

# Immune responses to *Helicobacter pylori* in children with recurrent abdominal pain

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## Abstract

The systemic immune response to *Helicobacter pylori* was examined in 69 children with recurrent abdominal pain and upper gastrointestinal symptoms. Twenty one (30%) children were histologically positive for *H pylori*. Eighteen of the 21 positive subjects and two *H pylori* negative subjects (one with normal mucosa, one with lymphocytic gastritis) were positive for *H pylori* IgG antibodies by enzyme linked immunosorbent assay (ELISA) (86% sensitivity, 98% specificity). In children with *H pylori* associated gastritis, there was a significant positive correlation ( $p < 0.05$ ) between IgG antibody titres and patient age. Intra-assay comparison of sera from histologically negative adults with those of histologically negative children showed that the cut off for positivity in the ELISA for adults was greater than that for children. Immunoblotting showed IgG positivity in 20 of the 21 patients with *H pylori* infection (95% sensitivity). Both ELISA and immunoblotting for IgA and IgM *H pylori* antibodies had poor discriminatory value for determining infection.

Serological detection of *H pylori* IgG antibodies seems to be valuable in the assessment of children presenting with recurrent abdominal pain and other gastrointestinal symptoms, but assays must first be validated in paediatric populations.

The strong association between gastric *Helicobacter pylori* colonisation, gastritis, and peptic ulcer disease in adults is now firmly established.<sup>1,2</sup> *H pylori* colonisation results in a systemic IgG response,<sup>3-8</sup> and serology has been strongly advocated as a suitable means of screening adult dyspeptic patients.<sup>9,10</sup> Epidemiological studies have shown that childhood infection with *H pylori* in the West is generally low (less than 5%).<sup>11</sup> In underdeveloped countries, however, infection with this organism is more common in children and is associated with a higher overall incidence of seropositivity in adults.<sup>11</sup>

In children the association between gastric colonisation with *H pylori* and antral gastritis has also recently been established.<sup>12-16</sup> The association of *H pylori* with recurrent abdominal pain in children<sup>17</sup> introduces a further identifiable organic cause of this condition. Consequently, non-invasive serological detection of *H pylori* infection may be impor-

tant in the differential diagnosis of such children. Recently systemic immune responses to *H pylori* in children undergoing endoscopy were examined by enzyme linked immunosorbent assay (ELISA).<sup>18-21</sup> Some studies suggest a possible diagnostic role for *H pylori* serology in children with chronic abdominal pain. These have, however, been based on only a very limited number of positive children<sup>21</sup> in largely adolescent populations.<sup>20,21</sup> The development of a full systemic immune response to *H pylori* can take several months following initial infection.<sup>22</sup> As *H pylori* infection in children is likely to be relatively recently acquired, the systemic antibody response to this bacterium may not be fully established, particularly in younger subjects. For accurate diagnosis in children seropositivity should be determined using sera from local children of known *H pylori* response rather than asymptomatic control sera.

## Methods

Sixty nine children (mean age 10.8 (SEM 2.9), range 1 to 16 years) undergoing upper gastrointestinal endoscopy were investigated. The project was approved by the local research committee (ethics) of Leeds Eastern Health Authority. Sera obtained at the time of endoscopy were stored at  $-20^{\circ}\text{C}$  until assayed. Endoscopy was performed using paediatric endoscopes (Olympus GIF P3, Fujinon UGI PE) under intravenous sedation with Diazemuls (0.5-1.0 mg/kg, maximum 20 mg). In all patients a minimum of two biopsy specimens were taken from the gastric antrum for histological examination. Sections of antral biopsy specimens were stained with haematoxylin and eosin to determine the presence of gastritis using Whitehead's criteria.<sup>23</sup> *H pylori* were detected histologically using a modified Giemsa stain.

## ELISA

Sera were assayed for *H pylori* IgG, IgA, and IgM antibodies by an ELISA. Flat bottomed microtitre plates (Falcon 3912, Becton Dickinson, Oxford) were coated with 100  $\mu\text{l}$  ultracentrifuged (100 000  $\times g$  for one hour) sonicated antigen preparation (3.5  $\mu\text{g/ml}$ ) from one strain of *H pylori* diluted in coupling buffer (0.1 M bicarbonate buffer, pH 9.6) for 24 hours at  $4^{\circ}\text{C}$ . After washing (phosphate buffered saline (PBS) containing 0.1% Tween-20) and blocking with 0.5% bovine serum albumin (BSA), sera diluted in PBS-Tween containing 1% BSA were assayed in triplicate. Serum dilutions were 1 in 200 for IgG and IgM, and 1 in 50 for IgA. After incubation

