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RESEARCH ARTICLE

Assessment of Gastric *Helicobacter* spp. in Fresh Gastric Samples of Naturally Infected Dogs by Scanning Electron Microscopy

Ali SHABESTARI ASL [1] Hadi AMANIZAD [1] Alireza NEJAD PARTOVI [1]
Mehdi JALILI [2] Dariush MOHAMMAD NEZHAD [3] Mohammad Hosein SOROUSH [4]
Abolfazl BARZEGARI [5] Daryoush BABAZADEH [6]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, Tabriz Branch, Islamic Azad University, Tabriz - IRAN
[2] Department of Veterinary Medicine, Karaj Branch, Islamic Azad University, Karaj - IRAN
[3] Drugs Applied Research Center, Tabriz University of Medical Sciences, Tabriz - IRAN
[4] Department of Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz - IRAN
[5] Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz - IRAN
[6] Young Researchers and Elite Club, Tabriz Branch, Islamic Azad University, Tabriz - IRAN

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Summary

Different species of gastric *Helicobacter*-Like Organisms (GHLO) reported from dogs' stomach. The aim of present study was to morphological evaluation of gastric *Helicobacter* spp. in fresh gastric samples of naturally infected dogs. Thirty two gastric samples of the stray dog were taken at necropsy. The specimens were used for rapid urease test, light microscopy, scanning electron microscopy (SEM) and polymerase chain reaction (PCR). Light microscopy examination confirmed the presence of GHLO in 90.5% of stray dogs. 87.5% and 94% of gastric samples were positive in rapid urease test and PCR, respectively. Four distinguishable *Helicobacter* organisms were confirmed by SEM. Three strains of these organisms were inditified as *H. felis,* candidatus *H. heilmanii* and *H. bizzozeronii* because of their apparent morphological differences and PCR results. The last strain of these bacteria was not distinguishable with routine studies. The large-scale studies with fast and simple recognition methods are recommended to confirm the different types of canine gastric *Helicobacter*. The results of present study showed further investigation in canine GHLO is required because new species of *Helicobacter* reported.

Keywords: *Canine Helicobacter, SEM, PCR, Smear*

Doğal Enfekte Köpeklerin Taze Mide İçeriği Örneklerinde Gastrik *Helicobacter* Türlerinin Taramalı Elektron Mikroskopi İle Değerlendirilmesi

Özet

Köpeklerin midesinde Gastrik *Helicobacter*-benzeri Organizmalar (GHBO)'ın üç farklı türü olduğu bilinmektedir. Bu çalışmanın amacı, doğal enfekte köpeklerin taze mide içeriklerinde gastrik *Helicobacter* türlerinin morfolojik özelliklerinin belirlenmesidir. Sokak köpeklerinden nekropsi sırasında otuz iki adet mide içeriği örneği alındı. Örnekler Hızlı Üreaz Testi, Işık Mikroskopisi, Taramalı Elektron Mikroskopisi (SEM) ve Zincirleme Polimeraz Reaksiyonu (PCR) ile incelendi. Işık Mikroskopisi ile köpeklerin %90.5'inde Gastrik *Helicobacter*-benzeri Organizmaların varlığı belirlendi. Köpeklerin %87.5'i ve %94'ü sırası ile Hızlı Üreaz Testi ve PCR pozitif bulundu. Dört adet *Helicobacter* bakterisi SEM ile tespit edildi. Bu bakterilerden 3 suş, PCR ve morfolojik farklılıklarına göre *H. felis,* candidatus *H. heilmanii* ve *H. Bizzozeronii* olarak tanımlandı. Bakterinin son suşu ise rutin metodlar ile identifiye edilemedi. Köpeklerin Gastrik Helicobacterlerinin tiplerini belirlemek için hızlı ve basit tanı metodları üzerinde yapılacak geniş çaplı çalışmalara ihtiyaç vardır. Bu çalışmanın sonuçları yeni *Helicobacter* türlerinin varlığının bildirilmesinden dolayı köpeklerin GHBO'nın daha detaylı araştırılması gereğini ortaya koymaktadır.

