Comparison and Detection of *Helicobacter pylori* in Stool Specimens by use Polymerase Chain Reaction technique and immunochromatographic assay

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Abstract

There are several types of immunological tests available for the diagnosis and management of *Helicobacter pylori* infection. Most commercially available serological kits use the immunochromatographic assay, and has been developed for the detection of *Helicobacter pylori* in faeces as well as the polymerase chain reaction for the same purpose. The aim of the study is therefore to detect *H. pylori* from the stool of patients by PCR and compare results obtained with ICT. A total of 42 stool samples from patients presenting were screened for immunochromatographic assay (ICT) and the presence of *H. pylori* DNA using stool-PCR. Out of 42 stool samples analyzed, 31 (73.8%) were positive for *Helicobacter pylori* in ICT and 20 (47.61%) positive for *H. pylori* by PCR, through amplification of cagA samples and VacA genes respectively. the cagA gene was detected in 12 (38.7%) and VacA was detected in 8(25.8) samples. The sensitivity and specificity of the cag A gene compared with ICT is 38.7% and 100% respectively while of the VacA genes compared with ICT is 25.8% and 100% respectively. It is concluded that PCR may be useful for the diagnosis of *H. pylori* from stool in comparison with Immunochromatographic.

Keyword: PCR, cag A, Vac A, immunochromatographic, *H.pylori*.
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المقارنة والكشف عن الملوية البوابية في عينات البراز باستخدام تقنية سلسلة تفاعلات البلمرة والفحص المناعي

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الخلاصة

هناك عدة أنواع من الاختبارات المناعية المتاحة لتشخيص العدوى ببكتيريا الملوية البوابية، ومعظم المجموعات المصلية المتاحة تكون تجارية وتستخدم للفحص المناعي، ولقد تم تطويره للكشف عن الملوية البوابية في البراز فضلا عن سلسلة تفاعل البلمرة لنفس الغرض. إن الهدف من هذه الدراسة هو الكشف عن بكتيريا من البراز من المرضى عن طريق PCR ومقارنة النتائج التي تم الحصول عليها مع تقنية الفحص المناعي. تم فحص ما مجموعه 42 عينة من البراز للمرضى الذين يعانون اعراض الاصابة ومن أصل 42 عينة من البراز، كانت 31 (73.8٪) إيجابية للهيليكوباكتر بيلوري باستخدام الفحص المناعي و20 (47.61٪) إيجابية للبكتيريا عن طريق PCR، من خلال تضخيم الجين cagA والجين vacA على التوالي. تم الكشف عن الجين cagA في 12 (38.7٪)، والكشف عن vacA في 8 (25.8) عينة. ان درجة الحساسية والخصوصية للجين cagA باستخدام الدوال الاحصائية ومقارنتها مع الفحص المناعي كانت 38.7٪ و100٪ على التوالي في حين ان الجين vacA مقارنة الفحص المناعي كانت 25.8٪ و100٪ على التوالي. وخلص إلى أن PCR تكون مفيدة لتشخيص بكتيريا من البراز بالمقارنة مع الفحص المناعي.

الكلمة المفتاحية: سلسلة تفاعلات البلمرة، جين cagA، جين vacA، الفحص المناعي، ببكتيريا الملوية البوابية.

