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Prevalence of *Helicobacter pylori* among residents and their environments in the Nara prefecture, Japan

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ABSTRACT

Background: Chronic infection with *Helicobacter pylori*, specifically *cagA*-positive strains, is associated with gastric cancer. Thus, measures to prevent *H. pylori* infection are required. This study was conducted to clarify the prevalence of *H. pylori* in the community to identify the infection source and comprehensively assess the risk of *H. pylori* infection.
Methods: We collected 90 human faecal samples and 73 environmental samples (water, vegetable, and animal faecal samples) from the residents in an area with a high incidence of gastric cancer in Japan. Polymerase chain reaction assay was performed to detect the *glmM* housekeeping gene and the *cagA* virulence gene of *H. pylori*. A questionnaire survey was conducted, and the responses were analyzed statistically.
Results: The *glmM* gene was detected in 18 of 90 (20%) faecal samples obtained from residents; among them, the *cagA* gene was detected in 33.3% (6/18), and in all who had undergone eradication therapy. *H. pylori* was not detected in environmental samples. However, contact with dogs (OR 3.89, 95% CI 1.15–13.15, $P < 0.05$) was associated with higher odds for *glmM* gene positivity in the questionnaire survey.
Conclusions: The prevalence of *H. pylori* and *cagA*-positive strains among the residents was low. However, the study results suggest a correlation between recurrent infection and *cagA*-positive *H. pylori* strains. Although *H. pylori* genes were not detected in living environments, an association between contact with dogs and a *glmM* positive status was revealed. Further investigations targeting community-dwelling healthy people and their living environments would be required for *H. pylori* infection control.

Introduction

Gastric cancer is one of the most common causes of cancer-related death worldwide and remains a global health problem. More than half of all cases of gastric cancer occur in East Asia, particularly in South Korea and Japan [1]. Gastric cancer is a complex disease, as many risk factors (including genetic and environmental risk factors) are associated with the disease onset [2].

Infection with *Helicobacter pylori* (classified as a group I carcinogen in humans by the International Agency for Research on Cancer) is considered as one of the most significant risk factors for gastric cancer. More than 50% of the global population is infected with *H. pylori*, although the infection rate varies between countries. *H. pylori* infection is mostly acquired during early childhood, and the infection persists throughout a lifetime without eradication [3]. Chronic *H. pylori* infection is associated with a significantly increased risk of gastric cancer [4]. The cytotoxin-associated gene A (*cagA*) is one of the virulence factors and contributes to the development of gastric cancer. Moreover, the infection ratio of the *H. pylori cagA*-positive strain is higher than that of *cagA*-negative strain worldwide [5].

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Table 1
PCR primers and conditions used in this study.

Target gene (product size)	Primer sequence (5′-3′)	PCR condition	Reference
glmM (294 bp)	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	95 °C for 10 min; 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min	[17]
cagA (400 bp)	AATACACCAACGCCTCCAAG TTGTTGCCGCTTTTGCTCTC	95 °C for 10 min; 40 cycles of 95 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min	[18]
cagA (variable)	ACCCTAGTCGGTAATGGG GCTTTAGCTTCTGAYACYGC	95 °C for 10 min; 40 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; final extension: 72 °C for 7 min	[19]
16S rRNA (1465 bp)	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	95 °C for 10 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; final extension: 72 °C for 2 min	[20]

PCR: polymerase chain reaction.

More than 130,000 cases of gastric cancer were diagnosed in Japan in 2017 [6]. Early screening and eradication therapy for *H. pylori* infection has been effective in preventing and reducing the incidence of gastric cancer [7,8]. Therefore, a national program for the screening and prevention of gastric cancer is underway. The national health insurance system covers the treatment of *H. pylori*-associated diseases among adults in Japan [9]. However, eradication therapy for *H. pylori* infection has not been proven to be safe in children [10]. An increasing number of local governments are conducting programmes for screening *H. pylori* infection in children and adolescents, although this remains limited.

Further, *H. pylori* infection occurs orally. Therefore, measures aimed at preventing *H. pylori* infection by blocking the transmission route (which are non-invasive and safe, particularly among children) are required. Many reservoirs and transmission routes of *H. pylori* infection in humans have been suggested. *H. pylori* has been detected in water, foodstuffs, soil, and animals, such as pets and livestock [11,12]. The faecal-oral transmission of pathogens present in faeces has been considered an important infection route [13]. However, the source of infection and transmission routes are incompletely understood and remain controversial.

