would logically increase the likelihood of failure to clear bacteria from the blood and other sterile sites. These sterile site infections involving common toxigenic bacteria could be a “footprint” of a brief bacteraemic episode happening just prior to death. It is plausible that thymomegaly (evidenced by comparative organ weight and simulated organ growth analysis) seen consistently in SIDS has its origins in prenatal life and is probably generated via in utero infection/exposure to bacterial/viral antigen with consequential thymocyte “priming”. Overall, bacteriological and pathological observations appear to be consistent with an infectious aetiology for unexplained SUDI.

**Funding:** The authors gratefully acknowledge research grant support from the Foundation for the Study of Infant Death, UK.

**Competing interests:** None.

**Provenance and peer review:** Not commissioned; externally peer reviewed.

**REFERENCES**

Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2
Distribution of Interleukin-1 receptor antagonist genotypes in Sudden Unexpected Death in Infancy (SUDI); unexplained SUDI have a higher frequency of allele 2

Running title: IL-1RN genotype in unexplained SUDI

Authors: Amanda R. Highet¹, ² (Corresponding author), Anne M. Berry¹, and Paul N. Goldwater¹, ².

¹Department of Microbiology and Infectious Diseases, SA Pathology at the Women’s & Children’s Hospital, 72 King William Road, North Adelaide, South Australia, Australia

²University of Adelaide School of Paediatrics and Reproductive Health, Discipline of Paediatrics, North Adelaide, South Australia, Australia.

Corresponding author: Amanda Highet, Department of Microbiology and Infectious Diseases, SA Pathology, Women’s and Children’s Hospital, 72 King William Road, North Adelaide, South Australia, Australia. amanda.highe@adelaide.edu.au

Abstract

Aims: This investigation was designed to explore the role of IL-1RN genotype in unexplained infant deaths (including SIDS), non-infectious infant deaths and infectious infant deaths, and to investigate whether IL-1RN genotype is related to the finding of organisms in normally sterile sites in infant deaths.

Methods: IL-1RN 89bp variable number of tandem repeat polymorphism genotype was determined using PCR for 49 cases of unexplained sudden unexpected death in infancy (uSUDI), 13 cases of infectious sudden unexpected death in infancy, 10 cases of non-infectious sudden unexpected death in infancy and 103 live control infants. IL-1RN
genotype was then compared with the presence of bacteria in normally sterile sites in infant deaths.

**Results:** An association was found between the homozygous A2 allele and uSUDI \((p=0.007\, 95\%\text{CI }1.41-17.67)\) where carriage of the 2/2 genotype was 4.85 times more likely to increase risk of uSUDI compared with the predominant 1/1 genotype.

**Conclusions:** The role of infection in uSUDI and SIDS may be via an immune response pathway where \(IL-1RN\) A2 affects IL-1 regulation. These results are consistent with previous research where polymorphic genotypes conferring more severe pro-inflammatory responses are found more frequently in uSUDI/SIDS infants than controls.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SIDS</td>
<td>Sudden Infant Death Syndrome</td>
</tr>
<tr>
<td>SUDI</td>
<td>Sudden unexpected death in infancy</td>
</tr>
<tr>
<td>-iSUDI</td>
<td>Infectious sudden unexpected death in infancy</td>
</tr>
<tr>
<td>-niSUDI</td>
<td>Non-infectious sudden unexpected death in infancy</td>
</tr>
<tr>
<td>-uSUDI</td>
<td>Unexplained sudden unexpected death in infancy</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
Keywords

Infection

Interleukin 1 receptor antagonist protein

Polymorphism, genetic

Sudden infant death

Key Messages

The role of infection in uSUDI and SIDS may be via an immune response pathway where

*IL-1RN* genotype plays a critical role.

In this study an association was found between the homozygous A2 allele and uSUDI

(*p*=0.007) where carriage of the 2/2 genotype gave a 4.85 times increased risk of uSUDI

compared with the predominant 1/1 genotype.

