Bangladesh J Microbiol, Volume 26, Number 1 & 2, June and December 2009, pp 9-14

Original Article

Association Between Malnutrition and Other Possible Risk Elements with the Acquisition of *Helicobacter pylori* Infection Among the Children Population in Bangladesh

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[Received 20 February 2009; Accepted 15 May 2010]

The acquisition of *Helicobacter pylori* infection occurs mostly in early childhood and results in hypochlorhydria which may facilitate the subsequent attainment of other enteropathogens. Infection with *H. pylori* has also been implicated in micronutrient deficiencies and malabsorption of vitamins. These impacts could play a role in childhood malnourishment. To determine the age related seroprevalence of *H. pylori* infection among well nourished and undernourished children population in Bangladesh and to examine the relevant risk factors, this case-control study was conducted. A total of 149 (0 - 12 years) malnourished (case) and age and socioeconomic condition matched 151 nonmalnourished (control) children were individually interviewed (for children below 3 years of age, parents were interviewed) on the basis of a standardized questionnaire during the collection of serum. Case population was further classified into 3 different categories according to the degree of severity of their malnourishment. The presence of *H. pylori* specific antibody in serum samples was detected by in house ELISA and the cut-off value was determined by immunoblotting. The overall seroprevalence was found to be 66% among the children population (n = 300). The seroprevalence was lowest among nonmalnourished children (60%) and gradually increased with the increase of severity in malnourishment. Seroprevalence of *H. pylori* infection has been found to be related significantly with stratified age groups among case ($p = 0.008$) and control ($p = 0.003$) children, and this seroprevalence increased with increasing age. Seroprevalence of *H. pylori* was found to increase with increased number of siblings but was not found to be associated with gender. Seroprevalence of *H. pylori* infection was similar amongy children from poor and medium socioeconomic status (68%) but comparatively lower among children from upper socioeconomic class. Associated malnourishment was significantly related to gender ($p = 0.037$). Furthermore, use of nonsanitary toilet augmented the risk of malnourishment. The findings in this research reveal that improvement of socioeconomic condition, hygiene situation and eradication of *H. pylori* infection could contribute to the improvement of the nutritional status of the children in Bangladehs and thus assist them to grow to their full potential of physical and mental abilities.

Keywords: *Helicobacter pylori*, malnourishment, risk factors

Introduction

The acquisition of *Helicobacter pylori* infection occurs mostly in early childhood and results in hypochlorhydria, which may facilitate the subsequent attainment of other enteropathogens and may cause diarrhea. Infection with *H. pylori* has also been implicated in the development of iron deficiency anemia, micronutrient deficiencies and malabsorption of vitamins. The combined impact of these extragastric manifestations of *H pylori* infection ultimately results in impaired childhood growth and cognitive function caused by the comorbidity associated with malnutrition[2].

Human population can be infected by *H. pylori* by ingesting contaminated food and water and through person to person contact. In the developing countries like Peru, Bangladesh and Gambia, children start to become infected in the first few months of life and in some communities with poor sanitation, as many as 50% of the children can be infected[1]. Once infected by *H. pylori*, it usually persists, probably continue to grow in low level in the stomach through out the host's life. The diagnostic potential of direct microscopic, culture and rapid urease test (RUT) of endoscopic antral biopsy tissues, urea-breath test and enzyme linked immunosorbent assay (ELISA) of anti *H. pylori* antibody in serum for the detection of *H. pylori* was evaluated[4-5]. Several studies have implied that the immune response correlates with the histological findings in biopsy specimens. The detection of antibodies specific for strains of *H. pylori* has demonstrated the value of serology for providing evidence of infections[6]. Detection of anti *H. pylori* antibody by ELISA can provide a simple, rapid and inexpensive test with the eye sensitivity (85-95%) and the specificity (95%)[5-7]. A number of different serological techniques have been used to detect antibodies in human population. These include haemagglutination, complement fixation coagglutination, indirect immuneofluroscense and latex agglunitations[8]. However ELISA and immunoblotting have been emerged as the two most frequently used techniques for their high sensitivity and specificity. Recently in house

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ELISA for the detection of anti- *H. pylori* antibody has been validated by several works[6-9].

