Value of the ^{13}C -Urea Breath Test for Detection of Gastric *Helicobacter* spp. Infection in Dogs Undergoing Endoscopic Examination

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ABSTRACT. Urea breath test (UBT) using an infrared spectral analyzer is widely used for non-invasive and rapid detection of gastric *Helicobacter* spp. in human, but not veterinary medicine. The main purposes of this study were to determine the reference range of the UBT in dogs and to evaluate its clinical usefulness. To address the first aim, 6 healthy laboratory beagles were subjected to UBT and upper gastrointestinal endoscopy. Gastric endoscopic biopsy samples from the antrum, corpus and fundus were examined for *Helicobacter* spp. by polymerase chain reaction (PCR) testing, rapid urease test (RUT), histology and cytology. Amoxicillin, metronidazole and omeprazole were given to infected dogs for 14 days, and dogs that became *Helicobacter*-negative were used to determine the reference range for UBT. To address the second aim, 32 canine patients underwent UBT before upper gastrointestinal endoscopy, and the sensitivity and specificity of UBT were calculated based on our newly determined reference range using PCR as the gold standard for detection of *Helicobacter* spp. Initially, all 6 laboratory beagles were infected in all gastric regions and became uninfected after eradication. The mean $\pm$ 2 SD UBT value after eradication was $0.6 \pm 1.8\%$, and the reference range for UBT was determined to be less than 2.5%. UBT was completed successfully in 27 patients. Using our reference range, UBT displayed 89% (16/18) sensitivity and 89% (8/9) specificity, indicating that UBT was quite useful for the detection of gastric *Helicobacter* spp. infection in dogs.

KEY WORDS: canine, *Helicobacter*, sensitivity, specificity, urea breath test.

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Helicobacter spp. colonizes the stomach and intestine of humans and several animal species [8]. The *Helicobacter* genus currently includes about 38 formally named members with numerous other putative species under investigation [11]. In humans, *Helicobacter pylori* is known to be the major agent of chronic diffuse superficial gastritis, plays a causative role in peptic ulcers and is considered a co-factor in the development of gastric malignancies [15, 28, 36]. A variety of gastric non-*H. pylori* helicobacters (NHPH) can infect the stomachs of dogs. The majority of the infections in the canine gastric mucosa are mixed infections with *Helicobacter* spp. such as *H. felis*, *H. bizzozeronii*, *H. salomonis* and “*Candidatus Helicobacter heilmannii*” [8]. *H. pylori* infection of canine gastric mucosa has been reported in only 2 dogs [1]. A new species, *H. cynogastricus*, was recently isolated from the stomach of a dog [39]. The reported prevalence of *Helicobacter* spp. in dogs is between 61 and 100%; it is found in 61–82% of dogs with recurrent vomiting [6, 13, 41], 67–86% of clinically healthy pet dogs [4, 41] and almost 100% of laboratory beagles and shelter dogs [4, 12],

but insufficient epidemiological data are available to estimate the prevalence in Japan. In addition, although *H. felis*, *H. bizzozeronii*, *H. salomonis* and “*Candidatus Helicobacter heilmannii*” are also found in the human stomach [3, 38, 40] and are suggested to be associated with gastric diseases in humans [18, 19, 37, 42], their pathogenic significance in dogs is controversial [10, 16, 29–34].

Diagnostic tests for gastric *Helicobacter* spp. in dogs and cats include polymerase chain reaction (PCR) testing, rapid urease test (RUT), histology and cytology; all of these require anesthesia and gastric biopsy [9, 21, 22]. The ^{13}C -urea breath test (UBT) is a non-invasive test with high sensitivity and specificity that is widely used in human medicine [7, 14, 23, 35]. In a multicenter trial in Japan, UBT using an infrared spectral analyzer showed 98.1% sensitivity and 97.9% specificity in human when a cut-off value of 2.5% was used to distinguish between patients with and without *Helicobacter* infections [23]. In veterinary medicine, UBT using an isotope ratio mass spectrometer has been used only experimentally in dogs and cats [2, 20, 22], and UBT using an infrared spectral analyzer, despite being much more rapid and economical than gas isotope ratio mass spectrometric analysis [24], has not yet been applied in the clinical setting.

The main purposes of this study were to determine the reference range of UBT using an infrared spectrograph in dogs and to evaluate its validity in clinical cases.

