Helicobacter pylori serology in two MOH areas of the Western Province of Sri Lanka

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The Ceylon Journal of Medical Science 2003; 46: 35-43

Abstract

Thus far five studies on H. pylori show a prevalence range from 3 – 70% in Sri Lanka. However there are no published studies based on serology and virulence factors.Cytotoxin associated protein A(CagA), urease and vacuolating cytotoxin A (VacA) have been described as virulence factors to H. pylori. This study was done to determine the sero-prevalence of IgG to Helicobacter and CagA in a representative population attributable to age groups and to determine the host response to virulent antigens such as CagA, urease, VacA and other major antigen found on H pylori.

Three hundred and fifty nine healthy volunteers between the ages of 1 to 94 years, with equal distribution of males to females participated in this study. The sero prevalence of IgG to H. pylori and CagA was determined using enzyme linked immunosorbent assay. The presence of IgG antibodies to several major antigens of H. pylori were determined in 48 samples (positive by IgG to H. pylori and/or positive by IgG to CagA) using an in-house western blot assay. Data was analysed by a chi square test.

The study consisted of 359 serum samples from 180 males and 179 females. Only 37 (10.3%) of the 359 serum samples were positive for IgG to H. pylori. Anti CagA was detected in 29/359 (8.1%). In the 48 samples studied by immunoblotting for major antigens (CagA-120Kda, VacA-89Kda, Urease-66Kda, 35Kda, 26 Kda, 19 Kda) the commonest western blot band was VacA(89Kda).

In conclusion, the prevalence of 10.3% seems to be low, but the presence of CagA antibody in H. pylori negative sera, indicates that responses in the host may not be always detected by routine assays. This may be due to a different strain used in assay, genetic differences in the host not enabling the host to mount a response, or to cross reactivity.

Key Words: Helicobacter pylori, serology, Sri Lanka

Introduction

The prevalence of H. pylori differs according to geographical location, with a higher prevalence rate in developing countries and a lower rate in developed countries.

Thus far, five studies have reported on the prevalence of H. pylori in Sri Lanka. All were from the Western province: in one study using the biopsy urease test a prevalence rate of 46% was recorded in an adult population (1) whilst more recently, in a population aged 18-80 years, using culture and the biopsy urease test, only 12% were recorded as colonized (2). The third study which also used the biopsy urease test, showed only a 3% prevalence in a dyspeptic group of patients (3).

In more recent studies using stool antigen detection and salivary antibody, only 12/184 (6.5%) children had detectable H. pylori antigen in their stools and were considered infected with H. pylori, while 51/184 (27.7%) had H. pylori IgG in saliva(4). Fernando et al detected a prevalence of 70.1% with 47.5% CagA positive in a dyspeptic population from Sri Lanka; s1am1 was the most common VacA allelic type prevalent in this population(5).

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Studies carried out in neighbouring countries show the following prevalences: in Bangladesh, 61% of 1-3 month old infants tested positive for H. pylori; this rate declined steadily to 33% in children aged 10-15 months and then increased to 84% in children aged 5-8 years (6). In India, prevalence rates have been reported ranging between 31%-84% (7) with sero-prevalence in children ranging from 22% in 0-4 year old to 87% in 10-19 year olds(8). In China, which has one of the highest rates of cancer in the world, one study reported a prevalence of 69% in children between age of 3 to12 years (9).

The exact reasons for pathogenesis of Helicobacter related disease is not known and it is thought that it is a combination of host, environmental and microbial factors. Vacuolating cytotoxin, urease (VacA), cytotoxin associated gene protein (CagA), phospholipases A and C, catalase, motility and adherence have all been implicated in disease states. CagA has been found to have a type IV secretary system that injects this protein to the eukaryotic cell where it becomes tyrosine phosphorylated and this triggers profound changes in the host cyto-skeleton along with hijacking of cell signaling. This new information will support the role of CagA in research on H. pylori virulence. Antibodies developed against this protein are used diagnostically(10).

Overall, the published data for the prevalence of H. pylori in dyspeptic adults in Sri Lanka seems to correspond more to that of an industrialized country (ranges from 20-50%) rather than a non-industrialized country, such as the geographically adjacent India. There the prevalence of H. pylori in patients with dyspepsia and those asymptomatic was 65% and 46% respectively, and the age related prevalence in these two groups was seen to increase with age (50 to 70% and 45 to 60%) leading to the conclusion that exposure occurs early and is widespread in both symptomatic and asymptomatic individuals.