Introduction

Helicobacter pylori is the major causative agent of chronic active gastritis in humans, and infection with this organism is an important etiological factor in the pathogenesis of peptic ulcer and gastric cancer (1). However, most people harboring *H. pylori* are asymptomatic, and only a few patients infected with this bacterium develop peptic ulcer or gastric cancer. One possible explanation for this phenomenon is that patients with serious gastroduodenal lesions are infected by virulent *H. pylori* strains, whereas those with simple chronic gastritis and no ulcer or cancer are infected by organisms with low pathogenic potential. One important virulence factor is a vacuolating cytotoxin (VacA) that induces the formation of intracellular vacuoles in eukaryotic cells in vitro (2, 3, 4) Thus, individual infected with strains that express these virulence factors probably will develop sever local inflammation that may induce the development of peptic ulcer and gastric cancer(5). *H. pylori* consists of a large diversity of strains, and the genomes of three have been completely sequenced (26695, J99 and a chronic atrophic gastritis strains) (6, 7). The genome of the strain "26695" consists of about 1.7 million base pairs, with some 1.550 genes. The two sequenced strains show large genetic differences, with up to 6% of the nucleotides differing. Study of the *H. pylori* genome is centered on attempts to understand pathogenesis, the ability of this organism to cause disease. Approximately 29% of the loci are in the "pathogenesis" category of the genome database. Two of sequenced strains have an approximately 40-kb-long Cag pathogenicity island (a common gene sequence believed responsible for pathogenesis) that contains over 40 genes (8). The genotypes of *H. pylori* clinical isolates vary in many genetic loci, including the presence or absence of a pathogenicity island (9) and allelic variation of the vacuolating cytotoxin gene (vacA) (10) and genes encoding adhesion molecules such as BabA2, which binds the Lewisb fucosylated moiety found on human gastric tissue (11). Epidemiological studies suggest that strains expressing the pathogenicity island, those expressing high levels of VacA, and those expressing functional BabA2 correlate with more severe disease (12). The cagA gene codes for one of the major *H. pylori* virulence proteins. Bacterial strains that have the cagA gene are associated with an ability to cause ulcers (13). The pathogenicity of *H. pylori* may be increased by genes of the cag pathogenicity island. About 50-70% of *H. pylori* strains in Western countries carry the cag pathogenicity island (cag PAI) (14). Western patients infected with strains carrying the cag PAI have a stronger inflammatory response in the stomach and are at a greater risk of developing peptic ulcers or stomach cancer than those infected with strains lacking the island (15). Following attachment of *H. pylori* to stomach epithelial cells, the type IV secretion system expressed by the cag PAI "injects" the inflammation-inducing agent, peptidoglycan, from their own cell wall into the epithelial cells. The injected peptidoglycan is recognized by the cytoplasmic pattern recognition receptor (immune sensor) Nod1, which then stimulates expression of cytokines that promote inflammation (16).

Material and Methods

Convenient sampling stool samples from 41 patients at the private laboratory all the patients were complain various gastrointestinal symptoms. The stool samples were collected using sterile toothpicks into eppendorf tubes containing 700 µl absolute ethanol at room temperature. The samples was considered *H. pylori*- positive when VacA gene or both genes (VacA and cagA) were detected by PCR, and the results compared with those from ICT as the standard and confirmatory test (17).

