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RESEARCH ARTICLE

DETECTING *HELICOBACTER PYLORI* IN WATER OF PET TURTLES AT PUEBLA, MÉXICO

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ABSTRACT

H. pylori is a microaerophilic bacterium that causes chronic gastritis in half the world's population. *H. pylori* is the etiological agent of 95% of duodenal ulcers and 70-80% of gastric ulcers and 60-70% of cases of gastric cancer. Routes have been proposed for the transmission of *H. pylori* infection are: fecal-oral, oral-oral, gastro-oral routes. Also it has been proposed water as a means for the transmission of *H. pylori*. There are few reports about whether *H. pylori* can be transmitted from animal reservoirs. Present study aimed to seek evidence the occurrence of *H. pylori* through detection of *16S rRNA* gene of this bacterium in water where domestical turtles are kept.

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INTRODUCTION

H. pylori is a microaerophilic Gram negative bacterium; it causes chronic gastritis in half the world's population, also it is the etiological agent of 95% of duodenal ulcers and 70-80% of gastric ulcers (Brown, 2000; Bruce and Maaroos, 2008; Hagymási and Tulassay, 2014). *H. pylori* plays an important role in 60-70% of cases of gastric cancer (one of the most common cancers worldwide) (Graham *et al*., 1991; Hagymási and Tulassay, 2014; Herrera and Parsonnet, 2009; Kim *et al*., 2011; Lee, 1994; Polk and Peek, 2010; Watar *et al*., 2014). Epidemiology and prevalence of *H. pylori* infection is different in developed and developing countries: it is higher in developing countries than in the developed countries (Ahuja *et al*., 2002; Brown, 2000; Cullen, 1993; Glynn *et al*., 2002; Graham *et al*., 1991; Khalifa *et al*., 2010; Pounder and Ng, 1995). Several routes have been proposed for the transmission of *H. pylori* infection: fecal-oral, oral-oral, gastro-oral routes; it has also been considered water as a means for the transmission of *H. pylori* (Allaker *et al*., 2002; Anand *et al*., 2014; Bahrami *et al*., 2013; Brown, 2000; Madinier *et al*., 1997; Parsonnet *et al*., 1999; Pounder and Ng, 1995; Safaei *et al*., 2011; Thomas *et al*., 1992; Valdez-González *et al*., 2014; Vincent, 1995).

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H. pylori appears to have multiple routes of transmission. There are few reports about whether *H. pylori* can be transmitted from animal reservoirs. Por example, *H. heilmanni* is a zoonotic bacterium found in humans and in animals such as cats, dogs and pigs. There are reports about children with chronic gastritis and presence of *H. heilmannii*, which reported having dogs with chronic vomiting. The animals were subjected to endoscopy and biopsy finding microorganisms of the genus *Helicobacter sp.* (Happonen, 1996; Hernández and Gallón, 2004; Van, 2005). Studies have reported the presence of *H. pylori* in feces, saliva and dental plaque secretions of cats, which led to suggest that this bacterium could be a zoonotic agent to humans (Hernandez and Gallón, 2004). In reptiles, the reports concerning the presence of *H. pylori* is very limited. A study shows pathological findings of *Helicobacter* sp. associated to sepsis of a turtle (*Malacochersus tornieri*) (Brian, 2010).

MATERIALS AND METHODS

Water samples and recovery of microorganisms

For this study 20 samples of water were collected from glass and plastic containers where turtles (*Trachemys scripta elegans*) are kept routinely. The sites for water monitoring were selected randomly in different neighborhoods of the city of

Puebla. Water samples were collected with the permission of the people who had containers of pet turtle at home. 3 samples of water (900 mL) were collected from each monitored site. Each water sample was collected directly from container of pet turtle in bottles of 1000 mL wide-mouthed sterile polypropylene, being transported to laboratory within 2 hours and stored under refrigeration (4°C) until analysis. To remove feces and food waste, water was filtered using sterile filter paper. Each water sample, 1 mL aliquot was taken and isolation of bacteria was carried out. Then samples of water were filtered through 0.22 µm filter membrane (Millipore Co) to collect the microorganisms in them. Each membrane was placed in a sterile 50 mL conical tube and it was washed with 1mL of TE buffer (10 mM Tris-CI pH 7.5, 1 mM EDTA). Washing solution was recovered into a 1.5-mL Eppendorf tube, which were stored at -30°C for later analysis. After this, DNA extraction was performed.

