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.**

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Keywords: *Helicobacter pylori*; sudden infant death syndrome

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Sudden infant death syndrome (SIDS) is the major cause of postneonatal death in the developed world,[1] 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".[2] 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.[3]

There is a substantial body of evidence for the hypothesis that infection plays a pathogenetic 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.[4] Maternal smoking is a risk factor for SIDS,[5] and as prone sleeping is reduced, the relative importance of smoking is increased[6]; children of smokers have more respiratory tract infections and may be at risk of acquiring organisms from the mother's oropharynx.[7] SIDS victims have higher than normal γ globulin concentrations, both in the circulation[8] and in the lung,[9] suggesting greater exposure to infections. Many SIDS victims have hyperthermia, suggesting a pyrexia prior to death which is consistent with infection and/or overwrapping.[10 11] There is also evidence that these infants have systemic endotoxaemia.[12]

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,[13] 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.[7] SIDS victims also have increased numbers of plasma cells in the tracheal and duodenal mucosa, suggesting cytokine involvement.[14 15] 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.[16]

Helicobacter pylori has recently been proposed as a possible cause of SIDS, based on epidemiological evidence.[17] Both *H pylori* and SIDS are more common in poor communities, in single parent families, in males, and in overcrowded living conditions.[18–23] Growth retardation is common to both SIDS[24] and *H pylori* victims.[22 25] Both SIDS[26] and *H pylori* infection[27] 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.[19 28] *H pylori* has been found in dental plaque[29] and saliva[30] 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,[31] which may assume additional significance in conditions of overcrowding. Human milk IgA against *H pylori* can protect infants from early acquisition of infection,[32] 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 death within 1 year of age, and with a known cause of death: *Streptococcus pyogenes* meningitis, ileal perforation, *Escherichia coli* septicaemia, pneumonia, necrotising enterocolitis, prematurity (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 were prepared on coated slides for haematoxylin and eosin (H&E) staining, and 10 μm sections were prepared in sterile Eppendorf tubes for subsequent PCR. H&E sections were examined for the presence of bacteria using both low (×40) and high (×100) power light microscopy.

SPECIFIC PRIMERS FOR *H PYLORI*

For the *ureC* region, first round primers were 5'-AAG CTT TTA GGG GTG TTA GGG GTT T-3' corresponding to positions 784–808 and 5'-AAG CTT ACT TTC TAA CAC 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 GCC ATC GCC 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 CGC CTC CAA GAG TCC TGA T-3' corresponding to positions 1539–1563 and 5'-TGT TGC CGT TTG GTC 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 GTA 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 precipated 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 $(NH_4)_2SO_4$, 20 mM Tris/HCl pH 8.8, 2 mM $MgSO_4$, 0.1% vol/volTriton X-100, 15 mM $MgCl_2$). Template DNA (10 μl) was added to the mixture and denatured at 94°C for 10 minutes, cooled on ice, and 2.5 U taq DNA polymerase (Perkin Elmer) added prior to 40 amplification cycles (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.

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 GAA GCA TTG CGC GAT TGG GGA TAA GTT TGT GAG CGA AT-3' corresponding to positions 904–946.[37] For the *cagA* gene, the probe was 5'-ACA AGA AAG CTA ATA AGC TTA TCA AAG 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 a range of 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.[40] A 100 µl aliquot of each dilution was plated onto Columbia agar with 5% (vol/vol) horse blood (Oxoid, Basingstoke, Hants, UK) and incubated at 37°C in a microaerophilic atmosphere. The DNA was then extracted from each dilution using guanidium thiocyanate, using the method of Pitcher *et al*.[41] Bacterial strains were suspended in 10 ml sterile water and the cells lysed by the addition of 0.5 ml of GES reagent (5 M guanidium thiocyanate (Sigma), 100 mM EDTA, and 0.5% vol/vol sarkosyl). Then 25 ml 7.5 M ammonium acetate was added with mixing, the mixture held on ice for 10 minutes, and 50 ml chloroform/2-pentanol (24/1) was added. The mixture was centrifuged for 10 minutes, the supernatant removed, and 50 ml 2-propanol added. The tube was centrifuged for 20 seconds to pellet the DNA, which was then washed twice with 70% ethanol. DNAs were redissolved overnight at 4°C in sterile water and used as template in the above PCR–ELISA tests for *H pylori ureC* and *cagA* genes. The highest dilution giving a positive PCR–ELISA test was used to calculate the sensitivity of the PCR–ELISA reactions. Positive (DNA extract from a plate culture of *H pylori* NCTC 11637) and negative (sterile water) controls