- **Immunochromatographic assay:** The Linear *H. pylori* Ag is a qualitative immunochromatographic assay for the determination of Helicobacter pylori in faeces samples. The membrane is pre-coated with monoclonal antibodies, on the test band region, against *H. pylori* antigens. During testing, the sample is allowed to react with the colored conjugate (anti-H. pylori monoclonal antibodies-red polystyrene micro spheres) which was pre-dried on the test strip. The mixture then moves upward on the membrane by capillary action. As the sample flows through the test membrane, the colored particles migrate. In the case of a positive result the specific antibodies present on the membrane will capture the colored conjugate. The mixture continues to move across the membrane to the immobilized antibody placed in the control band region, a green coloured band always appears. ImmunoCard STAT! HpSA test (Meridian Bioscience-German) is an invitro qualitative procedure based on a lateral flow chromatographytechnique that detects bacterial antigens using monoclonal anti-*H. pylori* antibodies. Using an applicator stick, a small portion of the stool sample is transferred into a diluent vial. After vortexing or hand-shaking for 15 second, four drops are dispensed into the window of the device containing immobilized anti-*H. pylori* monoclonal antibodies and the result is read after 5 min.ImmunoCard STAT! HpSA results were interpreted according to the manufacturer's instructions. The test was considered negative if only one blue colored band (control line) appeared across the central window of the device, and positive when, in addition to the control line, a distinguishable pink-red band (test line) also appeared across the central window of the device. As specified by the manufacturer's recommendations', any pink-red line, even weak, was considered as positive result. Tests were also considered as invalid if the control band was absent. The biologist who performed ImmunoCard STAT! HpSA (FP) was blinded to the results of all other tests carried out.
- **DNA analysis:** The QIAamp DNA Stool Mini Kit is designed for rapid purification of total DNA from up to 220 mg stool and is suitable for both fresh and frozen samples. A special protocol is provided for isolating DNA from larger amounts of stool. Purification requires no phenol chloroform extraction or alcohol precipitation, and involves minimal handling. DNA is eluted in low-salt buffer and is free of protein, nucleases, and other impurities or inhibitors. The purified DNA is ready for use in PCR and other enzymatic reactions, or can be stored at -20°C for later use.
- **Determination of DNA concentration, yield, and purity:** DNA yields are determined from the concentration of DNA in the elute, measured by absorbance at 260 nm. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7-1.9. Absorbance readings at 260 nm should lie between 0.1 and 1.0 to be accurate. Sample dilution should be adjusted accordingly. Use elution buffer or water (as appropriate) to dilute samples and to calibrate the spectrophotometer. Measure the absorbance at 260 and 280 nm, or scan absorbance from 220-320 nm (a scan will show if there are other factors affecting absorbance at 260 nm).

Concentration (µg/ml) = (A260 reading – A320 reading) × dilution factor × 50µg/ml

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume. DNA yield (µg) = DNA concentration × total sample volume (ml)

cagA gene (325 bp)

PCR amplification using this primer was carried out using the primer set cagA-

F: 5' - CAAGCAGGCTTCCCTTTGAG- 3' R: 5' -TGCCGGTCGCTTTTTCATTG - 3' NCBI. The length of primer was 20 bp and the Product length will have 325. The 25 µl reaction

mixture consisted of x1 PCR buffer, 1.5 mM Magnesium Chloride, 200 µM of each dNTP, 20pmol of each primer and 1U Taq DNA polymerase (Promega company). Amplification was carried out in an Eppendorf Mastercycler gradient using the following cycling parameters: an initial denaturation at 94°C for 5 min and 40 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min. This was followed by a final extension of 72°C for 5 min. The PCR product was separated on a 2% Agarose gel and 100 bp ladder was used as DNA molecular weight standard (18).

VacA gene (216 bp)

The following primers were used:

F: 5' - ACCCTTGGGGCACATCAAAA -3' R: 5' - CGGTCGCGCTGTCTATATCA -3' NCBI(2013) F started at (1910-1929) and R was started at (2125-2106). The 25 µl reaction mixture consistedof x1 PCR buffer, 1.5 mM Magnesium Chloride,200 µM of each dNTP, 20pmol of each primer and 1U Taq DNA polymerase (Promega company). The following were the conditions for amplification: one cycle of denaturation at 94°C for 5 min; 35 cycles at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 2 min, followed by a final elongation step by 1 cycle at 72°C for 7 min. 15°C and amplification was carried out in an Eppendorf Master cycler gradient (Hamburg). The PCR product was separated on a 2% Agarose gel with 100 bp of DNA ladder (19).

- **Detection and analysis of amplified PCR product Southern hybridization:** Ten microliter amounts of each PCR product were electrophoresed on agarose of 2% gel (Bangalore Genie, India) which was stained with ethidium bromide and visualized under UV light. Southern blots were performed on randomly selected PCR positive samples to confirm that the amplified product was from H. pylori. The sensitivity and specificity were calculated in the traditional methods.