Isolation of bacteria

For isolation of bacteria from water samples was performed using microbial methods. Thus it was placed 1mL of sample water in Petri dishes containing tryptone soy agar (Difco Co), it incubated at 37°C for 24 hours. For the identification of bacteria present in the water samples different microbial biochemical tests were used. Isolation and bacterial identification tests were performed both water samples than are routinely placed to domestical turtles as water samples taken from the containers of the turtles.

DNA extraction

Genomic DNA was extracted according to methodology described by Ho *et al*., (1991). It was added 30 µL of 10% sodium dodecyl sulfate and 3 µL (2 mg/100 µL) of proteinase K to each aliquots of 1mL, previously obtained and it was incubated at 37°C for 1 h. The mixtures were extracted with an equal volume of phenol-chloroform-isoamyl alcohol and centrifuged at 12,000 x g in a microcentrifuge for 3 min; the aqueous layer was transferred to a fresh Eppendorf tube. Two further extractions were performed with equal volumes of phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. The DNA was precipitated with 0.7 volume of isopropanol at -20°C. The genomic DNA was pelleted by microcentrifugation at 13,000 x g for 5 min, washed with -20°C 70% (vol/vol) ethanol, desiccated for 30 min, and dissolved in 50 µL of molecular biology-grade water. The DNA was quantified spectrophotometry. Genomic extracted DNA was stored at -30°C.

Assay conditions for gene *16S rRNA* amplification by nested PCR

Amplification of the DNA template was carried out using primers Hp1, Hp2 and Hp3 previously described by Ho *et al*., (1991) and Mazari-Hiriart *et al*. (2001). Thus, it was added 0.5 µL of each oligonucleotide primer (50 pmol/µLfor each primer) in an Eppendorf tube, 5 µL of extracted DNA, 2.5 µL of 10X PCR buffer (500 mM KCl, 100 mM Tris-Cl, 15 mM $MgCl_2$, pH 8.3), 1 µL of deoxynucleoside triphosphate mixture (final concentration, 1.25 mM each dATP, dCTP, dGTP, and dTTP). 0.3 µL of Taq DNA polymerase and molecular biology-grade distilled water were added to make a final reaction volume of 25 µL. For nested PCR, 30 cycles were used for each round of amplification. The temperature profile was as follows: 4 min at 94°C, 45 s at 94°C, 45 s at 60°C, 45 s at 72°C. The last cycle was identical, except that the 72°C extension period was increased to 7 min and the mixture was subsequently refrigerated at 4°C before analysis. Primers were used to amplify *H. pylori* by nested PCR with the following sequences: Hp1: 5′-CTG GAG AGA CTA AGC CCT CC-3′, Hp2: 5′-ATT ACT GAC GCT GAT TGT GC-3′, and Hp3: 5′-AGG ATG AAG GTT TAA GGA TT 3′.

Analysis of PCR products

Aliquots of each PCR product were separated by electrophoresis in a 1.5% (w/v) agarose gel (Ultra Pure, Invitrogen) with the MBI Fermentas (Amherst NY) 100 bp DNA Ladder Plus used as a size marker, in TAE buffer (90 mM Tris-HCl, 90 mM acetic acid, 2 mM EDTA and stained in ethidium bromide at 0.06 µg/mL). Positive and negative controls were included in all assays to monitor specificity and laboratory contamination during the analyses. The specificity of the PCR assays has been previously reported by Ho *et al*., (1991). Assays on all samples were repeated in duplicate. Samples were interpreted as being positive for the presence of *Helicobacter* DNA if one or more of the assays produced a fragment comparable in size to that of the positive control DNA (*H. pylori* 26695).