The epidemiology of *H. pylori* infections is clearly distinct between the developed and the developing world. Prevalence of *H. pylori* varies widely in different parts of the world with an average rate of 40-50% in western countries rising to more then 90% in the developing world[10]. The prevalence of *H. pylori* infections in individuals depends on the country where the individuals live, his or her socio-economic status and age. The prevalence of infection in asymptomatic cases increases with age in developed countries[11].

The clinical outcome of *H. pylori* infection is most likely of complex interactions among host, bacteria and environmental factors; being clustered in families and institutions in the developed world, suggesting transmission due to close contact of infected individuals[12].

The present study was undertaken to analyze the relationship between seroprevelence of *H. pylori* infection and severity of malnourishment. This investigation also explores the influence of several sociodemographic and hygiene associated factors for the acquisition of *H. pylori* infection as well as the development of undernourishment in children population.

Materials and Methods

Study population and sample collection

The study population included children (0 - 12 years) from various socioeconomic backgrounds attending a children-based hospital in Dhaka city for treatment or vaccination or blood grouping. From each participant 3 ml of blood was drawn from which serum was separated using standard method[9]. Nutritional status of each participant children was assessed by comparing their weight, height with the medians of the NCHS[13] (National Center for Health Statistics) reference population of the same age and sex group. Children whose weight-for-age were >90% of median of the NCHS, weight-for-height were ≥90% of median of the NCHS, and mid arm circumference were >14 cm[14] were classified as nonmalnourished or control. But whose weight-for-age were <90% of median of the NCHS, weight-for-height were ≤90% of median of the NCHS, and mid arm circumference were <14 cm were graded as malnourished or case children. The degree of malnourishment was further stratified by ranking into first (mild), second (moderate) and third (severe) degree of malnutrition[15].

A precoded questionnaire was filled up for each enrolled child or his/her parent to obtain relevant demographic, anthropometric, socioeconomic, clinical and nutritional data. The whole study population was categorized into seven age groups. Socioeconomic condition was measured by using the international poverty line (monetary cut-off points separating the poor from the non-poor) proposed by the World Bank set at US$1.00 per day per capita in purchasing power parity (PPP) terms[16].

Preparation of H. pylori (locally isolated strain) whole cell antigen for ELISA

H. pylori cells were suspended in phosphate buffered saline (PBS) containing 1% (v/v) formalin and kept at 4°C for 1 hour. Then it was centrifuged (12,500 g, 5 minutes) and the pellet was resuspended in 1 ml of PBS. It was washed with PBS 4 times to remove the formalin. Finally, a suspension of 1mg ml^{-1} cells in PBS was prepared.

In-house ELISA

One hundred microliter of formalin-fixed bacterial antigen (diluted to 1 µg per 100 µl of coating buffer) was added to each well of the 96 welled polystyrene microtitre plate. The plate was covered with a sealer and incubated overnight at 4°C. On the following day, the plate was washed 3 times with PBS containing Tween-20 (0.05%). The wells were blocked with 200 µl of 1% (w/v) bovine serum albumin (BSA) in PBS, and then the plate was incubated at 37°C for 30 minutes. The PBS-BSA was discarded and the plate was washed 3 times with PBS - Tween-20. Then, 100 µl of diluted serum sample (1:500 in PBS) was added to all the wells except wells A1 and B1, which were used to calibrate the ELISA reader. The plate was covered and incubated at room temperature for 2 hours. Afterward, the plate was washed three times again and 100 µl of diluted antibody conjugate (1:30,000 in PBS) was added (antihuman polyvalent immunoglobulin-peroxidase; SIGMA - 8794), and incubated at room temperature for an additional 2 hours. The plate was again washed three times with PBS-Tween-20 and 200 µl of enzyme substrate (H_2O_2, O-phenylenediamine in 0.1M sodium citrate buffer) was added to each well. The plate was placed in the dark and optical density (OD_{490}) was measured by an ELISA reader (BioRad, Japan, model No.680) exactly after 30 minutes.