Result

The sample were positive for immune-chromatographic assay that detects bacterial antigens using monoclonal anti-H. pylori antibodies, Out of 42 stool samples analyzed, *H. pylori* positive were detected in 31 (73.8%), by immunochromatographic assay (ICT). The detection of cagA positivity by PCR was observed in 31 out of 42 (73.8) samples were positive for *H.pylori* by ICT (Table 1) . those simultaneously positive for stool- PCR (cag A gene) and ICH were 12 (38.7%) while 19 (61.3%) were positive for ICT and negative for stool- PCR using Cag A gene . On the other hand, the Vac A gene was positive for stool- PCR and ICT for 8 sample out of 31 (25.8%) while 23 sample (74.2%) were positive for ICT and negative for stool-PCR using Vac A gene. Table 2 shows the evidenced genes (cag A and Vac A). The sensitivity and specificity of the gene cag A compare with ICT is 38.7% and 100 respectively while the Vac A gene compare with ICT is 25.8% and 100 respectively (Fig. 1 and 2).

Table (1) Results of stool PCR test using cagA and gene VacA with ICT

ICH (n-42)	**Results for stool-PCR, cag A gene (n = 42)**					
		Positive	**Negative**	**Total**	**Sensitivity**	**Specificity**
	Positive	12	19	31	38.7%	100%
	Negative	0	11	11		
	Total	12	30	42		
	Results for stool-PCR, Vac A gene (n = 42)					
		Positive	**Negative**	**Total**	**Sensitivity**	**Specificity**
	Positive	8	23	31	25.8%	100%
	Negative	0	11	11		
	Total	8	34	42		

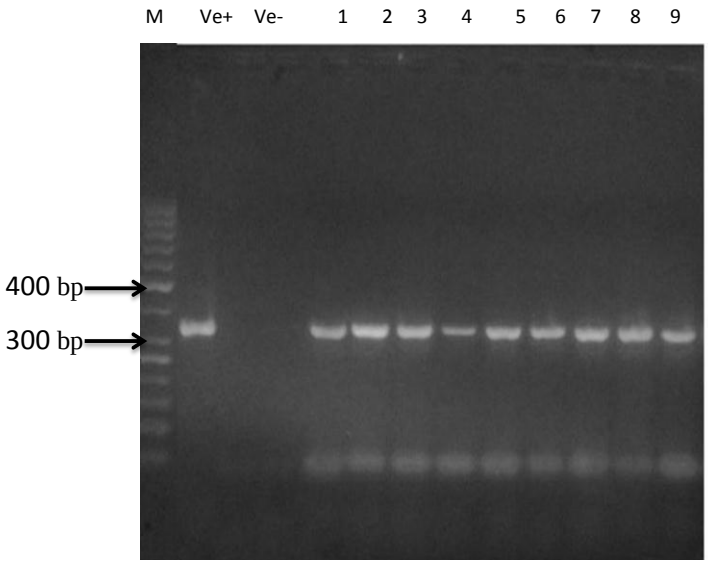

Fig. (1). Gel electrophoresis 2% of PCR products amplified using the cagA gene of *Helicobacter pylori*: M: molecular weight marker; ve+: positive control, ve-: negative control; 1-9 samples.

