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Helicobacter pylori : Is it Transmitted Through Faecal-oral or Oral-oral Route?

Rupa Mulchandani[1,2], Hans-Olof Nilsson[2], Torkel Wadström[2] and B.R. Joshi[1]

[1]Department of Microbiology, Smt. Chandibai Himathmal Mansukhani College, University of Mumbai, P.B. No. 17, Opp. Railway Station, Ulhasnagar – 421 003, Thane, Maharashtra, India.
[2]Department of Infectious Diseases and Medical Microbiology, Lund University, Solvegatan 23, 223 62, Lund, Sweden.

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The association of *Helicobacter pylori* with gastroduodenal diseases is well established. The mode of transmission of the organism is still unclear, although the faecal-oral route has been proposed. *H. pylori* has been detected in gastric antrum, dental plaques, faeces and water. In the light of these studies we attempted to detect *H. pylori* in antral biopsies, dental plaques, and stool specimens of patients who were suffering from upper GI complaints. These patients resided in Mumbai city, India. Clinical specimens from different sources were subjected to RUT, Culture, Histopathology and Polymerase chain reaction (PCR). We found *H. pylori* in antral biopsies and faeces by culture and PCR but absent in dental plaques. Therefore, faecal-oral is the possible route of transmission of this bacterium. Dental plaque may not be a permanent reservoir of this bacterium because this organism needs strict microaerophilic conditions to survive.

Key words : *Helicobacter pylori*, PCR, faeces, HPU, VacA, CagA.

Helicobacter pylori is recognized as an important cause of active chronic gastritis in humans; it plays a significant pathogenic role in the development and recurrence of gastric and duodenal ulcers, gastric cancer and MALT lymphoma[1,2]. The mode of transmission, the natural history and other aspects of epidemiology of *H. pylori* infection are still unclear[3,4]. Available epidemiological studies support oral-oral or faecal-oral transmission. *H. pylori* has been cultured in the dental plaques from a high proportion of subjects in India by conventional microbiological techniques[5] and abroad by polymerase chain reaction (PCR) in other studies[6,7,8], suggesting a possibility of oral-oral transmission. The organism has also been cultured from faeces in Gambian children and adults, and adults in U.K. and seems to survive in water in non-culturable coccoid forms[9,10]. The fact that these bacteria can exist outside the body suggests that the environment may serve as a reservoir for bacteria[11,12]. Waterborne and foodborne transmission has therefore been suggested in the few studies on this subject[13]. However, some investigators have failed to detect it in faeces even by PCR[14], due to the complex inhibitors present in samples. Immunomagnetic separation (IMS) using super-paramagnetic particles or beads coated with antibodies against surface antigens of cells has been used to recover eukaryotic cells from body fluids as well as for the separation of prokaryotic pathogens from heterogeneous samples such as blood, stool, food and water[15].

Because of the fastidious growth requirements and lengthy (3-7 days) incubation

* To whom all correspondence should be addressed.
Tel.: +91-2512232162; Mob.: +91-9323643554;
E-mail : mulchandaniroopa803@gmail.com

period necessary to isolate *H. pylori*, nucleic acid techniques have been developed for its detection in clinical samples[16,17]. PCR assays based on the gene sequences encoding urease structural genes, a specific antigen and 16SrRNA have been used to detect *H. pylori*[18,19]. PCR has also been useful in detecting CagA positive strains giving inflammatory response and antibiotic resistant strains of *H. pylori*[20,21].

We cultured *H. pylori* from antral biopsies, dental plaques, faeces, and performed PCR on the clinical samples. We found *H. pylori* in antral biopsies and faeces by PCR thereby confirming faecal-oral route of transmission in humans.

MATERIALS AND METHODS

Collection of Clinical Specimens

Antral biopsies were collected from 58 patients who were suffering from upper GI complications and who underwent endoscopy. The biopsies were subjected to routine rapid urease test (RUT), Culture, Histopathology. Dental plaques (25) and stool samples (23) were collected from the same patients who had undergone endoscopy and volunteered themselves for the study. Dental plaque samples were analysed by RUT and culture and stool specimens by culture. Only 11 gastric biopsies, 11 dental plaques, 10 stool specimens from the same patients whose antral biopsies were histopathology positive for *H. pylori* status were also analysed by PCR method. In addition 25 antral biopsies were analysed by PCR. All these samples were provided by the Gastroenterologists for *H. pylori* analysis at KEM hospital, Mumbai and B.Y.L. Nair Ch hospital, Mumbai, India as we had a collaboration with them on this project. Transport mediums used for antral biopsies were Stuart's and for dental plaques 25% glycerol brucella broth. Stool samples were collected in clean, sterile bottles. The samples were preserved on ice until processed.

