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Bacterial flora of Tasmanian SIDS infants with special reference to pathogenic strains of *Escherichia coli*

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SUMMARY

The general bacterial flora of 38 Tasmanian SIDS infants was examined together with faecal flora of 134 comparison infants ranging in age from birth to 6 months. The microflora of all specimens received was investigated with special emphasis on the toxigenic *Escherichia coli* (TEC). Samples were examined for verocytotoxigenic *E. coli*, free faecal verocytotoxin (FVT), heat labile toxin (LT) and heat stable toxin (ST) producers with the use of a Vero cell assay and commercial kits. The findings of this study revealed a high isolation rate (39%) of TEC from SIDS infants as compared to 1·5% from the healthy comparison infants. Atypical *E. coli* strains were also identified during the study, including *E. coli* A–D. An analysis of the same specimens for rotaviral and adenoviral antigens indicated that 30% of the SIDS cases were positive as compared to 20% in the comparison group.

INTRODUCTION

Sudden Infant Death Syndrome (SIDS) is the most common cause of postneonatal mortality in the developed world. It has been defined as the 'sudden death of any infant or young child which is unexpected by history and in which a thorough post-mortem examination fails to demonstrate an adequate cause for the death' [1] and thus is a diagnosis of exclusion [2]. Epidemiologically, the syndrome has certain consistent features, including a peak winter incidence with 60–80% of deaths occurring in the winter months [3] and an age incidence curve which rises from birth to a maximum at 2–3 months, then falls rapidly [4]. Geography is no barrier to SIDS, although there is a disparity in incidence, varying from 1·5 to 3·5 per 1000 live births in Europe and the United States, but dropping to around 0·19 per 1000 live births in Hong Kong [5]. The average SIDS rate in Australia is 2·0 cases per 1000 live births, but on average the Tasmanian rate has been nearly twice the national rate during the last decade [6] nearing 3·5 per 1000 livebirths.

A number of common factors have been established and may offer arguable clues as to possible causes of SIDS. A preceding history of infection is a common finding in babies dying of SIDS and can be present in 40–75% of cases [7]. Most of these infections comprise minor respiratory tract infections, which can be caused by either viruses or bacteria, although no association between SIDS and

any one particular organism has been identified [8]. The age distribution of SIDS has also been described as peaking at the time of maximum susceptibility to infection, which coincides with the decline in maternal antibodies [4, 9–11]. Consequently it has been speculated that a combination of infection and other environmental factors that cause thermal stress may contribute to death in over a third of SIDS victims [8]. Recently, there has been increasing evidence that the prone sleeping position is related to the risk of Sudden Infant Death Syndrome [12].

Several findings have suggested respiratory or other viruses such as echovirus, respiratory syncytial virus, adenovirus, influenza A and B, and other enteroviruses [1, 13] may also be involved. It has been postulated that viruses, particularly pneumotrophic species, may trigger an increase in sensitivity to bacteria [3], but few have postulated a direct role for bacteria. However, it has been proposed that common toxins produced by bacteria growing in the respiratory tract following a viral infection in hypoimmune infants may be a cause of SIDS [10, 14]. Organisms such as *Escherichia coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, and haemolytic streptococci (a and b) have all been attributed with possible roles [11, 14]. Anaerobes such as *Clostridium botulinum* and *Cl. difficile* have also been identified as potential agents [15].

It has further been suggested that a disordered nasopharyngeal flora may be associated with SIDS, as a result of a significantly larger population of *Staph. aureus*, streptococci, and Enterobacteriaceae in SIDS cases than in control groups [11]. It has been hypothesized that bacterial populations may be a cause of death between the time of losing maternal immunoglobulin and the acquisition of specific immunity. McKendrick and colleagues [14] found that 68% of the SIDS cases they examined had at least one potentially lethal toxigenic organism in their nasopharyngeal flora, as compared to 36% of controls, providing further support for these postulates.

Other studies have implicated staphylococcal toxins in SIDS – either alone [10] or acting synergistically with *Escherichia coli* [4, 17]. More specifically, during a pilot study by Bettelheim and colleagues [18] a strong association between heat labile enterotoxins (LT) and Verocytotoxin (VT) producing *E. coli* and SIDS was noted. Bettelheim and colleagues [18, 19] found toxigenic *E. coli* (TEC) producers of LT or VT in 46% of SIDS cases in South Australia, and this increased to 63% from an examination of Victorian cases [20]. By contrast, no TEC were found in comparison non-SIDS infants of South Australia, whilst 22% of such comparison infants in Victoria possessed these strains.

