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PCR based identification of *Helicobacter pylori* infection in saliva samples

Komal Aman[1], Bashir Ahmad[*1], Sajid Ali[2], Saad Ahmad Khan[4], Md Motiar Rahman[3], Farhana Jabeen Shah[4], Muslim Raza[3*] and Huang Linfang[5]

[1]*Centre of Biotechnology and Microbiology, University of Peshawar, Khyber Pakhtunkhwa, Pakistan*

[2]*Department of Biotechnology, Abdul Wali Khan University, Mardan, Khyber Pakhtunkhwa, Pakistan*

[3]*Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Shenzhen, China*

[4]*Community Medicine Department, Kabir Medical College, Gandhara University, Peshawar, Khyber Pakhtunkhwa, Pakistan*

[5]*Institute of Medicinal Plant Development (IMPLAD), Peking Union Medical College, Chinese Academy of Medical Sciences (CAMS), Malianwa, HaiDian, Beijing, P.R. China*

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Abstract

Helicobacter pylori (*H. pylori*) are recognized as fastidious chronic bacterial infections in humans, commonly leads to gastritis, peptic ulcer, gastric cancers and gastric malt lymphoma. Currently, around the globe a massive increase of gastric cancer occurs. It could be reduced by identifying and eradicating of *H. Pylori,* therefore it is an urgent need to develop an accurate diagnostic method. The current study focused on the diagnosis of *H. pylori* infection without performing endoscopy highlight the importance of noninvasive tests. In first step molecular studies for *H. pylori* infection were conducted on genomic DNA that were extracted from salivary secretions to detect *H. pylori* in saliva by targeting two genes such as 16srRNA and Urec gene but we were failed to detect any *H. pylori* DNA in saliva. In second step we determined the accuracy of *H. pylori* culture and then its PCR sequencing. Saliva samples from 60 anti-*H. pylori*-positive patients were cultured on *H. pylori* selective media, then colonies were isolated and screened through different biochemical tests. Moreover, *H. pylori* colonies were amplified on PCR using 16srRNA for validation, our preliminary results were further confirmed on gel electrophoresis and automated sequencer ongoing. Moreover, this study in the future will give information for more accurate and molecular diagnosis of *H. pylori* infection through noninvasive methods.

* **Corresponding Author:** Bashir Ahmad ✉ bashirdr2015@yahoo.com

Introduction

H. pylori is a gram-negative, curved or spiral-shaped bacterium with microaerophilic features. It is an endemic pathogen responsible for mortality and morbidity throughout the world. Currently 50% (over 3 billion) of the world populations are infected with *H. pylori*, mainly in the developing countries (Khasag *et al.*, 2018). *H. pylori* infection is a common chronic infection responsible for the major cause of gastritis (Toscano *et al.*, 2018). It can make colonies in the stomach mucosa resulting to chronic inflammation and severe complications such as dyspepsia, peptic ulcers and finally in extreme cases, it results to gastric malignancies There are different mechanisms reported for *H. pylori* gastric carcinogenesis. Mostly can leads to the chronic gastric inflammation, it might progress to precancerous changes of atrophic gastritis and intestinal metaplasia (Eusebi, Zagari, and Bazzoli 2014). Gastric inflammation, other gastric disorders, and development of gastric mucosal damage may be resolved by eradication of *H. pylori.* A massive disease burden of gastric cancer could be reduced by identifying and eradicating of *H. Pylori,* but before its treatment, an accurate diagnostic method is needed (Savoldi *et al.*, 2018, Graham 2015). There are different techniques available for its identification such as rapid urease test which is a reliable method for the detection of *H. Pylori* in which carbon-14 is detached from the urea molecule by the action of urease enzyme, there are certain limitations of this method, such as their cost, time consuming. and the radiations used in this approach are harmful to human health (Wang *et al.*, 2015, Patel *et al.*, 2014). It can also be detected by direct culturing or histological examination of *H. pylori*-infected tissue biopsy (Sarma *et al.*, 2016). Recent reports suggested that the endoscopic examination is also dangerous for healthy subjects, it has been noted as a of cross-contamination and infection transmission in KPK/Pakistan. *H. pylori* can also be diagnosed by serological test of serum antibodies produced against *H. pylori* (Tonkic *et al.*, 2012). This method has limitations of not governing full information about active and previous infection. However, for diagnosis of *H. pylori,* rapid urease test, and histological examination are suggested as gold standards by Maastricht consensus report (Okullu *et al.*, 2017). These methods are costly and painful to humans; therefore, we cannot rely on these diagnostic methods.