RUT and Culture

Antral biospsies and dental plaques were analysed immediately for rapid urease test in Christensen's urea agar butts. Culture of antral biopsies, dental plaques and stool samples was performed on Columbia blood agar plates supplemented with 7% sheep blood and Skirrow's antibiotic supplement (Hi-Media, Mumbai, India). Cultures grown were identified by catalase, oxidase, urease tests and Gram's staining.

DNA Extraction from antral biopsies

Antral biopsies were cut into small pieces with a sterile scalpel and were subjected to lysis and extraction procedure. For lysis of bacterial cell membrane, digestion buffer [(250µl of 1% Triton X-100, 10mM TNE and 12.5µl of lysozyme (10mg/ml)] was added and incubated at 37°C for 45 minutes. After incubation 25µl of proteinase-K (10mg/ml) was added and the tubes were incubated at 37°C for 48 hours. The tubes with lysate were then kept at 95°C for 5 minutes in a water bath to inactivate proteinase-K. The samples were centrifuged in a microcentrifuge at 12000xg for 5 minutes in a cold room. After centrifugation, the supernatant was transferred to a fresh sterile microcentrifuge tube. DNA was extracted by the conventional phenol:chloroform:isoamylalcohol, centrifuged at 12000xg for 15 minutes. The upper phase was transferred to a new tube and further extracted with equal volume of chloroform:isomylalcohol. The DNA was then precipitated with 0.6 vol. of isopropanol and 0.1 vol. of 3M Na-acetate at -20°C. The DNA was pelleted by microcentrifugation at 12000xg for 15 minutes, washed with 70% (vol/vol) cold ethanol, desiccated for 30 minutes at room temperature and dissolved in 100µl of molecular biology-grade water and preserved at -20°C until the PCR was performed[22,23].

Immunomagnetic separation (IMS) and DNA extraction for dental plaques and stool samples: Immunomagnetic beads are activated magnetic particles covered with polyacroline distributed in water phase. Coupling of immunomagnetic/paramagnetic particles with polyclonal anti-*H. pylori* rabbit antibodies was performed. One ml of paramagnetic particles (10mg/ml) (Bional, Tartu, Estonia) was mixed with 700µl of 0.06M PBS, pH-7.2, 100µl of anti-*H. pylori* IgG (2mg/ml), purified in a Pharmacia MAB Trap G system (Pharmacia LKB Biotechnology, Uppsala, Sweden) was added to the mixture and allowed to rotate on a rotar for 1 hour at room temperature. Ten mg/ml of $NaBH_4$ was added to the above mixture and it was allowed to rotate overnight on a rotar. The coupled bead-antibody mixture was separated from the solution by using a magnetic device (MPC-M,

Dynal AS) and the supernatant was discarded. The bead-antibody mixture was washed with sterile distilled water, physiological saline, 0.5M NaCl and 0.06M sterile PBS. The washed magnetic beads coated with antibodies were suspended in 400µl of 0.06M PBS and 0.05% sodium azide as a preservative and kept at 4°C until use[15]. Magnetic bead-bacteria complex was subjected to lysis prior to extraction of DNA by the procedure mentioned above for antral biopsies.

PCR Primers

The primers used were HPU (Ure A) I and II, CagA 1 and CagA 2 and VacA 1 and VacA 2. HPU I (5' GCC AAT GGT AAA TTA GTT 3') and HPU II (5' CTC CTT AAT TGT TTT TAC 3') are 18 bp primers, have Tm=48°C and give a 411 bp sequence on amplification. CagA 1 (5' GAT AAC AGG CAA GCT TTT GAG G 3') CagA 2 (5' CTG CAA AAG ATT GTT TGC GAG A 3'), Tm = 60°C are 22bp primers and give 349bp product on amplification[18-20]. HPU and CagA primers were bought from Scandinavian Gene Synthesis, Kebo Lab, Sweden and VacA primers were developed in-house, at the Department of Medical Microbiology, Lund University, Sweden.

