Determination of the Prevalence of Helicobacter heilmannii-Like Organisms Type 2 (HHLO-2) Infection in Humans and Dogs Using Non-Invasive Genus/Species-Specific PCR in Korea

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ABSTRACT. Helicobacter spp. may have multiple routes of transmission. It is unclear, however, whether the agent is zoonotic and therefore transmitted from an animal reservoir, including dogs. The aim of this population-based study was to assess the relationship between pet ownership or frequent exposure to dogs and Helicobacter spp. infection, especially focusing on HHLO-2 (Helicobacter heilmannii-like organisms type 2) in saliva and feces samples in Korea, using non-invasive genus/species-specific PCR. One hundred twenty-four eligible human subjects and 39 dogs participated in this study. Relativity of contact with dogs and Helicobacter spp. infection diagnosed by genus-specific PCR showed a statistically significant result (P<0.01), but in the relativity analyses between contact with dogs and H. pylori, H. felis and H. bizzozeronii infections diagnosed using species-specific PCR, only Helicobacter felis showed a statistically significant result. Although H. pylori infection showed a statistically significant relativity, no statistically significant association was found between veterinarian subjects and Helicobacter spp., H. felis and H. bizzozeronii infections. On performing risk factor analyses of HHLO-2 infection by transmission, using matching species, between HHLO-2-positive dog owners and HHLO-2-positive dogs, Helicobacter felis infection showed an extremely significant relativity (P<0.0001), and Helicobacter bizzozeronii may also be a possible significant risk factor (P<0.01). These results suggest that HHLO-2 infection might be a zoonotic infection, because continuous contact with dogs was proved to be correlated with human H. felis and H. bizzozeronii infections in this study.

KEY WORDS: Helicobacter bizzozeronii, Helicobacter felis, Helicobacter pylori, HHLO-2 (Helicobacter heilmannii-like organisms type 2), PCR.

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Helicobacter spp. are gram-negative and spiral-shaped bacteria. Till now, almost 24 Helicobacter spp. have been reported, and most are suspected or proven gastric or hepatic pathogens [1]. Spiral organisms, such as Helicobacter spp., have been observed in domestic feline and canine gastric tissues since the last 100 years. These gastribacteria have received special attention, and it seems that the stomachs of many types of domestic or companion animal species harbor their own Helicobacter spp. with long-term evolution. The Helicobacter spp. infection rate is 40 to 80% throughout the world in humans, and the most common Helicobacter spp. infection in humans in half of the world’s population is H. pylori [2]. Recently, H. pylori was identified in domestic cats obtained from a commercial breeder, which suggests that pets may be a potentialsource of H. pylori infection [3].

General diagnostic methods for Helicobacter infection are divided into invasive and non-invasive methods [4]. ELISA has been developed and used for making the diagnosis of Helicobacter species, and direct observation of Helicobacter spp. organisms in biopsied specimens usually requires the use of special stains, e.g. Giemsa, WSS, Genta and alcian yellow-toluidine blue stains. Typically, H. pylori is not visualized by H&E staining [5]. Culture with biopsy of the historically affected region is the gold standard in invasive measures; however, this method is less sensitive in most Helicobacter species. In non-invasive methods, the PCR assay has a higher sensitivity and specificity than the other non-invasive methods, such as serological tests, and nested PCR analysis for Helicobacter spp. increases both the sensitivity and specificity than a single PCR analysis [6].