The results of Bettelheim and his team stimulated a prospective study of SIDS cases in South Australia [17] and the present study of Tasmanian SIDS cases for the presence of enterotoxigenic and verotoxigenic *E. coli*.

MATERIALS AND METHODS

SIDS infants

During our study period a total of 38 babies died suddenly and unexpectedly. All except one were born in Tasmania and were aged between 1 month and 1 year at the time of death. All infants had a full postmortem examination using a State

pathology protocol and were given the SIDS label by pathologists at the time of necropsy. These postmortem examinations were performed in hospitals throughout the State of Tasmania. Specific information on case histories of all SIDS infants, including details of feeding, was provided by the Menzies Centre for Population Health and Research.

Each hospital was supplied with appropriate materials to obtain specimens, and a standard protocol to follow. The pathologists were requested to provide colonic and ileal intestinal segments, as well as faecal and pernasal swabs. Faecal specimens were collected with the aid of a swab and placed in a container of Stuart's Transport Media (Media Makers) and Buffered Peptone Water (Oxoid CM509). 3 cm segments of both ileum and colon were excised and separately placed in sterile Universal containers containing Peptone Water (Oxoid, CM9). All specimens were used for virological and bacteriological studies using routine procedures as detailed below. Peptone Water was chosen for maintenance of the nasal swab and intestinal sections due to its ability to sustain a fairly broad range of bacteria, including the Enterobacteriaceae [21, 22].

Control infants/comparisons

Faecal specimens were obtained from 134 comparison infants randomly chosen from the Queen Alexandra Maternity Hospital in Hobart, between late 1989 and 1991. The specimens were obtained with parental consent for bacteriological and virological investigation. Stool specimens were collected 3 days after birth to avoid the meconium.

Upon leaving the hospital these children attended Child Health Clinics in the southern part of the state. As part of their surveillance service the Child Care nurses recommend clinic attendance for routine checks at 3 weeks, 3 months, and 6 months to advise mothers on feeding and management of their children. During these visits, parents and nurses were requested to collect a stool specimen from the child. They were provided with sterile containers and swabs for collection and specimens were kept at 4 °C until collected. Nurses provided information on any antibiotics used and, in addition, the feeding habits of the child during the periods of collection were noted. Infants were described as breast fed if they were breast fed without supplements at the age of 3–4 weeks. A small number of infants who died from accidental causes were also included as comparisons to the SIDS cases.

Isolation of bacteria

The contents of the small and large bowel segments from the SIDS cases and dead comparison infants, and faeces from the live infants were identically cultured within 1 h of collection. Each specimen was inoculated onto MacConkey Agar (Oxoid CM7), Xylose-Lysine-Desoxycholate Medium (XLD Medium; Oxoid CM469), Hektoen Enteric Agar (Oxoid CM419), Sorbitol MacConkey (Oxoid CM813), Sorbose MacConkey, Violet Red bile Agar with MUG (BBL) and Blood Agar (Tasmania Laboratory Services) for non-specific aerobic growth. Both intestinal and faecal samples were assessed for anaerobic growth on Blood Agar and incubated for 18 h at 37 °C. All swabs were also inoculated into enrichment media, including: Cooked Meat Medium (Oxoid CM81), Nutrient Broth (Oxoid CM3), Tryptone Soya Broth (TSB; Oxoid CM129), and Hajna media (Difco,

Detroit, USA) and incubated overnight at 37 °C. After incubation, a loopful from each of the broths was spread onto the described media and incubated overnight at 37 °C for further screening. All plates were examined for possible enteric pathogens, and for *E. coli*.

The isolates obtained from SIDS samples were examined in varying detail. Organisms considered normal commensals were identified by colonial characteristics and Gram morphology whilst any potential pathogens were thoroughly examined and identified biochemically using various diagnostic aids. These aids included the Roche Enterotube II (Roche Diagnostics, New South Wales), Microbact 24 E System (Disposable Products Pty Ltd) and Analytical Profile Index (API) 20 E test kits (bioMérieux s.a., France). All isolates identified as *E. coli* were stored in Protect vials at −20 and −80 °C.

E. coli *strains*

All *E. coli* isolates were confirmed by an assessment of their biochemical characteristics using commercial kits, then tested for the ability to produce the heat-labile enterotoxin (LT) by both the Phadebact ETEC-LT Test (Karo Bio diagnostics, AB, Sweden) and VET-RPLA (Oxoid TD920). These strains were also screened for both free faecal verocytotoxin (FVT) and the ability to produce verocytotoxin (VT).