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

Case no.	Age at death (wk)	Cause of death	*H pylori ureC gene*			*H pylori cagA gene*		
			Stomach	*Trachea*	*Lung*	*Stomach*	*Trachea*	*Lung*
T1	2	SIDS	0.265	0.310*	NT	0.444*	0.363*	NT
T2	3	SIDS	0.474*	0.340*	0.163	0.099	0.107	0.107
T3	4	SIDS	0.297	NT	0.575*	0.404	NT	0.434*
T4	4	SIDS	NT	0.302*	0.278	NT	0.222	0.239
T5	5	SIDS	0.355*	0.278	0.321*	0.537*	0.427*	0.477*
T6	6	SIDS	0.268	0.125	0.213	0.368	0.109	0.429*
T7	7	SIDS	0.132	0.374*	0.163	0.410	0.247	0.369*
T8	7	SIDS	0.124	0.112	0.113	0.088	0.099	0.120
T9	8	SIDS	0.236	0.240	NT	0.583*	0.294*	NT
T10	8	SIDS	1.020*	0.483*	1.040*	0.497*	0.417*	0.496*
T11	8	SIDS	0.270	0.336*	0.406*	0.365	0.369*	0.280
T12	8	SIDS	0.406*	0.270	0.310*	0.570*	0.576*	0.375*
T13	8	SIDS	2.231*	2.918*	0.249	0.117	0.360*	0.102
T14	9	SIDS	0.470*	0.417*	0.500*	0.541*	0.431*	0.525*
T15	10	SIDS	0.282	NT	0.137	0.104	NT	0.096
T16	10	SIDS	0.319*	0.243	0.260	0.181	0.260	0.255
T17	11	SIDS	1.040*	0.500*	0.464*	0.496*	0.496*	0.488*
T18	12	SIDS	0.317*	0.299*	0.220	0.506*	0.378*	0.364*
T19	12	SIDS	0.179	0.147	0.130	0.106	0.102	0.102
T20	13	SIDS	0.286	2.570*	2.827*	0.709*	1.922*	0.389*
T21	14	SIDS	0.127	0.155	0.190	0.105	0.113	0.097
T22	15	SIDS	0.336*	0.248	0.220	0.333	0.442*	0.352*
T23	16	SIDS	NT	0.240	0.373*	NT	0.364*	0.348*
T24	16	SIDS	0.358*	0.295*	0.437*	0.306	0.304*	0.852*
T25	16	SIDS	0.490*	0.516*	0.485*	0.484*	0.286	0.441*
T26	19	SIDS	0.285	0.209	0.225	0.426*	0.472*	0.386*
T27	19	SIDS	0.267	0.302*	0.390*	0.287	0.253	0.379*
T28	21	SIDS	0.375*	0.310*	0.331*	0.401	0.431*	0.436*
T29	24	SIDS	0.375*	0.331*	0.248	0.332	0.433*	0.338*
T30	24	SIDS	0.233	0.319*	0.406*	0.435*	0.518*	0.652*
T31	28	SIDS	0.292	0.293*	0.327*	0.420*	0.504*	0.432*
T32	28	SIDS	0.304*	0.307*	0.321*	0.333	0.331*	0.263
C1	3	Prematurity	0.100	0.150	0.180	0.120	0.130	0.090
C2	4	Prematurity	NT	0.200	0.090	NT	0.120	0.100
C3	7	Ileal perforation	0.265	0.298*	0.283	0.414*	0.303*	0.317
C4	7	Necrotising enterocolitis	0.200	0.150	0.180	0.120	0.200	0.150
C5	20	*E coli* septicaemia	0.170	0.160	0.177	0.150	0.120	0.160
C6	24	Suffocation	0.210	0.080	NT	0.150	0.090	NT
C7	32	Pneumonia	NT	0.130	0.180	NT	0.150	0.180
C8	44	Pneumococcal septicaemia	0.100	0.140	NT	0.120	0.090	NT
Mean (SD)			0.174 (0.065)	0.163 (0.063)	0.181 (0.060)	0.179 (0.116)	0.150 (0.071)	0.166 (0.080)

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. SIDS, sudden infant death syndrome; T1, test case number 1; C1, control case number 1; NT, not tested.

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'-GAA GAG CCA AGG ACA GGT AC-3') and PC04 (5'-CAA CTT CAT CCA 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 $MgCl_2$, 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 the *ureC* region, 25 SIDS cases were positive in one or more tissues compared with one of the controls (Yates's corrected $\chi^2 = 9.40$; $p = 0.0022$). For the *cagA* gene, 25 SIDS cases were positive in one or more tissues compared with one of the controls (Yates's corrected $\chi^2 = 9.40$; $p = 0.0022$). Considering both gene sequences together, 28 SIDS cases were positive in one or more tissues compared with one of the controls (Yates's corrected $\chi^2 = 14.49$; $p = 0.0001$). Results were also calculated using a cut off of the mean optical density plus three 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,[17] 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%,[42] while figures of 7.5%[43] and 19%[44] 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, all tissues (both from cases of SIDS and controls), tested positive for human DNA in both human β globin gene PCRs.

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.[43] Once infection with *H pylori* has occurred, this will probably be asymptomatic initially,[45] but because of the almost universal gastro-oesophageal 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.[46] The incubation period for *H pylori* from inoculation to symptoms is three to seven days,[47 48] followed by 7–49 days of achlorhydria[49] 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.[50–52] IL-1 is highly inflammatory, synthesised in vascular tissue,[53] 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.[54] Cardiac electrical instability may predispose to *H pylori* induced SIDS, on the basis of an increased sensitivity to the sympathetic effect of IL-1.[55]

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.[56 57] Firstly, *H pylori* produces large amounts of urease, which will be fully active in the neutral pH of the *H pylori* infected stomach.[58] 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[56]; unlike the case of ammonia production within the gastric mucosa. Intravenous administration of ammonia is known to be fatal.[59] 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.[60] 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.[57] Against this background, minor infection, overwrapping, or prone sleeping position may then lead to terminal hypoxaemia.

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