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Recovery of *Helicobacter Pylori* from Sewage in Basrah Governorate and its Confirmation by 16S rRNA

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Abstract

A total of one hundred and fifty six sewage samples were collected from three regions in Basrah governorate namely Five miles, Al-Jame'eyat and Al-Ashar from February 2008 to February 2009. This study examined the possibility that sewage harbors *Helicobacter pylori*. Because of existing methods for the direct culture of *H. pylori* from sewage are unsuccessful, a modified Columbia urea agar and Monophasic-Diphasic culture set up have been exploited. As a result 76 isolates were identified as *H. pylori* using conventional techniques. Polymerase chain reaction technique was used to confirm the identity of *H. pylori* isolates using primers for 16S rRNA and Ure A gene. Only 6 isolates out of 76 gave positive results for the identification by PCR using 16S rRNA, and all of them were from Five miles region. Also, these 6 isolates have an identical biochemical profile. On other hand the 76 *H. pylori* isolates have not been confirmed by PCR using Ure A gene.

These findings suggest that sewage may represent a reservoir for this bacterium and reveal that conventional tests for the identification of *H. pylori* are not sensitive enough to rely on.

The fact that all the six isolates were from one region and one of them showed an RFLP pattern similar to those from clinical and drinking water may be of an epidemiologic significance.

Keywords: *Helicobacter pylori*; Ure A; 16S rRNA; Polymerase chain reaction; Restriction fragment length polymorphism

Introduction

Helicobacter pylori has been the subject of intensive investigation since its culture from a gastric biopsy in 1982. *H. pylori* has been associated with chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach [1].

Water supplies contaminated with fecal material may be a potential source of *H. pylori* transmission [2]. This is particularly relevant in developing countries where municipal water supplies are not adequately treated and water is obtained from rivers and other untreated sources [3].

Enumeration of *H. pylori* in environmental samples is confounded by the lack of a standard procedure for the detection of this organism. Attempt to culture the organism directly from water samples [2] has been unsuccessful due to the over growth of interfering organisms on the rich media used for this organism. In addition, *H. pylori* has been shown to enter viable but nonculturable (VNC) state in water. Under these circumstances, the organism would not be recovered by traditional cultural techniques. Both polymerase chain reaction and fluorescent antibody staining have been used successfully for the detection of *H. pylori* in clinical or environmental samples [4].

This study aimed at the recovery of *H. pylori* from sewage in Basrah governorate, which has a population of about 3 million with no function at sewage treatment plant.

Materials and Methods

Samples collection

A total of 156 samples of sewage were collected from three regions in Basrah governorate namely Five miles, Al-Jame'eyat and Al-Ashar during the period from February 2008 to February 2009. Samples were collected in a sterile 250 ml glass bottles and transported on ice to the laboratory for analysis.

Samples filtration and culturing

Aliquots of 10 ml and 25 ml from each sample were filtered by membrane filtration technique, using 47 mm cellulose acetate filters with a nominal pore size of 0.22 µm (Sartorius, Germany).

The filters were cultured by using Monophasic-Diphasic culture set up (MDCS) method [5]. The MDCS is a system in which a slant of modified Columbia urea agar (MCUA) was prepared in test tubes (160 mm size) followed by the introduction of 2 ml of Tryptic Soy Broth (TSB) to cover the lower portion of the slant only. The lower portion of the test tube represents diphasic environment i.e. a liquid phase in contact with solid one, while the upper portion is a single phase, solid only. To cultivate and isolate *H. pylori,* the filters were placed in 2 ml TSB for 30 min with shaking. After that, the TSB medium was poured into the test tubes containing the slant, mixed well and then tilted once or twice to cover the upper portion of the slant prior to incubation. The test tubes were incubated under microaerophilic condition (5% CO_2, 85% N_2, 10% H_2), at 37°C for 3-5 days.

Identification tests of *H. pylori*

Morphological characteristics of the isolated bacteria such as shape, size, color of colonies on culture media after isolation were recorded.