Here in this study, we conducted molecular detection of *H. pylori* in DNA (extracted from saliva) and then culturing of salivary secretions. *H. Pylori* were isolated and cultured by using their specific media and identified by using morphological, biochemical and molecular characterization. *H. pylori* is a microorganism that grows in the complex nutrient environment and it can usually grow on nutrient-rich media such as blood agar base supplemented of sheep/horse blood with selective antibiotics for this microorganism. These cultures may take seven days to achieve maximum growth. Our main focus in this study was to avoid the invasive method of *H. pylori* diagnosis, to accurately and rapidly diagnose this infection through noninvasive means such as molecular identification of *H. pylori* in saliva and culturing of oral secretions afterwards.

Material and methods

2.1. Patients' selection

We selected a total of 60 patients with confirmed *H. pylori* infection of different gender (36 women and 24 men), the blood samples were collected at clinical laboratory Lady Reading Hospital (LRH) Peshawar Khyber Pakhtunkhwa, Pakistan. Informed consent was taken from all of the patients and this research work was approved by the ethical committee of the Centre of Biotechnology and Microbiology, University of Peshawar.

Inclusion criteria

Patients with severe gastric ulcer complaints and who had been diagnosed previously for anti-*H. Pylori* antibodies were included in this study.

Exclusion criteria

Those patients that taking proton pump inhibitor or antibiotics within the last four weeks were excluded from the study.

Samples Collection and Culturing

The fresh saliva samples were collected with DNA genotek oragene saliva collection device (OG-500)

from patients previously tested for *H. pylori* antibody and with severe symptoms of gastric ulcer. These saliva samples with sterile swabs were inoculated on freshly prepared selective nutrient enriched medium supplemented with sheep blood (35 mL) amphotericin B (0.25mg/100ml), cefsulodin (1.6mg/100ml), trimethoprim (2mg/100ml) and vancomycin (0.6gm/100ml). The selection of media was done on the basis of our study that *H. pylori* grow best on blood agar base with selective supplements (Kolaylı *et al.*, 2017). The plates were placed in a container having microaerophilic conditions, incubated at 37°C and after 5 days small bacterial colonies were observed in culture media.

Confirmation of Helicobacter pylori colony

Even though after the use of selective antibiotics, there is less chance for the growth of bacteria other than *H. pylori,* but it is important to confirm that organisms isolated are actually Helicobacter species (Blanchard and Nedrud 2012). There were some morphological and biochemical tests carried out to confirm the growth of *H. pylori*. In the current study, the microorganisms were identified on the basis of morphology by gram staining, and then these, these colonies were confirmed biochemically by using urease, oxidase, and catalase activity.

Urease, oxidase and catalase assays

In the current work, two types of media were used to confirm the urease activity. One is the Christensen's urea agar and the other is Stuart's urea broth. Catalase enzyme is produced by cytochrome containing microorganisms which breaks down H_2O_2 into oxygen and water. Bubbles of oxygen form when a small amount of catalase producing microorganism is introduced into H_2O_2 as a result of the enzyme's activity. As *H. pylori* are microaerophile therefore 15% hydrogen peroxide was used to determine the oxidase activity of these organisms. A biochemical reagent (McLeod oxidase (R026-100 ml)) was used for the confirmation of cytochrome oxidase activity.

Extraction of genomic DNA

The bacterial DNA was extracted from fresh bacterial colonies that were grown on sheep blood selective media. These bacterial colonies were then inoculated in broth media, incubated at 37°C for 24hrs. Up to 1mL of bacterial broth culture were centrifuged for 5min at 8000rpm and the supernatant was removed. From pellet bacterial DNA was extracted by using the Macherey Nagel DNA extraction kit (catalog number - 06/2014, Rev. 14) using a standard protocol. Confirmation of template DNA was done on 2% agarose gel electrophoresis stained with ethidium bromide for qualitative analysis and the DNA was checked on nanodrop for quantitative analysis.

Molecular identification of Helicobacter pylori

There are different techniques available for the detection of *H. pylori.* Among all techniques, only polymerase chain reaction is a reliable approach due to its sensitivity and specificity (Patel *et al.*, 2014). It can detect *H. pylori* from a number of clinical samples such as biopsy, dental plaque, gastric juice and saliva (Singh *et al.*, 2008, Vianna *et al.*, 2016, Saez *et al.*, 2012). 16srRNA and Urec genes of *H. pylori* were subjected to amplification through conventional PCR for molecular screening of *H. pylori* infection in saliva using primers listed in Table 1. The specific genes were subjected to optimization using the master mix, DreamTaq Green PCR kit (Cat#K1081; Thermo Scientific, Waltham, MA, USA) with different concentrations of genomic DNA template such as 0.5µL, 1µL, 2µL. Forward and reverse primers (Macrogen, Seoul, South Korea) were used at a concentration of 1µL. 0.5µL of Taq DNA polymerase, 10µL of PCR Master Mix, and 5.5µL of PCR-grade water with a final reaction volume of 25µL were used at different annealing temperatures (50-60˚C) for gene optimization.

