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Research Article

Determination of Helicobacter Infections with ^{14}C Urea Breath Test (^{14}C UBT) and Polymerase Chain Reaction (PCR) in Dogs and Treatment [1] [2] [3]

Yücel MERAL [1] Güvenç GÖKALP [1] Sibel UÇAK SEMİRGİN [2] Mehtap ÜNLÜ SÖĞÜT [3]

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[3] *This study is summarized from the PhD Thesis of Güvenç GOKALP*

[1] University of Ondokuz Mayis, Faculty of Veterinary, Department of Internal Medicine, 55139, Atakum, Samsun - TURKEY
[2] University of Ondokuz Mayis, Faculty of Medicine, Department of Nuclear Medicine, 55139, Atakum, Samsun - TURKEY
[3] University of Ondokuz Mayis, Health School, 55139, Atakum, Samsun - TURKEY

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Abstract

It was aimed to determine that if ^{14}C UBT (^{14}C Urea Breath Test) application as a "Gold Standard" in the human practice for diagnosing Helicobacter infections could also be used in an easy, practical, and a reliable method in the veterinary practice. Three groups were allocated in the study. Dogs (n=41) were detected as clinically healthy in their physical examinations were grouped as healthy (Group I). Dogs (n=32) had clinical symptoms of vomiting after feeding, anorexia, epigastric pain during abdominal palpation were grouped as diseased and these diseased dogs (Group II) that were treated grouped as treatment (n=32) (Group III). Helicobacter staining, Polymerase Chain Reaction (PCR) and ^{14}C UBT were compared for diagnosis of Helicobacteriosis before and after therapy. Based on the results of the present study ^{14}C UBT was found 2 times more reliable when compared to PCR and 65 times more reliable than Helicobacter staining technique. Sensitivity for ^{14}C UBT, PCR and Helicobacter staining technique was found as 96.55%, 93.75%, and 59.38%, respectively. Moreover specificity of the ^{14}C UBT, PCR and Helicobacter staining technique was detected as 97.73%, 97.56% and 92.68%, respectively. Consequently, it was thought that ^{14}C UBT test could be used as a reliable method in veterinary practice.

Keywords: *Dog, Helicobacter spp, Diff-quick staining, PCR, ^{14}C Urea breath test*

Köpeklerde Helikobakter Enfeksiyonlarının ^{14}C Üre Nefes Testi (^{14}C UNT) ve Polimeraz Zincir Reaksiyonu (PZR) ile Belirlenmesi ve Tedavisi

Özet

Çalışmamızda; insan hekimliği pratiğinde Helikobakter enfeksiyonlarının teşhisinde "Altın Standart" olarak tabir edilen ve başarıyla uygulanan ^{14}C UNT (^{14}C Üre Nefes Testi)'nin Veteriner Hekimlik pratiğinde de kolay, pratik ve güvenilir bir yöntem olarak uygulanabileceğini belirlemeyi amaçladık. Çalışmamız klinik muayenesinde sağlıklı olan köpekler (n=41) (Grup I), anoreksi, yemeği takiben kusma ve epigastric ağrı şikâyeti ile kliniğimize gelen helikobakter enfeksiyonlu köpekler (n=32) (Grup II) ve tedavi edilen bu helikobakter enfeksiyonlu köpekler (n=32) (Grup III) olmak üzere 3 grup oluşturuldu. Helikobakteriyosizin karşılaştırmalı tanısı için, Helikobakter boyama, Polimeraz Zincir Reaksiyonu (PZR) ve ^{14}C Üre Nefes Testi (^{14}C UBT) tedavi öncesi ve tedavi sonrası köpeklere yapıldı. Çalışma sonucunda, ^{14}C UNT'nin PZR'a göre 2 kat ve Helikobakter boyama yöntemine göre 65 kat daha güvenli bir tanı yöntemi olduğu bulundu. Sensitivite; ^{14}C UNT'de %96.55, PZR'de %93.75 ve Helikobakter boyamada %59.38 bulunurken, spesifite sırasıyla; %97.73, %97.56 ve %92.68 olarak bulundu. Sonuç olarak, ^{14}C UBT'nin Veteriner Hekimlik pratiğinde güvenli bir metod olarak kullanılabileceğini düşünmekteyiz.

