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Application of Molecular and Cultural Methods for Identification of *Helicobacter* spp. In Different Animal Sources

Mohammad Tabatabaei

School of Veterinary Medicine, Shiraz University, Shiraz, Iran

Abstract: It is known that *Helicobacter pylori* is a cause of chronic gastritis and peptic ulcer disease in humans. However the origin and transmission of this bacterium has not been clearly explained. One of the suggested theories is transmission from animal sources such as milk from animals to human beings. In this study, the presence of *Helicobacter spp and H. pylori* was investigated in different animal samples such as milk, feces and abomasal contents of cow, sheep and goats. For this purpose, a total of 80 milk samples and 60 samples from feces and abomasal contents collected from the south west regions of Iran were examined by specific cultural procedures and PCR. *H. pylori* was not isolated from any sample except one goat fecal sample as indicated by culture and PCR. It is suggested that *H. pylori* is not present in animal sources collected from different region of Iran and transmission of *H. pylori* from animal sources such as milk is not a primary factor in infection related to this bacterium in humans, but the possibility of other modes of transmission from environmental samples to humans should be considered.

Key words: *Helicobacter* spp · *H. pylori* · Chronic Gastritis · Iran.

INTRODUCTION

The *Helicobacter* genus consists of a group of microaerophilic, nonsporulating, Gram-negative rods that colonize the mucus layer covering the epithelial surface of the gastrointestinal tract of humans and a variety of animal species. There are currently 6 validated *Helicobacter* species isolated from gastric tissue and 16 validated entero hepatic species. Some *Helicobacter* species may be commonly (*H. aurati*) or occasionally (*H. bilis* and *H. muridarum*) isolated from both gastric and entero hepatic sites [1].

The type species of the genus, *H. pylori* is the principal cause of chronic gastritis and is strongly associated with peptic ulcer disease [2, 3]. Current knowledge implies that *H. pylori* infection occurs during childhood or adolescence both in developing and developed countries. Its occurrence may increase the risk of the development of gastric cancer [4]. Prevalence of *H. pylori* in children ranges from below 10% to more than 80% [5]. Prevalence is low in developed countries and high in developing countries. More than 50% of children in our country are infected with *H. pylori* [6].

Although *H. pylori* is present in the stomachs of about half of world's population, the routes of transmission are still unclear and non human reservoirs have not been identified. The prevalence of *H. pylori* infection increases with age and is inversely related to socioeconomic and hygiene status, suggesting person-to-person transmission [7-9]. Several studies have shown high prevalence of antibodies against *H. pylori* in abattoir workers, such as veterinarians, butchers and slaughterers, suggesting that *H. pylori* might be transmitted from animals to man [10-12]. Isolation of *H. pylori* from domestic cats, obtained from a single commercial source of research animals, also raised the possibility that the infection may be transmitted from cats to humans (or *vice versa*) [13, 14]. Dogs and sheep have also been implicated in the transmission of *Helicobacter* infection [15, 16]. For example, Goodman *et al.* [16] observed an excess prevalence of *H. pylori* infection among children in the Columbian Andes who had contact with sheep. Dore *et al*. [17] observed a very high prevalence of *H. pylori* infection in school children, living in rural area as in Sardinia and multiple regression analyses revealed that contact with dogs was a powerful independent risk factor for *H. pylori* infection in that population.

Corresponding Author: Mohammad Tabatabaei, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.
Tel: +98711 6138696 / 6138660.

Recently, the recovery of *H. pylori* from cow's and sheep milk and gastric tissue samples has suggested that cow and sheep are natural ancestral hosts of *H. pylori* and *H. pylori* infection in humans that is associated with contact with cow and sheep [18-20].

The aim of the present study was to investigate whether or not *Helicobacter spp and H. pylori* are present in different animal samples such as milk, feces and abomasal contents of cow, sheep and goats in Iran.

MATERIALS AND METHODS

Sampling Details: Sixty raw milk samples were collected from cows, sheep and goats (20 samples of each) in different geographical regions of south west of Iran. From each udder of each animal, approximately 5-10 ml of milk was pooled into sterile falcon tubes, after first cleaning and disinfecting the teats. All milk samples were transported to the laboratory ice cooled within 2h of the collection. These samples were frozen at -80°C until processing. Twenty commercial pasteurized and sterilized milk samples (10 of each) from 10 companies were also investigated immediately after shipping from the factories.

