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Helicobacter Pylori Detection by Nested PCR in Rapid Urease Negative Gastric Biopsy Samples

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Abstract

Background: Helicobacter pylori infection is the most prevalent in developing countries. It is an etiological agent of peptic ulcer, gastric adenocarcinoma, and mucosal-associated lymphoid tissue (MALT) lymphoma. Despite the development of different assays to confirm H. pylori infection, the diagnosis of infection is challenged by the precision of the applied assay. Hence, this study aims to assess the diagnostic accuracy of Nested PCR using a highly specific and sensitive new untested Primer pair, especially against the routinely and commonly performed chairside Rapid Urease card test. Methods: Gastric biopsy samples were collected from patients exhibiting Helicobacter pylori infection during endoscopy, and a rapid urease card test was conducted. From twenty out of those rapid urease negative samples, DNA extraction was performed, followed by amplification using two primer pairs (Pylo A and Pylo AN) synthesized from the 16s rRNA gene of Helicobacter pylori genome. Results: Twelve (60%) of the rapid urease card test-negative gastric biopsy samples tested positive with Nested PCR. Conclusions: The results indicate that the Nested PCR is more sensitive and specific than the Rapid urease test for detecting Helicobacter pylori in gastric biopsy samples. Consequently, due to its superior diagnostic accuracy, it should be consistently utilized for patients with Gastroduodenopathy to avert any potential misdiagnosis.
Keywords: - Helicobacter Pylori, Rapid Urease Test, Nested PCR.

1. Introduction

Helicobacter pylori (H. pylori) is a Gram-negative bacterium that significantly contributes to the development of gastrointestinal disorders, including peptic ulcers, low-grade B-cell lymphoma (MALT lymphoma), and gastric cancer [1, 2]. Numerous epidemiological studies have also demonstrated that individuals infected with H. pylori exhibit a higher incidence of gastric carcinoma [3]. The prevalence of H. pylori varies significantly among different populations and countries. This variation is influenced by socioeconomic conditions. In developing nations, the prevalence is about 90%, whereas in developed countries, it is around 50% [4, 5]. Moreover, both gastric cancer and peptic ulcers account for over a million deaths annually worldwide, thus underscoring their significance as major public health issues [6, 7].

Diagnostic methods for H. pylori encompass both invasive and noninvasive approaches that employ either direct or indirect in nature techniques. Microscopic detection of the bacteria and culture represent direct methods, whereas demonstrating urease production and detecting stool antigen or antibodies are considered indirect methods that serve as markers of infectious disease response. Advancements in molecular methods are now widely employed as reliable tools for diagnosing infectious diseases, owing to their improved sensitivity and specificity [8]. Due to resource limitations, diagnostic methods such as the urea breath test or invasive bacterial culture of biopsied tissue are not conducted in our setting. Similarly, the reliability of immunological tests is often a subject of debate.

In recent years, the utilization of molecular methods like polymerase chain reaction (PCR) has transformed diagnostic approaches for detecting H. pylori. Furthermore, it enables the tracking of various genetic alterations in the bacteria, thus facilitating understanding of drug resistance characteristics [9] and coinfection with other pathogens in gastric diseases [10].

The molecular approach has facilitated the comparative analyses between conventional methods such as microscopy and the rapid urease test (RUT) with PCR in resource-limited settings, thus enhancing the effectiveness of diagnosis and treatment. In our setting, to leverage the availability of molecular methods, a comparison between RUT and PCR was conducted to assess the efficacy of each method, hence contributing to further evaluation of the study. Identifying H. pylori infection in gastroduodenal diseases is of paramount importance as it prevents potential gastrointestinal malignancies. In developing countries like India, the prevalence of H. pylori is significantly higher in cases of duodenal ulcer, gastric ulcer, and gastritis. Hence, this study aims to assess the efficacy of two techniques, namely Nested PCR and RUT in detecting H. pylori in gastric biopsy specimens.

