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Short communication

PCR-based detection of *Helicobacter pylori* and non-*Helicobacter pylori* species among humans and animals with potential for zoonotic infections

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Abstract

Helicobacter species have been reported in animals, some of which are of zoonotic importance. This study aimed to detect *Helicobacter* species among human and animal samples using conventional PCR assays and to identify their zoonotic potentials. *Helicobacter* species was identified in human and animal samples by genus-specific PCR assays and phylogenetic analysis of partial sequencing of the 16S ribosomal RNA gene. The results revealed that *Helicobacter* species DNA was detected in 13 of 29 (44.83%) of the human samples. *H. pylori* was identified in 2 (15.38%), and *H. bovis* was detected in 4 (30.77%), whereas 7 (53.85%) were unidentified. *H. bovis* and *H. heilmannii* were prevalent among the animal samples. Phylogenetic analysis revealed bootstrapping of sequences with *H. cinaedi* in camel, *H. rappini* in sheep and humans, and *Wollinella succinogenes* in humans. In conclusion, the occurrence of *non-H. pylori* infections among human and animal samples suggested zoonotic potentials.

Key words: *Helicobacte*r spp., zoonotic infections, detection, identification

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Introduction

Helicobacter species were initially considered to be members of the genus *Campylobacter* before being classified in a new genus named *Helicobacter* (Goodwin et al. 1989). Humans have been reported to be one of the primary hosts of different *Helicobacter* species, most of which are common and potentially pathogenic and are likely to be zoonotic infections from contact with animals (Fox 2002). *Helicobacter pylori* has been shown to be the primary cause of acid peptic conditions of the stomach. *H. pylori* is considered to be a significant risk factor in the development of stomach neoplasms (Van den Bulck et al. 2005).

Following the discovery of *H. pylori* in humans, *Helicobacter* species were detected in pets (Buczolits et al. 2003). *Helicobacter* species were then reported in wild and domestic mammals of different dietary habits (Solnick 2003). Zoonotic infections by *Helicobacter* species have been proposed (Fox 2002, Mladenova-Hristova et al. 2017). However, the roles of these species in the pathogenesis of gastric conditions in companion animals remain unknown (Amorim et al. 2015).

The data on the prevalence of *Helicobacter* species infections in humans and animals remain few. This study aimed to determine the prevalence of *Helicobacter* infection among humans and animals and to identify their zoonotic potentials.

Materials and Methods

Sample collection

Human sample collection

A total of 29 samples were collected from human patients; 21 fecal specimens from patients who were suffering from dyspepsia, nausea, vomiting, abdominal pain, and hematemesis, and 8 gastric mucosal biopsies from cases admitted to the Department of Internal Medicine, Suez Canal University Hospital, Ismailia, Egypt. The patients were asked to read and sign an informed consent form immediately before the endoscopy. The biopsies were inoculated into sterile Eppendorf tubes containing 1ml phosphate buffer saline (PBS). The samples were frozen at -20°C until DNA extraction. This study was approved by the Research Ethics Committee, Suez Canal University.

Animal sample collection

A total of 35 samples (14 fecal and 21 saliva samples) from dogs and 23 samples (9 fecal and 14 saliva samples) from cats were collected. These pets were hosted in private pet clinics in the Ismailia governorate, Egypt. Saliva swab samples were collected in tubes containing one ml sterile PBS solution**.** In addition, a total of 44 fecal samples were collected from 15 cattle 12 buffaloes, 8 sheep, and 9 camels from private farms located in the Ismailia governorate. Fresh fecal samples were collected from apparently healthy animals.

Ethical Approval

This study was approved by the Research Ethics Committee, Suez Canal University.

DNA extraction and PCR reactions of Helicobacter species

Bacterial DNA was extracted from fecal samples using the QIAamp DNA fecal Mini Kit (Qiagen) as per the manufacturer's instructions**.** Bacterial DNA was extracted from tissue samples of the collected gastric biopsies and saliva samples using an EZ-10 Spin Column Genomic DNA Minipreps Kit (Biobasic, Canada) following the manufacturer's instructions.