RESULTS

20 samples of water (each triplicate) were collected from containers where routinely domestical turtles were kept. Water samples were taken from different places at Puebla city. Feces and other organic wastes were eliminated by filtrating. From water samples, isolation and culture of bacteria was performed both water samples that were routinely added to containers as water samples taken from containers containing turtles. The results are shown in Table 1. As shown in Table 1, 5 of 20 water samples (routinely used in containers) were positive for the presence of bacteria. In all cases it was possible to isolate *Escherichia coli*. While in 12 of 20 water samples taken from containers containing turtles, it was recorded positive bacterial growth. In this case, the bacterial genera found were: *Salmonella* sp., *Proteus* sp., *Klebsiella pneumoniae* y *E. coli*. Therefore, 25% of water samples that were routinely added to containers were positive for the presence of *E. coli* enterobacterium, while that 60% water samples taken from containers containing turtles recorded other bacterial genera, also medically important.

Subsequently, it proceeded to the extraction of DNA from water samples that showed bacterial growth. For that, water samples in bottles of 1000 mL, removing feces and food waste and filtered through 0.22 µm filter membrane to collect bacteria. After this, DNA extraction from water samples was performed according to comun protocol using phenol-chloroform-isoamyl alcohol and ethanol. DNA concentrations determined from water filtred samples are shown in Table 2.

Table 1. Growth registred in culture dishes and isolation of bacteria

Water samples	Growth in culture dishes: Added to containers (without turtles)	Taken from containers (with turtles)
1	Negative	Positive (*Salmonella* sp.)
2	Negative	Positive (*Salmonella* sp.)
3	Negative	Positive (*Klebsiella pneumoniae*)
4	Positive (*Escherichia coli*)	Positive (*Salmonella* sp.)
5	Positive (*Escherichia coli*)	Positive (*Escherichia coli*)
6	Positive (*Escherichia coli*)	Positive (*Salmonella* sp.)
7	Negative	Positive (*Salmonella* sp.)
8	Negative	Positive (*Salmonella* sp.)
9	Positive (*Escherichia coli*)	Positive (*Proteus* sp.)
10	Positive (*Escherichia coli*)	Positive (*Escherichia coli*)
11	Negative	Positive (*Salmonella* sp.)
12	Negative	Positive (*Salmonella* sp.)
13	Negative	Negative
14	Negative	Negative
15	Negative	Negative
16	Negative	Negative
17	Negative	Negative
18	Negative	Negative
19	Negative	Negative
20	Negative	Negative

Table 2. Concentrations of DNA extracted from water samples

Water samples	DNA concentrations (ng//µL)[a]: Added to containers (without turtles)	Taken from containers (with turtles)
1	250	225
2	125	765
3	155	295
4	120	625
5	163	163
6	-	313
7	-	200
8	-	320
9	-	415
10	-	413
11	-	235
12	-	713