These results are consistent with previous research where polymorphic genotypes conferring

more severe pro-inflammatory responses are found more frequently in uSUDI/SIDS infants

than controls.
Introduction

_Bacteria and their toxins in SUDI and SIDS_

Unexplained SUDI (uSUDI) is a cause of death defined by exclusion of other recognized causes. Infant deaths that would previously have been termed SIDS constitute the majority of uSUDI cases. Pathological, epidemiological and genotypic findings in SIDS infants suggest an infectious aetiology with involvement of bacteria (1-6). Staphylococcal enterotoxins have been demonstrated in tissues or sera in over half of SIDS infants from five countries including Australia (6) and _Staphylococcus aureus_ and coliforms have been found in normally sterile sites (heart blood, spleen and/or cerebrospinal fluid) in 10.76 and 17.7% respectively of uSUDI cases (7). Host factors, including genetic determinants, influence the outcome of interactions between toxin and host. These have been the focus of many recent SIDS investigations (8). Age-dependant susceptibility to sudden death has been demonstrated in a mouse model of endotoxic shock where the animals displayed gross pathological findings consistent with SIDS in humans (9). The role of bacteria in SIDS pathogenesis may be via an immune response pathway. Interleukin-1 (10) is a cytokine responsible for causing rapid vasodilation in septic shock. Given that _S. aureus_ toxins are powerful inducers of interleukin-1 (10) an investigation of this aspect of the immune response pathway was considered worthwhile.

_Interleukin-1 receptor antagonist_

Interleukin 1 receptor antagonist (IL-1ra) is a competitive inhibitor that binds to IL-1 receptors without inducing intracellular response, by blocking receptors it is an important endogenous regulator of inflammation and actively limits septic shock and reverses hypotension (11). Additionally, IL-1ra suppresses the release of IL-1β and TNFα from mononuclear cells (12). Clinical (13) and pathological findings in SIDS are similar to
endotoxin-induced shock (9, 14) suggesting that high levels of circulating IL-1 may be implicated in their death. The known genetic variations in the IL-1β gene are not major regulators of in vitro production of IL-1β, rather the IL-1RN (gene encoding IL-1ra) allele type has the decisive role (15). Assessment of the genetic basis of IL-1β production IL-1RN polymorphic sites, rather than IL-1β polymorphic sites, was considered apposite. In a study by Moscovis et al. a single nucleotide polymorphism in IL-1RN (T+2018C) was found not to be differently distributed between SIDS infants and controls (16). A length variation within intron 2 of IL-1RN was first reported by Steinkasserer et al (17). Currently five alleles, A1-A5, are recognised. The A1 allele, and the 1/1 genotype, are predominant (18). The A2 variant reportedly enhances in vitro IL-1β production almost two-fold (15), and contributes to susceptibility to severe sepsis (19). At present there are conflicting reports that A2 can increase (20), or have no effect (21), on IL-1ra levels. It is generally accepted that homozygous carriers of A2 have a more severe and prolonged pro-inflammatory immune response than do those with other IL-1RN genotypes (22). This investigation was designed to 1) explore the role of IL-1RN genotype in unexplained infant deaths (uSUDI) including SIDS, non-infectious infant deaths (niSUDI) and infectious infant deaths (iSUDI), and 2) to investigate whether IL-1RN genotype is related to the finding of organisms in normally sterile sites in infant deaths (which might trigger an uncontrolled pro-inflammatory response).
Materials and methods

Ethics approval: The study was approved by the Research Ethics Committee of the Children, Youth and Women’s Health Service, South Australia, and the Victorian Institute of Forensic Medicine (VIFM), Victoria.