Anahtar sözcükler: *Köpek, Helicobacter spp., Diff- quick boyama , PZR , ^{14}C Üre nefes testi*

İletişim (Correspondence)
+90 555 5128917
ymeral@omu.edu.tr

INTRODUCTION

Helicobacter pylori was firstly described as a cause of b type human gastritis in 1982 in West Australia by Warren and Marshall [1]. Later it is named as *Campylobacter pyloridis* and its etiologic relations with gastritis were confirmed by applications of Coch Postulates [2].

The spiral shaped microorganisms in the dog stomach had been discovered a century before than the bacteria known as *H. pylori* in humans and later named as *Helicobacter* spp. It is believed that early identification of *Helicobacter* species in dogs related to their bigger morphological structures [3].

Among the gastric *Helicobacter* spp. species *H. heilmannii* group is detected in numerous mammalian species after *H. pylori*. They are presented most commonly in dog, cat, cheetah, pig, wild cats with multiple primate species and humans [4-6]. The clinical studies are obviously reported that spiral bacteria have been detected in the clinically healthy dogs' and cats' stomachs as much as their gastrointestinal diseased counterparts [6].

There are at least four spiral microorganisms- *H. felis* [5], *H. salomonis* [7], *H. bizzozeronii* [8,9]. *H. heilmannii* known that colonized in the dog and cat stomach.

In the last decade, O'Rourke et al.[10] succeeded in distinguishing the *H. felis, H. salomonis, H. bizzozeronii* ve *H. heilmannii* by sequence analyze of some of the urease gen complex. Later experimental studies are showed that *H. pylori* have similar disease procedures in dogs like humans [11].

The prevalence of *Helicobacter* spp. have been reported very high in the dogs and cats. It is reported in the healthy dogs as 67-100%, and 74-90% as in the infected dogs. On the other hand, in cats this prevalence is determined as 40-100% in healthy or infected cats [12].

However, it has not been clearly known how the *Helicobacter* spp. transmitted to people. It is propounded that *H. pylori* could be transmitted by oral-oral or fecal-oral way. Especially puppies in the lactation period have a very closer social life. Therefore, they could have exposed to *Helicobacter* spp. transmissions in their early lives [13].

Dogs and cats are propounded as reservoirs for transmission of *H. heilmannii* like organisms (HHLO) to people because of the progressed intimate life with humans and the companion animals [12].

Beyond the invasive approaches like endoscopy, rapid urease test, histology, culture, PCR (Polymerase Chain Reaction) that based on the phase-contrast microscopy of the stomach tissues, non-invasive approaches like serology, urea breath test, "*H. pylori* Stool Antigen test (HpSA)", stool, salvia or dental plaques are also could be used for detecting the microorganism with molecular techniques [14].

Urea breath is a non-invasive test that has a high sensitivity and specificity is applied with a radioactive method. Even it is a slightly expensive method; it could be used in both diagnosis and treatment [15].

Within the present study we tried to emphasize that ^{14}C UBT application in the human practice for diagnosing Helicobacter infections remains as a "Gold Standard" also could be used in an easy, practical, and a reliable method in the veterinary practice.

MATERIAL and METHODS

Dogs (n=41) belonging to Group I were clinically healthy and negative for *Helicobacter* spp by PCR and ^{14}C UBT. Inclusion criteria for Group II (before treatment) (n=32) were presence of clinical symptoms of vomiting after feeding, anorexia, epigastric pain during abdominal palpation and positivity of *Helicobacter* spp. by staining, PCR and ^{14}C UBT. All dogs in Group II were treated according to Aytuğ et al.[16] (amoxicillin, 20 mg/kg per orally, tid and with omeprazole, 0.75 mg/kg for one week). This group were classified later as Group III (after treatment) (n=32). On the other hand, care was taken that all groups had not received any antibiotics and active acid inhibitors one month before the procedure.