Biochemical identification tests: Isolates have been subjected to the following biochemical tests i.e. catalase, oxidase, urease, H_2S, nitrate

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Primer	Sequence
Forward (F)	5'-GCTAAGAGATCAGCCTATGTCC-3'
Reverse (R)	5'-TGGCAATCAGCGTCAGGTAATG-3'

Table 1: The primer of the 522 pb, product of the 16S rRNA sequence.

Primer	Sequence
Forward (F)	5'-GCCAATGGTAAATTAGTT-3'
Reverse (R)	5'-CTCCTTAATTGTTTTTAC-3'

Table 2: The primer of 411 pb, product of the Urea gene sequence.

District	No. of samples	No & % of samples with *H. pylori*	% of sample with *H. Pylori* out of total samples
Five mile	95	(56) 58.9	35.8
Al-Jame'eyat	35	(2) 5.7	1.2
Al-Ashar	26	(18) 69.2	11.5
Total	156	76	

Table 3: Number of samples taken from each region, and percentage of samples with *H. pylori* at each region.

reduction, growing on PSD agar, growing at different temperature, growing in 3.5% NaCl, growing on 1% Glycine, sensitivity to nalidixic and cephalothin antibiotics.

Identification of *H. pylori* using Polymerase Chain Reaction (PCR):

16S rRNA identification of isolates: The identified isolates have been confirmed by using primer, specifically designed for identification of *H. pylori* and based on 16S rRNA sequence [6]. The primer of the 522 pb, product of the 16S rRNA sequence comprised of the following nucleotides (Table 1).

Ure A gene identification of isolates: Another primer was used for the identification of *H. pylori* that is Ure A gene [7]. The primer of 411 pb, product of the UreA gene sequence comprised of the following nucleotides (Table 2).

Extraction of genomic DNA from bacteria isolates: Isolation of genomic DNA was carried out according to Peek et al. [8] as follows: Genomic DNA of the isolates from the solid phase was prepared by vortex, mixing loopful of pure culture in 1 ml of PBS, centrifuged at 14,000×g for 2 min, then boiled the pellet in 1 ml of distilled water for 1 min. The samples centrifuged at 12,000×g for 4 min at 4°C, and the supernatants were stored in sterilized vials at -20°C until used as PCR templates.

DNA from the liquid portion was extracted according to the same procedure of solid portion, whereas 1 ml of the inoculated liquid medium was centrifuged, and the precipitate is then processed through the same steps for extraction of DNA from solid medium. The concentration of the isolated DNA was calculated according to Sambrok et al. [9].

Amplification conditions: The PCR cycle for 16S rRNA sequence was set up as the following according to Giri et a1. [10]. While the PCR cycle for Ure A gene sequence was set up as the following according to Notarnicola [7].

The PCR product was electrophoresed in 2% agarose and the products were detected and examined under transilluminator.

Results

The number of samples was 95 samples from Five miles, 35 samples from Al-Jame'eyat and 26 samples from Al-Ashar region. Table 3 shows the number of samples, taken from all regions and the number of *H. pylori* isolated on MCUA medium by using MDCS method and identified according to conventional tests (Table 4). From the district of Five miles 56 out of 95 samples harbor *H. pylori* with a percentage of 58.9%. The percentage of samples with *H. pylori* to total samples number taken from all districts is 35.8%. Only two samples showed *H. pylori* in Al-Jame'eyat region with a percentage of 5.7%. The percentage of samples of this region with *H .Pylori* to the samples number taken from all regions is 1.2%, which is the lowest percentage among samples of the three regions. Al-Ashar district revealed 18 samples with *H. pylori*, and a percentage of *H. pylori* to the samples number taken from this region was 69.2%. This was the highest among the three regions. The percentage of samples number identified associated with *H. Pylori* presence from this region to the total samples number taken from all regions was 11.5%.