Anahtar sözcükler: *Köpek, Helicobacter, SEM, PCR, Sürme preperat*

INTRODUCTION

Helicobacter-Like Organisms (HLO) are live in stomachs of dogs, cats, pigs and other carnivores [1-8], and this genus contains several species from a wide range of hosts [8-10]. HLO are assigned to cause gastric disease in humans

İletişim (Correspondence)
+98 912 3356659
shabestari@iaut.ac.ir

and animals [1,8,11,12], but the exact role of these fastidious bacteria is not confirmed yet [2,7,8]. Until now, different *Helicobacter* species have been isolated from canine stomachs [2,4,13-15]; but it is not known whether these species are representative of all canine gastric helicobacters or which of them are most common [8,14]. Different studies have reported dissimilar ranges of contamination [3-6,16] and some of these reports were showed high prevalence (over 90%) of contaminations [17-19].

In spite of possibility to culture of canine gastric helicobacters, some of these organisms are not cultivable [8]; or we are not able to culture them. Candidatus *H. heilmanii* is a zoonotic microorganism which is a common cause of the chronic gastric inflammation in human (0.2- 6%) [8,11,20]; but the definitive culture of this organism has not been achieved to date and only two *H. Heilmannii*-like strains *have been* cultured from *human* gastric tissue [8]. The identification of these fastidious requires specific culture techniques and due to presence of non-cultivable species further diagnostic methods are needed [8,21].

The aim of present study was to *evaluate the morphological* characteristics of canine gastric *Helicobacter* spp. and investigate the presences of atypical *Helicobacter* strain(s) in fresh gastric samples of naturally infected dogs.

MATERIAL and METHODS

Admission and Selection of Dogs

Thirty two dogs were randomly selected among the stray dogs that were euthanized in dog population control program which was organized by municipal employees in Tabriz city (East Azerbaijan province, Iran). All dogs of *both sexes* were seven months of age or older and lived in different locations of the city.

Sample Collection

Gastric *samples were taken at necropsy immediately after death* (between April and October, 2010). Four gastric samples were used in diagnostic tests. First sample was immediately fixed and used in cytological study; second sample was placed in normal saline and stored in -20°C for Polymerase Chain Reaction (PCR) assessment; Third sample was used for Rapid Urease Test (RUT) and the fourth sample was placed on microtubule for Scanning Electron Microscopy (SEM).

Impression Smear and Urease Test

Impression smears of gastric mucosa were prepared on an air-dried slide which followed by methanol fixation and stained by Giemsa (Merck, Germany) for detection of GHLO's at 1000 × magnifications. The rapid urease test (Difco, USA) was read within 12 h.

Scanning Electron Microscopy

SEM was performed on fresh samples of canine gastric mucosa. Gastric samples were immediately fixed in 2.5% glutaraldehyde phosphate-buffered solution (pH 7.2) For SEM examination. Samples were dehydrated in a graded ethanol series. After vacuum drying and gold coating, the samples were studied by using a Leo-440i-SEM (Cambridge, UK) at Islamic Azad University in Tehran, Iran.

PCR Amplification of 16S rRNA

Gastric samples were investigated by PCR amplification based on 16S rDNA sequences. The samples were thawed and DNA was extracted by using the DNP™ KIT (Cinna Gen, Iran). PCR analysis on the 16S rRNA gene was performed in an Eppendorf Mastercycler (Bacteriology Laboratory of Veterinary Faculty, Islamic Azad University, Tabriz, Iran) using specific primers *(Table 1)*. Finally, PCR products were examined using agarose gel electrophoresis.

RESULTS

Impression Smears

Presence of canine Gastric *Helicobacter*-like organisms in 29 of 32 stray dogs (90.5%) was confirmed by Light

Table 1. *Oligonucleotide primers which were used for PCR amplification and sequencing of 16S rRNA*
Tablo 1. *PCR amplifikasyonu ve 16S rRNA dizin analizi için kullanılan oligonükleotid primerler*

Target Gene	Reference	Primer Sequence (5'…….. 3')	Amplified Fragment
16Sr RNA genes of *Helicobacter* spp.	Germani [22]	(f): AAC GAT GAA GCT TCT AGC TTG CTA (r): GTG CTT ATT CGT GAG ATA CCG TCA T	399 bp
ure B gene of *H. felis*	Germani [22]	(f): GTG AAG CGA CTA AAG ATA AAC AAT (r): GCA CCA AAT CTA ATT CAT AAG AGC	241 bp
ureB gene of *H. heilmannii*	Neiger [23]	(f): GGG CGA TAA AGT GCG CTT G (r): CTG GTC AAT GAG AGC AGG	580 bp
ureC gene of *H. pylori*	Labinge [24]	(f): GGA TAA GCT TTT AGG GGT GTT AGG GG (r): GCT TAC TTT CTA ACA CTA ACG AGG	294 bp
ureB gene of *H. bizzozeronii*	Neiger [23]	(f): ACT AGG CGA TAC CAA CCT GAT TT (r): TTC TTC AGC TGC GCG GAG CAT GC	499 bp