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MATERIALS AND METHODS

Animals: Six healthy laboratory beagles (1 male and 5 females) kept in the animal facility of the Veterinary Medical Center of the University of Tokyo (VMC-UT) were used for the determination of the reference range of the UBT. The dogs' ages ranged from 2.8 to 6.7 years and their body weights from 7.8 to 14.2 kg. No dog showed any gastrointestinal clinical sign or other abnormality upon physical examination. To evaluate the validity of the reference range of the UBT, dogs that visited VMC-UT and underwent upper gastrointestinal endoscopy for various reasons between March 2011 and August 2012 were enrolled in this study. Dogs were excluded from the study when collection of breath samples failed or endoscopic samples could not be obtained, because of incidents during anesthesia. The experiments and animal care procedures were approved by the Animal Use and Care Committee of the University of Tokyo (approval number: P11-527).

Experimental design: For the determination of the reference range of the UBT, 6 laboratory beagles underwent UBT and upper gastrointestinal endoscopy in that order. Dogs were judged to be *Helicobacter* spp.-negative when all of the gastric biopsy samples from the gastric antrum, corpus and fundus were negative by all of the tests performed (PCR, RUT and histology/cytology). For *Helicobacter* spp.-positive beagles, amoxicillin (250 mg/dog, PO, q 12 hr), metronidazole (250 mg/dog, PO, q 12 hr) and omeprazole (10 mg/dog, PO, q 24 hr) were administered for 14 days [2, 27], and dogs that became *Helicobacter* spp.-negative were used to determine the reference range.

In the canine patients, the UBT and upper gastrointestinal endoscopy were performed in that order, and the sensitivity and specificity of the UBT based on the results of PCR as the gold standard for the detection of *Helicobacter* spp. were calculated.

UBT: After overnight fasting, a breath sample was collected using a close-fitting anesthesia mask and a breath sampling bag (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) as shown in Fig. 1. A breath sample was collected until the sampling bag was inflated adequately without disconnecting the mask from a mouth. The laboratory beagles were given ^{13}C -urea UBIT[®] tablets (Otsuka Pharmaceutical Co., Ltd.) orally at a dose of 50 mg/dog based on a previous report [2]. The canine patients were dosed with ^{13}C -urea UBIT[®] tablets as follows: 25 mg/dog for dogs weighing less than 6 kg, 50 mg/dog for dogs weighing 6 kg or more but less than 15 kg and 100 mg/dog for dogs weighing 15 kg or more [2]. A second breath sample was obtained 30 min after administration. These paired breath samples were analyzed using a POcone device (Otsuka Pharmaceutical Co., Ltd.), an infrared spectral analyzer that measures the change in the carbon isotope ratio ($^{13}\text{CO}_2/^{12}\text{CO}_2$) in the exhaled air. The difference between the ratios before and after ^{13}C -urea administration was expressed as $\Delta^{13}\text{CO}_2$.

Endoscopic biopsy: After the UBT, an intravenous catheter was placed, and anesthesia was induced with propofol and maintained with isoflurane by endotracheal intubation.

Fig. 1. The procedure for collecting a breath sample using a closefitting anesthesia mask and a breath sampling bag (Otsuka Pharmaceutical Co., Ltd.).

Dogs were restrained in left recumbency and subjected to gastroscopy. Endoscopic biopsy samples of the stomach were obtained from the antrum, corpus and fundus in this order using an endoscope designed for animals, the OLYMPUS VQ-8143B (Olympus Medical Systems Corp., Tokyo, Japan) and a biopsy forceps (FB-54Q-1, Olympus Medical Systems Corp.). The biopsy forceps was intensively washed with water and 70% ethanol every time the targeted gastric regions were changed. The samples obtained were subjected to PCR, RUT and histology/cytology.