However, epidemiologic data of other Helicobacter spp., such as H. felis and H. bizzozeronii, are lacking to support the animal and public health implications. Helicobacter spp. have been detected continuously in humans, mainly H. pylori, and Helicobacter spp., such as H. felis and H. bizzozeronii, have been detected in pets in Korea [7]. Despite the advances in understanding the pathophysiology and virulence determinants of H. pylori infection, knowledge of...
H. felis or H. bizzozeronii is lacking till now [8]. Animal and public health implications of Helicobacter species have been continuously debated since their introduction to the seroepidemiologic studies of H. pylori with transmission occurring from cats to humans [9] or from sheep milk to humans [10]. Recently, circumstantial evidence has suggested that these bacteria, also referred to as “Helicobacter helimanni-like organisms” (HHLO), may be transmitted through animals [11]. At least two genotypically different types of HHLO have been identified based on the analysis of 16S rRNA genes, and type II was found to be highly related to H. felis and H. bizzozeronii. Although HHLO-2 (HHLO type II) was found in a small percentage of 0.2 to 0.6% in humans, approximately 50% of non-pylori Helicobacter species inducing gastritis, gastric ulcer and MALT lymphoma in humans are associated with HHLO-2 [12]. The infection with HHLO-2 is common in domestic animals with or without gastric diseases, and various studies have reported the prevalence rate as 41 to 100% in dogs and 57 to 100% in cats [13, 14]. The identification of HHLO-2 in humans that have mostly been identified in pets arouses interest in the zoonotic potential of HHLO-2; however, this finding is not clear as yet.

The aims of this study were to evaluate the prevalence and morphologic types of Helicobacter spp. using Helicobacter genus-specific PCR and Helicobacter species-specific PCR and to assess the relation of exposure to pets with HHLO-2 prevalence in a population-based study of pet owners in Korea.

MATERIALS AND METHODS

Study population and sample preparation: One hundred and 24 human subjects were included in the “dog-contact risk factor group”. In detail, the dog-contact risk factor group was composed of 41 dog owners and 43 veterinarians who regularly contact dogs. In this group, 12 veterinarians who were themselves dog owners. Each of the dog owners including veterinarians was matched with their own 39 dogs by labeling for the statistical analyses. Forty human subjects with no history of pet ownership were included for the negative dog-contact factor (Supplemental Fig. 1). Saliva and feces samples were taken from all subject populations. The feces and saliva samples were taken by using sterilized cotton swabs and subsequently submerged in 500 µl of autoclaved phosphate buffered saline. DNA was extracted from 20 to 30 µl of each sample by using DNeasy Tissue Kit (Qiagen, Santa Clarita, CA, U.S.A.). The DNA samples were eluted in 200 µl volume and stored in a −20°C freezer until PCR was conducted.

Genus-specific PCR: Each DNA sample was amplified on Helicobacter 16S rRNA gene using C70 and B37 outer primers, and subsequently, the PCR products were conducted nested PCR using C97 and C98 inner primer pair (Supplemental Table 1) [15]. The PCR mixture, total volume of 20 µl contained a final primer concentration of 0.5 µM, 1 µl DNA samples (0.3 µl for nested PCR) and 18 µl diethyl pyrocarbonate treated water, was added to Maxime PCR PreMix Kit (iNtRON Biotechnology Inc., Seoul, Korea). The PCR samples were heated at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec and extension at 72°C for 3 min and finally extended at 72°C for 15 min using outer primers by using programmed temperature control system (PC808, Astec, Fukuoka, Japan). For nested PCR, the PCR products were heated at 94°C for 2.5 min followed by 35 cycles of denaturation at 94°C, annealing at 50.5°C, extension at 72°C for 1 min each and a final extension at 72°C for 15 min. The PCR products were electrophoresed by 1.5% agarose gels containing ethidium bromide in 0.5x TBE buffer and visualized on ultraviolet light illuminator.

Species- specific PCR: PCR amplifications of H. felis and H. bizzozeronii were performed using primer (Supplemental Table 1) which amplify the urease B gene of them [16]. PCR assay specific for H. pylori is also performed. The total volume of PCR mixture 20 µl including a final each primer concentration of 0.5 µM, 1 µl DNA samples (0.3 µl for nested PCR) and 18 µl diethyl pyrocarbonate treated water was added to Maxime PCR PreMix Kit (iNtRON Biotechnology Inc.). DNA extracts from pure cultures of H. pylori (HpKTCC H. pylori strain 114), H. felis (ATCC 49179) and H. bizzozeronii (ATCC 70030) served as positive controls.