Selection criteria: The infants included in this study are those from the cohort of uSUDI, infectious SUDI and non-infectious SUDI described by Goldwater (7), where stored material was available. Small portions of intestinal contents from 39 SIDS in South Australia (SA) between 1989 and 1993 were collected at autopsy and stored in sterile containers at -80°C until required. South Australian SIDS diagnoses matched the criteria specified by the 1991 definition for SIDS (23). Another ten infants (one category 1a SIDS, seven category 2 SIDS and two unascertained/SUDI) infants had a bowel contents sample collected at autopsy at the VIFM, Victoria in 2007 and 2008. The samples were stored at -80°C and transported on cardice. Victorian SIDS met the 2004 definition (24). This gave a total of 49 unexplained SUDI cases. The median age of unexplained SUDI infants was four months, the interquartile range was 3.5 months and the proportion of male infants was 54%. The infectious SUDI infant group consisted of thirteen cases of death caused by infectious agents (sepsis, pneumonia, myocarditis, meningitis or gastroenteritis). Nine of the infants were from South Australia and four from Victoria. Their median age was 2.5 months, interquartile range was 5.7 and 62% were male. Non-infectious SUDI infants were cases of sudden death not caused by infectious agents (principally congenital heart disease and asphyxia). Six were from South Australia and four from Victoria. Their median age was three months, interquartile range was 8.6 months and 40% were male. Intestinal contents were sampled and stored as described above. As a comparison group for IL-1RN genotype frequencies, 103 whole blood samples (in EDTA tubes, surplus to clinical need) were
retrieved from infants who were not suspected of, or diagnosed with, infectious disease. This group primarily consisted of infants with specific organ system disease (renal failure, heart disease) haematological disturbances and infants undergoing surgical procedures. The median age of the live control infants was 5.5 months, the interquartile range was 6.3 months and the proportion of male infants was 62%. Fifty microlitre samples of blood were absorbed onto filter card and stored at room temperature until required.

**DNA extraction from intestinal contents:** A small portion (<1g) of intestinal contents or faeces was suspended in 1ml glycerol broth (3% w/v tryptone soya broth and 20% glycerol) and stored at -80°C until required. To extract DNA, 90μl of the suspension was aliquoted into a centrifuge tube with 10μl 10X chelex-lysis solution (50% w/v Chelex-100 (Biorad, Hercules California), 2% w/v SDS, 0.1M Tris, 5mM EDTA) and 4mg Proteinase K (Roche) and vortexed briefly. The mixture was incubated at 55°C for one hour, then 95°C for 30 minutes and centrifuged for one minute. The supernatant was diluted tenfold before use in PCR reactions.

**DNA extraction from dried blood samples:** A single 1.2mm punch biopsy was taken from each dried blood sample. Nucleic acids were extracted from the punches by treating with a lysis solution with 50% w/v Chelex-100 (Biorad, Hercules California), 1mg Proteinase K (Roche), incubation at 60°C for 30 minutes then 95°C 30 minutes. The Chelex was pelleted by centrifugation and 2μl of the supernatant was used in each PCR reaction.

**PCR amplification:** *IL-1RN* was amplified using the primer pair published by Tarlow *et al.* (18) Cycling conditions were as follows: 95°C 2 mins, 38 cycles of 95°C 30s, 59°C 30s, 72°C 1 min and a final extension of 72°C 3mins on a Bio-Rad IQ5 cycler. *IL-1RN* genotype
was determined by the amplicon size (variable number of tandem repeats) as described by Tarlow et al. (18). Each PCR reaction consisted of 0.05µM MgCl₂, 1X Colourless GoTaq Flexi Buffer, 0.0025µM dNTP mix, 0.05µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison WI), 2µl of extracted DNA and PCR-grade water to a total volume of 12.5µl. Amplification products were visualized on a 2% agarose gel stained with GelRed (BioTium, Hayward) alongside a pUC19/HpaII DNA molecular weight marker (Geneworks, Thebarton). Homozygous 1/1 and 2/2 controls for each genotype were sourced from the study cohort and confirmed by sequencing the amplification products (IMVS Sequencing Centre, Adelaide).