Table 1. Sequence and amplicon size of 16srRNA.

Gene		Primer sequence 5′→ 3′	Amplicon size	Tm	References (adapted from)
16srRNA	Forward	AGAGTTTGATCCTGGCTCAG	1545	59°C	Hirendra *et al.,* 2014
	Reverse	AAGGAGGTGATCCAGCCGCA			

Results

3.1. Molecular confirmation of H. pylori in saliva

The saliva specimens collected from 60 anti *H. pylori* patients of different age groups from Lady Reading Hospital, Peshawar for amplification of *H. pylori* specific gene but no specific band was obtained for 16srRNA and UreC gene even by changing different parameters such as annealing temperature from 50˚C to 60˚C, different volumes of primers and different concentration of templet DNA and the reasons were unknown.

Culturing and Identification

The saliva of 60 patients was cultured on blood agar base for around 5 days of incubation, then small round colonies were observed and the color of the media was changed from red to dull brown (Fig. 1).

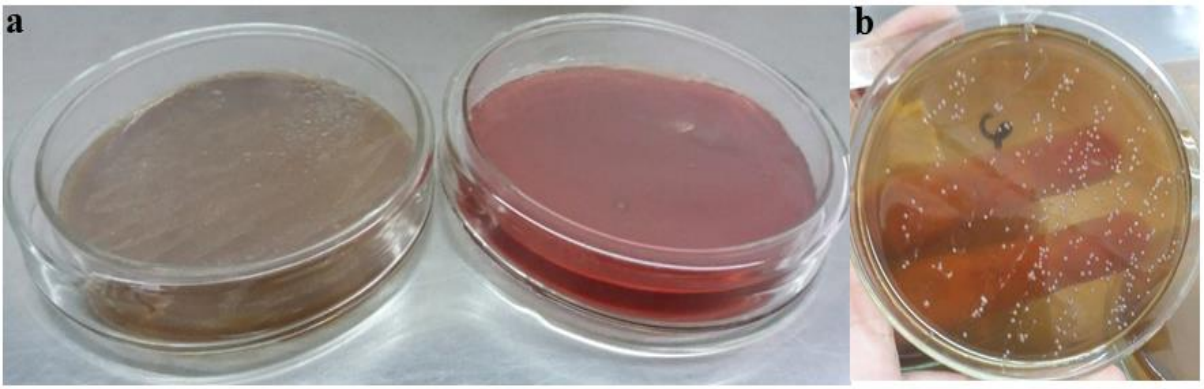

Fig. 1. (a) Illustration of the media color changes from red to dull brown, while the (b) refer to the cultured colonies of *H. pylori*.

The colonies were identified as *H. pylori* by microaerophilic growth requirements, their morphology and through biochemical analysis [Table 2]. Different biochemical tests were performed to confirm the *H. pylori* colonies (Abu-Sbeih *et al.*, 2014). These tests include catalase, oxidase and most important the urease test. It was observed that 56 colonies (93%) were gram-negative (remaining four-gram positive colonies were excluded from the study as *H. pylori* are gram-negative bacteria). Most of these gram-negative colonies shared these three biochemical characteristics and the results were noted that out of 56 gram-negative colonies total 51(91%) showed positive catalase activity upon checking at 15 percent H2O2 (Remaining 5 colonies which were catalase negative, were excluded for further processing as *H. pylori* are catalase positive). By testing on oxidase reagent, out of the 56-gram-negative samples, 48 (85%), showed positive oxidase activity due to the reaction of the colony with oxidase reagent (remaining oxidase negative were excluded from the study). When bacterial colonies were grown in urease medium, 43 (76%) out of 56 colonies gave urease positive result by changing the color of media from golden yellow to pink (Fig. 2) and these 43 colonies were gram-negative as well as oxidase, catalase, and urease positive.

Fig. 2. Detection of Urease activity through color changes.

Table 2. Result of gram staining and biochemical screening of isolated colonies.

