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Helicobacter pylori vacA gene detection in saliva of patients with upper gastrointestinal disorders in Accra, Ghana

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Abstract: *Helicobacter pylori* play an essential role in the pathogenesis of upper gastrointestinal disorders. The diagnostic role of the bacterium thus has been a subject of intense investigations. In this study we used an immune-chromatographic method and the polymerase chain reaction (PCR) to detect *H. pylori* in the saliva of patients with clinically diagnosed upper gastrointestinal disorders. Thirty such patients reporting to the Korle-Bu Teaching Hospital (Accra, Ghana) for upper gastrointestinal endoscopy consented for this study. Saliva samples were collected from each subject and analysed for *H. pylori* antibodies using a rapid immuno-chromatographic assay and *H. pylori* DNA by nested PCR using specific primers. Ten (33.3%) out of the 30 samples tested positive for the saliva antibody test with the most prevalent gastrointestinal disorders among the positive subjects being peptic ulcer (60%) followed by gastritis (30%) and esophagitis (10%). Following nested PCR analysis, a 346bp fragment of the *vacA* (m2) gene region of *H. pylori* was amplified in 9 (90%) out the 10 samples that were positive by the rapid immuno-chromatographic assay. Saliva samples could serve as a reliable non-invasive alternative to detect the presence of *H. pylori* infection in synergy with available diagnostic methods in Ghana.

Keywords: *Helicobacter Pylori,* Saliva, Polymerase Chain Reaction

1. Introduction

Helicobacter pylori bacteria are responsible for most upper gastrointestinal diseases. The bacterium causes gastritis, peptic ulcers, and over time, gastric cancer [1]. Over 80% of individuals infected with *H. pylori* are asymptomatic [2]. It is estimated that *H. pylori*-positive patients have a 10 to 20% lifetime risk of developing ulcer disease and a 1 to 2% risk of developing distal gastric cancer [3]. In developing countries *H. pylori* contribute to diarrhea, malnutrition and growth failure in young children. Extra gastric disorders such as coronary heart disease, iron deficiency anemia, dermatological disorders, autoimmune thyroid disease and thrombocytopenic purpura, have all been associated with *H. pylori* [4],[5].

Diagnostic tests available for *H. pylori* detection consist of invasive and non-invasive procedures with their respective advantages and disadvantages. The invasive test is based on gastric specimens from endoscopy for histology, culture, urease test or other methods. The non-invasive tests are based on peripheral samples such as blood, breath samples, stools, urine, or saliva for detection of antibodies, bacterial antigens, or urease activity [6],[7]. Though direct diagnosis of *H. pylori* via endoscopy is considered a gold standard method a major problem associated with this diagnostic method is inherent in the invasiveness of the procedure. The cost of endoscopy is also high in developing countries [8],[9].

Studies have shown that *H. pylori* DNA and antibodies could both be detected in saliva of infected patients using an immuno-chromatographic method and PCR [10],[11],[12]. Diagnostic procedures that avoid the use of endoscopy would therefore be beneficial to patients and researchers. With collective effort over the years, salivary

diagnostics have exhibited tremendous potential in clinical applications. This is because saliva has been demonstrated to be a promising body fluid for easy detection of diseases [13]. The salivary diagnostic method largely diminishes discomfort associated with endoscopy and predominant issues related to blood, stool and urine collection. The method avoids the need for blood or biopsy samples in epidemiological studies where large-scale *H. pylori's* screening is required. Unlike other diagnostic procedures, PCR does not only detect the bacterium present but also detects specific genes relevant to pathogenesis and mutations associated with antimicrobial resistance [14]. Further studies directed towards understanding of this diagnostic procedure would be beneficial to *H. pylori* diagnosis and its related epidemiological research.

In this study we used nested PCR analysis and immuno-chromatographic dip stick to detect *H. pylori* in saliva of patients with clinically diagnosed upper gastrointestinal disorders.

2. Materials and Methods

2.1. Study Subjects

Patients clinically diagnosed with upper gastrointestinal disorders and who reported to the Korle-Bu Teaching Hospital (KBTH) for upper gastrointestinal endoscopy were recruited.

2.2. Ethical Issues

Ethical clearance was obtained from the Ethics and Protocol Review Committee of the School of Allied Health Sciences, College of Health Sciences, University of Ghana. Appropriate approval was also obtained from the Medical Directorate of KBTH. All patients gave their consent.