**Statistics:** Unexplained SUDI genotypes were analysed against live control infants by comparing homozygous and heterozygous A2 carriage with the 1/1 genotype. Yates Corrected Probability Test using 2x2 contingency table software (Statcalc, Epiinfo v6) was used to calculate $p$ values, odds ratios and 95% confidence intervals. Sterile site bacteriology recorded in post-mortem reports, where available, was compared against *IL-IRN* genotype for uSUDI, iSUDI and niSUDI. Description of the bacteriological specimen collection using aseptic techniques has been described previously (7).
Results

Not all samples gave a valid result due to the presence of PCR inhibitors in blood. Ninety-four of the 103 live control infant, and all of the uSUDI, iSUDI and nSUDI samples gave a valid IL-1RN genotype result (*Table 1*). The allele frequencies of the live control infants was similar to those in the previously published literature (18), (±0.04). Heterozygous 1/2 and homozygous 2/2 genotypes were analysed against 1/1 genotype for uSUDI compared with live control infants (*Table 2*). Compared with the control group, 2/2 genotype distribution in the uSUDI cohort was not in Hardy-Weinberg equilibrium.

**Comparison of post-mortem bacteriology findings with IL-1RN genotype**

The 11 uSUDI infants with the 2/2 genotype had the following post-mortem bacteriology: six had no growth (one of which had been on antibiotics); one had coliform isolated from heart blood; one *S. aureus* from lung and coliform from spleen; one *Klebsiella oxytoca* from lungs and heart blood; one *Haemophilus influenzae* from lung, coliform from ear and non-fermentative Gram-negative bacilli (presumed contaminant) from heart blood and one *Haemophilus influenzae* from lung, non-fermentative Gram-negative bacilli (probable contaminant) from heart blood and cytomegalovirus from salivaries. Two infants showed signs of infection; one was treated with antibiotics for an upper respiratory tract infection and the other had lung inflammation reported at autopsy with unremarkable bacteriology. Infants with sterile site infection more frequently carried the 2/2 genotype (23%) than infants without sterile site infection (11%) (*Table 3*).
Discussion

The role of infection in uSUDI and SIDS may be via an immune response pathway where IL-1RN genotype plays a critical role. S. aureus toxins are powerful inducers of IL-1 (10), a cytokine responsible for causing rapid vasodilation in septic shock. The frequency of IL-1β polymorphic site C-511T has been investigated in a small group of Australian (n=18) SIDS infants where a trend ($p=0.59$) was observed towards the TT genotype, which confers higher IL-1β levels, compared with Australian adult controls (16). However, Santtilla et al (15) reported that the known variations in the IL-1β gene are not major regulators of in vitro production of IL-1β, rather the IL-1RN allele has this important role. The availability of IL-1ra for neutralization of high of IL-1 levels would influence the outcome of the suspected S. aureus toxaemia in uSUDI and SIDS. Moscovis et al. have investigated the single nucleotide polymorphism IL-1RN (T+2018C) in SIDS, but found it not to be differently distributed between SIDS infants and controls (16).

This study investigated the relationship between IL-1RN 89bp VNTR genotype and uSUDI, and looked at whether a particular genotype was associated with the finding of bacteria in a normally sterile site at autopsy. An association was found between the homozygous A2 allele and uSUDI ($p=0.007$) where carriage of the 2/2 genotype gave a 4.85 times increased risk of uSUDI compared with the predominant 1/1 genotype. These results are consistent with previous research in which polymorphic genotypes conferring more severe pro-inflammatory responses were found more frequently in SIDS infants than controls (16, 25, 26). Apart from competitively blocking IL-1 receptors, IL-1ra also suppresses the release of IL-1β and TNFα from mononuclear cells. It could be postulated that unchecked levels of IL-1β and TNFα (through IL-1RN polymorphism) could play a role in a sudden cardiogenic event leading to sudden unexpected death in an infant, as these cytokines act synergistically.
to depress human myocardial function (27). This mode of death is consistent with a non-respiratory, cardiogenic event as suggested by the observations of Poets et al. (13). The IL-1RN 2/2 genotype was more common in infants with bacteria found in normally sterile sites at autopsy than those with no growth from these sites. Although the interpretation of these results is limited by small numbers, the potential relationship is noteworthy and deserves further study with larger numbers of subjects and controls.