(f): *Forward sequence,* ***(r):*** *Reverse sequence,* ***bp:*** *base pairs*

microscopy. In most cases, large resemble of GHLO was related to *H. felis*, candidatus *H. heilmannii*; but the size of one of these bacteria was much smaller.

Rapid Urease Test

87.5% (n=26 dogs) among all necropsy samples were positive in RUT.

16S rRNA Sequencing

About 94% (n=30 dogs) of gastric samples were positive in PCR. The presence of *H. felis*, candidatus *H. heilmannii* and *H. bizzozeronii* *(Fig. 1, 2, 3)* was confirmed by PCR. One of the strains was not distinguishable as a common canine gastric *Helicobacter* organism; but it was identified as a *Helicobacter* strain because of its positive 16S rRNA and the positive results of RUT *(Fig. 4)*.

The results of PCR indicated that candidatus *H. heilmannii* was most recognized *Helicobacter*-like organisms (n=16) and other common recognized strains were *H. felis* (n=10) and *H. bizzozeronii* (n=6), respectively.

Scanning Electron Microscopy

Four different *helicobacter* organisms were confirmed by scanning electron microscopy. Three of these organisms were confirmed as *H. felis*, candidatus *H. heilmanii* and *H. bizzozeronii* *(Fig. 5, 6, 7)* by PCR and SEM. The fourth strain *(Fig. 8)* typically varied from others by its small size and different shape (with 2-3 helixes). This strain morphologically was similar to *H. canis* and H. pylori but the RUT of it was positive. Furthermore, there were no positive PCR results for *H. pylori* [16].

The immunological reactions of the gastric mucosa in some gastric samples were notable and they were detected by SEM *(Fig. 9)*.

DISCUSSION

The high incidence of *Helicobacter* organisms in dogs' stomach confirmed by different studies [2,4,7,13,17-19]. In spite of confirmation of *H. pylori* as a main cause

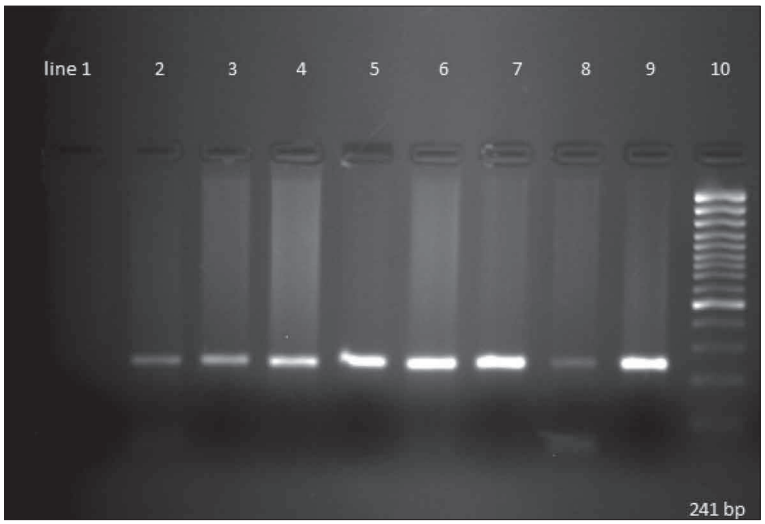
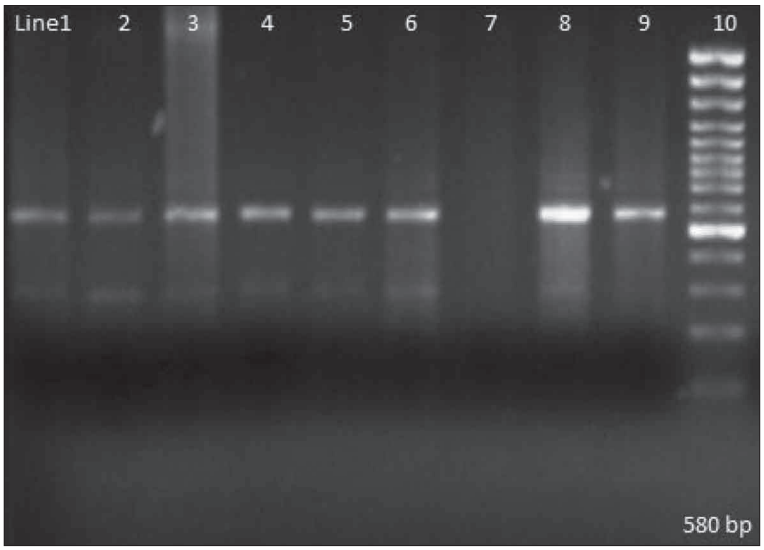