2. Methods

Gastric biopsy samples were obtained from 50 patients (33 males and 17 females) with an age range of 20-69 years who underwent endoscopy for upper gastrointestinal complaints at the Gastroenterology Department of PBM Hospital associated with Sardar Patel Medical College. Patients who had been prescribed non-steroidal anti-inflammatory drugs, bismuth compounds, proton pump inhibitors, oral anticoagulants, or antibiotics with efficacy against H. pylori within the preceding two weeks were excluded. Additionally, individuals who had recently undergone blood transfusions, gastric surgery, or had bleeding diathesis were also excluded from the study. From patients showing findings commonly associated with H. pylori infection (Antral Gastritis, Gastric and Duodenal Ulcers), two gastric biopsy specimens were collected from the antrum of the stomach and Rapid Urease test was performed chairside from one of them in the endoscopy room and the other sample was immediately frozen at -40 degrees Celsius in Gastroenterology department, to be used later for PCR after being transferred to the Multidisciplinary Research Unit (MDRU) Laboratory of Institute Sardar Patel Medical College.

One specimen was rapidly examined for the presence of H. pylori by RUT Card (Gastro Cure Systems, Kolkata, India). In the RUT, the biopsy material was placed into gel containing a pH indicator that changed color from yellow to red/pink within 2-10 minutes if H. pylori was present, due to the production of ammonia by the organism's urease enzyme. (Figure 1)

DNA extraction was performed from the other specimen according to HiPurA Multi-Sample DNA Purification Kit (HiGenoMB, HIMEDIA, Maharashtra, India) protocol. DNA extracts were stored at –20°C until used for PCR.

A highly sensitive and specific Oligonucleotide sequence Pylo A & Pylo AN from 16s rRNA of H. pylori genome was chosen and ordered to be synthesized by Bioserve Biotechnologies Company (Hyderabad, India). [11]

Five microliters from all DNA extract samples underwent a two-step nested PCR using two primer pairs from the 16s rRNA gene of the H. pylori genome. The outer primer pair Pylo A was 5'- TTGATCCTGGCTCAGAGTGAACG-3' and 5'-TGCAGCCTACAATCCGAACTGAG-3', and it amplified a 1274-bp product. After the Initial Denaturation at 96°C for 5 min, the amplification cycle consisted of 40 cycles at 95°C for 1 min, 56°C for 1 min and 74°C for 1 min. The final cycle included extension for 5 min at 74°C to ensure full extension of the product. After PCR, 1 microliter of the reaction mixture was transferred to the second-round reaction mixture containing 0.6 μM of each inner primer and the same buffer as in the first round. The nested inner primer pair Pylo AN was 5'-GGTGGAATTCTTGGTGTAGGGGT-3' and 5'-TAGCATCCATCGTTTAGGGCGTG–3', and it amplified a 160-bp product. The amplification cycle of the second round PCR was the same as in the first round. Ten microliters of the final PCR product were electrophoresed on a 1.2 % agarose gel containing 0.5 μg of ethidium bromide per ml. Following electrophoresis, the gel bands were visualized under ultraviolet light and the image was captured using a camera.

3. Data Analysis

McNemar's test was applied to analyze the disagreement between the tests performed. Kappa coefficient was used for qualitative analysis of categorical data.

K (kappa coefficient) = (OA-AC)/(T-AC)

where OA is Observed Agreement, AC is Agreement of Chance, T is total no.of samples

4. Results

Tables 1 & 2 present the results obtained with RUT and Nested PCR used for the detection of H. pylori infection in gastric biopsy samples. Out of the 50 biopsy samples, 30 were positive (60%) and 20 were negative (40%) by Rapid Urease Test (Figure 1). 42 patients (84%) were found to be positive for H. pylori by nested PCR and 8 (16%) were found negative (Figure 2).

In order to rule out the false-negative H.pylori PCR results, human beta-hemoglobin PCR was also performed to assure the quality of DNA extraction. Out of the 20 patients with RUT negative results, 12 were positive by nested PCR.

