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Short Communication

Comparing Methods and Finding *H. pylori* Infections and their Resistance to Beta Lactam Drugs as Seen in Peshawar, Pakistan

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Abstract: The aims of this study were to find and report pattern of resistance towards beta lactam antibiotics in patients infected with *H. pylori* by visiting Hayatabad Medical Complex (HMC). Diagnostic procedures like: gastroendoscopy, Rapid Urease Test (RUT), biochemical testing, microscopic examination, antibacterial sensitivity tests and molecular methods were utilized and compared. Polymerase chain reaction was used to find 16S rRNA gene fragment. Hundred forty-four patients were found *H. pylori* positive for urease and 182 were positive for catalase. Hundred and thirty-two were positive for oxidase while hundred eighty were Gram negative. By polymerase chain reaction, 16S rRNA gene fragment was found to be positive in 164 subjects. The pattern of antibiotics resistance in *H. pylori* as seen in Peshawar, Pakistan in 200 gastric biopsies. A poorer resistance level towards amoxicillin is noticeable as due to low socioeconomic conditions.

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INTRODUCTION

H. pylori was discovered in 1982. It is considered a great advancement towards understanding /managing gastrointestinal diseases (Megraud and Lehours 2007). A bent bar shaped, or short spiral shaped gram-negative bacterium which was initially named *Campylobacter pylori* (Al-Sulami et al.2010). Self-ingested experimentations and tests on volunteers showed that the pathogen is able to colonize in human stomach, causing gastric mucosal inflammation (Morris et al.1991). These experiments led to further investigations which demonstrated that H. *pylori* colonizing may be the reason of disorders in upper gastrointestinal tract, like peptic ulcer disease, chronic stomach inflammation; gastric mucosa associated lymphoma of lymphoid tissue and carcinoma of stomach. The inflammation severity can underlie diseases related to H.pylori (David et al.2009). All those infected and colonized with *H.pylori* are not necessarily facing the afore-mentioned consequences, 10-15% will suffer from some symptoms and the complex interactions between bacterial factors (Cogo et al.2011).

Its prevalence is common in humans and found from 30% to 60% in developed regions on the basis of age and socioeconomic standing (Vinette et al.2002). The epidemiologic aspects and transmission sources of *H. pylori* infection are yet to be properly understood (Tiwari et al.2005). There are several virulence factors supporting this pathogen to colonize host and to develop disease. Urease enzyme is such a factor which is required for survival in the acidic pH of stomach when pathogen enters into the gastric mucosa, having comparatively less acidic pH (Montecucco and Rappuoli 2001). Motility and chemotaxis genes are also required by this bacterium for moving, locating to its favoured infection position (Ottemann and Lowenthal 2002).

Diagnosing *H. pylori* infection is not an easy task (Tiwari et al. 2005), but correct diagnostics is also vital to successfully treat and manage it (David et al. 2009). Similarly culturing and finding *H. pylori* in stomach biopsies is a tough task requiring expertise and dexterousness. Invasive techniques like endoscopy with biopsies for histology, culture and rapid urease test (RUT) are useful in diagnosing infection caused by this pathogen (Vaira and Vakil 2001). Some less invasive techniques are getting more acceptability in contemporary practice due to the high cost of otherwise invasive methods. The non-invasive methods are urea breath test and blood serological examination (Zagari et al.1999). Nevertheless, a positive outcome of blood serological test does not ensure existing *H. pylori* infection and urea breath tests also necessitate use of costly specialized apparatuses and reagents. Furthermore, sometimes in culture negative patients, urea breath test gives positive results (Bazzoli et al.1997). Culturing has been used as the preferred method to detect causative pathogens. But slow growing organisms like *H. pylori* take several days before achieving a result (Smith SI et al.2004).