For PCR amplification of H. pylori, the samples were heated at 95°C for 5 min and followed by 35 cycles at 94°C for 45 sec, at 59°C for 45 sec and at 72°C for 45 sec and a final extension at 72°C for 10 min using outer primers. Second round of PCR was performed at 95°C for 5 min, 30 cycles followed at 94°C for 45 sec, at 54°C for 45 sec and at 72°C for 30 sec and final extension at 72°C for 10 min. For H. felis-specific PCR, samples were heated at 94°C for 2.5 min once, followed by 40 cycles of denaturation at 94°C, annealing at 45°C, extension at 72°C for 1 min each with a final extension at 72°C for 15 min using outer primers. Second round of PCR was performed at 94°C for 2.5 min, 30 cycles followed at 94°C for 45 sec, at 50°C for 45 sec and at 72°C for 45 sec and final extension at 72°C for 15 min. The H. bizzozeronii-specific PCR was carried out following conditions, heated at 94°C for 2.5 min once and 33 cycles of at 94°C for 1 min, at 57°C for 1 min and at 72°C for 1 min. Final extension was performed at 72°C for 16 min using outer primers. Second round of PCR was performed at 94°C for 2.5 min, 30 cycles followed at 94°C for 45 sec, at 50°C for 45 sec and at 72°C for 45 sec and final extension at 72°C for 15 min. The PCR products were electrophoresed on ethidium-bromide stained 1.5% w/v agarose gels in 0.5x TBE buffer and visualized on ultraviolet light illuminator.

Nucleotide sequence analysis: In order to confirm the identity of H. pylori, H. felis and H. bizzozeronii specific PCR assay products with their target genes, after the PCR products of the specific size were extracted by commercial gel extraction kit (MEAgQuick-spin, INTRON, Seoul, Korea), direct sequencing of the PCR products with specific primer was conducted by ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, Foster City, CA, U.S.A.). The result of sequencing was compared to those present in databases using BLAST software.
Table 1. Transmitted infection possibility of Helicobacter spp., Helicobacter pylori, Helicobacter felis and Helicobacter bizzozeronii in dog-contact risk factor

| Species | Dog-Contact |
|---------|-------------|
|          | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Subtotal | Total |
| H. pylori | 19 | 21 | 34 | 6 | 39 | 1 | 24 | 16 | 40 | 160 |
| H. felis  | 8 | 35 | 11 | 32 | 35 | 8 | 23 | 20 | 43 | 172 |

a) Pearson’s χ² test with Yates’ continuity correction: χ²=7.59, *P*<0.01. b) Pearson’s χ² test with Yates’ continuity correction: χ²=23.23, *P*<not significant. c) Pearson’s χ² test with Yates’ continuity correction: χ²=6.09, *P*<0.05. d) Pearson’s χ² test with Yates’ continuity correction: χ²=0, *P*<not significant.

Table 2. Transmitted infection possibility of Helicobacter spp., Helicobacter pylori, Helicobacter felis and Helicobacter bizzozeronii in veterinarian risk factor

| Species | Veterinarian |
|---------|-------------|
|          | Negative | Positive | Negative | Positive | Negative | Positive | Negative | Positive | Subtotal | Total |
| H. pylori | 10 | 31 | 20 | 21 | 31 | 10 | 28 | 13 | 41 | 164 |
| H. felis  | 8 | 35 | 11 | 32 | 35 | 8 | 23 | 20 | 43 | 172 |

a) Pearson’s χ² test with Yates’ continuity correction: χ²=0.14, *P*<not significant. b) Pearson’s χ² test with Yates’ continuity correction: χ²=3.59, *P*<0.05. c) Pearson’s χ² test with Yates’ continuity correction: χ²=0.14, *P*<not significant. d) Pearson’s χ² test with Yates’ continuity correction: χ²=1.35, *P*<not significant.

