An association between sudden infant death syndrome (SIDS) and Helicobacter pylori infection

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Abstract

Background—Helicobacter pylori has recently been detected in the stomach and trachea of cases of sudden infant death syndrome (SIDS) and proposed as a cause of SIDS.

Aims—To establish the incidence of H pylori in the stomach, trachea, and lung of cases of SIDS and controls.

Methods—Stomach, trachea, and lung tissues from 32 cases of SIDS and eight control cases were examined retrospectively. Diagnosis of SIDS was based on established criteria. Controls were defined by death within 1 year of age and an identifiable cause of death. Tissues were examined histologically for the presence of bacteria. Extracted DNA from these tissues was tested for H pylori ureC and cagA sequences by nested polymerase chain reaction and amplicons detected by enzyme linked immunosorbent assay (ELISA). The cut off for each ELISA for each of the tissue types was taken as the mean optical density plus two times the standard deviation of a range of negative controls.

Results—Ages of SIDS cases ranged from 2 to 28 weeks. Ages of controls ranged from 3 to 44 weeks. For the ureC gene, 25 SIDS cases were positive in one or more tissues compared with one of the controls. For the cagA gene, 25 SIDS cases were positive in one or more tissues compared with one of the controls.

Conclusions—There is a highly significant association between H pylori ureC and cagA genes in the stomach, trachea, and lung of cases of SIDS when compared with controls.

Keywords: Helicobacter pylori, sudden infant death syndrome

Sudden infant death syndrome (SIDS) is the major cause of postneonatal death in the developed world, however, the cause(s) of SIDS remains unknown despite extensive investigation. SIDS is defined as “the sudden death of an infant or young child which is unexpected by history and in which a thorough postmortem examination fails to show an adequate cause of death”.

The prone sleeping position has been shown to be the largest risk factor, however, it is not the only cause as infants have died in other sleeping positions.

There is a substantial body of evidence for the hypothesis that infection plays a pathogenic role in SIDS. Most SIDS cases occur at 2–4 months, the age at which infants are especially vulnerable to infection on the basis of immune system immaturity.

Maternal smoking is a risk factor for SIDS, and as prone sleeping is reduced, the relative importance of smoking is increased; children of smokers have more respiratory tract infections and may be at risk of acquiring organisms from the mother’s oropharynx. SIDS victims have higher than normal IgG and IgA concentrations, both in the maternal circulation and in the lung, suggesting greater exposure to infections. Many SIDS victims have hyperthermia, suggesting a pyrexia prior to death which is consistent with infection and/or overwrapping.

There is also evidence that these infants have systemic endotoxaemia.

Evidence supporting the role of a gastrointestinal infection in SIDS includes the reduction in incidence of SIDS by avoiding the once popular prone sleeping position, possibly by reducing inhalation of refluxed gastric contents which is more likely in the prone position. There is an increased incidence of SIDS with maternal smoking, associated with increased risk of acquiring organisms from the mother’s oropharynx. SIDS victims also have increased numbers of plasma cells in the tracheal and duodenal mucosa, suggesting cytokine involvement. Interleukin 1, which can cause fever, activation of the immune system, and increased deep sleep, has been proposed as a link between infection and prolonged sleep apnoea, leading to SIDS.

Helicobacter pylori has recently been proposed as a possible cause of SIDS, based on epidemiological evidence. Both H pylori and SIDS are more common in poor communities, in single parent families, in males, and in overcrowded living conditions. Growth retardation is common to both SIDS and H pylori victims. Both SIDS and H pylori infection show intrafamilial clustering.

H pylori infection most commonly occurs in childhood and children may already be infected with H pylori by the age of 3 months. H pylori has been found in dental plaque and saliva of infected persons, and therefore maternal smoking and salivary exposure during handling and on fomites (feeding bottle) are possible routes of transmission. It has been suggested that the natural route of transmission is by gastric juice as a consequence of epidemic childhood vomiting, which may assume additional significance in conditions of overcrowding. Human milk IgA against H pylori can protect infants from early acquisition of infection, and breast feeding may indirectly.
minimise possible exposure to *H pylori* from feeding bottles.