2.3. Saliva Sample Collection

The protocol for saliva collection and processing was derived from the "Salivary Proteome Handbook Procedures and Protocols" [15]. Saliva collection was done prior to endoscopy after subjects' demographic data consisting of age, sex and clinical diagnosis was taken. Each of the subjects was given a 10ml sterile Falcon tube with the pathological number correctly assigned to it.

Subjects were then given drinking water and asked to rinse their mouth out well without drinking the water. They were asked to refrain from talking and to bow their heads to let the saliva run naturally to the front of the mouth. Subjects were alerted not to cough up mucus as saliva is collected and not sputum. They were asked to spit whole saliva into the given sterile Falcon tubes. Collected samples were kept on ice at all times prior to processing. Saliva samples were then taken to the laboratory for investigation.

2.4. Saliva Antibody Test

A commercial kit (HiSens *H. Pylori* antigenic cassette, HBI Co. Ltd., Korea), a rapid immuno-chromatographic assay was used for the qualitative detection of saliva antibodies to *H. Pylori.* All specimens and test devices were brought to room temperature (25^{o}C) for 15-30 minutes before testing as recommended by the manufacturer.

2.5. Genomic DNA Extraction

Genomic DNA was extracted from the stored saliva samples using the QIAGEN DNA Mini Kit (QIAGEN Co. Ltd, USA) following the manufacturer's instruction. The extracted genomic DNA was stored at -20^{o}C until required for use.

2.6. Nested PCR Analysis

The *Helicobacter* genome *vacA* signal sequence and middle regions of the DNA were analyzed by PCR as previously described [16] with some modifications. The sequence details of the primers are given in Table 1. Each DNA amplification reaction was carried out in a final volume of 25µl and consisted of 1X PCR buffer plus $MgCl_2$ (OneTaq® Standard Reaction Buffer Pack, New England Biolabs, MA, USA), 1U of *Taq* polymerase, 200 µM of each dNTP and 500nM of each primer.

The first amplification reaction used 10µl of the extracted DNA as the template for each PCR whilst the second amplification was accomplished by using 1µl of the first product as the DNA template for each PCR. Each PCR reaction mix was thoroughly mixed before the amplification. The PCR reaction profile used in both the first and second amplifications involved an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 50s, annealing for 1 min at 55°C for vac1F-vac1R and 60°C for vac3F-vac3R and vac4F-vac4R, extension at 72^{o}C for 1 min, and a final extension at 72°C for 2 min.

The amplification products were analysed by gel electrophoresis in 2% agarose gels stained with 0.5µg/ml ethidium bromide. Ten microliters of each sample was added to 2µl of orange G (5X) gel loading dye for the electrophoresis. A hundred base pair DNA molecular weight marker (Sigma, MO, USA) was run alongside the PCR products. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system at 100 volts for one hour and the gel photographed over a UV trans-illuminator.

3. Results

3.1. Demographic Data

A total of thirty (30) patients involving fifteen (15) males and fifteen (15) females who were reporting for upper gastrointestinal endoscopy at KBTH consented for this study. Their ages ranged from five to 90 years. The mean age for males was 44.07 ± 0.05 years whilst that of females was 45.80 ± 0.02 years. The 40-49 age group had the highest number of patients (Table 2).

Table 1. *Oligonucleotide primer sequences used in amplification [16]*

Gene	Region amplified	Primer designation	Primer sequence	PCR product size (location)
vacA	s1[a]	vac1F	5'GAAATACAACAAACACACCGC3'	201 (800-1000)[b]
		vac1R	5'GGCTTGTTTGAGCCCCCAG3'	
	s2[a]	vac1F	5'GAAATACAACAAACACACCGC3'	228 (349-576)[c]
		vac1R	5'GGCTTGTTTGAGCCCCCAG3'	
	m1	Vac3F	5'GGTCAAAATGCGGTCATGG3'	388 (2741-3128)[b]
		Vac3R	5'CATCAGTATTTCGCACCACA3'	
	m2	Vac4F	5'CCAGGAAACATTGCCGGCAAA3'	346 (2290-2635)[c]
		Vac4R	5'CATAACTAGCGCCTTGCA3'	

[a]PCR products of regions s1 and s2 were differentiated on the basis of molecular size and restriction endonuclease digestion with NlaIII .[b]Location in published vacA sequence of strain 60190 (8). [c]Location in strain Tx30a (GenBank accession no. U29401).