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with goat-antihuman IgG, IgM, and IgA alkaline phosphatase conjugates bound antibodies were detected with p-nitrophenyl phosphate substrate (Sigma) solution at 1 mg/ml in diethanolamine-MgCl<sub>2</sub> buffer. The reaction was stopped with 3M NaOH and the absorbance read at 405 nm in a Bio-Rad 2550 EIA ELISA reader. A standard curve of positive control sera and negative controls were included on each plate. Sera were assayed without knowledge of the endoscopic and histological diagnoses.

To determine the sensitivity and specificity of the *H pylori* IgG ELISA, a cut-off point of 2 standard deviations above the mean value of *H pylori* negative children was used. To compare cut-off points for determining seropositivity in the *H pylori* IgG ELISA an intra-assay comparison of paediatric and adult sera was undertaken using sera of 40 endoscoped adults (mean age 45.3 (18.4)) with normal antral histology and no evidence of *H pylori* colonisation.

#### SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND IMMUNOBLOTTING

*H pylori* (NCTC 11637 strain) grown microaerobically for 72 hours on Columbia agar (Oxoid, Basingstoke) with 7% defibrinated horse blood was harvested into 10 mM TRIS buffered saline (TBS) (pH 7.4). Cells were washed twice in TBS and whole cell sonicates were prepared in sample buffer (80 mM TRIS hydrochloride, pH 6.8, containing 5% 2-mercaptoethanol, 2.5% SDS, 10% (vol/vol) glycerol and 2 mM phenylmethylsulphonyl fluoride). Samples containing 300 µg of protein were separated by SDS-PAGE using a 12.5% separating gel and 5% stacking gel and transferred to nitrocellulose paper by semi-dry blotting<sup>24</sup> using an LKB Nova-Blotter. Molecular weight markers (Sigma) were run on each gel. After blocking in TBS containing 0.25% Tween-20 the nitrocellulose was incubated for two hours in sera diluted in TBS-Tween containing 20% fetal calf serum in a miniblottor apparatus (Biometra, Manchester). Serum dilutions of 1 in 50 (IgG), 1 in 25 (IgM, IgA), and 1 in 10 (IgA, IgG) were used. After washing in TBS-Tween bound antibodies were detected by sequential incubation with alkaline phosphatase conjugated goat antihuman IgG, IgA, or IgM (1 in 500 dilution in TBS-Tween containing 20% fetal calf serum) and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, as described previously.<sup>25</sup>

Data are expressed as means (SD). Statistical comparisons were carried out using the Mann-Whitney U test for non-parametric data.

Table 1 Antral histology and *H pylori* positivity

Antral histology	N=	Age	Sex (F/M)	<i>H pylori</i> positive by histology
Normal	47	10.7 (2.9)	26/21	0
Gastritis	22	11.1 (3.6)	14/ 8	21

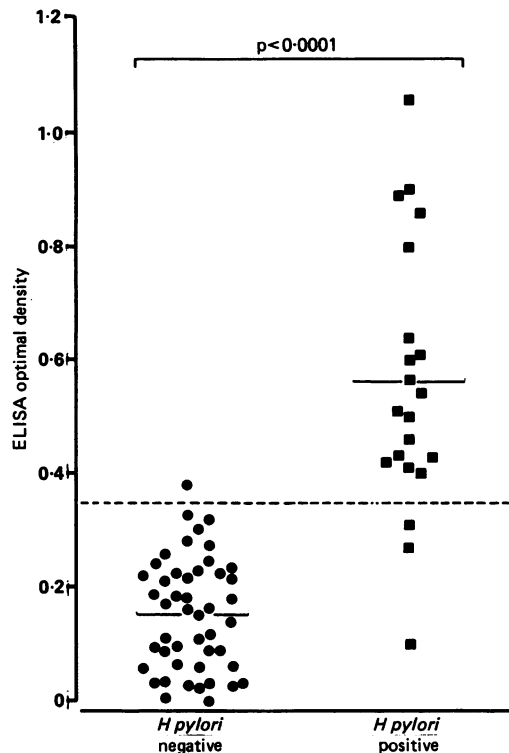


Figure 1 Serum *H pylori* IgG antibody titres in *H pylori* negative children with normal antral histology and children with *H pylori* associated gastritis.