**RESULTS**

Statistical analysis: Pearson’s chi-square test and Fisher’s exact test were used to find the dependence between 2 categories. To check for the independence of 2 categories, including positive/negative PCR detection, the results in human subjects/animal subjects were tested by chi-square analysis using contingency tables. After counting the positive results for Helicobacter spp., H. pylori, H. felis and H. bizzozeronii as the variables, the possibility of risk of transmission between animal and human subjects was assessed using Pearson’s chi-square test with Yates’ continuity correction or 2-tailed Fisher’s exact test for count data to rule out a variety of distribution assumptions. For this reason, Pearson’s chi-square test is vague when cell counts are less than 5. From the contingency tables, odd ratios for each Helicobacter spp. can be calculated for interpreting the possible risk factor analysis. Statistical analyses were conducted using the conventional statistical software R (version 2.15.2), and the significance was set at *P*<0.05.

**DISCUSSION**

The present study investigated the possible role of frequent contact with dogs or dog ownership in the transmission of Helicobacter spp. infection in a representative population sample comprising of dogs, dog owners, non-dog owners and veterinarians in Korea. We specifically tested the hypothesis that HHLO-2 may be a very important agent causing cross infection, because it has attracted attention as a zoonotic agent in recent studies [11, 17]. Dog owners and veterinarians were included as the subjects, because of their frequent contact with dogs. Overall, we found strong
evidence for an increased risk of HHLO-2 infections, such as \emph{H. felis} and \emph{H. bizzozeronii} infections, associated with the presence of dogs in the household (dog owners group), and co-infection rates were relatively high in dog-contact group (42.85%) than no dog-contact group (7.50%). In addition, \emph{H. felis} and \emph{H. bizzozeronii} co-infection rate was most frequent in both human and animal subject (42.85% in human subject and 55.12% in animal subject). In the previous study about \emph{H. pylori}, co-infection rate of \emph{H. pylori} L-form and vegetable-form in human subject was reported as 78.38% \cite{19}, but other form of \emph{Helicobacter} spp. co-infection rate report is extremely rare. Results indicated that in the group that was in frequent contact with dogs inside or outside the house (group in frequent contact with dogs), genus \emph{Helicobacter} spp. (P<0.01) and \emph{H. felis} (P<0.05) infection could be considered as a zoonotic infection. However, frequent contact with dogs did not have relativity as a risk factor in \emph{H. pylori} and \emph{H. bizzozeronii} infections, and this result supported the result of the previous studies \cite{8}. Interestingly, the veterinary group in Korea showed a high prevalence of \emph{H. pylori} infection with statistical significance as shown in Table 2.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\multicolumn{2}{|c|}{\emph{Helicobacter felis} \textsuperscript{a})} & \multicolumn{2}{|c|}{\emph{Helicobacter bizzozeronii} \textsuperscript{b})} & \\
\hline
Dog-owners & Negative & Positive & Dog-owners & Negative & Positive \\
\hline
\text{Dog-owners} & 23 & 0 & \text{Dog-owners} & 18 & 1 \\
\text{Positive} & 7 & 9 & & 7 & 13 \\
\hline
\end{tabular}
\caption{Risk factor analyses of \emph{Helicobacter felis} and \emph{Helicobacter bizzozeronii} by matching species between dog-owner and dog}
\end{table}

\textsuperscript{a}) Dogs
\textsuperscript{b}) Helicobacter bizzozeronii

\textsuperscript{a}) Pearson’s $\chi^2$ test with Yates’ continuity correction: $\chi^2=13.79$, $P<0.0001$, Fisher’s exact test for count data: odds ratio=Inf*. *Infinite value describes the faulty cell data by excel program (0/0). \textsuperscript{b}) Pearson’s $\chi^2$ test with Yates’ continuity correction: $\chi^2=12.62$, $P<0.001$, Fisher’s exact test for count data: odds ratio=30.04, $P<0.001$.