PCR amplification for clinical samples

DNA of all the clinical samples was amplified by HPU–PCR assay. The oligonulceotide primers HPU-1 and HPU-2 correspond to the Urease A gene sequence of *H. pylori* giving a 411bp product on amplification. The PCR mixture consisted of 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.01%BSA, 0.2mM of each deoxynucleotide triphosphate (Boehringer GmbH, Mannheim) was added and the reaction mixture was overlaid with 40µl of mineral oil (Sigma). PCR was performed in a reaction volume of 50µl in a thermal cycler (PTC – 100 MJ Research, Mass, USA). Amplification with the primer pairs HPU-1 and HPU-2 was carried out for 35 cycles (initial cycles of denaturation at 94°C for 45 seconds, subsequent cycles: denaturation at 94°C for 1 minute, primer annealing at 48°C for 30 seconds and extension at 72°C for 45 seconds, and the last cycle : denaturation at 94°C for 1 min, primer annealing at 48°C for 30 seconds and extension at 72°C for 10 minutes). Amplified products (13µl) mixed with gel-loading buffer (0.25% bromophenol blue, 30% glycerol, 40mM EDTA pH-8.0) and were analysed by 2% agarose gel electrophoresis and 1XTBE buffer stained with ethidium bromide (0.5µg/ml). DNA of *Helicobacter pylori* –CCUG 17874 standard strain (obtained from Culture Collection University of Gothenberg, Sweden) was run simultaneously as a positive control and sterile double-distilled water was used as a negative control. PCR products were visualized by excitation under UV light. Similarly, 7 HPU-PCR positive antral biopsy samples, 7 stool samples from same patients were also analysed for CagA and VacA genes. Amplification for CagA and VacA was carried out as described by Peek et al[20].

RESULTS

Rapid urease test

Results were observed within one hour. Change of colour of the medium from orange to pink denoted a positive test. RUT was found to be positive in 23/58 antral biopsies, 8/25 dental plaques collected.

Culture

The samples were streaked on Columbia blood agar plates and plates observed after 3 days. If no growth was observed, the plates were further re-incubated for 2-4 more days. Dental plaques and stool samples showed a mixed growth after 3 days so it was difficult to identify *H. pylori* colony. *H. pylori* like colonies which were catalase, oxidase, urease positive and showed Gram-negative curved rods or rods or cocci were subcultured. Cultures could not be preserved as there was no facility for deep freeze. However, culture reports found to be

Table 1. HPU–PCR assay of dental plaques, antral biopsies and stool samples

Patient No.	Dental plaques	Antral biopsy	Stool
1.	-	+	-
2.	-	-	+
3.	-	+	-
4.	-	+	-
5.	-	+	-
6.	-	+	+
7.	-	+	+
8.	-	+	+
9.	-	+	NT
10.	-	+	+
11.	-	+	+

Table 2. Results of VacA–PCR assay for antral biopsies and stool samples

Patient No.	Antral biopsy	Stool Sample
1.	+	-
2.	-	-
6.	+	+
7.	-	+
8.	-	+
10.	-	-
11.	+	+

Table 3. Result of CagA-PCR assay for antral biopsies and stool samples

Patient No.	Antral biopsy	Stool Sample
1.	+	-
2.	-	-
6.	+	-
7.	-	-
8.	+	-
10.	+	-
11.	+	-

Table 4. Comparison of HPU-PCR assay of antral biopsies with VacA and CagA PCR assay

Patient No.	HPU	VacA	CagA
1.	+	+	+
2.	-	-	-
6.	+	+	+
7.	+	-	-
8.	+	-	+
10.	+	+	+
11.	+	+	+

Table 5. Comparison of HPU-PCR of stool sample with VacA and CagA PCR

Patient No.	HPU	VacA	CagA
1.	-	-	-
2.	+	-	-
6.	+	+	-
7.	+	+	-
8.	+	+	-
10.	+	-	-
11.	+	+	-

H. pylori positive were 38/58 from antral biopsies, 3/15 dental plaques and 9/12 stool samples as *H. pylori*-like colonies were observed and confirmed by biochemical tests.

