Similarity of *Helicobacter pylori* isolated from drinking water and peptic ulcer patients.

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Abstract:

*In this study two stomach biopsy samples from antrum were obtained from each of 110 patients. One sample is for rapid urease test and the other for bacterial culture. The results showed that rapid urease test (RUT) was positive in 46(41.8%) cases, while in culturing method, **H.pylori** was isolated from 68(62%) based on oxidase, urease, catalase and Gram stain. There were no significant differences (P>0.05) of **H.pylori** among males and females. The highest detection rates of the bacterium , (80.76 %) was recorded in the age group 31- 40 years , while no case was recorded in the age group below 20 years. Statistically, the differences were significant (p<0.05) among these age groups. The higher percentage of resistance was against erythromycin (93%) for **H.pylori** isolated from biopsy and (100%) for **H.pylori** isolated from water, whereas the lowest percentage of resistance was against tetracycline (20%) for **H.pylori** isolated from biopsy and (29%) from water samples. There are no significant differences (P>0.05) between drinking water and biopsy to the antibiotics.16SrRNA PCR was used for the confirmation of isolated **H.pylori** . PCR results showed the presence of the 500 bp 16SrRNA sequence in 8 out of 15 tested clinical isolates. Similarity between clinical and water isolates, obtained from a previous study is indicated by RFLP using restriction enzyme (EcoRI) for whole DNA. This bears an epidemiological significance as it indicates a possible source of this pathogen outside the human stomach.*

1. Introduction

The first description of ***Helicobacter pylori*** (***H.Pylori***) by Marshall and Warren [1] as a fastidious, gram – negative, flagellate bacteria recognized as an important gastroduodenal pathogen. ***H.pylori*** infection recognized as the major cause of gastritis and peptic ulcer and it has been classified as a carcinogen class I [2] and gastric mucosa – associated lymphoid tissue (MALT) gastric lymphoma [3] . Infection occurs mainly in childhood and infected individuals who usually carry it for life unless treated [4,5].The route of transmission of this pathogen is still unknown. Fecal-oral, gastric –oral , or oral-oral may be the route of transmission of infection [6,7].

Transmission of a fecal oral bacterial route , and the presence of ***H.pylori*** in drinking water systems which detected by PCR has been reported from the United States , England , Germany , Japan , Sweeden , Mexico , Gambia , Peru and Iraq [8,9,10,11,12,14,14,15,16 and 17] . Hegarty ***et al***[18] also demonstrated the presence of respiring ***H.pylori*** from U.S. surface water .Several studies have demonstrated that ***H.pylori*** can survive in water under a variety of conditions for a period of days to weeks [19].Patients harboring ***H.pylori*** was reported by Al-Sulami,***et.al*** [20] and it was also isolated from drinking water [21].

The aim of the study:-was isolating and identifying ***H.pylori*** from biopsy in Basrah governorate using culturing and PCR (16SrRNA) and finding out if there is a similarity between ***H.pylori*** isolates from water to those of clinical origin by restriction fragments length polymorphism (RFLP).

2. Methods

2.1 Sample collection and processing :-

In this study two kinds of samples have been obtained, water and biopsy samples. A total of 110 patients with various dyspeptic symptoms attending endoscopy unit at Basrah teaching hospital during the period from April 2008 to March 2009 , underwent endoscopic examination . They were 62 males and 48 females . The age of the patients ranges from 18 years to 69 years. Information obtained from the patient which includes:- name, age, sex, address, drugs, alcohol drinking, smoking, occupation and kind of drinking water. Two stomach biopsy samples

From antrium were obtained from each patient. One sample is for rapid urease test and the other for bacterial culture .For ***H.pylori*** culture biopsy samples were transported to the laboratory of microbiology by using Tryptic Soy Broth as a transport medium (1ml/vial).Biopsy samples were cultured according to [22].

Biopsy samples: were homogenized by using sterile fine glass rod then streaked on the chocolate agar medium by using sterile cotton swab, and incubated in microaerophilic conditions in anaerobic jar at 37 C^{o} for 5-7 days. For water samples: isolates of ***H.pylori*** from Al-Sulami,***et.al*** [17] were used.