For the diagnosis of serum samples by in house ELISA using formalin fixed *H. pylori* whole cell antigen and AP (alkanine phosphatase) substrate system, absorbance ≥1 at 405 nm were interpreted as positive for *H. pylori* infection by previous researchers[9]. Ninety-six serum samples were evaluated by both HRP (horseradish peroxidase) and AP substrate system in this study to determine the cut-off value for HRP substrate system for the serodiagnosis of *H. pylori* infection. Accordingly, ≥0.2 at 490 nm were considered as indicative of the presence for significant levels of anti-*H. pylori* antibodies, while the test serum samples were examined by HRP substrate system. This cut-off value was further validated ($p = 0.0000$) by immunoblotting technique, employing *H. pylori* (the same strain used to make whole cell antigen for ELISA) whole cell extracts.

In the AP substrate system, the similar procedure of the HRP substrate system was followed with a suitable secondary antibody (anti-human polyvalent antibody conjugated with alkaline phosphatase diluted 1:800 times in PBS), and the substrate was p-nitrophenyl phosphate in diethanolamine buffer. The colour reaction was measured at 405 nm.

Each test serum was analyzed in duplicate wells to minimize the handling error. For each ELISA plate, the average of blank values was subtracted from OD value for each test serum.

Preparation of whole cell extracts for SDS-PAGE

After confluent growth, *H. pylori* cells were harvested and taken into pre-weighed screw-capped eppendorf tubes. Bacterial cells were sedimented by centrifugation (12,5000x g for 2 min) and suspended in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) solubilizing buffer[24] to a concentration of 500 µg /5 µl. The suspension was incubated at 100°C for 5 min to denature bacterial proteins and DNA was disrupted by brief sonication (30 sec). The suspension was reheated at 100°C for 2 min and diluted in SDS-PAGE solubilizing buffer to produce a final concentration of 70µg whole cell extract per 5µl. The aliquotes were stored at -20°C until required.

SDS-PAGE

The SDS-PAGE profile of *H. pylori* whole cell extract was prepared using the method of Laemmli[24] as described previously[25]. Gels comprised of a 4.5% (w/v) acrylamide stacking gel and 12.5% (w/v) acryamide separation gel were made. Samples were applied to the gel alongside protein molecular weight standard (1610305, Bio-Rad, UK). Electrophoresis was performed using a mini-gel system (Consort, UK) with a constant current of 35 mA for 60 min. Gels were used for immunoblotting.

Immunoblotting

H. pylori sero-status for the serum samples were determined by immunoblotting technique as described previously[17]. The SDS-PAGE protein profiles were transferred onto nitrocellulose sheets[26] using a semi-dry electrotransfer apparatus (Bio-Rad, UK). Individual protein profile was prepared by cutting nitrocellulose sheets into strips. The strips were incubated separately with human serum (1: 200 dilution) samples (primary antibody). Antibody-antigen complexes were detected using an antihuman polyvalent antibody conjugated with alkaline phosphatase (A-5034, Sigma, UK), diluted 1:1000 in PBS. Colour development was carried out in a polyethylene bag at 37°C in the dark for 10 min. Serum samples containing at least five types of anti-*H. pylori* antibodies were considered as seropositive for *H. pylori* infection[17].

Statistical method

Data was analysed by SPSS (statistical package for social services) version 11.5. Proportions were compared by two tailed x^2 - test. A 'p' value <0.05 was taken as significant. 'p' value <0.001 was taken as highly significant. Frequency was analyzed and seroprevalence for *H. pylori* infection was calculated. Proportions were compared by two tailed x^2 - test, and odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated.

Results

A total of 149 children (0 - 12 years) with malnourishment (case) and 151 children (0 - 12 years) without malnourishment (control) attending a children based hospital in Dhaka for check up, vaccination or treatment were enrolled in this study during the nine-month period from July 2007 to April 2008.