The incidence and mortality rates attributable to gastric cancer in Japan have been on the decrease; however, the rates continue to remain high in a few areas [14]. The current status of *H. pylori* infections among the residents in these areas is unclear. Moreover, none of the studies has investigated the prevalence of *H. pylori* in the living environment of residents assessed for risk of *H. pylori* infection. The present study focussed on the southern area of the Nara prefecture, Japan, with high gastric cancer mortality of standardized mortality ratios of 131.4 in men and 112.7 in women, as well as standardized incidence ratio of 147.6 in men and 143.1 in women [14]. We aimed to clarify the prevalence of *H. pylori* infection (including the *cagA*-positive strain) among the residents and in their living environments to comprehensively identify the source of infection and assess the risk of *H. pylori* infection among residents, following infection control.

Material and methods

Sample collection

The study was conducted between December 2017 and December 2019 in a small area of the Nara prefecture, an inland province located in the central part of Japan. More than 90% of the survey area comprised of mountains and forests. A total of 163 samples, including 90 human (44 men and 46 women) faecal samples and 73 environmental samples (water, vegetable, and animal faecal samples) were obtained. The mean age of the participants was 67.8 (range, 0–91) years.

In accordance with the Declaration of Helsinki, written informed consent was obtained from all participants before enrolment in the study. The study protocol was approved by the Ethical Review Committee of the Nara Medical University (project identification no.1574).

Faecal samples obtained from residents and DNA extraction

A total of 90 faecal samples were collected from healthy participants. For DNA extraction, each faecal sample was processed using the ISOSPIN Fecal DNA kit (Nippon Gene Co., Ltd., Tokyo, Japan) or MORA-EXTRACT (AMR Co., Ltd. Gifu, Japan), according to the manufacturer's instructions.

Water samples and DNA extraction

A total of 29 water samples (17 from tap water and 12 from untreated water [one from spring water and 11 water samples drawn from mountain and valley]) were collected. These samples were obtained from water sources used mainly for drinking and daily use. Furthermore, water sources were also sampled if they were consumed by the participants during childhood, and had been changed, but were available.

Approximately 1 l of each water sample was filtered through a filter membrane with a pore size of 0.45 μm using a suction pump (Millipore Corp., Massachusetts, USA). The membrane was immersed in 2 ml of Brucella broth and vortexed and heated in Brucella broth at 100 °C for 10 min.

Vegetable samples and DNA extraction

A total of 30 vegetable samples, which were homegrown for raw eating, were collected. Approximately 5 g of each vegetable sample was 5-fold diluted using Brucella broth and shaken. Further, the broth was heated at 100 °C for 10 min.

Animal faecal samples and DNA extraction

We collected 14 faecal samples (from 3 pet dogs and 11 wild animals, including 9 deer and 2 macaques in the living environment such as the garden and field). The ISOSPIN Fecal DNA kit (Nippon Gene Co., Ltd.) was used to extract DNA from 0.2 g of each faecal sample, according to the manufacturer's instructions.

Detection of H. pylori *using polymerase chain reaction assay*

Polymerase chain reaction (PCR) assays were performed using extracted DNA from faecal, water, and vegetable samples. To detect *H. pylori*, the *glmM H. pylori* housekeeping gene, which is the most diagnostic candidate genes detectable via PCR [15,16], and the virulence gene, *cagA,* were amplified with AmpliTaq Gold 360 master mix (Thermo Fisher Scientific, Waltham, MA) [17–19]. The primers and PCR conditions used in this study are summarized in Table 1.

Table 2
Detection of the *glmM* gene from human and environmental samples.

Detection method	Faeces		Water (n = 29)	Vegetable (n = 30)
	Obtained from residents (n = 90)	Obtained from animals (n = 14)		
PCR	18	0	0	0
Culture	0	0	0	0

DNA sequencing was performed using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA) and an ABI3730xl analyzer (Applied Biosystems). The Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to process the sequencing data and identify genes. We also amplified the 16S ribosomal RNA (16S rRNA) gene of the extracted DNA from faecal samples without amplified *glmM* or *cagA* genes to confirm the validity of the PCR results [20] (Table 1).