Total sample	Gram-negative	Catalase	Oxidase	Urease	Colonies sharing all the three biochemical tests
60	56	51	48 (catalase) + (oxidase)	43 (catalase) + (Oxidase) + (urease)	43 colonies out of 60 were catalase, oxidase, and urease positive.

3.2. Amplification and sequencing

Gram negative bacterial colonies having all three biochemical features were selected for PCR amplification using 16srRNA primer. The amplicon size was confirmed by gel electrophoresis and its size was 1.5kb. A specific band (1.5kb) was found in all samples (Fig. 3) A band of the amplicon of 43 samples was then sent to Macrogen Inc (Korea) for sequencing by automated sequencer and the sequence obtained was analyzed by NCBI-BLAST for checking the homology sequences were analyzed and observed as HSPrrnB16S ribosomal RNA [*H. pylori* strain 26695].

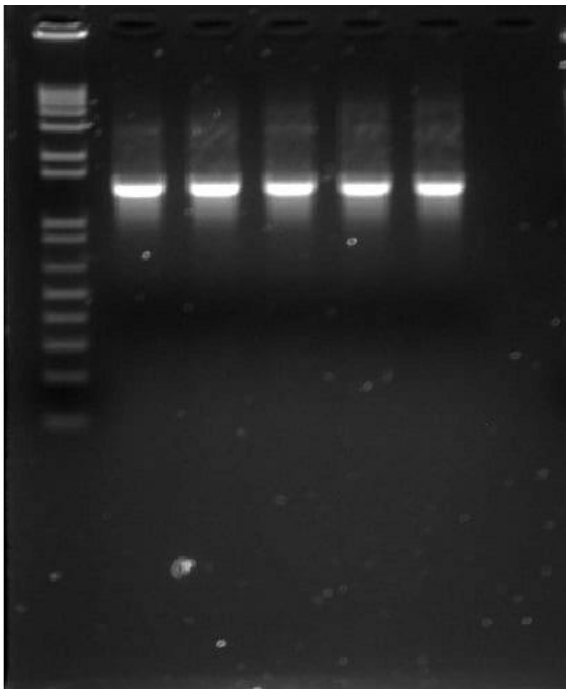

Fig. 3. PCR amplification of 16srRNA from *H. pylori*.

Discussion

Mouth plays a significant role in transmission of *H. pylori* and reappearance after being treated. It has been confirmed from previous reports that the mouth and stomach are significantly correlated with *H. pylori* infection (Samarbaf-Zadeh *et al.*, 2006). The reappearance rate of *H. pylori* infection is high in many countries, suggesting that the oral cavity act as a reservoir of *H. pylori*. The current study evaluated the molecular diagnostic method of *H. pylori* in saliva specimens. As our aim was to diagnose gastric disorders non-invasively. According to the previous reports *H. pylori* was diagnosed in more than 87% of cases, with the help of saliva samples and the results were comparable with the DNA obtained from biopsy of the gastric tissues of the infected patients. Furthermore, in saliva of asymptomatic subjects *H. pylori* infection was also diagnosed.

We collected the saliva samples from those patients who were tested previously for *H. pylori* antibodies. The DNA from saliva specimens were extracted soon after the collection and the extracted DNA was checked on gel electrophoresis and nanodrop to analyze the quality and quantity of DNA respectively. We targeted the two gene of *H. pylori* i.e 16srRNA and UreC gene for amplification. These genes were subjected to optimize by changing different parameters such as concentration of template DNA and primers and by changing different annealing temperatures from 50˚C-60˚C but we were failed to get any result after many unsuccessful trails. According to previous reports with proven gastric infections *H. pylori* was detected in the saliva of 87.5% of patients by PCR, but we are in contradiction with them because we didn't amplify any *H. pylori* gene in saliva as used by them.

According to some previous reports many attempts were made in the past to detect *H. pylori* in saliva (Tiwari *et al.*, 2005) but did not generate any clear evidence about diagnosis of *H. pylori* infection from saliva, and it may be due low detection power of the method used and low detection rates of *H. pylori* in the salivary secretions. It has been confirmed from a study that detection of *H. pylori* DNA in saliva is possible if

the DNA is extracted from the whole saliva rather only from pellets after centrifugation and if the saliva sample is collected directly into the digestion buffer. We are in agreement with this study because these might be the reasons as we collected the saliva samples in empty sterile tubes having no digestion buffers and the samples were pelleted out for DNA extraction.