Serum samples were analyzed by in house ELISA using formalin fixed *H. pylori* whole cell antigen and HRP (horseradish peroxidase) substrate system. Ninety-six serum samples were evaluated by both HRP (horseradish peroxidase) and AP substrate system in this study to determine the cut-off value for HRP substrate for the serodiagnosis of *H. pylori* infection. For AP (alkanine phosphatase) substrate system the absorbance ≥1 at 405 nm were interpreted as positive for *H. pylori* infection[9]. Accordingly, absorbance ≥0.2 at 490 nm was considered as the presence of significant levels of anti-*H. pylori* antibodies, while the test serum samples were examined by HRP substrate system. This cut-off value was further validated (p = 0.0000) by immunoblotting technique, employing *H. pylori* (the same strain used to make whole cell antigen for ELISA) whole cell extract as antigen.

The overall seroprevalence of *H. pylori* infection among the study population (n = 300; 0 - 12 yrs) was found to be 66%. *H. pylori* infection was considerably associated (p = 0.035) with the nutritional status of the children. Among the 149 case children, 107 contained anti-*H. pylori* antibodies, whereas 91 children possessed significant level of antibodies against *H. pylori* among the control population (n = 151). Severely malnourished group (Fig. 1) showed the highest seroprevalence (75%) rate of *H. pylori* infection. Seroprevalence of *H. pylori* infection gradually decreased for the moderately (72%) and mildly (70%) undernourished children population. While it was the lowest among the well nourished (60%) children.

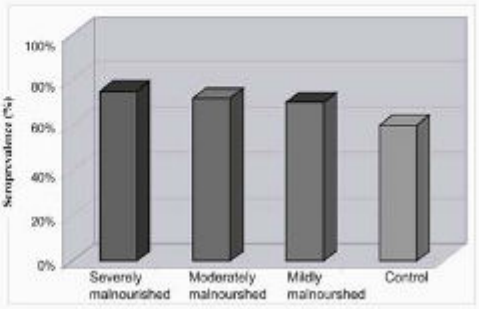

Figure 1. *Seroprevalence (%) of Helicobacter pylori infection among children (n = 300; 0-12 years) of different nutritional categories*

Seroprevalence of anti-*H. pylori* antibodies increases with the increasing age for both the case and control children population (Table 1). The *p*-value of the bivariate analysis of the stratified age groups and the presence of anti-*H. pylori* antibody in the serum samples was 0.003 and 0.008 for control and case children, respectively and that indicated highly significant association between seroprevalence of *H. pylori* infection and the age of the children.

Table 1. *Serum antibody response of 151 well nourished children (control) and 149 malnourished children (case) to Helicobacter pylori antigens*

Children without malnourishment (Control)				Children with malnourishment (Case)		
Age group	No. of children studied (n=151)	No (%) of children with anti *H. pylori* ^{x} antibody	(x^2 p-value)	No. of children studied (n = 149)	No (%) of children with anti-*H. pylori* antibody	(x^2 p-value)
0 – 6 mo*	30	14		20	9	
6 –12 mo	11	4		24	14	
1 – 2 y#	19	8	0.003	35	24	0.008
2 – 4 y	20	10		26	23	
4 – 6 y	26	10		23	20	
6 – 8 y	22	16		11	8	
8 – 12 y	23	21		10	9	
Total	151	91		149	107	

* mo refers to months

y refers to years

There was no significant variation (p = 0.365) of seroprevalence for *H. pylori* with respect to gender (Table 2). Although, a slightly higher seropositivity was found in male (67%) than in female (64%) children population.

Table 2. *Seroprevalence of Helicobacter pylori infection in different sex of the study population*

Sex of the children	Number of children	Number of children with anti-*H. pylori* antibody	Seropre-valence (%)	x^2 (p – value)
Female	131	84	64%	
Male	169	114	67%	**0.365**
Total/Ovrall	**300**	**198**	**66%**	

Per day per capita income was used as the indicator of socioeconomic status of the families of the children. The seroprevalence of *H. pylori* infection was similar among the children from middle and poor socioeconomic status (68%), while it was much lower (53%) among the upper

Table 3. *Seroprevalence of Helicobacter pylori infection among children from different socioeconomic status*

	Socioeconomic status of the children			
	Poor [a]	Medium [b]	Upper [c]	x^2 (p – value)
No. of children studied	185	69	46	
No. of children with anti-*H. pylori* antibody (%)	126 (68%)	47 (68%)	25 (53%)	0.193

[a] Per day per capita income below 1.00 $;
[b] Per day per capita income within 1.00 - 2.00 $;
[c] Per day per capita income more than 2.00 $

Nutritional status was found to be significantly linked to the gender of the children (p = 0.037). Number of female children was significantly higher (56%) in number among the malnourished group (Fig. 2) than the well nourished population (44%).