Polymerase chain reaction (PCR)

Antral biopsies

H. pylori DNA was detected in 17/36 antral biopsy specimens by HPU I and II primers, showed 411bp amplified products on the agarose gel by electrophoresis. The bands of antral biopsies correlate with the control *H. pylori* CCUG–17874 DNA and the molecular weight marker of 100 bp step-ladder showing 100-1000 bp. (Fig. 1).

Stool samples

H. pylori DNA was detected by the same primers only in six of ten stool samples. The positive control used was *H. pylori* strain CCUG – 17874 and the negative control used was sterile double distilled water.

Correlation between antral biopsies and stool samples

A good correlation was found between antral biopsies and stool samples. Five stool samples which were positive by HPU–PCR assay came from the same patients who were antral biopsy positive for *H. pylori* by HPU-PCR assay (Table 1).

Dental plaques

Dental plaques did not show any positive bands for *H. pylori*.

Amplification of DNA of antral biopsies and stool samples for VacA gene

DNA of seven antral biopsies and stool samples of the same patients was amplified for the detection of VacA using VacA primers. DNA of only three antral biopsies was found positive with VacA primers and four in stool samples (Table 2).

Amplification of DNA of antral biopsies and stool samples for CagA gene

DNA of seven antral biopsies and stool samples of the same patients was amplified for CagA gene using CagA primers. Five out of seven antral biopsies showed the positive bands for CagA primers on amplification. CagA was not detected in any of the seven stool samples analysed (Table 3).

Correlation of VacA and CagA primers for antral biopsies

Antral biopsies of 3 patients were found to be VacA and CagA positive, suggesting virulent strains of *H. pylori*.

Comparison of HPU, VacA and CagA primers

Antral biopsies

Only three of seven antral biopsies were found to be positive for all the primers i.e. HPU, CagA and VacA primers (Table 4).

Stool samples

Six of eleven stool samples were positive for HPU primers. Of these 4/7 were HPU and VacA positive. But all seven were CagA negative (Table 5).

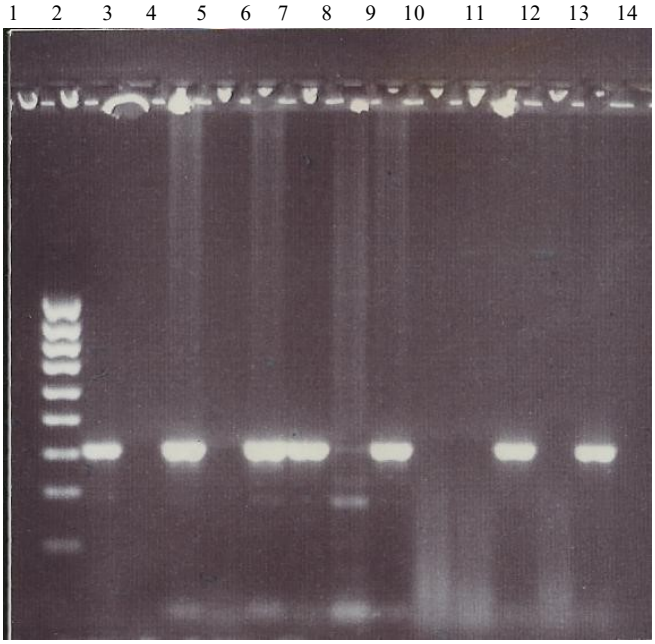

Fig. 1. Agarose gel electrophoresis of 11 antral biopsies showing HPU-PCR assay bands of 411bp DNA after amplification. Lane I shows Molecular weight marker of 100bp step ladder, Lane II positive control *H. pylori* CCUG-17874, Lane III shows negative control as dd/w, Lane IV-XIV shows positive and negative results for antral biopsies

DISCUSSION

Over 50% of the world's population is suffering from *H. pylori* infection. In India, a developing country, the prevalence of *H. pylori* in the general population is high as compared to the western countries. One of the suspected but hitherto unproved modes of transmission is faecal-oral. Over 80% of the population have IgG antibodies, 70% to 90% of patients with duodenal ulcer and 50% to 70% of those with non-ulcer dyspepsia harbor *H. pylori*[24]. The epidemiology of *H. pylori* has been widely studied but the exact mode of transmission still remains uncertain. Therefore, it is necessary to carry out epidemiologic studies to find out the exact mode of transmission of *H. pylori*, so as to carry out preventive measures. Several studies in the west have reported the presence of *H. pylori* in saliva, dental plaque, faeces and water[3,4,10,11]. However, in India such sort of studies were not performed in broad scale until we started with this project in 1995. In our study, different potential reservoirs like dental plaque, antral biopsies, faeces were tested for the presence of *H. pylori* by culture and polymerase chain reaction. We found *H. pylori* in 23/58 APD cases in antral biopsies by RUT and culture was positive for 38/58, in dental plaques by RUT (8/25) and culture (3/15), and in stool samples by culture 9/12 only. PCR was found to be positive for antral biopsies in 17/36 samples for urease primers, 6/10 stool samples for the same primers for *H. pylori*.