Assays on the basis of other techniques like Polymerase Chain Reaction (PCR) have been devised for discovering microbial DNA presence, inclusive of *H. pylori* DNA, by use of a number of gene targets directly from the biopsies. In contrast to other methods, here, the whole experiments are not sabotaged in case of power supply breakdown, because we can easily repeat them. The technique is also helpful in difficult environments conditions like in Russia (Smith SI et al.2004)). This molecular method is broadly used diagnosing *H. pylori* in gastric biopsy samples, saliva samples, faeces samples and archival samples (Zsikla et al.2006). The method can point to the prospective virulence markers presence in a particular strain, which may have consequences to develop more severe disease or on the eradication efficacy (Ricci et al.2007). Several primers have been used and some are also available in preformed commercial kits. Various loci can be used as the amplification targets like 16S rRNA, A-B and C urease, flaA, CagA, vacA and heat-shock protein (hsp) (Ricci et al.2007).

Therefore this work was conducted to test a number of invasive and non-invasive techniques for swiftly diagnosing *H. pylori* in infected individuals facing different gastric diseases using molecular techniques and biochemical testing and to find resistance pattern of the bug towards beta lactams.

MATERIALS AND METHODS

Biopsy specimens' collection

Two hundred biopsies from patients (110 males and 90 females) aged between 10-80 years from HMC Peshawar were collected. The patients suffering from dyspepsia and having clinical

indications that make physicians advise them for endoscopy. All patients were asked to fill a consent form before undergoing endoscopy. A collection of gastric tissue biopsies was made from the patient's stomach's antrum. Two biopsies were collected from every subject. The specimens were studied for gram staining, RUT and molecular techniques. The biopsy selected for culture was carried to the laboratory using 2 to 2.5milliliters 20% glucose solution as a transporting medium. The biopsies specified for RUT were immediately tested in the gastroenterology department of the hospital and the results examined within 1h.

Biochemical tests

Culturing *H. pylori* from specimens

The antral biopsies specimens were carried to the microbiology lab.in less than an hour in the transport medium. These specimens were homogenized by grinding and were cultured in Columbia Blood Agar (CBA) plates to which 8% sheep blood, trimethoprim lactate (5mg L^{-1}), polymyxin B (2500 units mL^{-1}), amphotericin-B (5 mg mL^{-1}), vancomycin (10 mg mL^{-1}) was added. The cultures were incubated at 37°C in a microaerophilic atmosphere (10 CO_2, 5% O_2, 85 N_2) (Sigma, Aldrich) for 5-7 days. The identification was made by colony morphology, Gram staining, urease reactions, catalase, and oxidase (MacFaddin 2000).

Rapid urease test

The pure isolates were spread deeply on the urea agar surface stabbing it with the help of a clean loop wire. The tubes were cultured in the incubator at 37°C. The purple colour formation was checked after 4h (Hamada et al. 2018).

Oxidase test

The isolates oxidase activity was tested according to standard method (Reference).

dissolved in 50µl TE buffer (10mM Tris HCL, 1mM EDTA) and were preserved at -20°C.

PCR Amplification

In *H. pylori* the complete nucleotide arrangement of the 16S rRNA gene has been determined. Two oligonucleotide primers designated Hp1-Hp2 (Hp1, 5́-GCG ACC TGC TGG AAC ATT AC-3́) (Hp2, 5́-CGTTAGCTGCAT TACTGGAG-3́) were used. The amplification result of the two primers with dNTPs (deoxynucleotide triphosphates) was a 109 bp fragment (Fig.1). The template DNA 5µl was added to 20µl of reaction mixture having 2X PCR buffer (0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP) and 10 pmol of each primer. The reaction mixture was then vertexed and centrifuged and kept in ice box. The PCR amplification was performed using thermo cycler (Select Bio Products USA) as per the following conditions: Initial denaturation at 94°C for five min. 30 cycles with each cycle consisting of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min. Final cycle was set at 5 min to ensure complete extension of the PCR products. The amplification product was analysed by 2% agarose gel electrophoresis. The ethidium bromide-stained gel was examined under UV trans-illuminator for the presence of the amplified DNA.