2.2 Primary diagnosis of *H.pylori* :-The suspected purified colonies were chosen according to Gram staining and cultural characteristics and tested for the production of catalase, oxidase and urease.

2.3 Antibiotic sensitivity test :-The method of Piddock [23] was used to test the sensitivity of 15 isolates of ***H.pylori*** chosen randomly,isolated from biopsy samples to seven types of antibiotics, kanamycin 30µg, erythromycin 15µg, tetracycline 30µg, ampicillin 10 µg, rifampin 5µg, amoxicillin 30µg and gentamycin 30µg.

2.4 16SrRNA PCR Identification of isolate: - From biopsy samples, 15 isolates were identified biochemicaly which chosen randomly to be confirmed by using primers specifically designed for the identification of ***H.pylori*** based on 16SrRNA sequence [24] . The primer for 500 bp product of the 16SrRNA sequence are represented by the forward primer sequence: 5 GCT AAG AGA TCA GCC TAT GTC C3 and the reverse one: 5 TGG CAA TCA GCG TCA GGT AAT G3.

2.5 Preparation of bacterial genomic DNA:-Genomic DNA from each isolate was prepared by vortex after suspending a loopful of colonies in 1ml of phosphate buffer saline (PBS) 7.6, centrifuging at 14000xg for 2 min ,and boiling the pellet in 1ml of distilled water for 1min [25]. Concentration and purity were measured spectrophotometrically at OD260 and OD280 respectively, to exclude any possible contamination, and a gel of 0.8% agarose was used for electrophoresis.

2.6 PCR amplification of 16SrRNA for *H.pylori* :-Amplification was carried out in a 25µl of reaction mixture containing 12.5µl master mix, 0.5µl forward primer, 0.5µl reveres primer, 5µl DNA sample, 6.5µl distilled water and 25µl mineral oil. PCR condition for 16SrRNA include: Denaturation step at 95 C^o for 5 min, followed by 39 cycles at 94 C^o for 1 min, annealing at 55 C^o for 1 min and extension at 72 C^o for 2 min, and an additional extension step at 72 C^o for 7 min. PCR products were electrophoresed in 2% agarose.

2.7 Restriction Fragment Length Polymorphism (RFLP) :-Whole genomic DNA of ***H.pylori*** isolated from clinical and water samples, were restricted by restriction enzyme (***Eco*** RI) .This enzyme recognize the sequence G*AATTC . The reaction mixture (20 µl) for RFLP containing 12.3 µl Sterile , deionized water, 2 µl RE 10X buffer,0.2 µl acetylated BSA , 10 µg/ µl, 5 µl DNA sample and 0.5 µl restriction enzyme, 10 U/ µl. The reaction was prepared for restriction digestion, mixing the first four reagents by pipetting them in Eppendorf tube , and then add the restriction enzyme .All were gently mixed by pipett then spun in a microfuge for few seconds .The reaction was incubated at the 37 C^o for 2 h. for the electrophoreseis to be .

3. Results

3.1 Peptic ulcer patients and *H.pylori* diagnosis: - A total of 110 patients with positive endoscopic diagnosis of peptic ulcer, 68 patients (62%) showed positive for ***H.pylori*** the remaining 42 patients (38%), were called non-***H.pylori***. Out of 110 patients, 46 (42%) showed positive rapid urease test . The suspected colonies of ***H.pylori*** were on chocolate agar translucent, rounded and tiny size**.** All ***H.pylori*** isolates were Gram negative , spiral , rods , or curved in shape.The biochemical tests performed to confirm the identity of ***H.pylori*** include : Oxidase , catalase and urease.

3-2 Frequency of *H. pylori* according to sex .

Of the total 62(56.36%) males, 37(54.41%) showed positive tests for ***H. pylori*** ,while 31(45.58%) out of 48(43.63%) females showed positive tests of ***H. pylori*** , but the difference was statistically not significant (P>0.05).

3-3 Frequency of *H. pylori* according to age .

(Table 1) shows the age groups distribution of the patients according to ***H. pylori*** and non ***H. pylori*** . The highest detection rates of the bacterium, (80.76 %) was recorded in the age group 31- 40 years , while no case was recorded in the age group below 20 years . Statistically, the differences were significant ($p<0.05$) among these age groups.

Table (1) :- Number and percentage of patients with ***H. pylori*** and non –***H. pylori*** ulcer according to age group .