In many countries, the reappearance rate of *H. pylori* infection is high, suggesting that the oral cavity act as a reservoir of *H. pylori* (Hammar *et al.*, 1992). The current study evaluated the diagnostic method of *H. pylori* in saliva specimens and we have isolated and identified *H. Pylori* from saliva. The detection of *H. pylori* in saliva provides a base that oral cavity has a significant role in the transmission of *H. pylori* infection, since many disagreements are there about this concept that whether oral cavity involved in *H. pylori* infection or not, in oral cavity *H. pylori* comes from stomach and from the stomach impulse due to high concentrations of oxygen are there in the oral cavity, therefore, the microaerophilic organisms can survive only for a few hours. If the anticipated idea is correct, then in saliva *H. pylori* antigens must be there that may be diagnosed through PCR. In our first method, we tried to detect *H. pylori* through PCR in saliva as the similar work was done by Tiwari *et al.,* they molecularly detected *H. pylori* in saliva (Mapstone *et al.*, 1993). *H. pylori*-specific DNA in the saliva of proven *H. pylori*-infected patients were detected by Hammar *et al.,* and its ratio was 47% (9/19) (Rasmussen *et al.*, 2012). Mapstone *et al.,* detected *H. pylori* DNA in saliva samples of the infected patients and its ratio was 31% (3/31) (Kim *et al.*, 2000), but we were failed to detect *H. pylori* DNA or antigen in the saliva of *H. pylori* infected patients directly through PCR and the reasons were unknown (Cutler *et al.*, 1995). In addition to it past few studies also failed to give any comprehensible information about *H. pylori* diagnosis directly in saliva. In our second method, fresh saliva samples were collected from the same patients and cultured on *H. pylori* selective media (sheep blood agar supplemented with selective antibiotics). From many studies, *H. pylori* culture is considered as the "gold standard" for the diagnosis of the infection (Majmudar *et al.*, 1990). In our study, *H. pylori* were detected in saliva of 71% (43/60) of patients after morphological and biochemical screenings. These colonies were then confirmed by PCR amplification and automated sequencing. In Indian population, Majmudar *et al.,* found *H. pylori* with a high prevalence of 40 saliva samples by culturing and CLO test (Desai *et al.*, 1991). Another study conducted by Desai *et al.*, in 98% of dyspeptic patients they confirmed the oral prevalence of *H. pylori,* but in their study, they used only the CLO test (Ferguson *et al.*, 1993). Similarly, *H. pylori* were isolated from saliva culture by Donald A *et al.,* in very low number, but in their work, they did not confirm these bacterial species through PCR and sequencing (Krajden *et al.*, 1989). Similarly in a study conducted by Krajden *et al.*, very low number of *H. pylori* was found in the oral cavity and confirmed only 1 out of 71 (1%) positive oral specimens by culturing. Another related research study conducted by Mapstone *et al.,* showed that 31% (3/33) of saliva samples delivered positive results on PCR.

The results of our study showed that the PCR assay of saliva culture is an accurate and reliable method for detection of *H. pylori* in a past study *H. pylori* like organisms were also detected in samples collected from the tongue and palate of a patient, both strains were grew in the microaerophilic environment and were oxidase, urease, and catalase positive, but its PCR analysis didn't show positive results and it represents the possibility of false identification the reason might be that in the oral cavity other urease producing bacteria are also present, such as streptococcus vestibularis and Actinomyces Viscosus, due to which the biochemical screening gives false positive results (Cheng *et al.*, 1996, Estrada-g *et al.*, 1988). In our study, all 43 patients showed culture positive results for *H. pylori* and PCR and the same results were mentioned by Hammer *et al.* They found that all of the patients who were culture positive were also positive for PCR. We only used a single universal primer that is 16srRNA and we performed Sanger sequencing of PCR product using 16srRNA because sequencing based on this primer is very satisfactory for phylogenetic analysis of bacteria (PASTER and DEWHIRST 1988). Urease gene was not targeted in

our study due to the fact that many other urease producing bacteria can also be present in the oral cavity. Many PCR assays detect *H. pylori* in oral cavity based on urease gene sequence. However, the prevalence of *H. pylori* in saliva detected by PCR using urease gene(Banatvala *et al.*, 1993) have been questioned because urease positive organisms are usually present in cultures from the oral mucosa and the same point was highlighted by SHENG-ANG HO *et al.,* (Ho *et al.*, 1991).

Conclusion

It is concluded that culturing of salivary secretions and then its further confirmation through PCR is an accurate and reliable method for detection of *H. pylori* infection and PCR of saliva samples for *H. pylori* DNA amplification without culturing is not a reliable method for *H. pylori* detection in saliva as initially we were failed to amplify any *H. pylori* DNA in saliva without its culturing moreover, the current work also reduces the risk of cross-contamination.

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