In this study, the combination of diagnostic methods RUT & N-PCR increased the test positivity from 60% (30/50) to 84% (42/50). In McNemar's analysis, it was found out that the H.pylori RUT results 76% ({30+8}/50) in agreement with Nested PCR results whereas 24% results between both diagnostic tests were in disagreement, but the kappa statistical analysis showed that the agreement is moderate (kappa coefficient = 0.44 with k=0 as No agreement & k=1 as Perfect agreement), as demonstrated in Table 3.
The Nested PCR is superior to Rapid Urease Card test in diagnosing the presence of bacteria in gastric biopsy tissues. However, the agreement between both assays shows them as of comparable diagnostic efficiency.

Table 1. Results of Rapid Urease Test to detect H. pylori.

Total (n)	RUT Positive	RUT Negative
50	30 (60%)	20 (40%)

Table 2. Results of Nested PCR to detect H. pylori

Total (n)	Nested PCR Positive	Nested PCR Negative
50	42 (82%)	8 (16%)

Figure-1 Chairside Rapid Urease Card Test showing Positive result for H. pylori.

Table 3. Comparison of diagnostic results for H.pylori (n = 50)

RUT	Nested PCR	Frequency	Agreement	Disagreement	Kappa Coefficient
Positive	Positive	30	76%	24%	0.44
Positive	Negative	0			
Negative	Positive	12			
Negative	Negative	8			