## Results

Twenty two of the children had histological gastritis and 47 had normal antral mucosa (table 1). *H pylori* was identified histologically in 21 of the patients with gastritis. The gastritis in seven *H pylori* positive patients (33%) was inactive. One patient with lymphocytic gastritis<sup>26</sup> was histologically negative for *H pylori*. None of the children with normal antral mucosa were *H pylori* positive on histological examination.

## ELISA

Children with *H pylori* associated gastritis had significantly higher titres ( $p < 0.001$ ) of *H pylori* IgG antibodies by ELISA, mean (SD) optical density 0.56 (0.23), than those with normal gastric histology and no evidence of *H*

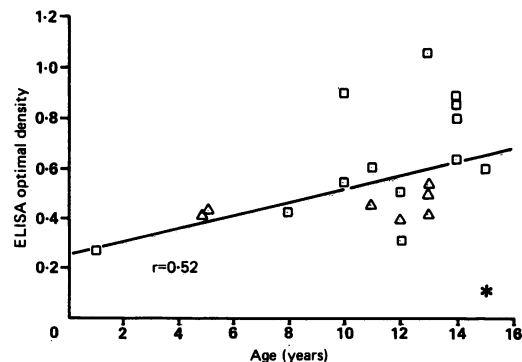


Figure 2 Relation between age and optical density in *H pylori* IgG ELISA in *H pylori* positive children:  $p < 0.05$ ,  $r = 0.52$ , excluding \* subject who was negative for *H pylori* IgG antibodies by immunoblotting.  $\Delta$  children with inactive gastritis.

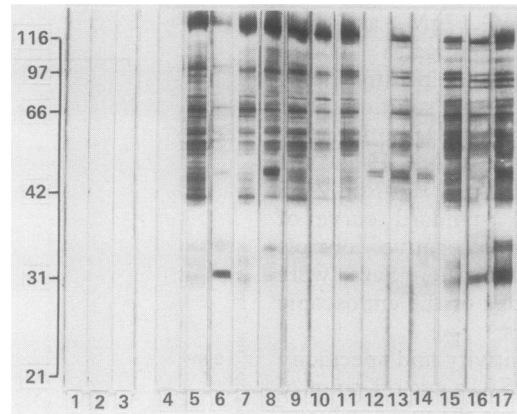


Figure 3 Western blot of IgG antibodies to whole cell proteins of *H. pylori*. Tracks 1-3 sera of *H. pylori* negative children with normal antral histology, tracks 4-17 sera of *H. pylori* positive children. Patient in track 4 showed some positivity in IgA and IgM immunoblots but was seronegative in the IgG ELISA. Left hand figures refer to molecular weight standards in kilodaltons.

*pylori* colonisation (0.15 (0.098)) (fig 1). The one histologically negative *H. pylori* subject with lymphocytic gastritis was strongly *H. pylori* seropositive by ELISA. The relation between age and optical density in the IgG ELISA for subjects with *H. pylori* associated gastritis is shown in fig 2. Increased optical densities were evident in older children with *H. pylori* infection. Exclusion of one seronegative subject (the only child with *H. pylori* associated gastritis negative for IgG *H. pylori* antibodies by immunoblotting) from analysis showed a significant positive correlation ( $p < 0.05$ ) between *H. pylori* IgG antibody titres and patient age ( $r = 0.52$ ,  $n = 20$ ). Subjects with inactive gastritis generally had lower *H. pylori* IgG antibody titres than those with active gastritis (fig 2). No significant increase in *H. pylori* specific IgM or IgA antibodies was evident in children with *H. pylori* associated gastritis.

Based on a cut-off of 2 standard deviations above the mean of *H. pylori* negative children with normal histology, three of the histologically positive subjects were seronegative and 18 were positive. Conversely, one histologically negative patient with normal gastric mucosa was seropositive. The sensitivity and specificity of the IgG ELISA were therefore 86% and 98%, respectively. Intra-assay comparison of adult *H. pylori* negative sera with negative paediatric sera showed an increased adult mean optical density (0.187 (0.17)) in the IgG ELISA relative to paediatric sera. The cut-off for positivity for adult sera was thus greater than that for paediatric sera. Use of the latter criteria for positivity would have resulted in only 10 of the 21 children with *H. pylori* gastritis being considered seropositive in the IgG ELISA.