PCR was performed using a 2X Emerald Amp GT PCR master mix kit (Takara Bio). Oligonucleotide primers (Metabion, Germany) were used to amplify targeted genes of *Helicobacter* species. The primers were used for the detection of *Helicobacter* spp by targeting the 16S rRNA gene for amplifying of 398bp (Germani et al. 1997), *H. pylori* by amplifying of 109 bp (Chong et al. 1996), *H. bovis* by targeting the 16S rRNA gene for amplification of 259 bp (De Groote et al. 2005), *H. felis* by targeting the ureA/ureB genes for amplification of 241 bp (Germani et al. 1997), *H. heilmannii* by targeting of the ureB gene for amplification of 580 bp (Neiger et al. 1998). The PCR reactions and the thermal profiles were followed as shown in the original references. The PCR reactions were electrophoresed and photographed using a gel documentation system (Alpha Innotech), and the data were analyzed through computer software.

Partial sequencing of 16S rRNA gene of *Helicobacter* species

The PCR was performed amplifying the 1200 bp of the 16S rRNA gene of *Helicobacter* species as previously described (Harper et al. 2002). Temperature and time conditions of the PCR reactions were used according to specific authors. PCR product purification of the amplicons was performed using the QIAquick PCR product extraction kit (QIAGEN Inc. Valencia CA). A second purification of the sequence DNA amplicon was performed using the spin column (Centrisep, Cat

Table 1. Prevalence and PCR-based species identification of *Helicobacter* spp. among positive *Helicobacter* species samples from humans and animals.

Item	*Helicobacter* spp. 16S rRNA (%)	*Helicobacter* spp.			
		H. pylori (%)	*H. helmennii* (%)	*H. bovis* (%)	Other *spp.* (%)
Human (n=29)*	13 (44.83)	2 (15.38)	0	4 (30.77)	7 (53.85)
Dog (n=35)**	13 (37.14)	0	2 (15.38)	1 (7.69)	10 (76.92)
Cat (n=23)***	5 (21.74)	0	1 (20)	0	4 (80)
Cattle (n=15)	6 (40)	0	0	5 (83.33)	1 (16.67)
Buffalo (n=12)	4 (33.33)	0	0	3 (75)	1 (25)
Sheep (n=8)	2 (25)	0	0	1 (50)	1 (50)
Camel (n=9)	2 (22.22)	0	0	0	2 (100)

* human samples included 7 (33.33%) out of 21 for fecal samples and 6 (75%) out of 8 gastric biopsy specimens were positive for *Helicobacter* species.
** dog samples included 8 (57.14%) out of 14 fecal samples and 5 (23.8%) out of 21 saliva samples positive for *Helicobacter* spp.
*** cat samples included 3 (33.33%) out of 9 fecal samples and 2 (14.28%) out of 14 saliva samples positive for *Helicobacter* spp.

number CS-901) according to the instruction of the manufacturer's instructions. The sequence reaction consisted of a total of 20 µl containing 2 µl of Bigdye terminator v.3.1 (Perkin-Elmer/Applied Biosystems, Foster City, CA), 1 µl of forward or reverse primers, and PCR grade water up to 20 µl. An Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) was used for sequencing reactions.

Genome blasting and phylogenetic analysis

Five samples (three gastric biopsies from humans, one fecal sample from a sheep, and one fecal sample from a camel) were submitted for sequencing of the 1200 pb of the 16S rRNA gene to determine the species of some unidentified *Helicobacter*. Partial sequences of the 16S rRNA gene of *Helicobacter* species and other reference *Helicobacter* strain sequences were used in the analysis. The reference strain sequences were retrieved from the GenBank database. The raw reads were assembled using SPAdes3.12, then analyzed using the National Center for Biotechnology Information (NCBI) BLAST analysis. BioEdit version 7.2 software was used for multiple sequence alignment. Sequence alignments of nucleotide sequences were performed using the CLUSTAL-W program**.** The pair-wise nucleotide percent identity matrix with the sequences of reference *Helicobacter* species sequences was calculated using the CLUSTAL-V algorithm in the MegAlign program of the Lasergene software suite (DNASTAR, Madison, Wisconsin). Phylogenetic analyses of partial sequences of the 16S rRNA gene were conducted by the neighbor-joining method using MEGA software version X. The robustness of the groupings in the neighbor-joining analysis was assessed with 1000 bootstrap replicates.

Results

Prevalence of *Helicobacter* species among human samples

Among 29 human samples examined, 13 (44.83%) were positive for *Helicobacter* species. Seven (33.33%) out of 21 fecal samples and 6 (75%) out of 8 gastric biopsy specimens were positive for *Helicobacter* species (Table 1). Species identification of 13 positive human samples of *Helicobacter* species by PCR revealed that 2 (15.38%) were positive for *H. pylori,* whereas 11 (84.62%) were positive for *H. Non-pylori. H. bovis* was positive in 4 (30.77%) of 13. However, *H. felis* and *H. heilmannii* were not detected among the examined samples (Table 1).

