

**Infection and immunogenetics in
unexplained infant deaths in Australia**

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Addendum

Chapter 1.

Table 1. SIDS deaths in Australia by age range and cases per year [2000-2006]

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

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 C600 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)

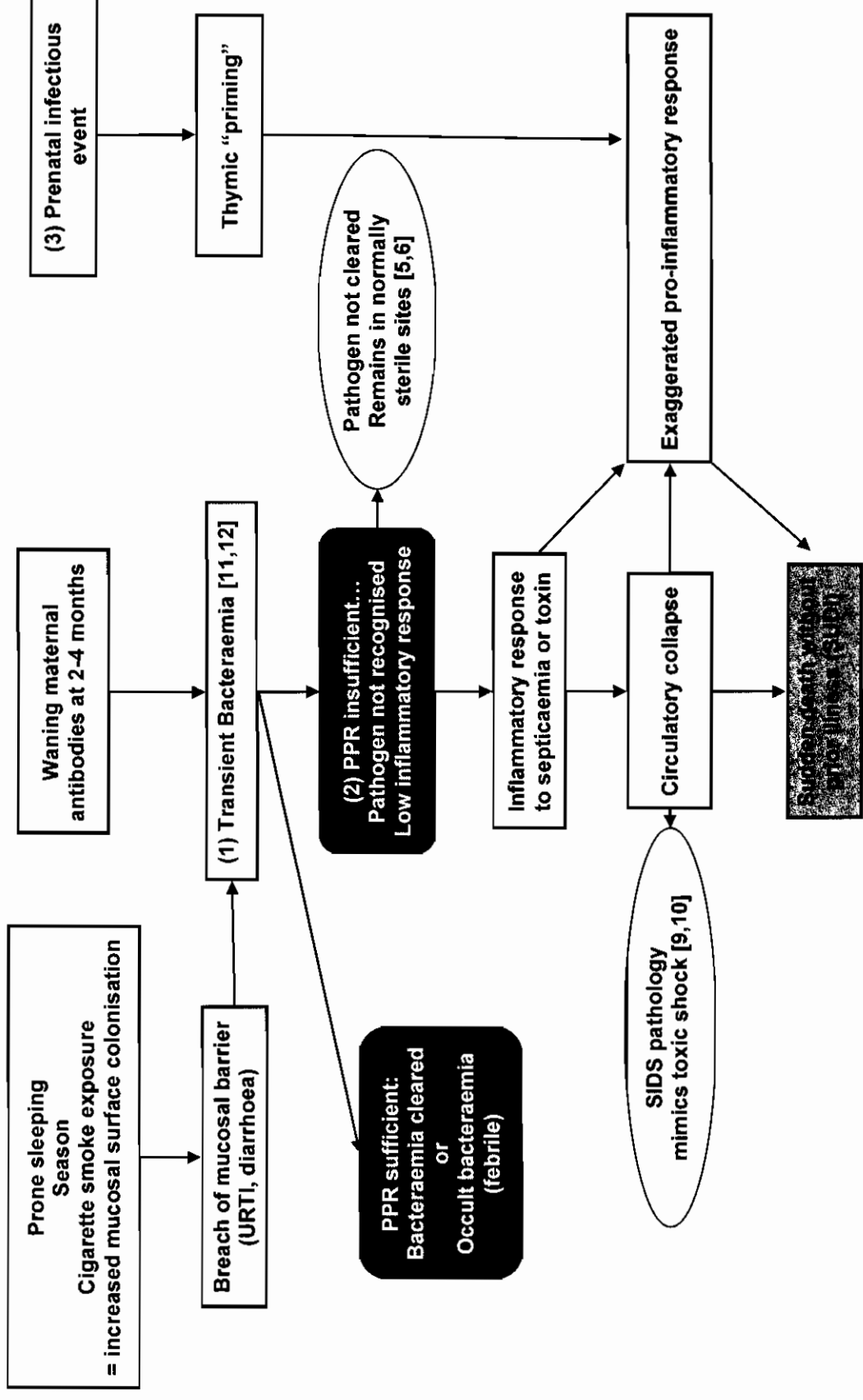


Figure 1. Proposed interactions between transient bacteraemia (1), PPR gene polymorphisms (2) and prenatal events (3) leading to sudden infant death

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

6. Zorgani A, Essery S, Madani O, Bentley A, James V, MacKenzie D, Keeling J, Rambaud C, Hilton J, Blackwell C, Weir D, Busuttill A. Detection of pyrogenic toxins of *Staphylococcus aureus* in sudden infant death syndrome. *FEMS Immunol Med Microbiol.* 1999;**25**:103-108

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-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."