Several small studies subsequently showed an association between *H pylori* and SIDS. Six of seven SIDS cases were shown to be positive for *H pylori* antigen by immunocytochemistry in either the gastric antrum or trachea.\(^33\)

Twenty-five of 37 (68%) SIDS cases had histological findings suggestive of *H pylori* infection in the gastric antrum and stomach,\(^34\) a method which was shown to be highly predictive when compared with polymerase chain reaction (PCR).\(^35\)

In view of these findings, we undertook a retrospective study to examine the prevalence of *H pylori* in the stomach, trachea, and lung tissue of SIDS cases and controls. Using formalin fixed, paraffin embedded tissue sections, we used nested PCR followed by detection using an internal probe in an enzyme linked immunosorbent assay (ELISA) format to detect *H pylori* ureC and cagA sequences. The *ureC* region was utilised because of its conserved nature and specificity for *H pylori*.\(^36,37\) The *cagA* region was utilised because of its association with *H pylori* disease.\(^38\)

Culture was not performed as we did not have access to these cases at presentation. Antigen detection was not performed as it is known to be unreliable and antibody detection was not performed as we did not have matching serum samples.

**Materials and methods**

**PATIENTS**

We retrospectively examined 32 cases of SIDS and eight controls. The diagnosis of SIDS was based on an adequate negative postmortem examination to exclude evidence of other causes of death. Ages of SIDS cases ranged from 2 to 28 weeks. Controls were defined by cause of death: pneumonia, necrotising enterocolitis, prematurity (n = 2), *S pneumoniae* septicaemia, meningitis, ileal perforation, *Escherichia coli* septicaemia, pneumonia, necrotising enterocolitis, pancreatitis (n = 2), *S pneumoniae* septicaemia, and suffocation. Ages of controls ranged from 3 to 44 weeks.

**TISSUE PREPARATION AND HISTOLOGY**

Formalin fixed, paraffin embedded specimens of stomach, trachea, and lung were examined from SIDS cases and controls. Tissue sections were prepared aseptically; 5 µm tissue sections from SIDS cases and controls. Tissue sections of stomach, trachea, and lung were examined by formalin fixed, paraffin embedded specimens. Using the specimen of human ureter was also used as a negative control. DNA extracted from the specimen of human ureter was also used as a negative control. Samples were run in duplicate and amplifications were performed on a 9600 thermal cycler (Perkin Elmer). DNA extraction and amplification was performed in a final volume of 50 µl containing 0.4 mM of each primer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP in PCR reaction buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 10 mM Tris/ HCl pH 8.8, 2 mM MgSO₄, 0.1% vol/vol Triton X-100, 15 mM MgCl₂). Template DNA (10 µl) was added to the mixture and denatured at 94°C for 10 minutes, cooled on ice, and dUTP was added to the mixture and denatured at 94°C for one minute; 55°C for one minute; 72°C for one minute), a 10 minute final elongation step (72°C), and holding at 4°C. For the second round reaction in each case digoxigenin labelled dNTPs were used (Boehringer Mannheim). DNA extraction from *H pylori* NCTC 11637 (positive control), and sterile distilled water (negative control) were incorporated in each run. DNA extracted from a specimen of human ureter was also used as a negative control. Samples were run in duplicate and amplifications were performed on a 9600 thermal cycler (Perkin Elmer). Contamination was minimised by utilisation of separate laboratory areas and pipettes for pre-PCR, PCR, and post-PCR stages of the procedure, use of sterile bunged pipette tips, and inoculation of the positive control as a last step in the pre-PCR preparation. Tissues were processed in a randomised and blinded fashion.

**DETECTION BY ELISA**

Amplicons were then detected semiquantitatively on the solid phase by ELISA using probes specific for the *ureC* and *cagA* genes. For the *ureC* gene, the probe was 5'-AGA ATT GCA ATT GTC ATC AGT TGG GAG TGC TCT CTA AAC CAC TAA CGC-3' corresponding to positions 829–849 and 5'-CAA GGC ATC GGC GGT TTT AGC-3' corresponding to positions 1012–1032, generating a 252 bp fragment.\(^37\)

For the *cagA* gene, oligonucleotide primers were designed using the nucleotide sequence of *H pylori* 26695.\(^39\) First round primers were 5'-CAC CAA GGC CTC CAA GAG TCC TGA T-3' corresponding to positions 1539–1563 and 5'-TGT CGT CGT TTG GTC TGC TCC AAT TTT-3' corresponding to positions 1905–1930, generating a 391 bp fragment; second round primers were 5'-AAG AGT CCT GAT AAG GTG TAA GGC-3' corresponding to positions 1552–1575 and 5'-CCA CTT CTT TCT CTA AAT GCT CTC-3' corresponding to positions 1877–1900, generating a 348 bp fragment.