PCR: Gastric biopsy samples were frozen at -20°C until DNA extraction. DNA was extracted from the specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Santa Clarita, CA, U.S.A.). PCR was performed with primers specific to *Helicobacter* 16S rRNA (forward primer: 5'-GCTATGACGGGTATCC-3', reverse primer: 3'-ACTTCACCCAGTCGCTG-5') that generate a 1,200-bp amplicon [5]. All PCR reactions consisted of 1 μl of extracted DNA, 12.5 μl of Hot-Star Taq Master Mix (Qiagen) and 0.5 μM of each primer in a final volume of 25 μl and were run using a TaKaRa PCR Thermal Cycler Dice[®] Standard (Takara Bio Inc., Otsu, Japan). PCR amplification was carried out according to the following protocol: 94°C for 10 min, followed by 30 cycles of denaturing at 94°C for 1 min, primer annealing at 58°C for 1 min and 30 sec, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. A 10- μl volume of each amplification product was analyzed by electrophoresis on a 1% agarose gel in Tris-acetate EDTA buffer. The gel was stained with ethidium bromide, and the DNA was visualized using a UV transilluminator.

RUT: All samples were analyzed for urease activity using CLOtest "KOKUSAI" (Sysmex Corporation, Kobe, Japan). Briefly, each biopsy specimen was incubated in a well of the kit for 24 hr, and the color change was evaluated. The result was considered positive when the color of at least 1 section of the well changed from yellow to red (due to the production of ammonia) within 24 hr and negative when the color remained yellow in all sections after 24 hr.

Histology/cytology: For histology, the gastric mucosal

Table 1. Results of PCR, RUT^{a)} and histology or cytology for *Helicobacter* spp. in gastric biopsy samples from 6 laboratory beagles

Dog no.	Before eradication ^{b)}			After eradication		
	PCR	RUT	Histology/Cytology ^{c)}	PCR	RUT	Histology/Cytology
1	+	+	+	–	–	–
2	+	+	+	–	–	–
3	+	+	+	–	–	–
4	+	+	+	–	–	–
5	+	+	+	–	–	–
6	+	+	+	–	–	–

a) Rapid urease test. b) *Helicobacter* spp. was eradicated by amoxicillin (250 mg/dog, PO, q 12 hr), metronidazole (250 mg/dog, PO, q 12 hr) and omeprazole (10 mg/dog, PO, q 24 hr) for 14 days. c) Positive when spiral-shaped bacteria were observed in at least 1 histologic section and/or cytology smear and negative when none was seen in all sections and smears. d) Total result and partial results (antrum/corpus/fundus); total result was positive when at least one region had positive results, and negative when all the three regions had negative results. Partial results are arranged in order of the obtention of endoscopic biopsy samples.

samples were fixed with neutral-buffered formalin, processed routinely and embedded in paraffin. Sections were cut, stained with hematoxylin and eosin and evaluated by light microscopy by 2 veterinary pathologists (K. U. and Y. M.). Impression smears of the gastric biopsy samples were stained with Wright-Giemsa stain and evaluated by light microscopy. The specimens were considered to be *Helicobacter* spp.-positive by histology/cytology when spiral-shaped bacteria were observed in at least 1 histologic section and/or cytology smear and negative when none was seen in all sections and smears.

Statistical analysis: The Mann-Whitney *U*-test was used to compare the numerical results of the UBT between the *Helicobacter* spp.-positive and -negative groups of canine patients. The analysis was performed using JMP version 5.0.1 (SAS Institute, Cary, NC, U.S.A.). The level of significance was set at $P=0.05$.

RESULTS

Determination of the reference range of the UBT in dogs: All of the gastric tissue samples obtained by endoscopy from the 6 laboratory beagles were positive for *Helicobacter* spp. by all tests (PCR, RUT and histology/cytology; Table 1). In 2 dogs, PCR detected *Helicobacter* spp. in the fundus and corpus of the stomach, but not in the antrum. After eradication using antibiotics, all dogs were negative in all regions by all examinations (Table 1).

The mean $\pm$ 2 SD value of UBT was $22.2 \pm 18.2\%$ before the eradication of *Helicobacter* spp. and decreased markedly after eradication (to $0.6 \pm 1.8\%$; Table 2). As all dogs were negative by all examinations after the eradication of *Helicobacter* spp., the reference range of the UBT in dogs was determined to be less than 2.5%, which is equivalent to the cut-off value for humans (2.5%).

Validity of the reference range of the UBT in canine patients: The UBT was tried to be performed for 32 dogs and succeeded in 27 dogs. Of these, 17 were female (13 spayed) and 10 male (6 castrated). The dogs' ages ranged from 1.2 to 13.1 years and their body weights from 1.8 to 25.4 kg.

