Intestinal Microbiota Composition in Sudden Infant Death Syndrome and Age-Matched Controls

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Objective To assess whether features of the infant intestinal microbiome, including the carriage of toxigenic bacteria, are associated with sudden infant death syndrome (SIDS).

Study design We undertook a case-controlled analysis of fecal microbiology in SIDS. Fecal material was obtained from 44 cases and 44 aged-matched controls. Microbiota composition was determined by 16S ribosomal RNA gene amplicon sequencing and comparisons between cases and controls made based on both bacterial alpha diversity measures and unconstrained ordination. Specific quantitative polymerase chain reaction assays were used to determine intestinal carriage of *Staphylococcus aureus*, toxigenic *Clostridium difficile*, and pathogenic and nonpathogenic *Escherichia coli*.

Results The microbial composition for the study population as a whole was consistent with previous studies of infants <12 months of age, with a correlation between alpha diversity and age ($r^2 = 0.08$; $P = .007$). However, no difference was observed in alpha diversity between SIDS cases and controls ($P > .4$). Nonmetric multidimensional scaling also revealed no evidence of differences in microbiota dispersal between SIDS cases and controls ($P = .4$, permutational multivariate ANOVA test; Pseudo-F = 0.9), nor was a difference observed in microbiota dispersion ($P = .19$, PERMDISP test; F = 1.9). There were no significant intergroup differences in the carriage of *S aureus*, toxigenic *C difficile*, total *E coli*, or pathogenic *E coli*.

Conclusions We found no evidence of an association between altered intestinal microbiology and SIDS, or to support the development of strategies to reduce the incidence of SIDS that target intestinal microbiology. *(J Pediatr 2017;191:63-8).*

A number of behavioral risk factors for sudden infant death syndrome (SIDS) have been identified, including prone and side infant sleeping positions, smoke exposure, overheating, and sleep surfaces,[1-4] whereas breastfeeding and room sharing without bed sharing seem to be protective.[5-7] Efforts to encourage safe sleeping practices, such as the release of the American Academy of Pediatrics safe sleep recommendations,[8] have been associated with a substantial decrease in the incidence of SIDS.[9] However, despite this progress, SIDS remains the leading cause of death of infants in the first year of life.[9]

Whether a common SIDS pathophysiological mechanism exists remains unclear. A number of models have been proposed that involve the interaction of multiple independent factors: an underlying vulnerability, a critical developmental period, and an exogenous stressor.[10-12] Given the influence of host–microbiome interactions on development and physiological regulation during this critical early life period,[13] it has been suggested that abnormal intestinal microbiology might represent a further contributor to SIDS risk.[14]

There are a number of pathways by which disruption of the infant intestinal microbiome could lead to an elevated SIDS risk. Neurochemical and developmental abnormalities of the brainstem have been suggested as an important underlying vulnerability.[15] Such abnormalities can lead to the loss of cardiorespiratory, arousal, and autonomic reflexes in the face of homeostatic stressors, such as hypoxia, increasing the risk of sleep-related death.[16] Although the precise manner in which neurophysiological abnormalities might contribute to SIDS remains unclear, the necessity of the intestinal microbiome for normal hippocampal development and regulation has been clearly demonstrated.[17,18] For example, analysis in germ-free mice has shown that the presence of the microbiome is required for the normal development of the hypothalamic–pituitary–adrenal axis, and its absence is associated with an elevated stress response.[18] Furthermore, the presence of enteropathogenic *Escherichia coli* enhances this increased stress response, whereas the presence of the commensal species *Bifidobacterium infantis* reverses it.[18]

PERMANOVA	Permutational multivariate ANOVA
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA
SIDS	Sudden infant death syndrome

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Reports of seemingly trivial infection around the time of death in approximately one-half of SIDS cases as well as mild tracheobronchial inflammation and altered serum immunoglobulin or cytokine levels and the presence of microbial isolates at autopsy[12,19] suggest infection might represent an important exogenous stressor. In vulnerable infants, such a mild infection could lead to death through the induction of a lethal cytokine cascade or toxic response.[12] The intestinal microbiome is an important regulator of systemic immunity, and its disruption of the intestinal microbiome in early life has been shown in animals to result in impairment of systemic innate and adaptive immunity.[20-25]