[a]Average value of DNA concentration from 3 measurements

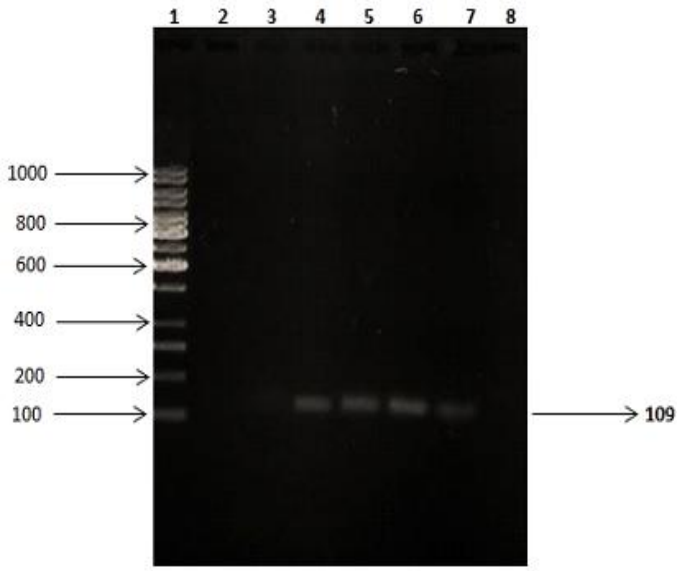

Fig. 1. Some *H. pylori* PCR products isolated from water samples. PCR products were analyzed by gel electrophoresis and ethidium bromide staining. Lane 1, MBI Fermentans 100 bp DNA Ladder used as a size marker; Lane 2, water sample No. 4; Lane 3, water sample No. 5; Lane 4, water sample No. 4; Lane 5, water sample No. 2; Lane 6, water sample No. 3; Lane 7, DNA amplified from *H. pylori* (NCTC 26695); Lane 8, negative control

As shown in Table 2, all samples of probed water were positive for the presence of DNA. It was a confirmatory test that there were bacteria in the water. As shown in Table 2, all analysed water samples containing different concentrations of DNA from 120 to 250 ng/µL of DNA from water samples than are routinely placed to domestical turtles, while water samples taken from the containers of the turtles have the highest concentrations of DNA: 163 to 713 ng/µL of DNA. DNA purity of each the samples of analyzed water showed average values of 1.15 (by rate OD260nm/280nm OD) (data not shown). Later, detection of *H. pylori* using Hp1, Hp2 and Hp3 primers that specifically amplify the *16S rRNA* gene was performed. Amplification conditions for *16S rRNA* gene detected by nested PCR were described in Material and Methods. PCR analysis amplified an approximately 109 base pair DNA fragments. In this study, *16S rRNA* gene of *H. pylori* was detected in 1 of 5 water samples that were routinely added to containers (data not shown) and in 6 of 12 water samples taken from containers containing turtles. It resulted novel because the DNA of *H. pylori* was found in 50% of water samples containing bacteria. Some *H. pylori* PCR products isolated from water samples taken from containers containing turtles are shown in Fig. 1. Lanes 4, 5 y 6 are examples of positive PCR; lane 7 represents positive control of *H. pylori*.

DISCUSSION

The relationship between humans and animals has been changing throughout history. Animals have been used as the working medium, as a food source, as protection for the home, as a symbol sacred instrument or object of worship, as models for research, as guides for persons with disabilities, as a source of affection for their owners (pets) (Gutiérrez *et al*. 2007). In recent years, it has increased the number of pets in large cities due to various factors. Sharp increase in pets in cities begins to pose serious problems of cohabitation, while requiring a review of the impact on public health (zoonoses) (Gómez *et al*. 2007). All population can contract a disease transmitted by animals, but their impact is much greater in children and immunocompromised persons; people who work with animals or handling contaminated products are also risk staff (Maguiña *et al*., 2004; Pacheco, 2003; Zúñiga-Carrasco and Caro-Lozano, 2007). In recent years, it has been frequently observed emerging and re-emerging infections transmitted through zoonoses, a phenomenon that has been favored by climate change, population migration and others.