The procedure for the detection of free faecal verocytotoxin (FVT) was adapted from the methodology described by Karmali [23]. A small portion of stool was emulsified in an equal volume of phosphate-buffered saline (PBS: pH 7·4, 0·01 M), and mixed thoroughly using a Vortex mixer. The emulsion was centrifuged at 10000 g for 10 min and the supernatant passed through a 0·22 μm (pore size) membrane filter (Dynagard, Microgon Inc). The samples were used in toxin assays performed using semiconfluent Vero cell monolayers (African Green Monkey Kidney; Commonwealth Serum Laboratories (CSL), Melbourne).

The procedure to detect verocytotoxin-producing strains was adapted from the methodology of Karmali [24]. The procedure for extraction of VT was initially performed in 20 ml of TSB at 37 °C. The cultures were later grown at various temperatures, 30, 35, 40 and 45 °C, and VT extractions performed. This procedure involves centrifuging the grown cultures at 10000 g for 10 min and resuspending the pellet, using a Vortex mixer, in 1 ml of polymixin B (0·1 mg/ml; Sigma Chem. Co.) and incubating for 30 min at 37 °C to enhance toxin detection. After further centrifugation, the supernatants were filtered using 0·2 mm (pore size) filters and toxin activity was assayed immediately using Vero cells, in 96-well microculture trays.

The Vero cell line was maintained as described by Bettelheim and colleagues [18]. Cytopathic effects were scored semiquantitatively on the basis of percent cell rounding; 50% or greater cell rounding was regarded as positive [18]. All FVT extracts were kept and stored at −80 °C.

Anaerobes

Separation of true anaerobes from facultative anaerobes was performed by incubating these organisms anaerobically on blood agar in 37 °C, in conjunction with metronidazole disks (5 mg) (Oxoid MTZ 5). The susceptible isolates were

further identified using the Microbact 24 AN System (Disposable Products Pty Ltd, Australia), Analytical Profile Index (API) 20 A and Rapid ID 32 A (bioMérieux s.a., France). Strains identified as *Clostridium perfringens* underwent additional testing using Nagler plates (Royal Hobart Hospital, Microbiology Department) and specific Antitoxin A (Wellcome).

Viruses

Faecal specimens from both the swab stored in Stuart's Transport Media and specimens taken with the ileal and colonic segments were used to detect rotaviral and adenoviral antigens with the use of Rotalex and Adenolex Test kits (Orion Diagnostica, Espoo, Finland).

RESULTS

SIDS

Thirty-three SIDS cases were examined between August 1989 and December 1991 and a further five cases were examined in 1992. A total of 134 comparison infants were examined during the period of 1989 and 1991.

Of the SIDS cases examined five (39%) were found to contain TEC strains. In contrast, only two (1·5%) of the comparison cases contained ETEC (Table 1). The null hypothesis that there is no significant difference between the incidence of toxigenic *E. coli* in SIDS and comparison cases from 1989–91 was tested using $c^2y(c^2y = 44{\cdot}41, \mathrm{df} = 1, P < 0{\cdot}001)$ and can be rejected at the 1% level. It can thus be concluded that a significantly higher proportion of SIDS infants harboured toxigenic *E. coli* compared with comparison infants.

Tests were conducted for a number of the toxins elaborated by *E. coli* and various proportions of toxins were found in SIDS cases (Table 2). Most of the toxigenic strains found elaborated either VT or LT. All strains that elaborated toxins produced low titres of the various toxins. The majority of the VT producers identified caused 50% cell necrosis, whereas a few induced 75% cell death.

There were two SIDS cases which possessed both ST- and VT-producing strains of *E. coli*, whilst several cases containing LT-producing *E. coli* gave positive (cytotoxic) FVT results on Vero cells. A few of the NLF strains isolated from SIDS cases were also found to be cytotoxic to Vero cells. As this is not a feature normally associated with these strains, they were designated as ?VT producers.

A number of non-lactose fermenting (NLF) *E. coli* which are normally classified as 'biochemically inactive' and ignored by routine laboratories were also isolated. Late lactose fermenting *E. coli* (some of which took about 60 h to ferment lactose), were isolated from a 1992 SIDS case with several unexpected organisms including *E. coli* Alkalescens-Dispar (A–D). NLF *E. coli* were found in 6 (16%) of the SIDS infants examined from 1989 to 1992 and in 5 of these cases *E. coli* (A–D) strains were also present (Table 3). NLF *E. coli*, including *E. coli* (A–D), were isolated from 6 (4·5%) of the 134 comparison cases examined from 1989 to 1991.