Table 2. The $\Delta^{13}\text{CO}_2^{\text{a)}$ values of UBT^{b)} in 6 laboratory beagles before and after eradication of *Helicobacter* spp. (‰)

Dog no.	Before eradication	After eradication
1	15.1	–0.8
2	35.9	0.6
3	26.8	0.7
4	13.9	0.0
5	27.1	1.5
6	14.3	1.6
Mean $\pm$ 2 SD	22.2 ± 18.2	0.6 ± 1.8

a) The change of the carbon isotope ratios ($^{13}\text{CO}_2/^{12}\text{CO}_2$) in the exhaled air between before and after ^{13}C -urea administration. b) Urea breath test.

The 19 pure breeds represented were Miniature Dachshund (n=5), Yorkshire Terrier (n=3), Chihuahua and Shih Tzu (n=2 each), and Pug, French Bulldog, Japanese Shiba Inu, German Shepherd Dog, Toy Poodle, Toy Manchester Terrier, Boston Terrier, West Highland White Terrier, Jack Russell Terrier, Cairn Terrier, Miniature Schnauzer, Siberian Husky, Pekingese, Kaninchen Dachshund and Pembroke Welsh Corgi (n=1 each).

By PCR, 18 dogs were positive and 9 negative for *Helicobacter* spp. (Table 3). *Helicobacter* spp. was detected by PCR in the fundus region specimens of all 18 positive dogs, but not in the antrum specimens of 10 or the corpus specimens of 2 of these dogs (Table 3). The median value of the UBT in the positive group (n=18) was 21.1‰ (range: 0.8–157.3‰), which was significantly higher than that of the negative group (n=9, median: 0.6‰, range: 0.2–6.4‰; Fig. 2, $P<0.01$). Based on the reference range of 0–2.4‰ determined in the first part of the present study, 17 dogs were judged to be positive and 10 to be negative for *Helicobacter* spp. by the UBT. Of the 18 dogs that were *Helicobacter*-positive by PCR, 16 were also positive by the UBT, while among the 9 dogs that were *Helicobacter*-negative by PCR, 8 were also negative by the UBT (Fig. 2). Therefore, UBT exhibited 89% (16/18) sensitivity and 89% (8/9) specificity.

Table 3. Results of PCR for *Helicobacter* spp. in the stomach of canine patients (n=27)

Dog no.	Total ^{a)} (antrum/corpus/fundus) ^{b)}
1	- (-/-/-)
2	+ (+/+/+)
3	+ (+/+/+)
4	- (-/-/-)
5	- (-/-/-)
6	+ (-/-/+)
7	+ (+/+/+)
8	+ (+/+/+)
9	+ (+/+/+)
10	+ (+/+/+)
11	- (-/-/-)
12	- (-/-/-)
13	- (-/-/-)
14	+ (+/+/+)
15	+ (+/+/+)
16	+ (+/+/+)
17	- (-/-/-)
18	+ (+/+/+)
19	- (-/-/-)
20	+ (+/+/+)
21	+ (+/+/+)
22	+ (+/+/+)
23	+ (+/+/+)
24	- (-/-/-)
25	+ (+/+/+)
26	+ (+/+/+)
27	+ (+/+/+)

a) Total result was positive when at least 1 region had positive results and negative when all the three regions had negative results. b) Partial results are arranged in order of the obtention of endoscopic biopsy samples.

DISCUSSION

Helicobacter spp. has been reported to be widespread in dogs regardless of their clinical signs and/or disease states [4, 6, 13, 41], and the prevalence is also reportedly high in laboratory and shelter dogs [4, 12]. In the present study, all laboratory beagles (n=6) were initially judged to be *Helicobacter* spp.-positive by all examinations (PCR, RUT, histology and cytology), indicating that *Helicobacter* spp. was prevalent in the kennel housing the laboratory beagles used in the present study.

Helicobacter spp. was eradicated from the stomachs of the infected dogs in the present study by administration of amoxicillin (250 mg/dog, PO, q 12 hr), metronidazole (250 mg/dog, PO, q 12 hr) and omeprazole (10 mg/dog, PO, q 24 hr) for 14 days; this protocol was based on previous reports [2, 27]. As all dogs were *Helicobacter* spp.-negative by all examinations after eradication, this protocol seemed to be quite efficacious for the eradication of *Helicobacter* spp. from the canine stomach.