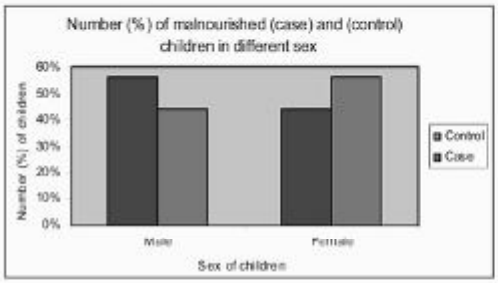

Figure 2. *Relationship between nutritional status and gender (p = 0.037) among malnourished (case) and nonmalnourished (control) children*

Thirty seven percent children (Table 4) of the study population (n = 300) were the only child of their parents, 42% had one sibling, 13% got two and only 8% of the study population had more than two siblings. The distribution of >2 siblings group was two-fold higher (13%) among the undernourished population than the properly nourished children (6%).

Table 4. *Relationship between status of nourishment and number of siblings*

No. of siblings	No. (%) of well nourished children (control)	Degree of malnourishment among case children (n = 149)			Total (%)	x^2 (p – value)
		Mild	Moderate	Severe		
none	58	35	9	8	110	
1	64	27	14	22	127	
2	20	10	5	3	38	0.090
>2	9	5	4	7	25	
Total	151	77	32	40	300	

Correlation (p = 0.000) was found between nonsanitary toilet usage and the associated malnourishment. The risk of development of malnourishment was 4.291 (OR = 4.291; 95% CI = 2.439 - 7.549) times more among the children using nonsanitary toilet in comparison to the children using sanitary toilet (Figure 3).

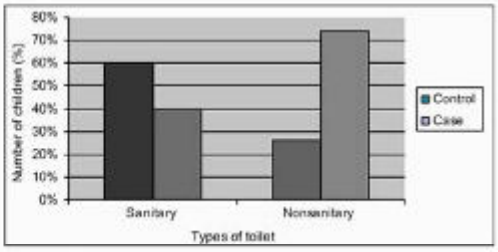

Figure 3. *Association between nonsanitary toilet usage and the associated malnourishment {p = 0.000; OR = 4.291(2.439-7.549)} among case and control population*

Discussion

H. pylori is often associated with acute and chronic gastritis, peptic ulcer disease, non-ulcer dyspepsia, gastric MALT (mucosa-associated lymphoid tissue) lymphoma and extragastroduodenal disorders[3]. Infection with *H. pylori* leads to the development of hypochlorhydria, which may increase the risk of acquisition of other enteropathogens and subsequently direct to frequent diarrhea. *H. pylori* infection also causes malabsorption of vitamins and minerals and thus may play role in the development of childhood malnourishment[2, 27]. Therefore, it is necessary to diagnose this infection among human population specially children because eradication of this infection might improve their nutritional status[1].

The overall seroprevalence of *H. pylori* infection was 66% among the study population (0 - 12 years). Rahman *et al.* (2005)[17] reported a similar seroprevalence (66.7%) of *H. pylori* infection among Bangladeshi children of 1-9 years of age. Malnutrition among young children may influence their linear and mental growth and intelligence quotient, and is synergistically linked to subsequent child morbidity and mortality[19]. It has been reported that 74% of the children under 12 years of age are suffering from chronic undernourishment in rural Bangladesh[18]. In our study, seroprevalence of *H. pylori* infection was significantly lower (60%) among the well nourished children than the malnourishment group (Fig. 1).

Acquisition rate of *H. pylori* infection increased gradually with the degree of severity of malnourishment. Mildly and moderately malnourished children showed 70% and 72% of seroprevanence respectively. Severely malnourished children group represented the highest seroprevalence (75%) rate of *H. pylori* infection among the entire study population.