There were 3 German shepherds, 4 Golden Retrievers and 34 mongrels of which 31 were male and 10 were female in Group I. Group II were consisted of 22 Mongrels, 8 Golden Retrievers, 1 Doberman and 1 Anatolian Shepherd dog of which 15 dogs were male and 17 were female. Dogs in Group III were as in group II.

Complete blood count was performed in all groups. All blood samples were analysed by using an Abacus Vet Junior Hematology Analyzer (Diatron, Austria). All data were recorded into their individual protocols.

^{14}C - UBT test was applied to all groups. These animals, which were given the test, were starved for 6 h, free from antibiotics and active acid inhibitors for at least a month. At the same time, dogs of Group II were given ^{14}C- Urea Breath Test after therapy in order to detect the existence of Helicobacter *spp*. Before the test, the animals were made to swallow a ^{14}C- Urea capsule (HELICAP®)-with plenty of water- which requires no preparation, is easy to swallow, has no risk of spilling and is safe. After putting over the capsule, 2 mg/kg of 2% Xylazine hydrochloride was administered to the patient. There after this procedure breath collector was connected to the dry cartridge within 10 minutes. Suitable endotracheal tube was applied with sedation. The breath is collected with the attached system until the membrane's color of the cartridge changed to yellow from orange within 20 min (approximately this period continues 20 min). Then, dry cartridge system was scanned at an analyser (Heliprobe Analyser Nosterkibion System 2223- A™), and the results were received in 250

sec. The results evaluated as GRADE 0 meaning infection negative, GRADE 1 meaning suspicious; GRADE 2 meaning infected *(Table 1)*. GRADE 1 results were rechecked.

Gastroscopy was administrated to every dog that allocated in the study after applying the UBT test. Starting from the pharynx, in addition to the systematic examinations of oesophagus and antrum, the examination of the stomach was completed with the scrutinization along the angulus antrum and pyloric canal as a whole. Gastric smear and juice were collected during the examination of stomach with gastroscopy. The gastric smear was then put into the eppendorf tubes that filled 0.9% saline and samely gastric juice were collected into the eppendorf tubes and stored at -18°C until analysis. Every signalmen's and clinical data of the dogs were grouped and recorded in the individual protocols and all samples were numbered.

The smear applied on microscope slides were dried in the air and fixed with methanol, it was stained with diff-quick stains II and I and dried in the air for 2 min. Next, it was examined for S or spiral-shaped organisms by the light microscope with active x400 magnification. The results were recorded in the protocol [12].

The stomach contents and biopsy materials were used for DNA extraction. For this aim, commercially available DNA extraction kits (PureLink Genomic DNA Kits, Invitrogen, Canada) were used according to the manufacturers' instructions. The 100 µl of extracted DNA was kept frozen at -20°C until molecular tests were carried out. Genus-spesific PCR analysis was conducted as reported by Riley et al.[17] and the observation of a 375 bpm band was considered positive. Species-specific PCR was performed on DNA that belongs to the samples found out to be positive in species-specific PCR result. For that purpose, PCR was conducted separately for 16S rRNA gene [17,18], which is specific to *H. pylori*, for UreB gene [18,19], which is specific to *H. heilmannii,* and for 16S rRNA gene [19,20], which is specific to *H. felis* and *H. bizzozeronii*. The DNA bands of 295, 580, 577-596 and 636-656 bp were evaluated as positive for *H. pylori*, *H. heilmannii, H. felis* and *H. bizzozeronii*, respectively. All PCR analyses were conducted according to the methods and under the conditions stated in the related literature.

Data obtained from the study is the threshold character data obtained in existing (+) and non-existing (-) type. Hence, the most suitable mathematical modelling was investigated by initially determining the structure and the distribution of Binomial character data. In categorical data analysis, χ^2 test were used statistically [21]. The ethical committee approval numbered 2009-38 was taken from Ethical Committee for Experimental Animals of Ondokuz Mayıs University.