Ethical approval of the study

The study was approved by the medical ethics committee of Hayatabad Medical Complex vide letter reference No. EC-06/HMC-18

RESULTS AND DISCUSSION

Research was done on total of 200 patients in HMC Peshawar. They were informed of the study objectives and their consents sought. Patients were required to fill case history data including information like their (sex/age), the symptoms they faced (e.g. nausea, vomiting, epigastric pain) and their medical history (other ailments and drugs they used). In this study, 55% (110 in 200) were males and 45% (90 in 200) were females, their ages ranged 10-80 years. Out of them 97% did not use medications while 3% infrequently used drugs like aspirin, famotidine, NSAIDs before coming to HMC. Eighty-eight patients suffered from symptoms like epigastric pain for last 6-12 months, 134 were under the distress of heartburn and 110 faced symptoms after eating.

Catalase test

Slide method was used according to standard method (Reference) for testing catalase activity of isolates with 3% H_2O_2 and observed for immediate oxygen bubble formation.

Molecular techniques

DNA Extraction from bacterial culture

DNA was extracted from the culture after biochemical tests performed to identify *H. pylori*. For this purpose, 1.5ml sterile water was put in Eppendorf tube and pure cultures were picked with the help of sterile wire loop to place in the Eppendorf tube and collected after centrifugation at 12,000 rpm for three minutes in a mini centrifuge (GYROZEN). Lysis buffer 200µl was added to the cell pellet to re-suspend and lysed by vigorous pipetting. The buffer solution was composed of (40mM Tris-acetate, pH 7.8 (AppliChem) 20mM Sodium-acetate, 1mM EDTA and 1% Sodium Dodecyl Sulphate). Then 66µl sodium chloride (5 M) (DAEJUNG, CHINA) solution was added to remove proteins and debris of cells. The resulting viscous mixtures were centrifuged for 10 minutes at 12,000 rpm. After centrifugation, two layers were formed from which the clear supernatant part was shifted into a new Eppendorf tube which was added with same volume of chloroform (Merck Germany). To form a completely milky solution, we rotated Eppendorf tubes for at least fifty times. Milky solutions centrifugation was done for three minutes at 12,000 rpm, again producing two layers, from which the separated supernatant layer was shifted into another Eppendorf tube. Same volume of 100% ethanol (LAB-SCAN) was added to precipitate DNA and then it was centrifuged for 5 minutes at 12,000 rpm until the formation of DNA pellet. The formed DNA pellet were washed twice with 70% ethyl alcohol and dried in desiccator after ethyl alcohol was eluted. The DNA pellet were re-

The prevalence of *H. pylori* varies from region to region and within states, and a higher infection rate is correlated to lower socioeconomic status and higher living densities (Goodman and Cockburn 2001; Hazel and Francis 2002). About, 40% and 80% adult population in the developed and developing countries is infected respectively (Timothy and Martin 1995). And this ratio increases with age, as 50% of the infected population is over 60 years in contrast to around 10% who are 18-30 years (Brown 2000). But the results of this study didn't show this pattern, because high ratio of the patients was younger (40-60 years). A larger study in France showed notably lower prevalence of *H. pylori* infection in females in contrast to males (Broutet et al. 2001). Our results also contradicted the (Broutet et al. 2001) statement as a higher infection ratio was seen in females. Our research also showed that out of symptomatic patients, 53.75% were *H. pylori* infected. An association of the infection was found with inconsistent gastrointestinal illnesses like chronic gastritis, intestinal metaplasia, and ulcerative conditions. This is in consensus with (Pilotto et al. 1998) who stated that chronic superficial gastritis due to *H. pylori* infection is a major predisposing attribute to cause atrophic gastritis, peptic ulcer, gastric lymphoma and gastric adenocarcinoma.

Table 1: endoscopic findings

Endoscopy Findings	Gastritis	Gastric Ulcer	Gastric Cancer	Deudenitis	Duodenal Ulcer	No Findings	Peptic Ulcer	Total
	98	18	16	16	16	24	12	200

Endoscopic examination with RUT test

To confirm *H. pylori* infected patients, RUT and endoscopy examination were used. Results showed that RUT positive patients were (72%) and RUT negative patients were (28%). Gastritis, duodenitis, gastroesophageal reflux disease, and the ulceration (Table 1) were the major endoscopic findings.