DNA EXTRACTION AND AMPLIFICATION

Paraffin was removed from the 10 µm sections using n-octane, and the DNA extracted in 2 ml of lysis buffer (20 mM Tris/HCl pH 8.3, 2 mM EDTA, 1% Triton-X, 0.5% sodium dodecyl sulphate, 0.5 mg/l proteinase K), and precipitated in ethanol and dried under vacuum. Amplifications were performed in a final volume of 50 µl containing 0.4 mM of each primer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP in PCR reaction buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris/HCl pH 8.8, 2 mM MgSO₄, 0.1% vol/vol Triton X-100, 15 mM MgCl₂). Template DNA (10 µl) was added to the mixture and denatured at 94°C for 10 minutes, cooled on ice, and dUTP was added to the mixture and denatured at 94°C for one minute; 55°C for one minute; 72°C for one minute), a 10 minute final elongation step (72°C), and holding at 4°C. For the second round reaction in each case digoxigenin labelled dNTPs were used (Boehringer Mannheim). DNA extracted from *H pylori* NCTC 11637 (positive control), and sterile distilled water (negative control) were incorporated in each run. DNA extracted from a specimen of human ureter was also used as a negative control. Samples were run in duplicate and amplifications were performed on a 9600 thermal cycler (Perkin Elmer). Contamination was minimised by utilisation of separate laboratory areas and pipettes for pre-PCR, PCR, and post-PCR stages of the procedure, use of sterile bunged pipette tips, and inoculation of the positive control as a last step in the pre-PCR preparation. Tissues were processed in a randomised and blinded fashion.

**SPECIFIC PRIMERS FOR *H pylori***

For the *ureC* region, first round primers were 5'-AAC AGT TTA GGG GTC TTA GGG GTT T-3' corresponding to positions 784–808 and 5'-AAG CTT TCT TTC TAA CAC CTC TAA CGC-3' corresponding to positions 1054–1085, generating a 301 bp fragment; second round primers were 5'-CTT TCT TCT CAA GCA ATT GTC-3' corresponding to positions 829–849 and 5'-CAA GGC ATC GGC GGT TTT AGC-3' corresponding to positions 1012–1032, generating a 252 bp fragment.\(^37\)

For the *cagA* gene, the probe was 5'-TAC ACA AAG GAA AAG CTA ATA AGC TTA TCA AAC ATT TTT TCA GCA GC-3' corresponding to positions 1722–1755.
The ELISA was performed by adding 35 µl of amplified PCR mixture to 40 µl of NaOH for 10 minutes at room temperature to denature the double stranded DNA. This was then mixed with 425 µl hybridisation solution containing the respective biotinylated probe at a concentration of 7.5 pmol/ml and the solution vortexed. This mixture was then transferred in duplicate to a streptavidin coated microtitre plate (200 µl/well) and incubated at 37°C with shaking for three hours. The wells were then washed five times, 200 µl of antidigoxigenin monoclonal antibody–horseradish peroxidase (HRP) solution added to each well, and the plate incubated at 37°C for 30 minutes. The wells were again washed, and 200 µl of a solution of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate–6] diammonium (ABTS) substrate solution added to each well, and the optical density (OD) read on a microplate reader (Titertek Multiskan Plus, Labsystems, Finland) at 405 nm. Positive results on PCR–ELISA were defined as those giving an optical density of greater than or equal to the mean OD plus two times the standard deviation of the negative controls (table 1).

DETERMINATION OF THE SENSITIVITY OF THE PCR–ELISAS

Sensitivity of the two PCR–ELISAs were evaluated using tenfold dilutions of an overnight broth culture of H pylori NCTC 11637. Viable counts were performed using the method of Miles and Misra.

Those specimens with a cut off value greater than or equal to the mean plus two times the standard deviation of the negative controls are marked with an asterisk.