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 210, Paragraph 2, Line 9. Added citation [8]

Page 211. Add the following to the reference list

8. Sheburn RE, Webb TE, Jenkins RO. Detection of toxigenic bacteria in polyurethane foam from cot mattresses by polymerase chain reaction. *Jpn J Infect Dis.* 2007;**60**:19-22

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.”

Abstract

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 *Escherichia coli*, *Staphylococcus aureus* and *Clostridium sordellii* from SIDS infants. In the case of *E. coli* and *S. aureus*, genes encoding potentially lethal virulence factors were detected in cultures from both SIDS and healthy infants, and *C. sordellii* lethal toxin detected in none. *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 V β 3.1 recombination signal sequence polymorphism (TCRBV3S1), 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 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.

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.

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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

1. Highet AR. An infectious aetiology of sudden infant death syndrome. *J Appl Microbiol.* 2008;**105**:625-635. **(Chapter 1)**
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 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. Novel hypothesis for unexplained Sudden Unexpected Death in Infancy (SUDI). *Arch Dis Child.* 2009;**94**:841-843. **(Chapter 6)**
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)**

7. Highet AR, Berry AM, Bettelheim KA, Goldwater PN. Bacterial toxins in the sudden infant death syndrome (abstract). *Forensic Sci Med Pathol* 2009;DOI 10.1007/s12024-008-9058-4:

Manuscripts in preparation

1. Highet, 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. Highet, 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
4. Highet, AR 2008, „Lethal bacterial toxins in sudden infant death syndrome“ Paper presented to the SIDS 10th International Conference 2008, Portsmouth UK.

Won Best Scientific Presentation by a Young Presenter
5. Goldwater, PN, Highet, AR 2008, „Sterile site infection at autopsy in sudden infant death syndrome (SIDS)“ Poster presented at the SIDS 10th International Conference 2008, Portsmouth UK.

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

7. Highet, A 2005, 'The incidence of AB5 subtilase toxigenic *Escherichia coli* in cases of sudden infant death syndrome (SIDS)', Paper presented to the Australian Society for Microbiology, South Australia general meeting, 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)
4. Highet AR, Berry AM, **Goldwater PN**. Novel hypothesis for unexplained Sudden Unexpected Death in Infancy (SUDI). *Arch Dis Child*. 2009;94:841-843. (Chapter 6)
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)

6. Highet AR, Berry AM, Bettelheim KA, **Goldwater PN**. Bacterial toxins in the sudden infant death syndrome (abstract). *Forensic Sci Med Pathol* 2009;DOI 10.1007/s12024-008-9058-4:

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)

2. Highet AR, **Berry AM**, Goldwater PN. Novel hypothesis for unexplained Sudden Unexpected Death in Infancy (SUDI). *Arch Dis Child.* 2009;94:841-843. (Chapter 6)
3. 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)
4. Highet AR, **Berry AM**, Bettelheim KA, Goldwater PN. Bacterial toxins in the sudden infant death syndrome (abstract). *Forensic Sci Med Pathol* 2009;DOI 10.1007/s12024-008-9058-4:

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. Highet AR, Berry AM, **Bettelheim KA**, Goldwater PN. The frequency of molecular detection of virulence genes encoding cytotoxin A, high-pathogenicity island and cytotoxic 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

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

-uSUDI	Unexplained sudden unexpected death in infancy
TCRBV3S1 RSS	T-cell receptor V β 3.1 gene recombination signal sequence
TNF α	Tumor necrosis factor alpha
VNTR	Variable number of tandem repeats