During the course of the investigation a diverse number of aerobic enteric flora were isolated from the majority of SIDS and comparison cases, these included: *Escherichia coli*, *Citrobacter* species, *Klebsiella* species, *Proteus* species, *Morganella* (*Proteus*) species, *Streptococcus* species, *Enterobacter* species and *Hafnia alvei*.

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Table 1. *The number of Tasmanian SIDS and comparison infants examined between 1989 and 1991, and SIDS cases studied in 1992, found to possess a toxigenic strain of* E. coli

Organism	SIDS cases Number (%) 1989–92	Comparison cases Number (%) 1989–91
Toxigenic *E. coli*	15 (39·0)	2 (1·5)
Total of cases	38	134

Table 2. *Various types of toxin-producing* E. coli *in Tasmanian SIDS and comparison cases, and evidence of the presence of toxigenic bacteria*

Toxin	SIDS cases Number (%) 1989–92	Comparison cases Number (%) 1989–91
Verotoxin	5 (13)	2 (1·5)
?Verotoxin*	4 (11)	0 (0·0)
Heat-stable toxin	2 (5)	0 (0·0)
Heat-labile toxin	8 (21·0)	0 (0·0)
Free faecal toxin	8 (18·0)	0 (0·0)

* Produced by NLF strains – requires confirmation.

Table 3. *The frequency of non-lactose fermenting* E. coli *and* E. coli *(A–D) in Tasmanian SIDS and comparison cases between 1989 and 1991, and SIDS cases during 1992*

Strain	SIDS cases Number (%) 1989–92	Comparison cases Number (%) 1989–91
NLF *E. coli*	6 (16·0)	6 (4·5)
E. coli (A–D)	5 (13·0)	6 (4·5)
Total of cases	38	134

Table 4. *The incidence of rotavirus, adenovirus, and overall infection in Tasmanian SIDS and comparison cases between 1989 and 1991, and in SIDS cases during 1992*

Pathogen	SIDS cases Number (%) 1989–92	Comparison cases Number (%) 1989–91
Rotavirus	6 (16·0)	16 (12·0)
Adenovirus	1 (3·0)	6 (4·5)
Rota- and adenovirus	4 (11·0)	5 (4·0)
Total infected	11 (30·0)	27 (20·0)

Staphylococcus species were also isolated from a number of pernasal swabs, which were taken from 1992 SIDS cases.

During the examination, both rotavirus and adenovirus were found in both comparison and SIDS infants between 1989 and 1991 (Table 4), with a trend towards a larger portion of SIDS infants being virally infected. These organisms were found in 30% of the SIDS cases examined, whilst 20% of the comparison infants were infected. However, a c^2y test indicated that there was no significant difference between the two populations.

Anaerobic organisms were also isolated from the intestinal and faecal flora of each SIDS and comparison case for completeness of the bacteriological examination for potential pathogens. A number of common commensal species were identified including, *Clostridium baratii*, *Cl. bifermentans*, *Cl. butyricum*, *Cl. fallax*, *Cl. perfringens*, *Cl. speticum*, and *Bacteroides* spp. A general trend towards greater diversity of anaerobic flora with increasing age was observed.

DISCUSSION

The main purpose of this study was to elucidate the role of TEC in SIDS infants compared with healthy infants living in Tasmania. It is well accepted that TEC are an important cause of morbidity and mortality among infants and children in developing countries [25], yet our findings revealed a surprisingly high isolate rate of TEC from SIDS cases in Tasmania.

Investigations by Bettelheim and colleagues [18, 19] and Luke and co-workers [20] indicated an alarmingly high prevalence of TEC from SIDS cases in South Australia and Victoria. Our survey of Tasmanian SIDS cases revealed TEC in 39% of SIDS cases and in 1·5% of comparison cases. The majority of these strains were observed to be either Verocytotoxin (VT) or Heat-labile toxin (LT) producers, similar to TEC isolated by Bettelheim and colleagues [18, 19]. The serotypes obtained by Bettelheim and colleagues [18, 19] were also not usually associated with toxigenicity in *E. coli*. Unfortunately, the resources required to serotype our TEC strains were not available in Tasmania.