Table 1. *Evaluation of the Heliprobe Analyzer Results*
Tablo 1. *Heliprob Analizer ile Alınan Sonuçların Değerlendirilmesi*

Grading	Infection Status	d (cpm)
0	Infection negative	d ≤ 25 cpm
1	Suspicious	25 cpm < d < 50 cpm
2	Infection positive	d ≥ 50 cpm

RESULT

There were significant differences between WBC, RBC and RDWc of Group I, Group II and Group III (P≤0.001). Other parameters were not found to be statistically important *(Table 2)*.

The values obtained via the ^{14}C- UBT from all dogs are shown in *Table 3*. D value, which is the sum of ^{14}C isotope values gathered in d1 (lower ped) and d2 (upper ped), were detected to be 28.03±10.95cpm and 109±163cpm (P≤0.001), respectively in groups. A statistical comparison of d1 and d2 parameters between groups was not performed.

With the gastroscopic examination performed in accordance with the method, hyperaemia in gastric mucosa and oedema (erythematous) were detected in Group II. Gastric ulcer was detected in two cases.

According to the clinical examination data of the animals, of 32 dogs considered to have *Helicobacter* spp.-

Table 2. *The distribution and the significance level of the blood count values of animals included in the study*
Tablo 2. *Çalışmaya alınan hayvanların kan sayımı değerleri dağılımı ve önem derecesi*

Blood Count Parameters	Group I Healthy Group (n=41)	Group II Before Treatment (n=32)	Group III After Treatment (n=32)
WBC (10^3 µl)	8.3±1.3^{b}	15.34±6.67^{a}	7.6±2.4 ^{b}
LYM (10^3 µl)	2.42±1.64 ^{a}	3.93±1.77 ^{a}	3.42±1.36 ^{a}
MONO (10^3 µl)	0.44±0,27 ^{a}	0.5±0.35 ^{a}	0.49±0.1 ^{a}
GRAN (10^3 µl)	8.22±2.9 ^{a}	8.2±3.6 ^{a}	7.2±1.6 ^{a}
LYM (%)	20.78±8.66 ^{a}	20.92±13.82 ^{a}	20.41±7.1 ^{a}
MONO (%)	4.48±1.43 ^{a}	3.93±2.19 ^{a}	3.22±0.9 ^{a}
GRAN (%)	68.46±23.96 ^{a}	71.27±20.68 ^{a}	66.21±14.6 ^{a}
RBC (10^6 µl)	6.08±1.17 ^{a}	5.13±1.27^{b}	5.77±0.8 ^{b}
HGB (g/dl)	14.09±2.44 ^{a}	13.34±4.28 ^{a}	13.71±2.94 ^{a}
HCT (%)	39.58±4.92 ^{a}	40.49±10.26 ^{a}	34±2.62 ^{a}
MCV (fl)	66.84±6.47 ^{a}	65.96±9.88 ^{a}	64.21±9.88 ^{a}
MCH (pg)	26.27±12.77 ^{a}	30.62±15.82 ^{a}	28.1±10 ^{a}
MCHC (g/dl)	36.31±8.82 ^{a}	36.21±8.21 ^{a}	36.9±9.2 ^{a}
RDWc (%)	19.93±4.7^{b}	25.6±3.55^{a}	21.2±2.4^{b}
PLT (10^3 µl)	414.52±166.24 ^{a}	405.24±191.26 ^{a}	366.3±117.2 ^{a}
PCT (%)	0.42±0.12 ^{a}	0.38±0.19 ^{a}	0.22±0.16 ^{a}
MPV (fl)	9.46±1.39 ^{a}	8.94±1.68 ^{a}	8.44±1.2 ^{a}
PDWc (%)	38.6±4.7 ^{a}	39.06±4.72 ^{a}	38.64±3.2 ^{a}