### Table 1 | Optical densities of PCR products from amplification reactions for H pylori ureC and cagA genes using as template DNA extracted from stomach, trachea, and lung from cases of SIDS and controls

<table>
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<th>Case no.</th>
<th>Age at death (wk)</th>
<th>Cause of death</th>
<th>Pneumococcal septicaemia</th>
<th>Pneumonia</th>
<th>SIDS</th>
<th>Prematurity</th>
<th>NECROtising ENTERocolitis</th>
<th>E coli septicemia</th>
<th>SUFFocation</th>
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Mean (SD)

0.174 (0.065) 0.163 (0.063) 0.181 (0.060) 0.116 (0.071) 0.080 (0.080)
were incorporated in each PCR run. DNA extracted from a specimen of human ureter was also used as a negative control.

PCR FOR TWO REGIONS OF THE HUMAN β GLOBIN GENE

To determine the presence of inhibitory substances, all clinical specimens were also examined for the presence of human DNA by two separate PCRs directed against the β globin gene. Primers GH20 (5’-GAG CCA AGG ACA GGT AC-3’) and PC04 (5’-CAA CTG CAT CGA CGT TCA CC-3’) produce a product of 268 bp; and primers KM29 (5’-GGT TGG CCA ATC TAC TCC CAG G-3’) and RS42 (5’-GCT CAC TCA GTG TGG CAA AG-3’) produce a product of 536 bp. Each of these reactions was performed using 200 µM of each primer and 1 U of Taq polymerase in a mixture of 67 mM Tris/HCl, 16 mM ammonium sulphate, 2 mM MgCl2, 0.02% gelatin, pH 8.4 and 200 µM deoxynucleoside triphosphate; incubation at 95°C for seven minutes was followed by 40 one minute cycles at 92°C, 55°C, and 72°C. Product detection was by 1.5% agarose gel electrophoresis.

STATISTICAL ANALYSIS

Differences in incidence of H pylori DNA positivity in tissues of SIDS versus controls were analysed with Fisher’s exact probability test; p < 0.05 was considered significant.

Results

HISTOLOGY

The histological examination of each tissue section from SIDS cases and controls confirmed the typical histological appearance of each respective organ. The prime purpose of this examination was to determine the presence of visible bacteria. Bacteria were not observed in any section of stomach, trachea, or lung from the cases of SIDS and controls.

PCR FOR HUMAN DNA

All tissues tested positive for both regions of the human β globin gene.

PCR–ELISA FOR H PYLORI DNA

Table 1 shows optical densities of PCR products from amplification reactions for H pylori ureC and cagA genes using template DNA extracted from stomach, trachea, and lung sections from cases of SIDS and controls. Results were calculated using a cut off of the mean optical density plus two times the standard deviation of the negative controls. For both the ureC and cagA genes, 19 SIDS cases were positive in one or more tissues compared with none of the controls (Yates’s corrected $\chi^2 = 6.82; p = 0.009$).

SENSITIVITY OF THE H PYLORI PCR–ELISAS

For both ureC and cagA assays, after nested PCR, the sensitivity of detection was 150 pg H pylori DNA which corresponds to 40 genome equivalents. In each case, the sensitivity of detection of the PCR–ELISA was 1–5 pg H pylori DNA which corresponds to approximately four genome equivalents.

Discussion

This study was undertaken following publication of a hypothesis proposing a link between H pylori and SIDS.3,37 and supportive preliminary data that H pylori occurs in a higher than expected incidence in cases of SIDS.33–35 Using the cut off of the mean plus two times the standard deviation of the optical density of the negative controls, 28 SIDS cases were positive for one or both genes (ureC and cagA) in one or more tissues compared with one of the eight controls (Yates’s corrected $\chi^2 = 14.49; p = 0.0001$); a highly significant result. The prevalence of H pylori infection in infants varies, depending largely on socioeconomic factors; estimates in developed countries are generally less than 2%,44 while figures of 7.5%35 and 19%45 are more typical of developing countries. Therefore, regarding the present study, an 88% H pylori DNA positivity in a group of SIDS cases from a developed country is very high by comparison. Although 68 of 90 tissue samples (from 28 SIDS cases) were PCR positive for either gene, no bacteria were visualised in these or any tissue section, in contrast to other studies in which the presence of bacteria in the gastric antrum and trachea from SIDS cases showed a high correlation with both immunocytochemistry for H pylori antigen and PCR for H pylori DNA.33–35