Out of the many tests accessible to diagnose *H. pylori* infection, RUT is most widely used in routine gastrointestinal endoscopy practice (Yakoob et al. 2005). In those having alarming symptoms or older patients with new onset dyspepsia, this is considered to be cost effective and reliable test that helps in

Table 2: biochemical tests results

Test	Positive	Negative	Total
Urease Test	144	56	200
Catalase Test	182	18	200
Oxidase Test	132	68	200

In this study, negative RUT results might be due to the fact that RUT results may be negatively affected by intestinal metaplasia and upper GI bleeding. (Megraud and Lehours 2007) had been reported even before now. Furthermore, urease genes expression is considered to be constitutive, since *H. pylori* regulate the expression and activity of urease enzyme according to the availability of nickel, a cofactor. And H. pylori cells may possess only cytoplasmic urease or both cytoplasmic and surface localized (extracellular) urease (Ernst et al. 2005).

Apart from this a change in the gastric lumen pH may also lead to a negative urease test. Achlorhydria causes negative RUT results, because the luminal pH of 7.0 can cause an enormously elevated pH nearby to the organism, such that its own urease destroys the *H pylori* (suicide) (Vaira et al.2002). Alkaline bile reflux into the stomach or excessive salivation might also contaminate the small biopsy sample because the resultant surface pH is above 6.0 (Ahmed and Sechi 2005). Moreover, urease presence in the gastric lining is also influenced by different drugs e.g antibiotics, sucralfate, bismuth, H_2 receptor antagonists and proton pump inhibitor drugs (Ahmed and Sechi 2005).

Microscopic examination, catalase and oxidase tests

The isolates were identified first on the basis of colony morphologic characteristics which appeared transparent with almost poor growth. Examined under microscope using Gram staining; (90%) of the isolates was found Gram negative, and (10%) Gram positive (Table 3). Catalase tests were performed for all cultured biopsy specimens, (91%) of the tested isolates was positive and (9%) negative for catalase test (Table 2). The biochemical test (oxidase test) for the *H. pylori* identification showed (66%) of the isolates was oxidase positive and (34%) was oxidase negative (Table 2).

Table 3: Gram Staining results

Test	Positive	Negative	Total
Gram Staining	180(Gram –ive)	20(Gram +ive)	200

***H. pylori* isolates detection by molecular techniques**

To confirm the outcomes of the above-mentioned *H. pylori* diagnostic methods, we also used PCR techniques. Genomic DNA was extracted out of the 200 *H. pylori* isolates and was tested for detecting the presence of 16S rRNA gene which was positive in 164 isolates. The 16S rRNA gene was amplified with particular primers (Hp1, 5́-GCG ACC TGC TGG AAC ATT AC-3́) and (Hp2, 5́-CGT TAG CTG CAT TAC TGG AG-3́). The amplified genomic DNA gave a single PCR band of about 109 bp in size.

Table 4: PCR results

Test	Positive	Negative	Total
PCR	164	36	200

To detect clarithromycin resistance and to diagnose *H. pylori* from gastric biopsy samples, saliva, feces and others. PCR technique has also been extensively used (Ricci et al. 2007). The targets of these PCR methods included ureaseA (ureA gene) cagA gene, phosphosamine mutase, (glmM gene) and 23S rRNA gene (Tomatari et al. 2010). In our work, technique of PCR was utilized to amplify 16S rRNA gene in *H. pylori.* Out of 200, 164 were positive for 16S rRNA gene (Table 4). Our results were in agreement with (Chong et al.1996), who detected *H. pylori* in 34 cultured samples in India. Use of PCR as a routine test has the disadvantages of being expensive and it is technically demanding test in contrast to histology, culture and the RUT. For PCR, extraordinary laboratory environment and separate facilities at every one step of the procedure are required, and even then, being a highly sensitive test, and due to the presence of chances of contamination, it may also give false-positive results. Positive results detection by a molecular technique will point to existing infection and will detect the DNA or dead organisms too (Lisby 1999). In the research on *H. pylori*, the current report of *H. pylori* genome brings molecular biology to the front position. PCR is thought to be the most sensitive method for detecting the microorganisms making this test particularly useful in the evaluation of eradication. Some more indications are *H. pylori* detection out of stomach which is present there in tiny numbers among many other microorganisms leading to false positive (Hassina and Ahmed 2013).