The current work revealed that *H. pylori* infection had significant relationship with age (Table 1) of the study population including both case ($p = 0.008$) and control ($p = 0.003$) population. Findings of the current investigation are in agreement with the previous reports where the seroprevalence of *H. pylori* increases with the increase of age. The prevalenceof *H. pylori* infection is effectively associated ($p = 0.023$) with age of the population in a study conducted by Islam *et al*[6]. In 1996, Mahalanabis *et al*[20], reported the prevalence of *H. pylori* infection among Bangladeshi children (6 - 9 years) as 84%. However, in our research, the similar age group (6 - 8 years) had overall seroprevalence of 73% including both case and control children. The highest seroprevalence for *H. pylori* infection was observed among the children group of 8-12 years for both case and control population (90% and 91% respectively). Lowest seroprevalence was found among the infants (0-1 year). Similar report was made by Mahalanabis *et al*, (1996)[20] where they found that the rate of *H. pylori* infection acquisition continued to rise to a significantly high level till the age of 8 years (more than 80%).

No statistical association was found between seroprevalence of *H. pylori* infection and sex of the children in a study conducted among the children of Turkey[21]. In the present research, seroprevalence of *H. pylori* infection was slightly higher in male (67%) than in female (64%) population. However, statistically significant relationship was not established between sex and *H. pylori* infection (Table 2). Malnourishment was found to be more prevalent among the female children (56%) than the male children (44%) (Fig.2) in this study. Acquisition of malnourishment was found to be significantly associated ($p = 0.037$) with the gender of the children. A male child in Bangladeshi society is usually more privileged than a female child. This cultural behavior and subsequent discrimination may explicate the difference in the nutritional status observed among these two sex categories.

Socio-economic status of a population directly or indirectly regulates the degree of hygiene, nourishment intake and standard of living condition. All these factors are related to the possibility of exposure to infectious agents like *H. pylori*[2]. Various parameters like parental education, occupation, household status, holding of assets and per capita per day income are used as measures of socioeconomic stratification for this investigation. Among these factors, per capita per day income had been documented to be the best indicator for socioeconomic status. In a research conducted among children in Turkey, it was demonstrated that seroprevalence of *H. pylori* infection was inversely related to economic status[21]. In the current work seroprevalence of *H. pylori* (Table 3) infection was similar among the children from middle and poor socioeconomic status (68%), which was much lower among the children from upper socioeconomic status (53%). Financial borderline between middle and poor socioeconomic status became thinner in recent times because of high price of essential commodities in urban life and there is little difference between life standard of people from middle and poor socioeconomic status living in big cities like Dhaka and this factor may contribute to the outcome recorded here.

It was also evident from this study that occurrence of malnourishment was higher among the children belonging to the families of more children. Furthermore, percentage of having more than two siblings group gradually augmented with the increase of degree of severity of malnutrition among the case group studied. Under strained financial situation, more number of children in the family can negatively influence the amount of food provided for them which may lead to the development of malnourishment. Mazumder *et al*.[22] documented increased number of siblings as an important predisposing factor for poor nourishment.

Use of nonsanitary latrine has been marked as putative predisposing factor among the children by making them more vulnerable to infectious diseases[23]. Nonsanitary toilet is unclean, foul smelling, lack adequate water for cleaning and often contain flies and mosquitoes. All of these parameters might contribute to the transmission and subsequent development of various contagious diseases. Highly significant association ($p = 0.000$) was found between using nonsanitary toilet and associated malnourishment in this work. The use of nonsanitary toilet was associated with 4.291 (95% CI = 2.439 - 7.549) times higher risk of developing malnourirshment than children using sanitary toilet.

This study demonstrated the high seroprevalence of *H. pylori* infection among the Bangladeshi children population and various environmental and socioeconomic factors were found to be significantly related to the risk of acquisition of *H. pylori* infection. Eradication of *H. pylori* infection and upgrading of socioeconomic condition and hygiene related factors would contribute to the improvement of the nutritional status of the children in developing countries and thus assist them to grow to their full potential of physical and mental abilities.

Acknowledgements

We are grateful to the participants of this study, and to the management and staff of Ad-din Hospital, Dhaka for their help and very kind cooperation.

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