Also thirty samples of abomasal contents of cow, sheep and goats (10 for each) were collected from Shiraz slaughter house and 30 fresh fecal samples from cows, sheep and goats (10 for each) were collected from different regions around the Shiraz city. All samples were immediately transferred to Microbiology Laboratory in Shiraz Faculty of Veterinary Medicine. These samples were then stored at -20°C until use.

Bacterial Growth Conditions: From each milk sample 100µL were cultured onto Brucella agar plates containing 7% defibrinated horse blood containing Skirrow's supplement (Vancomycin, 0.01%, Trimethoprim, 0.05% and Polymyxin B, 2.5IU/ml, Oxoid). Plates were incubated at 37°C with 95–99% relative humidity under microaerophilic conditions (5% O_2, 15% CO_2 and 80% N_2) with an anaerobic jar system and a Microbiology Anaerocult C (Merck, Germany) [2, 3, 21]. A high degree of humidity during incubation, obtained by placing wet paper towel in anaerobe jars. Plates were observed daily for 5-7 days and growth suggestive of a *Helicobacter* spp. was identified either as small (1 mm or less in diameter), clear, dome-shaped colonies or as a fine, translucent lawn [1].

Growing colonies were examined for *H. pylori* on the basis of colony morphology and positive biochemical reactions for catalase, urease, oxidase (although many entero hepatic species are urease negative) and negative Gram stain [2, 3]. To confirm the isolation techniques, 5 clinical isolates of *H. pylori* were obtained by biopsy from patients with suspected gastritis at the time of endoscopic examination at the Namazi Hospital, in Shiraz, Iran. Biopsy tissue was used to inoculate brucella agar plates containing 7% horse blood and Skirrow's supplement and the plates were cultured at the above conditions and checked for the growing colonies as above.

For isolation of entero hepatic *Helicobacter* species we used media and antibiotic supplements similar to those used for isolation of gastric *Helicobacter*. When entero hepatic *Helicobacter* were isolated from fecal samples or abomasal contents, 2 g of each fecal sample suspended in 3ml of sterilized phosphate buffered saline (PBS) pH 7.4 and slurry of fecal samples or abomasal contents were inoculated by the modified filter technique of Steele and McDermott [22]. Briefly, a sterile cellulose acetate membrane filter (0.45µm) was applied with a sterile pair of tweezers directly onto the surface of the agar. When the filter was totally absorbed on the selective agar, ~500 µl of the mixture was placed in the middle of the filter. The plates were incubated at 37°C for approximately two hours in a 5% CO_2, the filter was removed with a sterile pair of tweezers and the filtrate was streaked on the agar with a loop and the plates were incubated under the appropriate conditions for 5-7 days.

Genomic DNA Extraction: For extraction of genomic DNA from milk samples; 2 ml of each milk sample were centrifuged in a micro centrifuge (13000rpm/5min) and the precipitated cells were extracted using Accuprep Genomic DNA extraction kit (Bionneer, Korea).

For extraction of DNA from fecal samples and abomasal contents with high concentrations of PCR inhibitors, the following protocol was used; 100mg amount of feces or abomasal contents was thoroughly suspended in 1ml of lysis buffer (50mM EDTA, 50mM Tris-HCl, 0.5% Tween 20, 0.5% Triton X-100 [pH 8.0]) and the suspension was centrifuged (3000rpm/2min). Debris was discarded. The supernatant was boiled (10min) to ensure complete lysis and was centrifuged (8000rpm/10min) to remove particles. The DNA solution was incubated (30min, 37°C) in the presence of RNase A (0.2mg/ml) and proteinase K (0.5mg/ml). Then, a one-third volume of guanidine-HCl buffer (3M guanidine-HCl, 20% Tween 20 [pH 5.5]) was added and the specimen was incubated for 30min at 50°C. DNA was purified by column chromatography (Genomic Tip 20/G) according to the manufacturer's protocol (Fermentas, Germany), total

nucleic acids were purified and concentrated by standard phenol: chloroform extraction and sodium chloride/ethanol precipitation [23]. Precipitated nucleic acids were washed once with 70% ethanol, air-dried and resuspended in a final volume of sterile TE (40-60µl). DNA content and purity were determined spectrophotometrically by measuring the A260/A280 optical density ratio.