Prevalence of *Helicobacter* species among pet samples

A total of 13 (37.14%) out of 35 samples from dogs were positive for *Helicobacter* spp. including 8 (57.14%) out of 14 fecal samples and 5 (23.8%) out of 21 saliva samples. *H. heilmannii* spp was identified in 2 (25%) out of eight fecal samples and *H. bovis* was identified in one (20%) out of 5 saliva samples (Table 1). In cats, a total of 5 (21.74%) samples were positive for *Helicobacter* spp including 3 (33.33%) out of 9 fecal samples and 2 (14.28%) out of 14 saliva samples from cats. One out of 3 (33.33%) fecal samples were positive for *H. heilmannii*. *H. pylori* and H. *felis* were not detected among the *Helicobacter* species positive samples from pets (Table 1).

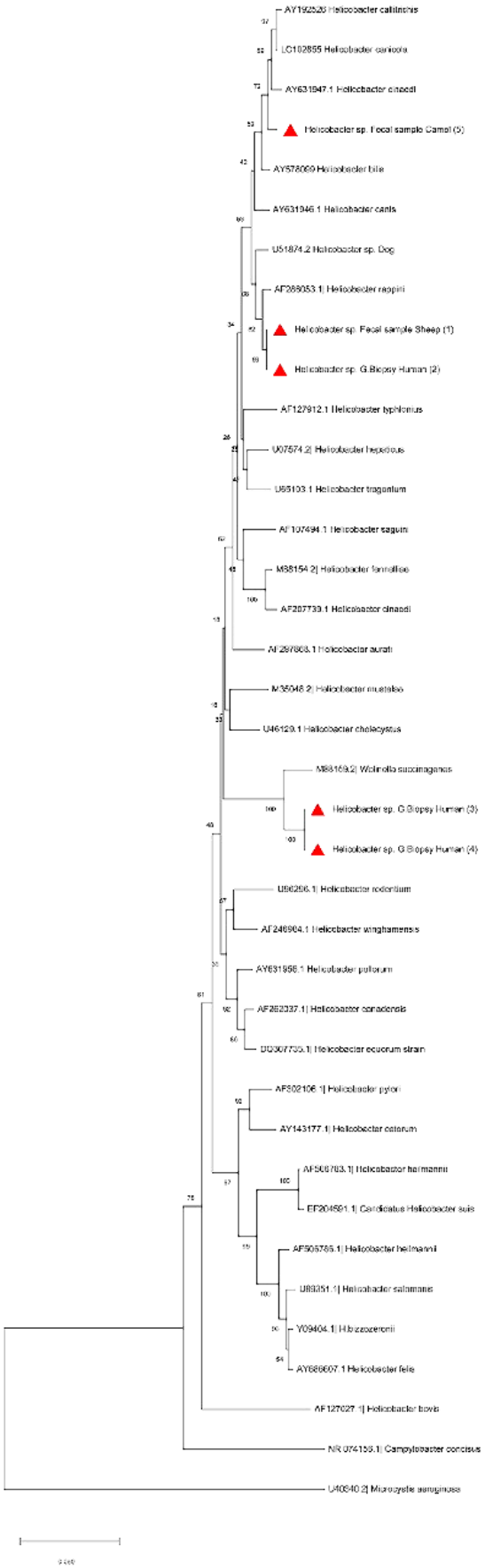

Fig. 1. Phylogenetic analyses of 5 partial sequences of the 16S rRNA gene conducted using the neighbor-joining method using MEGA software version X. The robustness of the groupings in the neighbor-joining analysis was assessed with 1000 bootstrap replicates.

Prevalence of *Helicobacter* species among samples from livestock animals

As shown in Table 1, 6 out of 15 (40%), 4 out of 12 (33.33%), 2 out of 8 (25%), and 2 out of 9 (22.22%) fecal samples of cattle, buffalo, sheep, and camels, respectively were positive for *Helicobacter* species. Species identification showed that 5 (83.33%) out of 6, 3 (75%) out of 4, and one out of 2 (50%) of cattle, buffalo, and sheep fecal samples were positive for *H. bovis*, respectively. However, *H. pylori*, *H. felis,* and *H. heilmannii* were not detected among the *Helicobacter* species positive samples.