Based on the post-eradication results of the UBT in the 6 laboratory beagles, the reference range of the UBT for dogs was calculated to be less than 2.5‰, which is equivalent

Fig. 2. $\Delta^{13}\text{CO}_2$ values from the urea breath test (UBT) for *Helicobacter* spp.-negative and -positive canine patients. Each point represents 1 dog. The reference range for the UBT is under the dotted horizontal line. The solid horizontal lines represent the median values of each group.

to the cut off value for humans. Next, the validity of this reference range was evaluated in 27 canine patients that underwent upper gastrointestinal endoscopy by determining the sensitivity and specificity of the UBT using PCR as the gold standard for the detection of *Helicobacter* spp. The UBT proved to be highly sensitive (89%, 16/18) and specific (89%, 8/9), indicating that the UBT could be quite helpful for detecting *Helicobacter* spp. in the canine stomach.

In the present study, RUT, histology and cytology were also performed on the laboratory beagles before and after eradication, and the results of all of these agreed with the results of the UBT and PCR, indicating that these examinations are also useful when gastric biopsy samples are available.

While *H. pylori* has been most frequently observed in the gastric antrum region in humans [17], several studies in dogs have reported that *Helicobacter* spp. has been identified at higher frequency in the fundus and corpus than in the antrum [9, 10, 34, 41]. In the present study, *Helicobacter* spp. was most frequently detected by PCR in the fundus in both laboratory beagles and canine patients (Tables 1 and 3). These observations indicated that gastric endoscopic biopsy samples should be collected from at least the gastric fundus for the detection of *Helicobacter* spp. in dogs. Though the possibility of the contamination between the gastric regions through the biopsy forceps cannot be completely denied, we believed it was minimized by intensively washing of the biopsy forceps with water and 70% ethanol every time the targeted gastric regions were changed.

In the *Helicobacter* spp.-positive patients, the UBT values of 3 dogs seemed to be relatively higher than those of the

rests. The number of *Helicobacter* spp. in the stomach of each patient wasn't counted in the present study, but there is no report which shows a positive correlation between the UBT value and the number of *Helicobacter* spp. in the stomach at present. Considering that the urease activities of *Helicobacter* spp. are different according to their species and strains [8], the identification of *Helicobacter* spp. by their specific sequences may be helpful for the investigation into the cause of the high values.

In the present study, 1 canine patient showed false-positive and 2 false-negative UBT results. The false-positive result may in fact have reflected the local presence of *Helicobacter* spp. in the stomach and the collection of endoscopic biopsy samples for PCR from areas in which there was no *Helicobacter* spp. In addition, oral or gastric urease-producing bacteria other than *Helicobacter* spp., such as *Enterobacter cloacae*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, might affect the results of the UBT, as has been reported in humans [25, 26]. The false-negative results may have occurred, because the number of *Helicobacter* spp. organisms was too small to detect by the UBT or because the ¹³C-urea did not sufficiently contact the organisms due to gastric hyperactivity.

During the study of canine patients, we failed to collect breath samples in 5 snub-nosed or small/large dogs: Pug (n=2), and Pomeranian, German Shepherd Dog and Belgian Shepherd Dog (n=1 each). Therefore, the UBT may require preparing a mask of appropriate shape and size for each dog. Furthermore, the UBT may be impossible to perform on very aggressive dogs.

The pathogenicity of *Helicobacter* spp. in the canine stomach is controversial at present. Some authors have denied the pathogenicity of *Helicobacter* spp. in dogs [10, 33, 34], but other reports have supported the relationship between *Helicobacter* infection and gastric disease [16, 29–32]. Therefore, treating *Helicobacter* infection in dogs is controversial, and whether treatment is needed in all cases or not is unclear at this point. Further studies of the relationships between infection with each NHPH species (as identified by their specific sequences) and canine gastric lesions with larger sample sizes are needed. The present study showed that the status of gastric infection with *Helicobacter* spp. in canine patients can be determined by the UBT with high sensitivity and specificity and without anesthesia and invasive procedure. UBT using an infrared spectrograph would thus be a useful screening test for domestic epidemiological studies of *Helicobacter* spp. in the canine stomach and would make the study of the pathogenicity of *Helicobacter* spp. in the canine stomach more efficient. In addition, as the UBT values decreased after eradication of *Helicobacter* spp., UBT could be useful for monitoring the gastric infection status in dogs treated with antibiotics for suspicious NHPH-related gastritis.

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