Age (years)	Patient	*H. pylori* ulcer	Non-*H. pylori* ulcer
20 or less	4	0	4 (100 %)
21-30	23	12 (52.17 %)	11 (47.82 %)
31 -40	26	21 (80.76 %)	5 (19.23 %)
41 -50	20	16 (80 %)	4 (20 %)
51 -60	19	11(57.89 %)	8 (42.10 %)
60 -69	18	8 (44.44%)	10 (55.55 %)
Total	110	68	42

3.4 Antibacterial drug susceptibility :- For ***H.pylori*** isolates from biopsy samples , Tetracycline was found to be the most effective antibacterial agent against ***H.pylori*** , 80 % of the tested isolates were sensitive to tetracycline followed by gentamycin (47%) , kanamycin (33%), amoxicillin (20%) and both ampicillin and rifampin showed (13 %) . Erythromcin was shown to be the least effective antibacterial drugs against ***H.pylori***. Statistically , the differences were significant ($p<0.05$) among these antibacterial drugs.

Figure (1) shows the results of antibacterial drugs resistance of 15 isolates of ***H.pylori*** from biopsy samples .

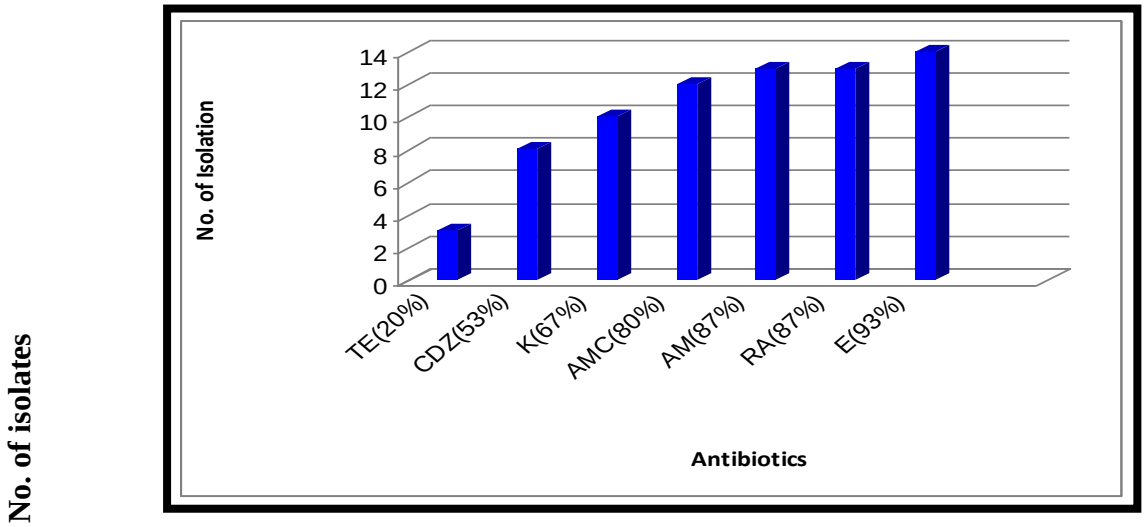


Fig. (1) :- Results of antibacterial drugs resistance of 15 isolates of ***H.pylori*** from biopsy samples**.**

Comparison between ***H.pylori*** isolated from drinking water [17] and biopsy to the antibiotics, showed that ***H.pylori*** from drinking water were more resistant than from biopsy (fig 2), but the difference was statistically not significant ($P>0.05$).