rapid determination of *H. pylori* infection stage (Megraud and Lehours 2007; Francesco et al. 2003).

In this work fifty-six out of two hundred gastric biopsy specimens obtained from patients was RUT negative, while one hundred and forty four out of two hundred were RUT positive (Table 2) in which this in agreement with the previous observations (Megraud and Lehours 2007). Our results are also close to (Ezzat et al.2012) who reported that 100% of the samples tested using RUT in the diagnosis *H. pylori* was positive.

Testing for susceptibility to antibiotics

The *H. pylori* isolates found positive after the biochemical analysis and Gram staining were subjected to test their susceptibility towards 9 type beta lactam antibiotics using disc diffusion method. The outcome of this exercise was a 100% of the isolates were Cefalexin and Cefpodoxime resistance, 84% Cefaclor resistance, 92% Cefixime resistance, 58% Cefotaxime resistance and 8% Ceftriaxone resistance. The pattern of sensitivity shown was, 89% of the isolates were sensitive to amoxicillin, 92% to Ceftriaxone, 42% to Cefotaxime, 27% to ampicillin and 100% of the isolates were Cefepime sensitive. Furthermore, 73% were susceptible to Amoxicillin/Clavulanic acid and 95% were sensitive to Piperacillin/Tazobactam. The results were interpreted according with the CLSI (formerly NCCLS) standard guidelines.

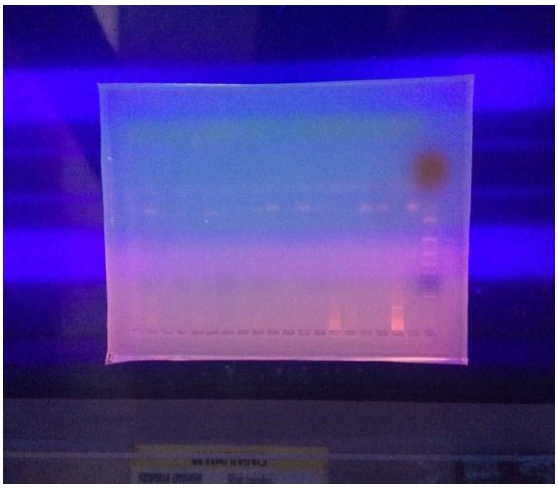

Fig.1 Showing the DNA bands of 109 bp in Agarose gel (2%) electrophoresis

The main cause of physicians not being successful to treat *H. pylori* infection is thought to be its resistance to antibiotics. The National Committee for Clinical Laboratory Standards (Performance standards for antimicrobial susceptibility testing, 1999) has developed guiding principles for conducting antibacterial sensitivity tests of *H. pylori*.

Antibiotic usage in the community, differences in local practice of prescribing antibacterials and mass eradication programs for *H. pylori* infections to prevent gastric cancer might cause hurdles in successful eradication therapy (Wong et al. 2002). In this report low to high levels of resistance to β-lactam antibiotics against H. pylori is reported. Sensitivity to β-lactam drugs to different degrees was shown by all the 200 isolates.

A low resistance to amoxicillin in Peshawar is astonishing because this region is considered a hot bed of bacteria resistant to different antibiotics. From this research work, the existence of a heterogeneous H. pylori population and their emerging trend of antibiogram in Pakistan is proved. It emphasizes to reconsider the treatment protocols according to local antibiotics susceptibility tests.

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Conflict of interest

Declared None

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