Dental plaques from all the eleven symptomatic patients (histopathology positive) were found to be *H. pylori* negative by culture and

IMS- PCR techniques but 8/11 were RUT positive. 3/15 dental plaques were culture positive which were confirmed by urease, catalase and oxidase biochemical tests. Bernander et al[25] cultured dental plaque from 94 consecutive patients attending an endoscopy clinic, 52 of whom had evidence of gastric *H. pylori* infection; none of them had evidence of *H. pylori* in dental plaques. Bickley et al[26] used urease C gene primers for the detection of *H. pylori* from dental plaques of 10 patients; five of these patients were urea breath test positive and 5 were negative. *H. pylori*-specific PCR product was not detected in any of these samples. In contrast to the above reports several authors have reported the presence of *H. pylori* in dental plaques. *H. pylori* has been reported to be isolated from dental plaques of healthy and dyspeptic subjects in India by conventional microbiological techniques and has also been detected in 98% of dyspeptics by RUT which is also in contrast to our studies[5,28]. We found culture positive dental plaques in two samples but PCR negative. Krajden et al[4] were also successful in isolating *H. pylori* from dental plaque of one patient with *H. pylori* positive antral biopsy. Olsson et al and others detected *H. pylori* by PCR in considerable numbers using different primers[6,7,28]. Our HPU primers specific for urease A gene sequence of *H. pylori* did not detect *H. pylori* DNA in any of the dental plaques tested, which suggests that dental plaque may not be a significant reservoir, we had concentrated bacteria by means of immunomagnetic bead separation technique as a pre-PCR step. Thus, it reveals that *H. pylori*–like other bacteria might be surviving in oral cavity which can be positive by conventional techniques like RUT and culture.

Culture of all the ten stool samples in our study gave mixed growth on the plates. It was still possible to identify *H. pylori*–like colonies. But we could not preserve these cultures because to lack of facilities. We identified *H. pylori* bacterium in 6 out of 10 stool samples by HPU-PCR assay and 8/11 culture positive from other adults who were not subjected to PCR test. Thomas et al[29] could isolate viable *H. pylori* from the faeces of an infected adult and 9 of 23 children aged 3-27 months from a Gambian village. They concentrated bacteria by centrifugation in a buffer equilibrated with a microaerophilic gas mixture. They found that strains isolated from stool samples were similar to the strain obtained from gastric biopsy on electrophoretic protein profiles. However, we mixed stool sample with 0.1M PBS, centrifuged and supernatant was streaked on the medium. We found a good correlation between antral biopsies and stool samples; that is 5/11 samples correlated in HPU-PCR assay. In another study by Kelly et al[9], *H. pylori* has been isolated from 12 to 25 adults with dyspepsia in UK. They confirmed 3/8 isolated cultures by PCR by using UreA primers to give a 411bp product and two cultures by CagA primers to give a 400 bp product. However, we found 4/7 stool samples *H. pylori* positive by VacA PCR. 3/7 samples correlated with antral biopsies. Absence of CagA in the stool samples may be due to deletion that covers several kilobases of chromosomal DNA upstream from the CagA gene of *H. pylori*. However, for the expression of VacA, CagA is not necessary and clinical isolates which express only one of two virulence factors have been reported[30]. Reports on direct detection of *H. pylori* DNA from clinical samples on the basis of VacA positivity have also shown that VacA is found in almost 100% of strains[31]. Shimada et al[32] analysed stool samples of 115 Japanese patients who visited their hospital for general check up. They used IMS-PCR method and VacA primers to detect *H. pylori* DNA. They found 29 of 115 samples positive for VacA primers amplifying 474 bp product. These patients were also found to be seropositve. Watanabe et al[33] also used VacA primers to detect *H. pylori* from saliva samples of 57 Japanese patients. They found 18 seropositive patients for *H. pylori* to be VacA positive from saliva samples. VacA and CagA together have also been detected from *H. pylori* strains. Birac et al[34] detected VacA gene in 100 *H. pylori* strains and CagA in 146 of 200 strains. Miehlke et al[35] used two different primer sets for the CagA gene: 297bp region and 1.4Kb region. The 297 bp PCR amplicon was identified on 59 of 60 *H. pylori* isolates from Korea and in 36/41 isolates from the Houston area. The second CagA primer set 1.4 Kb was found in only 1/60 *H. pylori* isolates from Korea and in 36/41 of isolates from the Houston area. They concluded that genetically different *H. pylori* strains may be circulating in different geographic regions. Pan *et al*.,[36] studied the prevalence of infection with CagA–positive *H. pylori* in Chinese patients with peptic ulcer disease