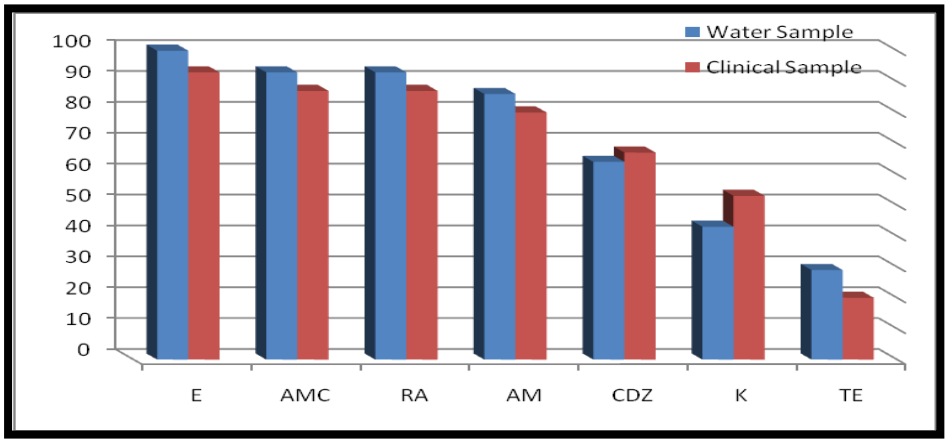
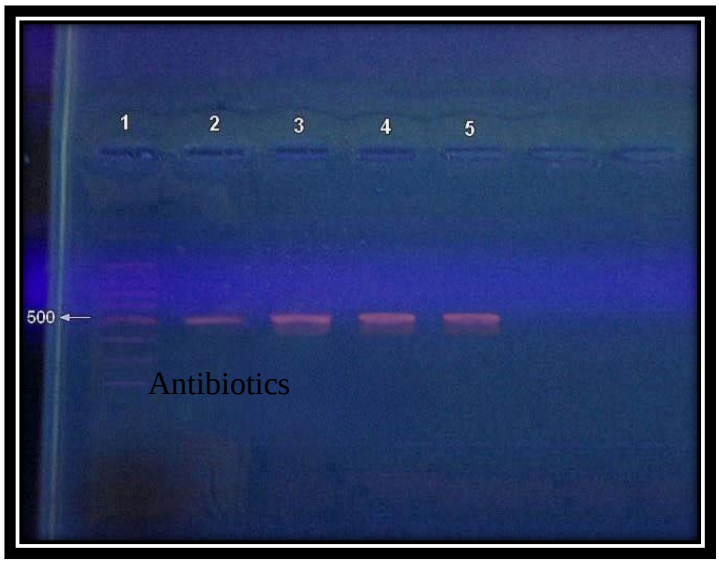


Fig. (2) :- Comparison of drug resistance of ***H.pylori*** isolated from drinking water and pationts to the antibiotics .

3.5 PCR results:-Out of 15 biochemically ***H.pylori*** positive isolates only 8 (53.3 %) were confirmed by 16SrRNA PCR. ***H.pylori*** from drinking water gave positive results by PCR [17]. PCR products for 16SrRNA based primers gave band on agarose gel corresponding to a 500 base pair product when compared to the molecular ladder , thus identifying the isolates as ***H.pylori*** as shown in (fig 3).

No. of isolates



Fig(3): PCR products for 16SrRNA based primers gave band on agarose gel corresponding to a 500 base pair product when compared to the molecular ladder. Lane 1 molecular ladder (1500-100) bp, lane (2-5) bands of PCR Product for ***H.pylori*** from clinical sample.

3.6 Restriction Fragment Length Polymorphism (RFLP) :-To our knowledge this is the first finding of its kind which is a confirmation that ***H.pylori*** isolated from R.O, tap water and biopsy were of the same source as shown in (fig 4) as indicated by the similarity of the fragments of DNA in numbers and locales for ***H.pylori*** isolated from R.O, tap water and clinical biopsy samples.

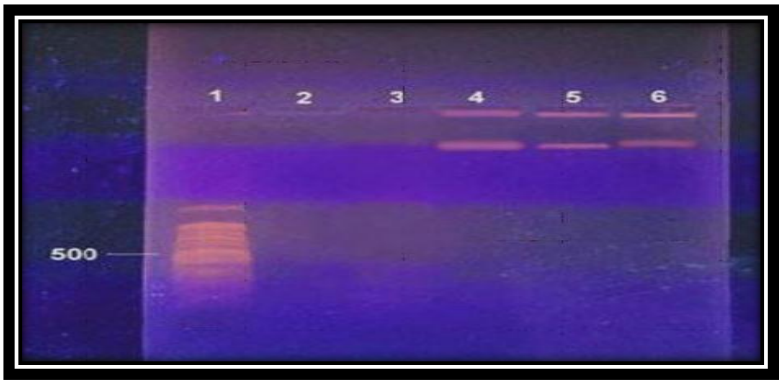


Fig (4) :- Restriction Fragments Length Polymorphism (RFLP) for DNA of ***H.pylori*** by restriction enzyme (EcoRI) of clinical and water isolates . lane 1 molecular ladder (1500 - 100)bp, lane 4 ***H.pylori*** isolated from tap water , lane 5 ***H.pylori*** isolated from biopsy samples and lane 6 ***H.pylori*** isolated from R.O water.