The reasons for the low prevalence of H. pylori in Sri Lanka is not at all clear and is a matter of interest. One explanation for the low prevalence in these studies may relate to the methods used to detect the organism. It is recognized that biopsy urease tests suffer from sampling errors and that prior antibiotics can affect both the biopsy urease test and culture results. Alternatively, the prevalence may be genuinely low, possibly due to various environmental factors and/or host factors inhibiting the colonization of the organism. It may also be due to ethnic differences as seen in previous studies. For example, individuals studied in Vietnam, Algeria and the Ivory Coast have higher sero prevalence rates compared to France (11).

Thus more extensive studies are required, encompassing different study groups: with typing of H. pylori to identify and compare the prevalent strains in the country with those from other regions; to study the host response to specific antigens and to correlate results with demographic data. The following study was carried out with samples from the Western Province of Sri Lanka, with the following aims.

1. Determining the sero-prevalence of Helicobacter in a representative population attributable to age groups using a commercial H. pylori IgG ELISA.

2. Determining the sero-prevalence of CagA IgG in a representative population attributable to age groups using a commercial ELISA.

3. Determining the host response to virulent antigens such as CagA, Urease and VacA and other major antigen found on H. pylori using western blot.

Material and Methods

Three hundred and fifty nine healthy volunteers (asymptomatic, specific clinical features for infection with H.pylori was not checked) between the ages of 1 to 94 years, with equal distribution of males to females, were selected by a multi-stage sampling technique. Two Medical Officer of Health (MOH) areas (Nugegoda and Kaduwela) in the Colombo District were randomly selected and 3 midwife areas were selected from

each MOH area(Delkanda South & North, and Udahamulla from the Nugegoda MOH area and Malasinhagoda, Wallaugiriya and Nawagamuwa from the Kaduwella MOH area); a lane from each midwife area was selected from the area map. The 3rd consecutive house starting from the midwife's office was included for the study. Different age groups based on the age distribution of the population given in the Annual Health Bulletin (1996) was selected. A sample of 359 individuals was sufficient to estimate a prevalence of 10% with an alpha error of 5%, a 95% confidence interval ranging $\pm$ 4% and a design effect of 1.5. After obtaining informed consent, a sample of blood (1to 5ml) was obtained from each subject for evaluation of IgG antibody to the high molecular weight cell associated proteins of H. pylori and cytotoxin associated proteins. In addition to collecting blood samples, factors such as age, gender, were noted. The serum was separated and stored at 4°C until use.

Sero -prevalence

The sero-prevalence of IgG to H. pylori was determined using EIA- SIA ™H. PYLORI (HMCAP ™ SIGMA) TEST KITS in 359 samples, according to the manufacturer's instructions and interpreted as described.

Sero-prevalence of cytotoxin associated protein (CagA)

The prevalence of IgG antibodies to CagA was determined in 359 samples, using an EIA-Helori CTX (Eurospital) test kits according to manufacturer's instructions as described.

Western blot analysis

The IgG antibodies to several major antigens of H. pylori were tested using an in-house assay (12). Helicobacter NCTC 11638 strain was grown for 5 days on 10 plates of Columbia horse blood agar (Oxoid, UK. cat P0812A). The growth was scraped off into 5 ml of distilled water, chilled on ice, and sonicated, using a vibra cell sonicator at an amplitude of 60rpm for 6 minutes. The protein concentration was estimated by the Warburg and Christian method(13). The appropriate concentration of antigen (50µg/well) (after chequer board titration) was determined and used in preparation of the western blot strips as decribed by Laemmli(12). The primary antibody (patients serum) was diluted at 1:100 (after chequer board titration to conclude the dilution factor) in 1% milk and 800µl added to each strip. These were placed in a BioRad tray and incubated on a shaker for 2 hours at room temperature. Blots were washed 3 times in PBS/ BSA/ Tween 20 for 5minutes, with a final wash in distilled water.

The secondary antibody peroxidase conjugated immunoglobulin to human IgG- (Harlan) was diluted 1:1000 (after chequer board titration) in PBS/ BSA/Tween 20, poured into wells and incubated for 1 hour on a shaker.

The strips were washed as before, given a final wash in PBS and rinsed in distilled water. The bands were detected with chloronapthol solution (made just before use) for 20 minutes at room temperature. Once the colour developed the dye was removed by rinsing the strips in tap water. The blots were dried, placed along the markers and stored.