Other proposed microbial mechanisms that may contribute to SIDS include transient bacteremia[26] or the carriage of toxigenic bacteria,[27,28] which may lead to death through the induction of toxic/septic shock.[26] In humans, disruption of the intestinal microbiome through antibiotic exposure has also been linked to reduced resistance to colonization by pathogenic bacteria[29-31] and to susceptibility to sepsis.[32]

Given the potential for alteration of the commensal intestinal microbiome in early life to contribute to SIDS, we hypothesized the fecal microbial composition would differ between SIDS cases and age-matched controls. To test this hypothesis, we characterized composition of the microbiome in 44 SIDS cases that occurred between 1989 and 1994, and in 44 healthy control infants, through a combination of 16S ribosomal RNA (rRNA) gene amplicon sequencing and species-specific quantitative polymerase chain reaction (qPCR).

Methods

The diagnosis of SIDS was in accordance with National Institute of Child Health and Human Development criteria.[1] Fecal samples were collected from the colon post mortem in 44 SIDS cases in South Australia between 1989 and 1994 and stored at -80°C in 50% v/v glycerol before processing. A control group was assessed that consisted of anonymous fecal samples were collected prospectively from live infants visiting the Women's and Children's Hospital, North Adelaide, South Australia, in 2006. Healthy infants were negative for routine microbiological (bacterial and viral) pathogen testing. None had been treated with antibiotics before sample collection. Forty-four control infants were selected at random to provide a comparison group whose age profile was not significantly different to the SIDS group ($P > .05$, χ^2 test). Sample storage and processing was performed in the same way for sets of samples. The study was approved by the Adelaide Women's & Children's Hospital Human Research ethics Committee, approval number 1944/4/2019.

DNA Extraction and 16S rRNA Gene Amplicon Sequencing

DNA extraction was performed using MoBio Powerlyzer Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, California) as described previously.[33] Briefly, samples that were immersed in solution were centrifuged at 13 000×*g* for 5 minutes and the supernatant discarded. Samples were washed with 1 mL of cold 1× PBS (pH 7.2) (Life Technologies, Melbourne, Australia) and centrifuged at 13 000×*g* for 10 minutes. DNA extraction was performed according to the manufacturer's instructions with the following modifications. Samples were placed into bead tubes with solution C1 and heated at 65°C for 10 minutes, before 2 cycles of bead beating at 6.5 m/second for 1 minute using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, California). Total DNA was eluted in 100 μL of sterile water. DNA concentration was quantified fluorometrically with a Qubit dsDNA HS Assay kit (Life Technologies).

The V4 hypervariable region of the bacterial 16s rRNA gene was amplified from fecal DNA extracts using modified universal bacterial primer pairs 515F and 806R, with an Illumina (Illumina Inc, San Diego, California) adapter overhang sequences as indicated by underline.[33] Amplicons were generated, cleaned, indexed, and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol with certain modifications. Briefly, an initial PCR reaction contained at least 12.5 ng of DNA, 5 μL of forward primer (1 μmol/L), 5 μL of reverse primer (1 μmol/L) and 12.5 μL of 2× KAPA HiFi Hotstart Ready Mix (KAPA Biosystems, Wilmington, Massachusetts) in a total volume of 25 μL. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies) using the following program: 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina) according to the manufacturer's instructions. The final library was paired-end sequenced at 2 × 300 bp using a MiSeq Reagent Kit v3 on the Illumina MiSeq platform. Sequencing was performed at the David R. Gunn Genomics Facility, South Australian Health and Medical Research Institute.

Sequence Data Processing

The Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0, Boulder, CO)[34] software was used to analyze the 16S rRNA sequence generated from paired-end amplicon sequencing using previously described bioinformatics pipeline.[35] Spurious operational taxonomic units were removed systematically using previous reports of common laboratory sequencing contaminants.[36] Reads were subsampled to a depth of 3290 for all samples. These data have been submitted to the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA380020).

Faith phylogenetic diversity was calculated using QIIME (version 1.8.0) and taxa richness, Shannon-Weiner Diversity index, and Simpson's evenness index were calculated using PRIMER (version 6, PRIMER-E Ltd, Auckland, New Zealand). Beta diversity was determined by weighted UniFrac similarity using the beta_diversity.py QIIME script. Principal coordinate analysis was used to visualize clustering of samples based on their similarity matrices. Distance from centroid was calculated as previously described, using PRIMER.[37] Permutational multivariate ANOVA (PERMANOVA)[38] on the beta-diversity matrices was used to test the null hypothesis of no difference among a priori-defined groups using