Gestational age data were not available for this cohort. Pre-term birth is a risk factor for SIDS and so may be over-represented in our SUDI cohort. However, this is unlikely to introduce bias to the IL-1RN genotype as only maternal, not neonatal carriage of A2 demonstrates an association with pre-term birth (28). The respective infants from the pre-term study showed no difference in A2 carriage, suggesting that in our study, infant A2 carriage is likely to be associated with uSUDI independent of pre-term birth. It has been reported, however, that IL-1RN A2 possession by the fetus leads to enhanced intra-amniotic IL-1β production, which in turn could lead to preterm labour. The live infant control group in the present study had an excess of males (62%) compared with the uSUDI group (54%). This also was unlikely to introduce bias to carriage of IL-1RN 2/2 genotype, as a previous study has found the 2/2 genotype to be unaffected by gender (29), although interestingly IL-1ra levels in amniotic fluid and urine of neonates are higher in females than males (30).

The possible association between IL-1RN A2 and uSUDI will be further investigated in a proposed study with a larger cohort, greater statistical power and ideally matched (for date of birth, gestational age, sex and birth weight) controls. This study will also include sterile site bacteriology data for a large number of infants, allowing us to observe if the trend indicated in Table 3, in which infants with sterile site infection had higher carriage of the
2/2 allele, is reproduced with a larger cohort, and also if the carriage of 2/2 genotype is associated with uSUDI independent of pre-term birth and gender.

**Acknowledgments/Disclosure of interests**

The authors wish to thank the Department of Histopathology, Women’s & Children’s Hospital Adelaide and The Victorian Institute of Forensic Medicine for providing samples and the Foundation for the Study of Infant Death, UK, for research grant support.

There are no competing interests to declare.
References


Table 1. Distribution of IL-1RN genotypes and alleles among infant deaths and live control infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>iSUDI</th>
<th>niSUDI</th>
<th>uSUDI</th>
<th>Live Control Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>6 (46%)</td>
<td>3 (30%)</td>
<td>22 (45%)</td>
<td>58 (62%)</td>
</tr>
<tr>
<td>1/2</td>
<td>3 (23%)</td>
<td>6 (60%)</td>
<td>13 (27%)</td>
<td>26 (28%)</td>
</tr>
<tr>
<td>2/2</td>
<td>2 (15%)</td>
<td>1 (10%)</td>
<td>11 (22%)</td>
<td>6 (6%)</td>
</tr>
<tr>
<td>1/3</td>
<td>2 (15%)</td>
<td>0</td>
<td>2 (4%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>1/4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>2/3</td>
<td>0</td>
<td>0</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>1/5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>10</td>
<td>49</td>
<td>94</td>
</tr>
</tbody>
</table>

A 1 17 (65%) 12 (60%) 59 (60%) 146 (78%)
A 2 7 (27%) 8 (40%) 36 (37%) 38 (20%)
A 3 2 (8%) 0     3 (3%)  1 (0.5%)
A 4 0       0     0     2 (1%)
A 5 0       0     0     1 (0.5%)
| Total    | 26      | 20      | 98     | 188     |
Table 2. *IL-1RN* 1/2 genotype and 2/2 genotype vs 1/1 genotype for uSUDI and live control infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>p-value (Yates Corrected)</th>
<th>95% Confidence interval</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2</td>
<td>0.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2/2</td>
<td>0.007</td>
<td>1.41-17.67</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>1/1</td>
<td>1/2</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td><strong>uSUDI with sterile site infection</strong></td>
<td>20</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td><strong>uSUDI without sterile site infection</strong></td>
<td>14</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td><strong>iSUDI with sterile site infection</strong></td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>iSUDI without sterile site infection</strong></td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>niSUDI with sterile site infection</strong></td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>niSUDI without sterile site infection</strong></td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total with sterile site infection</strong></td>
<td>30</td>
<td>12 (40%)</td>
<td>7 (23%)</td>
</tr>
<tr>
<td><strong>Total without sterile site infection</strong></td>
<td>19</td>
<td>9 (47%)</td>
<td>7 (37%)</td>
</tr>
</tbody>
</table>