In conclusion, we might suggest that HHLO-2 (\emph{Helicobacter heilmannii}-like organisms type 2) infection might be zoonotic, because continuous contact with dogs was proved to be correlated with human \emph{H. felis} and \emph{H. bizzozeronii} infections in this study. However, this report never intended to criticize pet ownership, but to carry out more intensive prevention, treatment and socio-epidemologic research of \emph{Helicobacter felis} and \emph{H. bizzozeronii} infections, which should be considered in both the medical and veterinary fields.

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Supplemental Fig 1. Study population.

**For dog-contact risk factor (for Table 1)**

Total human subject: 124

Dog-contact positive: 84 Vs Dog-contact negative: 40

Dog owner: 41

veterinarian: 43

**For veterinarian risk factor (for Table 2)**

Total dog-contact subject: 84

Veterinarian positive: 43 Vs Veterinarian negative: 41

Supplemental Fig 2. Detection of Helicobacter spp. DNA by genus-specific and species-specific nested PCR with specific primer (representative): Lanes 1 and 2 Helicobacter spp. genus positive samples (H. spp.) in human subject (H) and animal subject (A) (400 bp), lanes 3 and 4 H. pylori positive samples in human subject (H) and animal subject (A) (230 bp), lanes 5 and 6 H. felis positive samples in human subject (H) and animal subject (A) (160 bp), lanes 7 and 8 H. bizzozeronii positive samples in human subject (H) and animal subject (A) (207 bp) and each side marker (M); 50 bp DNA ladder.
### Supplemental Table 1. Primer sequences used for Helicobacter spp. polymerase chain reaction

| Target Gene | Species          | Oligonucleotides of PCR primer | Products (bp) | Reference Article No. |
|-------------|------------------|--------------------------------|---------------|-----------------------|
| 16S rRNA    | *Helicobacter* spp. | Outer primer                      | 1,506         | [15]                  |
|             |                   | F: 5′-AGA GTT TGA TYM TGG C-3’   |               |                       |
|             |                   | R: 5′-TAC GGY TAC CTT GTT ACG A-3’|               |                       |
|             |                   | Inner primer                     | 400           |                       |
|             |                   | F: 5′-GCT ATG ACG GGT ATC C-3’   |               |                       |
|             |                   | R: 5′-GAT TTT ACC CCT ACA CCA-3’ |               |                       |
| Urease B    | *H. pylori*       | Outer primer                      | 417           | [15]                  |
|             |                   | F: 5′-CCC TCA CGC CAT CAG TCC CAA AAA-3’ |       |                       |
|             |                   | R: 5′-AAG AAG TCA AAA ACG CCC CAA AAC-3’ |   |                       |
|             |                   | Inner primer                     | 230           |                       |
|             |                   | F: 5′-GGC AAA TCA TAA GTC CGC AGA A-3’ |   |                       |
|             |                   | R: 5′-TGA GAC TTT CCT AGA AGC GGT GTT-3’ | |                       |
|             | *H. felis*        | Outer primer                      | 1,150         | [12]                  |
|             |                   | F: 5′-ATG AAA CTA ACG CCT AAA GAA CTA G-3’ |   |                       |
|             |                   | R: 5′-GGA GAG ATA AAG TGA ATA TGC GT-3’ |   |                       |
|             |                   | Inner primer                     | 160           |                       |
|             |                   | F: 5′-TGT TAG ACT CGG CGA CAC TG-3’ |   |                       |
|             |                   | R: 5′-GGC GTT AGT GAG CAC ACC AT-3’ |   |                       |
|             | *H. bizzozeronii* | Outer primer                      | 420           | [12]                  |
|             |                   | F: 5′-GAA GTC GAA CAT GAC TG-3’   |               |                       |
|             |                   | R: 5′-GGT CGC ATT AGT CCC ATC AG-3’ |               |                       |
|             |                   | Inner primer                     | 207           |                       |
|             |                   | F: 5′-GGG ATG GCA CAA ACC AAT AG-3’ |   |                       |
|             |                   | R: 5′-AGC CAA AGC CTC AGT AGC AG-3’ |   |                       |

*F: forward, R: reverse*