Isolation of H. pylori

We adopted a culture method for the isolation of *H. pylori* from all 163 samples. Each human and animal faecal sample, 100 μl of the water sample, and Brucella broth obtained after washing vegetable samples were plated on Helicobacter agars (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Helicobacter agars were incubated at 37 °C for 4–7 days under microaerophilic conditions (O_2, 6–12%; CO_2, 5–8%). We selected violet-coloured colonies that were morphologically characteristic of *H. pylori*, according to the manufacturer's instructions. PCR assays were performed for all selected colonies to detect *glmM* and *cagA* in *H. pylori*.

Questionnaire survey and statistical analysis

A questionnaire survey was conducted for the 90 participants. Regarding sociodemographic data, 10 items were evaluated: age and gender, sanitation, water sources of daily life including drinking water, living environments and lifestyles, contact with animals and soil, and medical history of gastrointestinal tract disease.

We classified the 90 participants into two groups based on *glmM* positivity (whether or not the *glmM* gene was detected in faecal samples). Fisher's exact test was performed to compare the proportions of responses between the two groups. Moreover, the odds ratios and their 95% confidence intervals (CIs) were also calculated using the logistic regression analysis for the association between *glmM* status and the study variables. All statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). Variables with a *P-value* less than 0.05 were considered statistically significant.

Results

Prevalence of H. pylori *infection in faecal samples obtained from residents*

The *glmM* gene was detected in 18 (20%) of the 90 faecal samples (Table 2). The mean age of the participants positive for *glmM* gene was 64.9 years, and their ages ranged from 30 to 80 years; there were 8 samples from men and 10 from women. There was no difference in sex with respect to detection of the *glmM* gene ($P > 0.05$). Further, *cagA* was detected in 6 (33.3%) of 18 *H. pylori glmM* gene-positive faecal samples. To confirm the validity of the PCR results, we amplified the 16S rRNA gene in the 72 *H. pylori* gene-negative faecal samples. In all of these samples, the amplification of 1465 bp of the 16S rRNA gene was observed.

Additionally, colonies that were morphologically characteristic of *H. pylori* were isolated from 57 out of 90 faecal samples. However, *H. pylori* genes were not amplified by PCR (Table 2).

Table 3
Odds ratios for the associations between selected variables and *glmM* positivity.

Variables	OR	95% CI	*P*-value
Age	0.99	0.95–1.02	0.50
Contact with dogs	3.89	1.15–13.15	0.03
Drinking spring water or drawing water from a mountain or valley	1.12	0.24–5.25	0.89

OR: odds ratio, CI: confidence interval.

Prevalence of H. pylori *in the environmental samples*

The *glmM* and *cagA* genes were not detected in DNA extracted from 29 water and 30 vegetable samples. These *H. pylori* genes were also not detected in DNA extracted from 14 animal faecal samples (Table 2). The validity of the PCR was confirmed by the amplification of 16S rRNA in each of these samples.

Colonies that were morphologically characteristic of *H. pylori* were isolated from 26 out of 30 vegetable samples and 9 out of 14 animal faecal samples, but *H. pylori* genes were not amplified by PCR. Additionally, no colonies were isolated from water samples (Table 2).

Association between glmM *status and answers to the questionnaire survey*

The response rate of the questionnaire survey was 100%. A higher proportion of participants had contact with soil, animals, and water sources other than tap water in daily life in the *glmM*-positive group than in the *glmM*-negative group (Supplementary Table 1). Above all, contact with dogs (OR 3.89, 95% CI 1.15–13.15, $P < 0.05$) was associated with higher odds for *glmM* gene positivity (Table 3, Supplementary Table 1).

Further, 11.8% of participants in the *glmM*-positive group had undergone eradication therapy. All of these participants had come in contact with dogs in their daily life, and the *cagA* gene was detectable in their faecal samples.

Discussion

We conducted the present study to clarify the prevalence of *H. pylori* among residents and their living environments in an area with high gastric cancer incidence. Only a few number of studies have reported the prevalence of *H. pylori* in healthy people worldwide.