PCR Amplification and Detection of *Helicobacter* Spp. And *H. Pylori*: Extracted DNA template quality was confirmed by PCR amplification using universal bacterial 16S rDNA primers 46f and 519r (5'-GCY TAA CAC ATG CAA GTC GA-3' and 5'-GTA TTA CCG CGG CKG CTG-3') to amplify a 490bp fragment [24] (data not shown). This was necessary in order to determine whether negative PCR results were false due to the presence of PCR inhibitors. DNA extractions that yielded amplification products of the expected size (ca. 490bp) were used as template for PCR amplification with *Helicobacter* genus-specific and *H. pylori* species-specific primers. To detect the genus *Helicobacter*, we used PCR primers H276f [5'CTA TGA CGG GTA TCC GGC3' (nt 276-293)] and H676r [5'ATT CCA CCT ACC TCT CCC A3' (nt 676-658)] that amplify a 374bp fragment of the 16S rRNA gene from all known members of this genus [25]. Species-specific amplification of *H. pylori* was carried out using PCR primers HPU1 (5'GCC AAT GGT AAA TTA GTT GT3') and HPU2 (5'CTC CTT AAT TGT TTT TAC3') to amplify a 411bp product from *urease gene* A (nt 304 to 714) [26]. All reactions were performed in a 50µl volume with an automated CP2-003 thermal cycler (Corbett Research, Australia). Reaction mixtures contained each oligonucleotide primer at 1mM, PCR buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM Kcl [pH8.3]), 2.5U of *Taq* polymerase (Fermentas, Germany) and 1.25mg of template DNA, unless otherwise stated. Samples were heated at 94°C for 3 min and then subjected to 30 cycles consisting of denaturation (94°C, 45s), primer annealing (53°C, 45s) and extension (72°C, 60s). This PCR was performed in duplicate. Amplified DNA fragments were resolved by gel electrophoresis (5V/cm, 60 min) using 1.5% agarose gels in Tris Boric acid- EDTA (TBE) buffer. Gels were stained with 0.5µg of ethidium bromide (EtBr) per ml, visualized and photographed under UV illumination. 100bp DNA ladder (Fermentas, Germany) was used as a size reference for PCR assay.

RESULTS

Despite prolonged incubation for up to 10 days under microaerophilic conditions, no *Helicobacter* species were grown from the raw, pasteurized or sterilized milk and from abomasal content samples. But 4 of 5 cultures of biopsies from patients with suspected gastritis were grown as small, clear, dome-shaped colonies. Further analysis of this isolate by PCR with *Helicobacter* genus-specific rRNA primers and *H. pylori* species-specific primers amplified the expected 374bp and 411bp fragments respectively (Fig. 1 and 2).

In the other hand we were able to identify just one isolate from goat fecal samples as *Helicobacter* on the basis of growth as a thin watery film and positive biochemical reactions for catalase, urease, oxidase and negative Gram stain. Further analysis of this isolate by PCR with *Helicobacter* genus-specific rRNA primers amplified the expected 374bp fragment (Fig. 1).

Also DNA extracted from all milk sediment samples and homogenates of abomasal content were tested by *Helicobacter* genus-specific 16S rRNA primers and were negative.

These data indicate the absence of *H. pylori* in freshly collected non-pasteurized and pasteurized and sterilized milk and suggest that milk could not be an intermediate transmission vehicle of *H. pylori* infection in Iran.

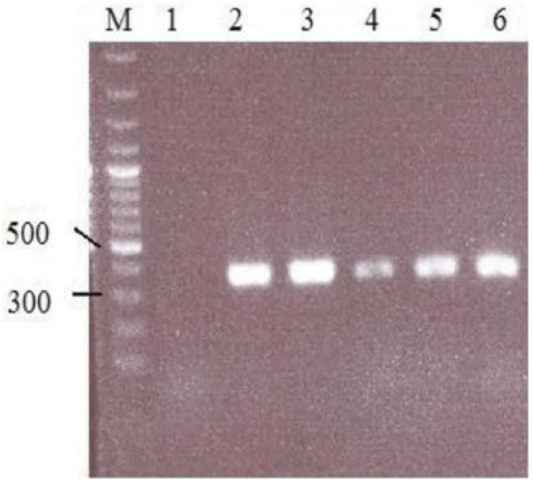

Fig. 1: PCR amplification products from DNAs of various samples with Helicobacter genus-specific rRNA primers. Lanes: M, 100bp DNA ladder, 1, negative control, no genomic DNA, 2-5 DNA isolated from gastric biopsies, 6, goat fecal isolate.