Reading of results

Six to seven commonly noted bands which were considered to be major bands were studied. The presence or absence of reactive band pattern was noted for each serum sample according to a standard curve plotted with known molecular weight proteins.

Statistical analysis

Data were analysed by the chi square test.

Results

The study consisted of 359 serum samples with 180 males and 179 females. Only 37 (10.3%) of the 359 serum samples were positive for IgG to H. pylori using the above serum assay. Anti CagA was detected in 29/359 (8.1%).

The age-correlated distribution of the prevalence of antibody to H. pylori and IgG to CagA is seen in (Fig 1). There was no significant difference between the age groups ($p>0.05$) for CagA and

H. pylori IgG but the prevalence was seen to increase with age from 15 years until 60 years with only a minor differences between the age groups (16 to 45 years) and to decrease after the age of 60 years.

No significant difference in prevalence between men and women was noted as seen in Table 1 ($p=0.114$; 0.859) for H. pylori and CagA respectively. Only 7 samples were IgG positive in both tests to H. pylori and CagA. Of the 37 positive for H. pylori, 30 were negative for anti CagA; 23 samples, although negative for IgG to H. pylori were positive for IgG CagA protein.

Western blot and results

Forty eight samples (positive by IgG to H. pylori and/or positive by IgG to CagA) were studied by immunoblotting for 7 antigens (Tables 2, 3). The commonest western blot band seen was an antibody response to the 89kDa protein. A heterogenic pattern of response was observed in the samples tested (Tables 2, 3).

Discussion

In this study based on the detection of IgG specific for H. pylori, the prevalence was found to be 10.3% when antibody to H. pylori was used, and 8.1% when IgG to CagA was used. These values indicate a low prevalence as seen in previous studies using other methods. It was interesting to note that only seven samples were positive by both tests.

The detection of anti-CagA antibodies in sera negative for anti H. pylori antibodies raises the question of whether these are false positives. The possibility of a cross-reaction with another microbe is unlikely according to published data (14). Also, the study of H. pylori by FASTA and BLAST search engines that detect similarities at the DNA and protein levels available at European Bioinformatics Institiue and National Centre for Biological Information internet sites respectively, excluded CagA as being similar to other polypeptides in other bacteria or humans (15). However there may be environmental organisms not yet included in these databases.

Recently Franceshi et al., (16) suggested that anti CagA antibodies cross-react with alpha tubulin of platelets from patients with idiopathic thrombocytopaenia, which suggests that there may be some human antigens which are similar to the CagA protein; and thus be responsible for false positive results.

Alternatively, these could be due to actual infections not detected by the H. pylori assay methods , perhaps representing an immunological memory due to a past contact, and/or a response that was directed against CagA and possibly unidentified components not been detected by the assay used. Such a mechanism could give a falsely low prevalence.

Previous studies have shown that some individuals who possess anti-CagA antibody in their sera have negative serology against H. pylori and have been shown to be H. pylori positive by other conventional methods (15). Therefore, the true sero-prevalence in this geographic locations may be higher than seen in the present study.

The assays used have been validated and have a sensitivity of 97.6% and specificity of 94.1% respectively, but the cut-off was determined for populations from developed countries. This study could not be optimized with known negative sera from Sri Lanka as this was the first such study to be conducted and lacked facilities to use conventional H. pylori diagnostic methods to obtain negative serum samples. Therefore, the cut-off used in this study may be too high to determine true *H. pylori* prevalence in Sri Lanka. Consistent with previous studies (17) the prevalence of *H. pylori* increased with age, thus confirming epidemiological variables as a risk factor which play a crucial role in acquiring the infection. It was also noted that there was a decline in sero-prevalence from the 7th decade, which might be the result of a decrease in immune response in the aged through the creation of a hostile environment to the organism as a result of *H.pylori*-induced chronic gastritis, leading towards atro-

phic gastritis (18). Alternatively, it might be the result of a physiological decrease in the immunological response in the aged (19).

As found in a majority of previous studies, there was no difference in colonization with gender, similarly no difference in colonization was found in the different study locations.

There was a heterogeneous response to *Helicobacter* in the sera which were positive for *H. pylori* (by IgG to either *H. pylori* or CagA) when tested by western blot. Bands 89kDa, and 66kDa were seen more commonly with a positive response to *H. pylori* antibody. It is known that human serum contains antibodies that react with a number of different *H. pylori* antigens. All sera tested in this study were tested against a reference strain (NCTC 11638) and the differences observed could be due to heterogeneity of the host. Another explanation is that infection could be with strains carrying different immunogenic antigens or a combination of these factors.