Table 2 Western blotting analysis of serum *H. pylori* antibodies in children with *H. pylori* associated gastritis

	IgG	IgM	IgA
Positive	20	4	6
Negative	1	17	15

#### IMMUNOBLOTTING

Twenty of the 21 children with *H. pylori* associated gastritis and the subject with lymphocytic gastritis were positive for *H. pylori* IgG antibodies by immunoblotting (95% sensitivity). No positivity was observed in *H. pylori* negative subjects with normal antral mucosa (fig 3). Two of the three ELISA seronegative children with *H. pylori* were positive in IgG immunoblots, including the youngest patient aged 13 months. The third subject, although negative for IgG antibodies by immunoblotting, showed a positive reaction with 31 and 61 kilodalton proteins in IgM and IgA immunoblots, respectively. Major *H. pylori* antigens recognised by the IgG response included 120, 90, 61, 53, 45 and 31 kilodalton proteins (fig 3). Two positive patients showed no IgG recognition of the 120 kilodalton protein (fig 3). Limited immunoreactivity in IgM immunoblots was evident in only four of the 21 subjects with *H. pylori* (table 2). At a serum dilution of 1 in 10 IgA antibodies were observed in six of the 21 children with *H. pylori* (table 2), but in four subjects reactivity was limited to only one antigenic band (61 kilodaltons).

#### Discussion

The identification of *H. pylori* infection in children and its association with gastritis<sup>12-16</sup> and peptic ulceration<sup>27</sup> makes non-invasive serological diagnosis of infection important in the management of children with recurrent abdominal pain. In this study we characterised the systemic immune response to *H. pylori* using ELISA and immunoblotting techniques. IgG immunoblotting, with its increased sensitivity, proved a useful adjunct for determining seropositivity of patients whose ELISA values were borderline.

The IgG ELISA sensitivity and specificity in this study are similar to those previously reported.<sup>18-20, 21</sup> Both the age of presentation and length of duration of symptoms are likely to influence the sensitivity of *H. pylori* ELISAs. A significant increase in *H. pylori* IgG antibodies, determined by ELISA, occurred with increasing age in positive children. One of the two ELISA negative subjects who was positive by immunoblotting was only 13 months of age and presumably infection had been recently acquired. Maximal concentrations of IgG are not evident until the ages of 7 to 10<sup>28</sup>; this is an important consideration when determining seropositivity in younger children.

The sensitivity and specificity of the ELISA will be influenced by the chosen cut-off point.<sup>3</sup> Our study clearly shows the importance of using age matched negative controls to achieve optimal sensitivities. With commercial *H. pylori* ELISAs now available,<sup>29</sup> it is important that they should be verified with control sera of children before being used in paediatric practice.

Although *H. pylori* specific IgA antibodies have been reported<sup>4, 18</sup> and a diagnostic role for IgA proposed, no specific *H. pylori* IgA antibodies were detected by ELISA in this

study, although limited IgA positivity was determined by immunoblotting in six subjects. Other studies have similarly not detected a strong systemic IgA response to *H pylori*.<sup>3</sup> IgM immunoblotting of adult sera has, as in this study, shown little evidence of a specific IgM response.<sup>30</sup> Interestingly, the only *H pylori* positive subject who had no detectable IgG response by immunoblotting, showed some positivity in IgM immunoblots that was suggestive of an early infection. The development of the systemic immune response to *H pylori* following infection has not been examined in detail. Our recent observations in a case of acute gastritis have shown that despite a rapid mucosal IgA and IgM response to the bacterium, systemic IgG responses take some time to develop fully and there was little evidence of a systemic IgM response.<sup>22</sup>

Western blot analysis of adult serum IgG responses to *H pylori* has shown the antigenicity of 110–120, 89, 61, 54 and 31 kilodalton proteins, although considerable variability in recognition in individual patients has been described.<sup>30–31</sup> Czinn *et al* observed an IgG response in children to 61, 54, and 32 kilodalton outer membrane proteins but no recognition of a 120 kilodalton protein as observed in this study.<sup>19</sup> This latter protein is not expressed in all strains of *H pylori*,<sup>32</sup> and in this study two positive children clearly showed no recognition of the 120 kilodalton antigen. The inclusion of the 120 kilodalton protein in ELISA antigen preparations has been shown to increase the sensitivity of the assay.<sup>33</sup>

In conclusion, *H pylori* ELISA and immunoblotting techniques are sensitive and specific tests for determining gastric infection with *H pylori*. Serological testing of children with recurrent abdominal pain for *H pylori* antibodies will identify those subjects who warrant further investigation and avoid unnecessary invasive investigations in seronegative children.

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