The commonest reasons underlying the request for upper gastrointestinal endoscopy among subjects included dyspepsia (20%), epigastric pain (10%), dysphagia (25%), and hematemesis and melena (45%).

3.2. Saliva Antibody Test

In this study, 10 (33.3%) out of the 30 patient samples tested positive for the saliva antibody test (Table 2). The most prevalent gastrointestinal disorder among the positives was peptic ulcer (n=6), followed by gastritis (n=3), then esophagitis (n=1).

3.3. Nested PCR Analysis

With the nested PCR analysis, only the 346 bp DNA fragment of the *vacA* (m2) gene region (Fig. 1) was amplified in 9 (90%) out the 10 samples that were positive by the saliva antibody test.

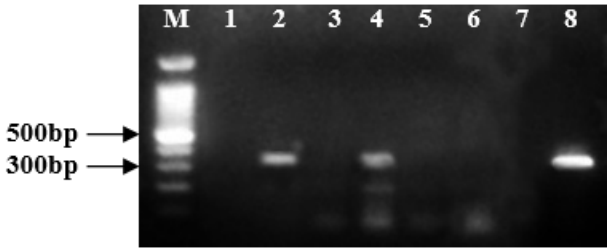

Figure 1. *Sample electrophoregram of ethidium bromide stained 2% agarose gel of PCR products. Lane M =100bp marker; Lanes 1,3,5,6 and 7 = PCR negative samples; Lanes 2, 4 and 8 = PCR positive samples.*

Table 2. *Distribution of H. pylori positive and negative subjects among the various age groups following the salivary antibody test*

Age group	No. of patients	*H. pylori* positive (%)
0-9	1	1 (100)
10-19	2	1 (100)
20-29	5	1 (20)
30-39	3	2 (67)
40-49	9	2 (22)
50-59	2	0 (0)
60-69	2	1 (50)
70-79	3	1 (67)
80-89	3	1 (33)
Total	30	10 (33)

4. Discussion

Helicobacter pylori are recognized as important cause of chronic gastritis, playing a vital pathogenic role in the development of peptic ulcer disease and gastric adenocarcinoma [17]. Polymerase chain reaction protocols have evolved over time in the detection of *H. pylori* in clinical specimens [18]. There is evidence that the mouth may serve as a reservoir for *H. pylori* infection [10].

Studies have shown that *H. pylori* DNA and antibodies could be detected in saliva of infected patients [10],[11],[12]. This is because saliva, a multi constituent oral fluid has been demonstrated to be a promising body fluid for easy detection of diseases [13]. In this work, ten (33.3%) out of thirty samples tested positive for *H. pylori* saliva antibody. Nine out of the ten samples were positive by the nested PCR analysis. The results agree with reports from similar studies [8],[12],[11]. According to these studies saliva could serve as a reliable non-invasive alternative for detection of *H. pylori* infection. Conversely, the results disagree with the reports by other researchers [9],[19] that saliva is not a suitable sample for *H. pylori* diagnosis. Other studies have shown variable prevalence of *H. pylori* DNA in patients with proven *H. pylori* infection: 47% [20] and 30% [21]. However, another PCR study had a high rate of detection of *H. pylori* DNA in saliva of 75% of patients with proven gastric *H. pylori* infection [10].

Possible explanations for these differences may be inherent in the variations in either saliva collection or the use of whole saliva samples instead of pellets from saliva for DNA extraction [10]. Methodology may therefore play a part in sensitivity of testing. Peptic ulcer (60%) and other upper gastrointestinal disorders like, gastritis (30%) and esophagitis (10%) were observed as the commonest disorders among the *H. pylori* infected subjects. These disorders are known to be associated with *H. pylori* colonization [3],[5].

The results showed no gender bias in the prevalence of the bacteria. Interestingly, the only sample from a 5 year old subject, tested positive in agreement with the hypothesis by Fiedorek et al. [22], Kuiper et al. [23] and Cover et al. [24] that *H. pylori* infection usually occurs at early childhood especially at the age of five.

5. Conclusion

Saliva samples could serve as a reliable non-invasive alternative to detect the presence of *H. pylori* infection in synergy with available diagnostic methods in Ghana.

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