** Groups marked with a different letter are significant among themselves (P≤0.001)*

Table 3. *The distribution of d, d1 and d2 values obtained Via ^{14}C- UBT method used in the study*

Tablo 3. *Çalışmada uygulanan C^{14} UNT yöntemi ile elde edilen d, d1 ve d2 değer dağılımları*

^{14}C Isotope Values (cpm)	Group I Healthy Group (n=41)	Group II Before Treatment (n=32)	Group III After Treatment (n=32)
d1	15.03±7.54	108.7±92.27	21.2±10.2
d2	13±5.29	1±0.2	8.4±7.9
d	28.03±10.95 ^{b}	109±163 ^{a}	30.2±4.1 ^{b}

** Groups with different letters are significant among themselves (P≤0.001)*

induced gastritis, positivity was detected in 22 animals via diff-quick staining method.

According to PCR, taking the number of patients under consideration, of 32 dogs, while 29 of them had *Helicobacter* spp, 3 of them had *H. heillmanni* species. None of the other *Helicobacter* species were detected in our study.

DISCUSSION

S-structure microorganisms in the stomach of animals were first discovered at the end of the 19th century by Rappin. Later on, in 1893, Bizzozero discovered a type of bacterium that causes inhibition in gastric glands and canals. Salomon, on the other hand, discovered these types of microorganisms in the stomach of cats and rats [22].

In defining helicobacter infection, invasive intervention (endoscopy), which requires rapid urease test, histology, culture, tests based on Polymerase Chain Reaction (PCR) and examinations, such as phase contrast microscopy of the gastric tissue and non-invasive serology, ^{13}C and ^{14}C Urea Breath Tests (UBT), Helicobacter stool antigen test (HpSA) are used [14].

Among the invasive examination methods which require endoscopy are histological examination, bacteriological culture, PCR and rapid urease test. Both groups of diagnosis methods have advantages and disadvantages in terms of operation, the choice of patients, time needed to get the result, and costs [23].

Urea breath test, which is accepted as one of the non-invasive tests in the diagnosis of Helicobacter infections, provides an accurate diagnosis without performing endoscopy. It is also a test with high sensitivity and specificity which can be used both in the initial diagnosis of an active infection in unhealed patients and to follow up the effectiveness of the treatment in post-treatment period [24].

In our study, the average leucocyte value is 8.3±1.3x10^{3}/µl in the whole blood count analyses of samples taken from Group I, and the average leucocyte value in dogs with *Helicobacter* spp-induced gastritis is 15.34±6.67x10^{3}/µl (P≤0.001). While the average erythrocyte value in the whole blood count analysis of healthy dogs is around 6.08±1.17x10^{6}/µl, this average value in dogs with *Helicobacter* spp-induced gastritis has been found out to be 5.13±1.27x10^{6}/µl (P≤0.001). While this value ranges between the values 19.93±4.70 (%) in erythrocyte distribution range of Group I, it ranges between 25.6±3.55 (%) in dogs with *Helicobacter* spp-induced gastritis *(Table 2)*. In a study conducted on 34 cats and dogs, Jennifer et al.[25] established that there could be a reduction in the erythrocyte number of *Helicobacter* spp. types and in the erythrocyte distribution range due to Vitamin B_{12} and iron deficiency.

We think that the reason why 96.55% obtained in our study is higher than what other researchers obtained is that we transferred the other lung air to dry cartridges via endotracheal tube. However, in their study, Kubota et al.[26] used mask to prevent the contamination. On the other hand, because it is difficult to make an animal breathe directly and spontaneously into the kit used as suggested in the method performed on people, it might reduce the sensitivity of the study, transferring air in dry cartridges through endotracheal tubes increases the sensitivity of our research.

In two of the samples taken to detect Helicobacter species, the result of ^{14}C-UBT was positive, and the negative results of these samples were seen in PCR. It is thought that this situation results from the fact that samples taken through endoscopy for PCR were not taken from the prepiloric antrum region of the stomach [27]. In the same way, in spite of this false positivity obtained via ^{14}C- UBT, Zotta et al.[28] describe these false positive results may occur according to the urease activity of the *Streptococcus thermophilus* which exists in the gastric mucosa with *H. pylori*.