Genome identity and phylogenetic analysis

Phylogenetic analysis of the five sequences showed clustering of *Helicobacter* species detected in the feces of the camel with *H. cinaedi*, which is supported by a bootstrap value of 72%. On the other hand, *Helicobacter* species detected in the feces of sheep and two of *Helicobacter* species detected in human gastric biopsies were clustered with *H. rappini* which was supported by a bootstrap value of 81%. However, *Helicobacter* species detected in human gastric biopsy revealed clustering with *W. succinogenes* that was supported by a bootstrap value of 100% (Fig. 1). Gene blasting of the sequences supported the phylogenetic analysis results.

Discussion

The success rate for the isolation of *Helicobacter* species from feces has been rather poor, and some of the *non-H. pylori* species are uncultivable (Makristathis et al. 2019). To overcome these diagnostic limitations, numerous non-invasive direct detection methods are recommended for *Helicobacter* species infections in clinical specimens using molecular assays (Germani 1997, Hong et al. 2015, Makristathis et al. 2019). Small amounts of gastric *Helicobacter* species in the feces might be related to the degradation of bacterial DNA in the large intestine or the presence of PCR inhibitors in the fecal matter such as complex polysaccharides (Hong et al. 2015). The use of PCR assays in this study enabled a wide range of detection of unculturable *Helicobacter* species from human and animal clinical samples.

The results of this study indicate a high percentage of detection of non-*H. pylori* species among humans and all the animal samples. This suggests that the non-*Helicobacter* species were prevalent and might be associated with clinical diseases. *Helicobacter* species DNA was detected in 75% of the gastric biopsy specimens. This finding was lower than that detected in previous studies which reported a prevalence of 88.62% (Van den Bulck et al. 2005) and 82% (Momtaz et al. 2014). The higher detection of *Helicobacter* species in gastric biopsies than in human fecal samples indicated that *H. pylori* in human cases could be associated with gastric disorders. Furthermore, gastric biopsy allows for direct sampling from the stomach, thus avoiding PCR inhibitors in the gastrointestinal tract.

This study showed a high prevalence of *Helicobacter* infection among dogs and cats with a high detection rate of *H. heilmannii* and *H. bovis*. Similarly, *Helicobacter* species among dog samples showed a prevalence of *H. pylori, H. felis*, and *H. heilmannii* amounting to 76.6%, 8.7%, 4.4%, and 95.7%, respectively (Jankowski et al. 2016). Another study showed that the *Helicobacter* species and *H. heilmannii* in the fecal samples of dogs were 62.5% and 37.5%, respectively (Hong et al. 2015). Additionally, Jankowski et al. (2016) obtained a percentage of *Helicobacter* species DNA in the fecal samples from dogs amounting to 23.3%, and a higher percentage of *H. heilmannii* (71.4%) and did not find *H. pylori* or *H. felis*. The detection of these species among pets could be a source of human infection and might represent a potential for zoonotic infections.

The results showed high detection rates of *Helicobacter* species among the fecal samples of livestock animals with a predominance of *H. bovis* among the positive samples. These findings agree with those of Sabry et al. (2016) who reported that the occurrence of *Helicobacter* species among different animal species in Egypt was 26.3%, 4.8%, and 0% in sheep, cattle, and buffaloes, respectively. The high detection rates of *H. bovis* among human and animal samples in this study indicate its zoonotic potential

Gene blasting and phylogenetic analysis revealed that the sequences of sheep fecal samples and one gastric biopsy sample were identified as *H. rappini* which was previously known as "*Flexispira rappini*." Both names are currently without formal taxonomic status, but phylogenetically, this organism is closely related to the genus *Helicobacter*, and it shares morphological features with some *Helicobacter* species, such as *H. bilis*, *H. canis*, and *H. trogontum*. *H. rappini* has been isolated from various animal sources, including aborted sheep fetuses. Two samples from gastric biopsies showed a 100% identity of the 16S rRNA gene sequence with *W. succinogenes* species. *W. succinogenes* was initially isolated from cattle rumen (Wolin et al. 1961). Phylogenetic analyses of one sample from the camel revealed that this sample was bootstrapped to *H. cinaedi*. This is the first record of this genus among

camels (dromedary camels). *H. cinaedi* infection has been reported in association with gastroenteritis in primates and immunocompromised patients (Mladenova-Hristova et al. 2017). Further epidemiological investigations are warranted to clarify *Helicobacter* species infection among the Camelidae family.

Conclusions

The detection of *H. pylori among* human samples and *non-H. pylori* among humans and animals suggested a potential for zoonoses. *H. bovis* was common among human and animal samples which might be zoonotic *Helicobacter* species. This study showed the first record of *H. cinaedi* infection in camels.

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