An immunoblot prepared with a Sri Lankan *H. pylori* isolate would help answer the question if there is no antigenic variation as hypothesized. Antibody responses to immunoblot were not studied in all subjects due to practical considerations. Although this test is expensive and time consuming it appears to be more sensitive, especially with sera with low levels of antibody that are not detected by ELISA (20), thus this was a limiting factor in this study.

The diversity of the immune response in these asymptomatic *H. pylori*-infected subjects indicates that sera from different clinical entities should also be studied in an attempt to find a specific profile or an individual antibody response which could be useful as a diagnostic tool. Development of the protein bands 19kDA, 66kDa and 89kDA were significantly positive with a positive *H. pylori* IgG result, and may be important immunological markers for a Sri Lanka population. The presence of anti-CagA was associated with other bands detected by western blot (89,66,35,30,26.5,19 kDa), and suggests that the ELISA based test for antibody to *H. pylori* may have missed some positive results, thus the very low prevalence contrasting to neighboring countries.

In conclusion, the prevalence seems to be low in keeping with other studies, but the presence of CagA antibody in *H. pylori* negative sera indicates that responses in the host may not be always detected by routinely used assays. This may be due to strain differences, genetic differences in the host not enabling the host to mount a response, or to cross reactivity.

Acknowledgements

We wish to thank Dr Helen Donoghue, Department of Bacteriology , University College London, United Kingdom for guidance in writing up this project and Dr Rajitha Wickramasinghe, Department of Community Medicine, University of Sri Jayewardenepura for his guidance and contribution in the statistical analysis.

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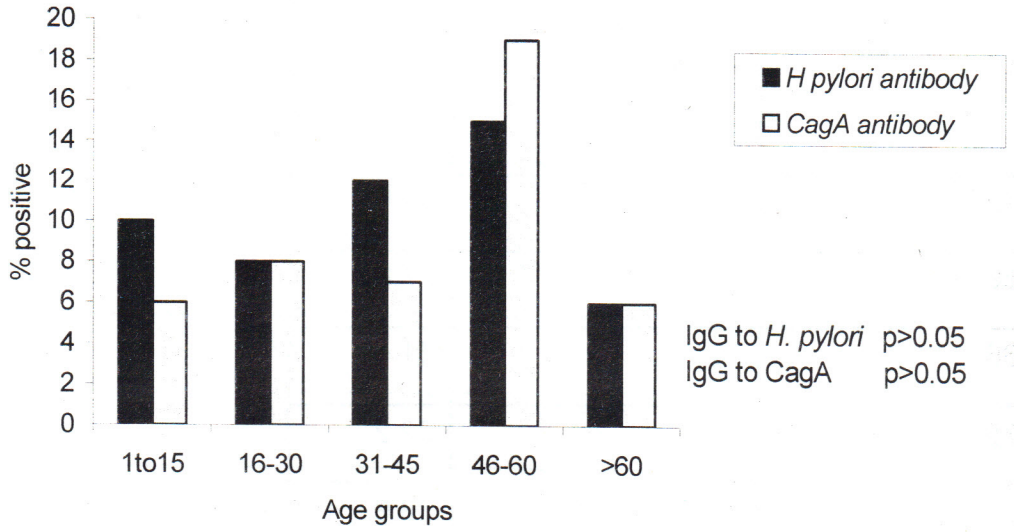
Figure1. Age related prevalence of antibody to *H.pylori* (IgG)and CagA(IgG)



*There was no significant difference between age groups

Table 1

The prevalence of IgG to *H. pylori* and CagA

Gender	Total	Number Positive for IgG *H. pylori*	percentage and p value	Number Positive for IgG CagA	Npercentage and p value
Male	180	14	7.78 (p>0.05)	14	7.78 (p>0.05)
Female	179	23	12.8 (p>0.05)	15	8.3 (p>0.05)
Total	359	37	10.3 (p>0.05)	29	8.1 (p>0.05)

Table 2
Western blot results in those positive to IgG *CagA*

Band (kDa)	Number positive for Cag A n=18	%
120	10	55.6
89	14	77.8
66	11	61.1
35	4	22.2
30	7	38.9
26	6	33.3
19	9	50.0

Table 3
Western blot results in those positive to IgG *H pylori*

Band (kDa)	Number positive for *H.* pylori n=37	%
120	19	51.4
89	35	94.6
66	33	89.2
35	11	29.7
30	15	40.5
26	1	27
19	23	62.2