4.Discussion

The natural reservoir for ***H. pylori*** is thought to be the human gastrointestinal tract [26]. However, the association of ***Helicobacter*** with nonhuman sources, such as livestock [27], domestic cats, [28], and vegetables [29], prompted researchers to look at environmental sources as reservoirs to humans, and in this context this study was carried out drinking water.

Two stomach biopsy samples from antrum were obtained of each patient , one for R.U.T and another for bacterial culture. Samples were taken from the antrum because ***H.pylori*** is found mostly in the gastric surface mucus gel layer and epithelial intercellular junction of the stomach [30]. The colonization is more frequent in the antrum than in the corpus [31], possibly related to the fact that inflammatory responses are more prevalent and more intense in the antrum than in the corpus [32]. Detection is dependent on the number and location of biopsy specimens [32] .Natural habitat of ***H.pylori*** is in the human stomach, other sources of ***H. pylori*** and its mode of transmission are unknown [33], water may be one route of the transmission of ***H.pylori***.

For the clinical samples, we used culture and rapid urease test as a non invasive test for the detection of ***H.pylori*** in patients. Out of 110 patients, 46(42%) showed positive rapid urease. This procedure is simple and it depends on the production of large amount of urease enzyme by ***H.pylori*** to confirm the presence of ***H.pylori*** in the biopsy sample [34].However the risk of a false positive results by urease – generating non-***H.pylori*** bacteria has been emphasized [35]. This test can be performed in the endoscope unit and usually administrated in the hospital outpatient setting because it requires time (15 to 20 min) and special equipment [36] . In the present study, the difference due to sex was statistically insignificant (P>0.05) considering the reported percent for the detection of ***H. pylori*** among males of 54.41 and females 45.58, Andre [37] and Ugwuga and Ugwa [38], showed no significance difference in ***H. pylori*** occurrence between males and females. ***H. pylori*** was not isolated from patients below 20 years of age, the highest rate of ***H. pylori*** 21(80.76) was recorded in the age group 31-40 years. Bernstein ***et al***. [39] and Nijevitch ***et al***. [40], showed that the prevalence was higher in adults than in children.

In present study, we tested antibiotic sensitivity for clinical and environmental isolates of ***H.pylori***, comparison between them showed that environmental isolates, are more resistant to antibiotics than clinical isolates but the difference was not significant. This is perhaps due to the stress of environmental condition.

4.1 16SrRNA PCR for *H.pylori* detection:-As mentioned earlier 8 isolates out of 15 biochemically identified ***H.pylori*** from biopsy samples, were confirmed by 16SrRNA. Lu ***et al***. [41] reported a total of 23 out of 37 isolates were confirmed to be ***H. pylori*** by 16SrRNA PCR, the reason why some putative ***H. pylori*** isolates could not be confirmed by PCR was not further investigated. Several ***Helicobacter*** species are biochemically closely related to ***Campylobacter*** and the urease reaction is a key reaction in identifying ***Helicobacter*** species, but some ***Campylobacter*** strains are urease positive and it may, therefore, be necessary to undertake protein profiles or genomic analysis to ensure the correct identification [42].

4.2 Restriction Fragment Length Polymorphism (RFLP) :-In the present study the similarity of the fragments of DNA in numbers and locales appeared for ***H.pylori*** isolated from R.O, tap water and clinical biopsy samples by using restriction enzyme (Eco RI). This is of important epidemiological consideration as it indicates a very significant source of this pathogen outside human stomach.

conclusion:-The isolation of ***H.pylori*** from drinking water , tap and R.O, by culture method and consequent identification by biochemical tests and PCR represents a solid proof for the presence of this dangerous but illusive pathogen in our consumable water .Also the similarity between these isolates from different sources and biopsy samples imply a connection between these sources. It certainly will impact our search for a better epidemiological understanding and measures of control.

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