Confirmation by PCR

For confirmation of *H. pylori* identity, the PCR technique was used to detect 16S rRNA gene. The product size of this primer is 522 bp. Out of 76 conventionally identified isolates only 6 isolates were found to harbor 16S rRNA gene of *H. pylori* (Figure 1). It is noteworthy that these 6 isolates were identical in being catalase, oxidase, urease positive, growing in 3.5% NaCl, in 1% glycine and on PSD medium as well as at 42°C but not at 25°C. They all reduced nitrate and failed to produce H_2S. Also they were sensitive to nalidixic acid but resistant to cephalothin.

Biochemical Tests										
Districts	Catalase	Oxidase	Urease	Growing at 3.5% NaCl	Growing on PSD	Nitrate Reduction	Growing at 25°C	Growing at42°C	Sensitivity to Nalidixic acid	Resistance to Cephalothin
Five mile	+	+	+	w	W	w	-	+	+	+
Al-Jame'eyat	+	+	+	w	+	w	-	+	+	+
Al-Ashar	+	+	+	w	W	w	-	+	+	+

+: Positive 100%; W: Weak 27-55%; -: Negative 0%

Table 4: Percentage of presumptive isolates as *H. Pylori* to biochemical tests.

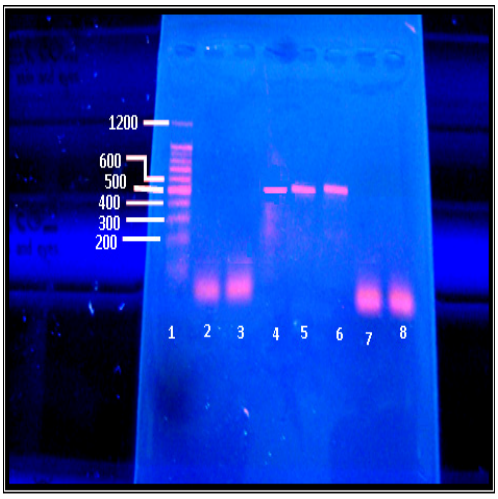

Figure 1: 16S rRNA PCR products from 7 *H. pylori* isolates. Lanes (4-6) denote the 522 base pair amplified product of the 16S rRNA sequence, Lane 1 molecular ladder, Lanes (2, 3, 7, 8) bands of 50 bp.

It should be pointed out that, these 6 isolates have the same biochemical profile and were obtained from Five mile district.

Extracted DNA from all of the 76 isolates was subjected to PCR for amplifying 16S rRNA gene. Amplified 16S rRNA gene was, then, subjected to gel electrophoresis. PCR products for the 16S rRNA based primers of only isolates gave sharp band on agarose gel corresponding to 522 bp product when compared to the molecular ladder, thus it identified the isolates as *H. pylori* (Figure 1).

Of the 76 isolates none was positive for Ure A gene as they gave DNA bands of 50-100 bp in weight, while the Ure A gene sequence is 411 bp.

Only one out of these 6 *H. pylori* isolates gave two fragments of DNA upon using ECO r_1 endonuclease similar to those of clinical biopsy and drinking water, which were taken from a parallel study done by Al-Abdullah [11].

Discussion

Based on conventional tests, *H. pylori* were found in the sewage of the three general regions in Basrah: Five miles, Al-Jame'eyat, and Al-Ashar. The percent of this bacterium is higher in Five-miles and Al-Ashar. This may be attributed to the socioeconomic status, which is, however, a broad criterion and encompasses factors such as level of hygiene sanitation, density of living, and educational opportunities, some of which have been reported and could influence the high levels of *H. pylori*. Malaty et al. [12], Murray et al. [13] and Veldhuyzen van Zanten [14] concluded that the low socioeconomic status to be associated with an increased prevalence of *H. pylori* infection.