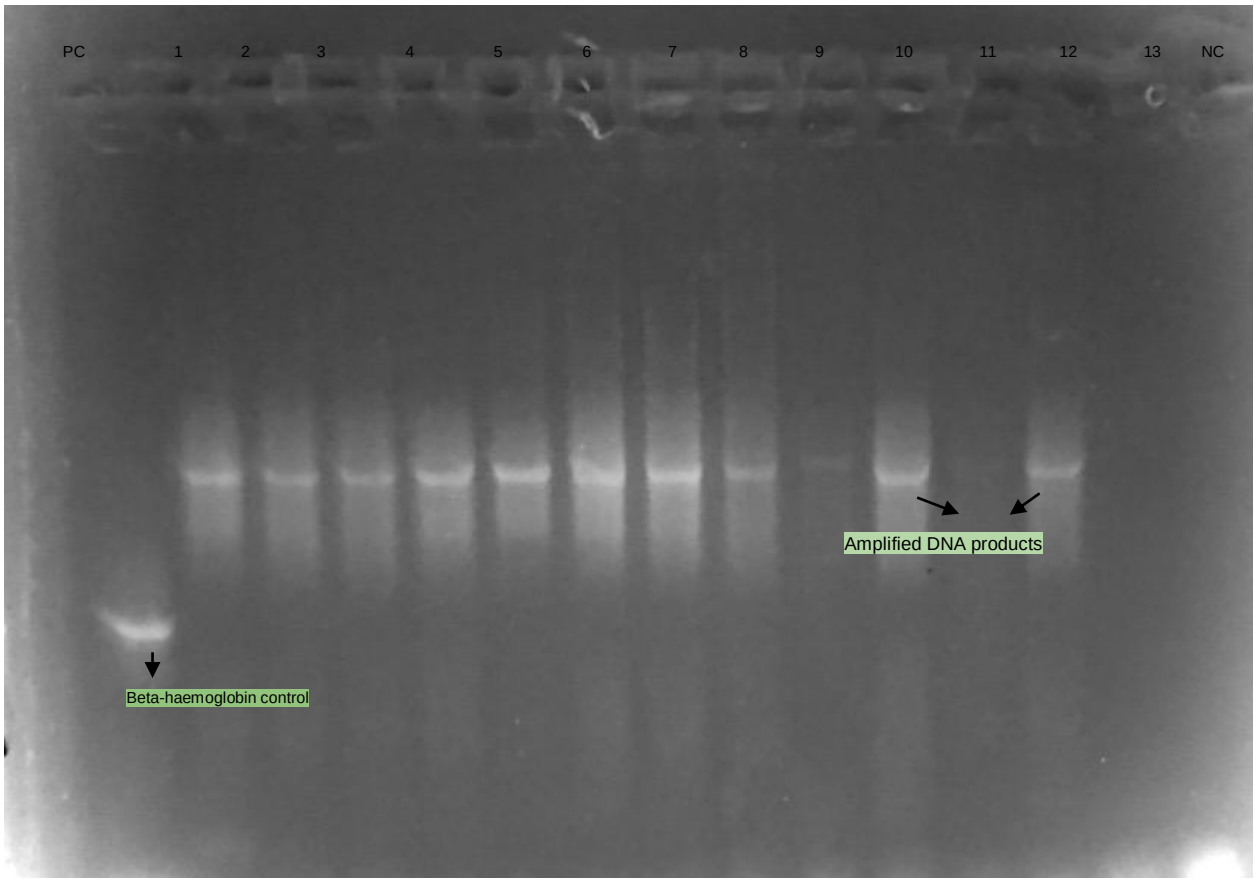

Figure-2 Gel Electrophoresis photograph showing the result of gene amplification where PC = Positive control, NC = Negative control (Distilled water), 1-13 slots were Amplified DNAs.

5. Discussion

To diagnose H. pylori infection, numerous methods are available, and the optimal choice should consider factors such as sensitivity, specificity, clinical condition, availability, and cost. Hence, several studies have compared and correlated H. pylori detection using various methods, both invasive and non-invasive.

In the current study, a comparison of the molecular method has been conducted through the target of the 16S rRNA with the RUT. Their sensitivity depends on some factors: the number of biopsies performed, the density of bacteria presents in each biopsy, the presence of *H. pylori* in endoscopic material and the presence of other microorganisms besides *H. pylori*. [12]

In this study, the sensitivities of the RUT and nested PCR results have been assessed. While both methods necessitate biopsy samples, not all biopsies contain a significant quantity of H. pylori organisms for detection. Since the one-step PCR lacks sufficient sensitivity, nested PCR has demonstrated superior specificity and sensitivity in detecting H. pylori [13], the latter method has been used in the present study. Nested PCR can indeed be applied to analyze H. pylori in various biological samples, including gastric tissue, saliva, feces, and others, provided that DNA extraction from the sample is feasible. The nested PCR shows high sensitivity (82%) in the current study while the RUT demonstrates less sensitivity at 60%. False-negative results from the RUT may have occurred when only a small amount of H. pylori is present in the samples, despite being subsequently detected by nested PCR. Lin et al. [14] reported that out of 82 gastric biopsy samples, 56 tested positives by RUT and 52 tested positives by PCR. They suggest that PCR could serve as a complementary assay to RUT. A comparison of the PCR, histology, culture and RUT methods shows that PCR has the highest diagnostic sensitivity (99.4%) for detecting H. pylori infection [15]. Archimandritis et al. also show that the rapid urease test was less sensitive than histology for diagnosing H. pylori infection [16]. In this study, the clinical samples are not examined histologically for presence of H. pylori bacilli. However, when compared with four diagnostic methods (RUT, PCR assay, culture and histological examination), it has been suggested that RUT is a rapid and sensitive method of screening for H. pylori infection and that PCR is more sensitive than other methods for detecting infection following treatment [17].

6. Conclusion

Rapid Urease Test being a fast and affordable method for reliably suggesting the presence of H. pylori can be used as screening test for many patients routinely in endoscopy rooms. The success rate in detecting H. pylori using the Nested PCR, at 82%, has been proved higher compared to the RUT. Molecular methods such as PCR and Nested PCR have not traditionally been a part of routine diagnosis for H. pylori. However, this trend has been evolving over the years due to the

achieved results, thus demonstrating high sensitivity and specificity. It is concluded from this study that the PCR assay with nested primers is a highly specific and sensitive method for detecting H. pylori DNA in gastric biopsy samples. Therefore, in the absence of a gold standard assay for identifying H.pylori, the combination of both assays RUT & N-PCR could be applied in order to reduce the false-negative H.pylori infection.

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Conflict of Interest

None

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