The ureC and cagA PCR–ELISAs were shown to have equal sensitivity of detection of H pylori DNA, however, there were discrepancies in the correlation of ureC and cagA positivity in single sections (table 1). This may be because of the fact that these tissues were formalin fixed, which is known to shear DNA. However against this, the expected incidence in cases of SIDS.33–35 Using the cut off of the mean plus two times the standard deviation of the optical density of the negative controls, 28 SIDS cases were positive for one or both genes (ureC and cagA) in one or more tissues compared with one of the eight controls (Yates’s corrected $\chi^2 = 14.49; p = 0.0001$); a highly significant result. The prevalence of H pylori infection in infants varies, depending largely on socioeconomic factors; estimates in developed countries are generally less than 2%,44 while figures of 7.5%35 and 19%45 are more typical of developing countries. Therefore, regarding the present study, an 88% H pylori DNA positivity in a group of SIDS cases from a developed country is very high by comparison. Although 68 of 90 tissue samples (from 28 SIDS cases) were PCR positive for either gene, no bacteria were visualised in these or any tissue section, in contrast to other studies in which the presence of bacteria in the gastric antrum and trachea from SIDS cases showed a high correlation with both immunocytochemistry for H pylori antigen and PCR for H pylori DNA.33–35

The outcome of neonatal and perinatal H pylori infection may depend on factors including immune system maturity and the level of passively acquired anti-H pylori antibodies at the time of acquisition. Transplacentally transferred maternal anti-H pylori IgG is detectable up to the third month of life and disappears in nearly all infants by six months.45 Once infection with H pylori has occurred, this will probably be asymptomatic initially,46 but because of the almost universal gastrooesophageal reflux in infants, may lead to microaspiration of H pylori, accounting for subtle histological changes in the upper airway and stimulation of immunoglobulins in the
lung and gastrointestinal tract. A recent study found *H pylori* in 10% of tracheal aspirates from patients with aspiration pneumonia. The incubation period for *H pylori* from inoculation to symptoms is three to seven days, followed by 7–49 days of achlorhydria with a possible enhanced infection risk from other organisms. Therefore, delayed handling by siblings combined with this incubation period and waning maternal antibodies may account for the absence of SIDS in the first month of life. Unfortunately, serum from cases in the present study was not available for testing.

Many immunological mediators are produced during *H pylori* infection, which elicit activation of neutrophils and other inflammatory cells (interleukin 8) and modulate the immune or inflammatory response (IL-1, IL-3, IL-4, IL-6, IL-8, tumour necrosis factor α, and interferon γ). These may be elicited by components of *H pylori* such as porins. IL-1 is highly inflammatory, synthesised in vascular tissue, and may account for petechiae formation in SIDS. Cortisol is important in inflammation because of reduction of capillary permeability and antibody synthesis, and stabilisation of lysosomal membranes, and is known to be increased in SIDS. Cardiac electrical instability may predispose to *H pylori* induced SIDS, on the basis of an increased sensitivity to the sympathetic effect of IL-1.

The pathogenesis of *H pylori* in SIDS is extremely difficult to study given that by definition cases are dead at presentation, and that there are no known indicators of SIDS prior to death. However, it has been proposed that death may occur as a result of one or both of two events, both of which have been shown in a rat model. Firstly, *H pylori* produces large amounts of urease, which will be fully active in the neutral pH of the *H pylori* infected stomach. Therefore, aspiration of this gastric juice may lead to large amounts of urease in the alveolae in close proximity to plasma urea. In this setting, urea hydrolysis may lead to ammonia production and supply directly to the systemic circulation where it cannot be detoxified by the liver; unlike the case of ammonia production within the gastric mucosa. Intravenous administration of ammonia is known to be fatal. Therefore, infant death may occur in the absence of obvious histological findings, as the presence of ammonia in the blood of a recently deceased baby would not be unusual. The presence of urease in the lung may account for the known biochemical abnormalities of lung surfactant in SIDS. Secondly, interleukin 1 production in the gastric mucosa may lead to fever, immune activation, and increased deep sleep, which in combination with a supply of ammonia to the systemic circulation may be lethal. Against this background, minor infection, overwrapping, or prone sleeping position may then lead to terminal hypoxaemia.

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