and those with chronic gastritis associated dyspepsia. They detected 99% CagA *H. pylori* isolates. Thus, CagA and VacA are important detection tools of virulent strains of *H. pylori*.

Clayton *et al*.,[19] for the first time in 1992, developed HPU-PCR for the sensitive and specific detection of *H. pylori* in gastric biopsies, with a single primer pair derived from the nucleotide sequence of the urease A gene of *H. pylori*. Their PCR-assay detected *H. pylori* in 15 of 23 gastric biopsies, whereas culturing and microscopy detected *H. pylori* in only seven samples which were also positive by PCR. In contrast to the above study, Zwet *et al*.,[37], using the same primers, reported culture to be as sensitive as PCR for the detection of *H. pylori* and found concordance in 97% of cases. Valentine et al[16] detected *H. pylori* in 13 of 14 (93%) culture positive tissues and 0 to 19 culture negative tissues with the same primers. Hammar et al[38] detected *H. pylori* in 19 antral biopsy specimens by PCR assay using the primers based on the sequences of a species-specific antigen of *H. pylori*. Only 15 of these samples were culture positive for *H. pylori* and 10 patients were positive by serology. In our study, 3/11 were culture positive and 10/11 antral biopsies were positive by using UreA gene sequence HPU-PCR from the same patients. Thus, HPU-PCR is a sensitive and specific method as compared to other methods.

H. pylori was detected by us in six of ten (60%) stool samples by IMS-PCR as an integrated method by using HPU-UreA primers. Enroth *et al*.,[11] also used IMS as a pre-PCR step to remove Taq-polymerase inhibitors from faeces and to facilitate direct detection of *H. pylori* in spiked stool, spiked water specimens and a patient's stool specimen. The primers used in their assay were the 375bp segment of the adhesion-encoding gene. Nilsson *et al*.,[39] used IMS–PCR for detecting *H. pylori* from stool samples of patients with gastritis and esophagitis. They detected *H. pylori* DNA in 11 of 17 (57%) samples by urea primers. Our results are consistent with this report. Other studies have also demonstrated the detection of *H. pylori* from faeces by PCR. Mapstone *et al*[10] detected *H. pylori* from 28 of 31 faecal samples from dyspeptic patients by 16S rRNA-PCR assay. Ho et al and others also detected *H. pylori* in faecal samples by the same primers but found to be non-specific[14,40,41]. It is probable that the organism has more than one route of transmission[42-45], but we believe that the faecal-oral route plays a predominant role as other studies also support. Reports regarding culture negative and RUT positive dental plaques have also been proved[46-48]. No significant association between *H. pylori* of dental plaque and the stomach has been found and also dental plaque cannot be used as a primary diagnostic aid for gastric infection[49]. A report with saliva 10.72%, dental plaque 0%, gastric biopsy 77.66% and stool 71.67% has shown that it supports faecal-oral route[50]. In another laboratory, it has been found a good correlation among gastric biopsies, oral and stool samples with 66.7% of patients positive for *H. pylori* antigen in stools[48]. Now-a-days, stool antigen test has become popular which is a non-invasive test specially for children showing symptoms of gastritis[56]. Stool antigen test has shown a good diagnostic performance as compared to serology and PCR[51-58]. In conclusion, we can say that our diagnosis for *H. pylori* from Mumbai patients in India confirms faecal-oral route of transmission as compared to oral-oral route. Further studies on more number of individuals will surely show a torch to combat with this infection.

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