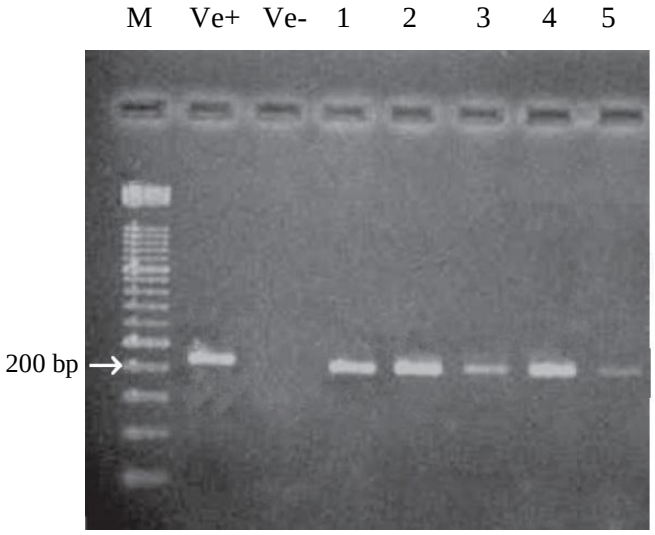

Fig. (2). Gel electrophoresis 2% of PCR products amplified using the VacA gene of *Helicobacter pylori*: M: molecular weight marker; ve+: positive control, ve-: negative control; 1-5 samples.

Discussion

H. pylori infections can cause serious clinical problems, such as atrophic gastritis, gastric or duodenal ulcers, and gastric cancer. Therefore, it is important that accurate diagnostic methods are available (20). There is increasing evidence that the genetic variability of *H.pylori* may also have clinical importance. Three different genes that are associated with bacterial pathogenicity have been described,i.e., cagA, vacA, and iceA (21,22) Immunochromatographic and PCR tests are play an important role in the diagnosis of *H. pylori* infections. However, all have their limitations in terms of cost, specificity, and sensitivity. the sensitivity and specificity was 38.7% and 100% respectively for stool-PCR, cag A gene and 25.8%, 100% respectively for stool-PCR, Vac A gene. The sensitivity is low. Our study result was agree with those observed by Aktepe *et al.* using five methods of detection of *H. pylori*, the stool-PCR had the lowest sensitivity (21%). However, Makristathis *et al*. developed a semi-nested PCR assay, targeted to aspecies-specific protein antigen which is present in all strains of *H. pylori*, for detection and follow-up of *H.pylori* infected patients and the sensitivity was as high as 93.7% and the specificity was 100%.

Another study by (23) corroborated the high sensitivity of stool-PCR in both pre-eradication and post-eradicationto be 72.5% and 97.1% and the authors concluded that stool-PCR was better indicator than HpSA test in posteradication assessment of infection. Another report by (24) revealed sensitivity of stool-PCR of 73%. In this study, cagA gene was present in 38.7% of the samples positive for *H. pylori* by ICT and in a related study by (25) cagA gene was found to be present in 22.7% this is lower than in our study. cagA positive *H. pylori* strains have been associated with severity of disease outcome and plays a critical role in the development of stomach cancer. From previous studies with gastric biopsy specimens, cagA positive *H. pylori* was found in 68% of the biopsy specimens (26), while from a related study by (25) 59% of the biopsy samples were positive for cagA gene, when compared with 22.7% from stool samples. While VacA gene was present in 25. 8% of the samples positive for H. pylori by ICT these results are consistent with other studies (27, 28, 29) and consistent with the findings of (30) who found a prevalence of 45% for VacA gene in Colombian patients with chronic gastritis (30) this is results more than in our study. The presence of *H. pylori* DNA was found in stool samples (64.5%). The results show that to some extent the cagA gene and VacA gene for detection of *H. pylori* DNA by PCR may be useful for the diagnosis of *H. pylori* from stool. In comparison with Immunochromatographic. **Conclusions,** Although there are many well validated methods that can be used to detect *H pylori*, there are nevertheless continued improvements to current tests and the development of new tests, driven by considerations of cost and a search for speed and technical simplicity. An increasing number of patients may be treated by their general practitioners and consequently there is a requirement for a rapid, simple, accurate, near patient test. Furthermore, the emergence of antibiotic resistant isolates has given an impetus to the development of rapid molecular techniques to identify such resistant organisms. The current main areas of development in diagnostic tests are in the use of non-invasive salivary antibody and faecal antigen tests, the use of rapid near patient antibody tests, and the development of DNA immunoassays for the detection of both antibiotic sensitive and resistant *H. pylori.*

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