The *glmM* gene of *H. pylori* was detected in 20% of faecal samples obtained from residents in their thirties to eighties. The detection rate of *H. pylori* in faecal samples obtained from healthy people worldwide ranged from 8% to 72.7% [21–24]. The prevalence rate of *H. pylori* infection in healthy Japanese adults aged 20–80 years was 27.2% [25], while that in children aged 0–18 years was 1.8–5.2% [26–28]. Our survey, based on a cross-sectional design, indicated a low *H. pylori* infection prevalence of 20%, which reflects the trend in Japan where the annual infection rate of *H. pylori* in all age groups has decreased [29]. This is due to improvements in environmental sanitation and the widespread screening and eradication treatment for *H. pylori* infection.

Further, it is unclear from previous studies whether there are sex-related differences in the prevalence of *H. pylori*. Tamura et al. reported a higher prevalence of *H. pylori* in men than in women [30], while Hirayama et al. found no such differences among healthy adults [29]. However, the variability in prevalence between subjects of different sexes reported previously should be considered while interpreting the results; the prevalence of *H. pylori* did not correlate with sex in our study.

The prevalence of *cagA* in *H. pylori*-positive faecal samples was 33.3% in our study. The prevalence of infection with *cagA*-positive strains of *H. pylori* was found to differ between countries (range, 20–100%) [31]. However, the infection ratio of *cagA*-positive *H. pylori* strains in subjects in East Asia is high, and the incidence of *cagA* seropositivity among patients with gastritis in Japan ranged from 53.7% to 83.3% [5,32]. Thus, the low *cagA* detection rate in the present study may be because our subject population comprised of healthy subjects.

CagA-positive *H. pylori* strains were detected in all participants who had undergone *H. pylori* eradication therapy in the *glmM*-positive group. Previously, *cagA* was detected in 97.5% of strains isolated from patients with recurrent *H. pylori* infection [33]. Moreover, the persistence of virulent *cagA* strains is considered the cause of frequent recrudescent infection [34]. However, we were unable to determine whether recrudescence or reinfection with *H. pylori* had occurred in subjects in the present study. Nevertheless, determining the presence of *cagA* from the genetic information of infectious *H. pylori* strains would assist in evaluating the risk of recurrence.

Water, vegetables and animals, particularly dogs, are considered sources of infection, although *H. pylori* was not detected in them. This could be attributed to the fact that the environmental samples investigated in the present study were not necessarily associated with what the participants ate, drank or came in contact with since their childhood. The reservoirs for *H. pylori* have been considered to be limited to humans, macaques, and cats [35]. However, the association between contact with dogs and status of *glmM*-positive was revealed in this study. Further, contact with dogs has been considered to increase the risk of *H. pylori* acquisition [36,37]. Moreover, a few studies have detected *H. pylori* in dogs [22,38]. Therefore, dogs could also be one of the possible reservoirs and sources of *H. pylori* infection, and further investigations are required to validate this.

Helicobacter agar selectively promotes the growth of *H. pylori* while inhibiting the growth of *Pseudomonas* spp. and fungi. Although colonies were isolated from 92 out of 163 human faecal and environmental samples, *H. pylori* genes were not amplified. This might be due to three reasons. First, *H. pylori* might not have existed. Second, other bacteria that are phenotypically similar to *H. pylori* existed in the gastrointestinal tract [39], and great competition with the numerous other bacteria present may have occurred. Finally, *H. pylori* may remain viable, but non-culturable under adverse environmental conditions. There have been many attempts to culture *H. pylori* from most PCR-positive samples worldwide, but it has been found difficult to isolate.

The present survey was conducted in a limited area; therefore, the results cannot reflect the conditions of all residents and the entire survey area, even in Japan. Thus, further studies are required to clarify the prevalence of *H. pylori* in healthy people and their living environments (including animals) to control *H. pylori* infection.

Conclusions

The overall prevalence of *H. pylori* and *cagA*-positive strains among the residents studied was low. However, a correlation between infection with *cagA*-positive *H. pylori* strains and recurrent infection was suggested. Although *H. pylori* genes were not detected in living environments, an association between contact with dogs and a *glmM*-positive status was revealed from the questionnaire survey. Further investigations targeting community-dwelling healthy people and their living environments would be required for *H. pylori* infection control.

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Competing interests

None.

Ethical approval

The study protocol was approved by the Ethical Review Committee of the Nara Medical University (project identification no.1574).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2020.11.018.

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