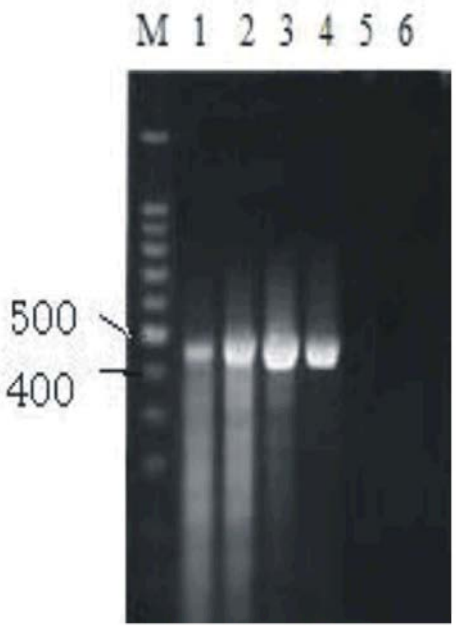

Fig. 2: PCR amplification products from DNAs of various samples with H. pylori species-specific primers. Lanes: M, 100bp DNA ladder, 1-4, DNA isolated from gastric biopsies growth samples, 5, DNA of goat fecal isolate, 6, negative control, no genomic DNA.

DISCUSSION

H. pylori infection is one of the most common infections worldwide. However, it is not known how *H. pylori* is transmitted and where in the natural environment the organism resides [27]. It is likely that raw sheep's milk could be an intermediate transmission vehicle of *H. pylori* infection [18]. It has been reported that *H. pylori* is almost always acquired in childhood [28]. In the other hand Sasaki *et al*. [29] reported that *H. pylori* was detected in cow's feces and soil and the bacteria could invade the teat channel of a cow when it was sprawled on ground including cow's feces and soil [30]. As reported by Fujimura *et al.* [20] that *H. pylori* gene is frequently detected in cow`s milk samples, the samples might have been contaminated with the organism from contaminated soil. Furthermore, they have shown the possibility that *H. pylori* survives in raw milk.

In this study, we failed to culture *H. pylori* from different raw milk samples and pasteurized and sterilized cow's milk. Comparing our study results to those obtained by Fujimura *et al.* [20] who found a high occurrence of *H. pylori* in cow's feces (50%), raw milk (72.2%) and in commercial pasteurized milk samples (55%). This apparent discrepancy could be due to cross-contamination with animal and human sources during milk processing. *H. pylori* may survive in cow's milk for a short time after delivery to the factory but in Iran, many manufacturers sterilize cow's milk at 120-140°C for 2-3s and this sterilization procedure apparently is effective against *H. pylori*. Also our results in some parts resembled to the work of Turutoglu and Mudul [31] as they could not isolated any *Helicobacter* from sheep milk samples. The absence of *H. pylori* in milk samples might be as a result of the geographical spread of the bacteria. In the other hand the presence of *H. pylori* in sheep milk has not been reported form anywhere, except Sardinia [18, 19]. *H. pylori* may not be cultured in pasteurized cow's milk because it may change to a coccoid form with sterilization [20]. In this study, we failed to culture *H. pylori* in pasteurized cow's milk. However, it has been shown that *H. pylori* survived in pasteurized skim milk up to 5 days after inoculation [32].

Thus our data suggest that sheep, goat and cow's milk can not play an important role in transmission of *H. pylori* infection in our study area.

H. pylori has been cultured from stool and has been identified in feces by PCR [33, 34]. *H. pylori* can survive in milk for several days, suggesting that milk contaminated with *H. pylori*-containing feces could be potentially infectious to humans [35]. Alternatively, animals might transmit the infection to humans through the environment contaminated by feces containing *H. pylori* or the coccoid forms [36]. But in the current study we failed to detect *Helicobacter* spp. by culture and PCR technique in feces of different animal except one goat`s fecal sample.

In general, Iranian people in urban area frequently drink pasteurized and sterilized cow's milk but in rural area people drink heated sheep and goat`s milk. However, the *H. pylori* prevalence in Iranian children is high [6]. It may be unreasonable to understand milk to be the major factor in *H. pylori* transmission. According to the report of Nouraie *et al*. [37] that low education of the study subjects; low father's and mother's education; poor tooth brushing habit; crowded families in childhood and lack of household bath, hygienic drinking water and swage disposal facility in childhood were determined as possible risk factors for transmission of *H. pylori* in human population in Iran. In addition, since the host factor is

important in colonization of *H. pyl*ori in the human, the infection may be established in a subset of individuals who drink cow's milk.

As a conclusion, it is suggested that *H. pylori* is not present in animal sources collected from different region of Iran and transmission of *H. pylori* from animal sources such as milk is not a primary factor in infection related to this bacterium in humans, but the possibility of other modes of transmission from environmental samples to humans should be considered.

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