It has been established in our study that there is a high incidence of accuracy of ^{14}C-UBT in the diagnosis of *Helicobacter* infection *(Table 4, Table 5)*, and in line with our study, Raju et al.[29] stated in their study (n=64) that the positive result rate was higher in ^{14}C-UBT than in other diagnosis methods used in the diagnosis of *H. pylori* infection. In analyses performed in order to detect a healthy animal or to determine the existence of spiral bacteria activity, diagnosis with the ^{14}C-UBT method has been found out to be sensitive at a rate of 96.55%, while the sensitivity rate has been found out to be 93.75% in PCR method and 59.38% in *Helicobacter* staining method *(Table 4, Table 5)*.

^{14}C-UBT, which is one of the leading non-invasive methods used in the diagnosis of *Helicobacter* spp. in carnivores, has been found out to be 96.55% in our urea breath test sensitivity rate study in the detection of the existence of spiral bacterium before the designation of species. Additionally, in the study conducted by Wong et al.[30] (n=68), this rate was found out to be around 94.5%, and around 95-97% in the study (n=85) by Vaira et al.[31].

Table 4. *Frequency (%), odd's ratio's, $\chi^2_{sd=1}$ and P value distributions of methods used in the study*
Tablo 4. *Çalışmada uygulanan yöntemlerin frekans (%), odd's ratio's , $\chi^2_{sd=1}$ ve P değer dağılımları*

Method	Frequency (%)		Odds Ratio's			$\chi^2_{sd=1}$	P Value
			Additional Risk	Relative Risk			
	Helicobacter (-)	Helicobacter (+)		Helicobacter (-)	Helicobacter (+)		
Clinical Signs	41 (60.27)	32 (39.73)	-	-	-	-	-
Diff-quick Stain	51 (69.86)	22 (30.14)	18.51	5.46±0.04	1.88±0.01	23.13	0.001
PCR	44 (60.27)	29 (39.73)	600	28.34±0.02	4.12±0.03	64.88	0.001
^{14}C UBT	42 (57.53)	31 (42.47)	1204	29.52±0.04	4.28±0.01	61.33	0.001

Table 5. *Frequency (n), sensitivity, specificity and Q value distributions of methods used in the study*
Tablo 5. *Çalışmada uygulanan yöntemlerin frekans (n), sensivite, spesivite ve Q değeri dağılımları*

Method	Frequency (n)		Sensitivity (%)	Specificity (%)	Q Value (1- Specificity) (%)
	Helicobacter (-)	Helicobacter (+)			
Clinical Signs	41	32	-	-	-
Diff-quick Stain	51	22	59.38	92.68	7.32
PCR	44	29	93.75	97.56	6.25
^{14}C UBT	42	31	96.55	97.73	3.45

When compared to each other, the urea test is 65 times more reliable than *Helicobacter* staining method, twice as reliable as PCR analysis method that it is a non-invasive method increases its suitability among other methods used for diagnosis *(Table 4, Table 5)*. Similarly, in the study of Kopanski et al.[32] it was reported that urea breath test as a non-invasive choice for detection of Helicobacter infections had higher sensitivity.

^{14}C UBT has superiority for the diagnosis of the existence of spiral bacterium in dogs, when compared with PCR analysis and Diff-quick test in our study. Cartridges that belong to ^{14}C Helicobacter Urea Breath dry cartridge system were anaesthetized and connected to the end of the intubation tube with a tape, and then dogs were made to breathe into this cartridge for 20 minutes. As a result of this, the existence of *Helicobacter* spp. was established as well as 96.55%, sensitivity rate and specificity at a rate of 97.73%.

Consequently, within the present study we tried to emphasize that ^{14}C UBT application in the human practice for diagnosing Helicobacter infections remains as a "Gold Standard" also could be used in an easy, practical, and a reliable method in the veterinary practice.

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