The success to culture *H. pylori* from sewage may be related to the method of isolation used, i.e. the MDCS [5]. The efficiency of this method may be ascribed to the simultaneous usage of two types of media, solid and liquid, to isolating *H. pylori*. The advantage of the MDCS lies in its versatility, as it permits a solid phase to be in direct contact with a liquid one. In this case, solid medium was MCUA and the liquid one was tryptic soy broth. It also allows phases with different compositions to be prepared as well as opposite slants of different ingredients. For example, it is possible to prepare one slant with the use of MCUA for primary isolation of *H. pylori*. MCUA contains a number of selective, nutritional, and differential components. This formulation permits growth of *H. pylori*, preventing the growth of many bacteria, as it contains vancomycin which has a broad inhibition spectrum for other bacteria. The novel combination consisting of urea and phenol red was to permit presumptive identification of *H. pylori* colonies by color change induced by urease activity. In the current study, the results showed that from 156 isolates, only 76 isolates were positive for the three major tests (catalase, oxidase, and urease). This reflects considerable presence of *H. pylori* in untreated sewage water.

With the exception of urease, catalase and oxidase, the results of these tests are different from one isolate to another, which may reflect strain differences. Only six out of 76 isolates are similar in the results of all biochemical tests, and these six isolates were also found to harbor 16S rRNA gene of *H. pylori*. All isolates that are positive to the three major tests are sensitive for Nalidixic acid and resistant for Cephalothin. This result is in accordance with Blaser [15], who said that most strains of *H. pylori* were sensitive to nalidixic acid and resistant for Cephalothin, and few of them gave opposite results. Also, these results were in agreement with Al-Sulami et al. [16] who found that all isolates of *H. pylori* from drinking water were sensitive for Nalidixic acid and resistant for Cephalothin.

The 16S rRNA primer was the best choice according to many researchers as 16S rRNA which is specific for the detection of *H. pylori* [6,17-22].

As reported in the result section, only 6 isolates from 76 biochemically identified isolates were *H. pylori* positive, i.e. giving DNA band of 520 bp. All of these isolates were from Five miles region, and the other 70 isolates gave negative results. These may be ascribed to the differences in the strains or may be related to the absence of intact template DNA, or in highly recombining genome like *H. pylori*, PCR primer annealing sites can pose problem and amplification may not be generated [23,24] or an insertion might have occurred, or polymorphism or mutation, or may be due to inhibitors. Liu et al. [25] said that PCR detection of *H. pylori* in biological specimens is rendered difficult by the extensive polymorphism of *H. pylori* genes and the suppressed expression of some genes in many strains. Consequently these results create less confidence in conventional tests.

The second primer that has been used in this study for detection of *H. pylori* in sewage is Ure A primer. The specificity of this primer for the detection of *H. pylori* was reported by some researchers [17,20,26,27]. As shown in the results, all 76 samples that are positive for the three major tests for the detection of *H. pylori*, gave negative results by giving DNA band of 50-100 bp, while the real weight was 411 bp. This result is identical to the result of Tiveljung et al. [28], when they found in their study, that some samples fail to detect the Ure A gene in two histologically normal controls, which were positive for *H. pylori* by 16S rRNA. Tsuda et al. [29] gave the same results, while Clayton et al. [30] used Ure A gene to detect *H. pylori* from clinical and environmental samples and found that the sensitivity shown by this technique was low to be applied to environmental samples. The specificity of 16S rRNA was 7.89% while for Ure A gene was 0.0%.

In this study, the negative results of Ure A gene may be due to sequence polymorphism in some location in Ure A gene [31-33].

Conclusions

From all results that are reported above, it is concluded that the detection of *H. pylori* should be confirmed by PCR technique with 16S rRNA primer because of the fact that it is more specific for this species of bacteria than Ure A primers. This is supported by Horiuchi et al. [17] when they found that PCR with 16S rRNA primers is more sensitive method for the detection of *H. pylori* than PCR with Ure A primers. In the present study, we found that PCR technique is rapid and highly sensitive to be used to detect the presence of *H. pylori* in sewage. However, PCR is expensive, the assay is difficult to setup. Specificity may be compromised by contamination, and it is not widely available outside the research laboratory.

The similar RFLP pattern of *H. pylori* isolated from sewage, clinical and drinking water could be of an epidemiological significance, which need extensive research.

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