The presence of free verocytotoxin (FVT) was noted in 18% of our SIDS cases, indicating that toxigenic strains, either ETEC or other toxigenic commensal, were present. In one of our SIDS cases, the FVT specimen induced cytotoxic changes in Vero cell, in conjunction with the isolation of LT-producing *E. coli*. These data may reflect an underestimation of the number of toxigenic strains in SIDS infants due to the logistic difficulties in isolating VTEC. In addition two distinct verocytotoxins have been isolated from human strains. These are antigenically distinct, bacteriophage-mediated toxins referred to as VT1 and VT2. The Vero cell assay is not considered ideal for identifying producers of VT-2, as their effect on these cells is considered 1000 times less than that of VT-1, yet they are currently believed to be the more significant pathogenically of the two [26].

Serotypes that are known to produce verocytotoxins have been clearly associated with haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). The important observation made is that verocytotoxin is very closely related to Shiga toxin from *Shigella dysenteriae*, while the terms now have become synonymous. It is also interesting to note that one difficulty in obtaining accurate epidemiologic data with VT producers is due to the fact that the organism or its toxin may have been lost by the time children present with haemolytic uraemic syndrome (HUS) [23, 27]. In instances where verocytotoxin-producers are not found, clinically finding FVT alone has been interpreted as an indication of recent infection by toxigenic strains [23].

A further interesting finding in the Tasmanian SIDS cases was the number of NLF isolates present in these infants, which included the *E. coli* (A–D) group. NLF *E. coli* strains in the past have caused difficulty in the diagnosis of enteric

infections, particularly by routine laboratories, often being mistaken for members of the genus *Shigella*. It is not proposed here that these organisms are directly involved in SIDS but approximately 11% of the NLF strains and *E. coli* (A–D) strains were found to produce toxins which were cytotoxic to Vero cells. A number of these strains were found to be capable of invading HEp-2 cells, an indication that they may be potential pathogens [22, 28]. Assays on these organisms have indicated the presence of P-fimbriae, known to be an important virulence factor in urinary trace infections (UTI) [29]. Since non-lactose fermenting *E. coli* strains were isolated at high frequency in the faeces of the SIDS children, further investigation is required to study a representative number of such isolates and screen for virulence factors.

An interesting number of other toxigenic species were isolated during the investigation. These included *Cl. perfringens* strains which were found to elaborate enterotoxin A on Nagler plates. Other potential toxigenic bacteria included klebsiella, staphylococci, streptococci, and proteus. It has been noted in English SIDS studies that significantly larger proportions of these strains possess toxin-producing capabilities as opposed to comparison infants [10, 11, 14]. The possible involvement of these organisms in Tasmanian SIDS cases has not been investigated.

There is a striking similarity between age distribution and seasonal incidence of severe cases of respiratory difficulties due to viral infection and SIDS [30]. It has been proposed that common toxins produced by bacteria growing in the respiratory tract following a viral infection in hypoimmune infants may be absorbed from the upper respiratory tract [10, 14]. An analysis of rotaviral and adenoviral antigen in conjunction with our bacterial survey indicated that 30% of the SIDS cases were positive, versus a 20% isolation in comparison cases. A viral infection of this nature has been suggested as being responsible for the unusual bacteriological flora observed in many SIDS cases [10], and studies on such viral infections have indicated their ability to disturb the commensal flora [31].

A major problem faced during the course of investigations of this sort was the incorporation of adequate controls. As Telford and colleagues [11] have commented: 'a problem with any investigation of SIDS is the lack of matched autopsy controls because there are few deaths in the first year of life in which one can be certain that infection has not played a part.' It is with similar reasoning to that of Telford and colleagues [11] that we have used healthy infants in the community and referred to them as 'comparison groups' rather than 'controls'. These individuals were used as an assessment of the intestinal flora from the time of birth. Another area of concern of many investigators is post-mortem colonization, as there is a variable interval between death and autopsy. To eliminate this concern, results have shown that there is a reduction in the isolation rate of enterobacteria with increasing delay in obtaining the sample [11].

In conclusion, the results obtained in the present study assess the occurrence of TEC organisms isolated from SIDS infants and lead to the suggestion that these strains should be monitored where possible in epidemiological studies on SIDS. The isolation of TEC in a substantial proportion of infants which were apparently healthy at the time of death indicates that the infants probably constitute a risk category for infection with these organisms, regardless of the country of origin.

Further investigations may be an attempt to identify the potential sources of infection and describe the modes of transmission of these enteric pathogens.

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