In addition to dogs and cats that are owned as pets, reptiles are carriers of microorganisms that cause serious damage to public health. It has been reported that 7% of *Salmonella* infections at USA are associated with possession of reptiles (at England is about 1%) (Zuñiga-Carrasco and Caro-Lozano, 2007). So cases of salmonellosis in young children who have reptiles as pets have been reported (Warwick *et al*., 2001). It has been estimated that approximately 93.8 million human cases of gastroenteritis and 155,000 deaths occur due to *Salmonella* infection around the world each year. In the USA alone, *Salmonella* causes an estimated 1,4 million human cases, 15,000 hospitalizations and more than 400 deaths annually (Hoelzer *et al*., 2011; Majowicz *et al.*, 2010; Mead *et al.*, 1999; Voetsch *et al.*, 2004). In this estudy, Table 1 shows positive results for the presence of bacteria in water samples, specially from water samples taken from containers containing turtles. The most frequently bacterial genus isolated was *Salmonella*, resulting in 66% of cases from water samples taken from containers containing turtles. This results are according to those reported by other authors (Hoelzer *et al*., 2011; Nakadai *et al*., 2004; Stam *et al*., 2003; Voetsch *et al*., 2004; Warwick *et al.*, 2001). In addition to Salmonella, other bacterial genera were found, such as *E. coli*, *Proteus* sp. and *K. pneumoniae.* Only some water samples that routinely were added to containers showed bacterial contamination by *E. coli* (Table 1). DNA found in the water samples was another important evidence to demonstrate the presence of microorganisms in the water samples, with a higher content in water samples taken from containers containing turtles (Table 2). It has reported the existence of enterobacteria in the water and on pets in Mexico, which are vehicles for the transmission of diseases by coexistence with animals (Mazari *et al*., 2001; Pacheco, 2003; Zúñiga-Carrasco and Caro-Lozano, 2007).

Furthermore, it proceeded to carry out the search for *H. pylori* in water samples using a test to amplify the *16S rRNA* gene using Hp1, Hp2 and Hp3 primers by PCR analysis. This method was used because it is highly sencible and can detect *H. pylori* easily and quickly in aqueous environments. It has been reported that PCR method used for this study could detect as little as 0.01-0.1 pg of *H. pylori* DNA, representing the detection of about 10-100 bacteria (Ho *et al*., 1991). In this study, microbiological methods were not used because takes much longer and in most cases and growth of *H. pylori* is inhibited due the faster growth of other enterobacteria in the environment. In this study, *16S rRNA* gene of *H. pylori* was detected in 50% of water samples taken from containers containing turtles and 20% of water samples that were routinely added to containers. The transmission of *H. pylori* occurs by fecal-oral, oral-oral, gastro-oral routes. Also water has been considered a means for transmission of *H. pylori* (Anand *et al*., 2014; Brown, 2000; Graham *et al*., 1991; Hagymási *et al*., 2014; Madinier *et al*., 1997; Michell *et al*., 1992 ; Parsonnet *et al.,* 1999; Thomas *et al.*, 1992). *H. pylori* can survive in water for months adopting the coccoid morphotype. *H. pylori* is subject to stressful environmental conditions, it acquires coccoid form (Azevedo *et al*., 2007; Bode *et al*., 1993; Bunn *et al.*, 2002; Chen *et al*., 2004; Fernández-Delgado *et al*., 2008; Flores-Encarnación *et al*., 2015; Percival and Suleman, 2014; Salama *et al*., 2013; Vincent, 1995). In the gastric mucosa of pet animals found spiral bacteria and them were considered normal flora. It described *H. heilmanni* as a bacterial specie found in humans and animals (such as cats, dogs and pigs). It knows of one case of a child infected with *H. heilmanni* from having dogs as pets. (Happonen, 1996; Hernandez and Gallón, 2004; Van, 2005). So presence of *H. pylori* in water samples taken from containers containing turtles, indicates a potential risk of *H. pylori* infection for manipulators for turtles, especially when it comes to children or great age or immunocompromised persons. Water taken from containers containing turtles is a vehicle for the storage and dissemination of pathogenic bacteria to humans (for example: *Salmonella, H. pylori* and other) because it has a poor microbiologic quality. Therefore it is important that pet owners like turtles know about biological risks have to possess

these animals at home. It is important to be careful with the handling of pet waste (eg turtle waste water), and prevent be disposed in the drain or gardens are irrigated with contaminated water with *Salmonella* or *H. pylori*.

Conclusion

The presence of *H. pylori* in water taken from containers containing turtles is a potential risk of human infection with *H. pylori* via handling of water with poor microbiological quality. It is important to carry out the sanitation of waste water before dumping to the drain. It is suggested that security measures are implemented to protect the health of people who have turtles as pets in their homes.

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