Fig 1. PCR detection of *H. felis*, Lane 1: Negative control, Lane 2-8: DNA samples, Lane 9: positive control, Lane 10: 100 bp DNA marker

Şekil 1. *H. felis*'in PCR tespiti, Şerit 1: Negatif kontrol, Şerit 2-8: DNA örnekleri, Şerit 9: pozitif kontrol, Şerit 10: 100 bp DNA marker

Fig 2. PCR detection of candidates *H. heilmanii*, Lane 1-6: DNA samples, Lane 7: negative control, Lane 9 and 10: positive control, 100 bp DNA marker

Şekil 2. *H. heilmannii*'nin PCR tespiti, Şerit 1-6: DNA örnekleri, Şerit 7: negatif kontrol, Şerit 9 ve 10: pozitif kontrol, 100 bp DNA marker



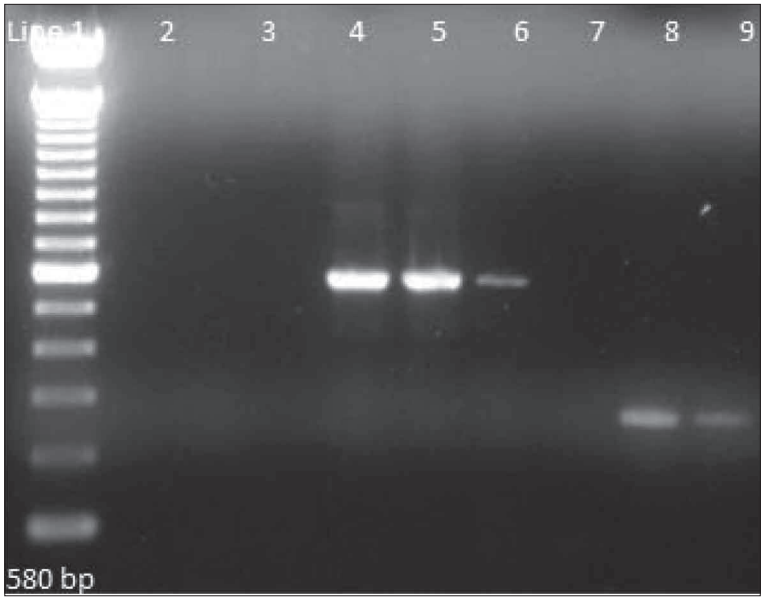
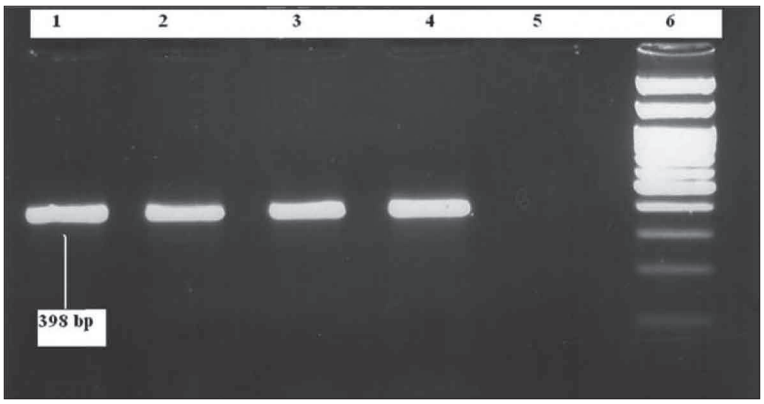


Fig 3. PCR detection of *H. bizzozeronii*, Lane1: 100 bp DNA marker, Lane 2 and 3: negative control, lane 4: positive control, lane 5-9: DNA samples

Şekil 3. *H. bizzozeronii*'nin PCR tespiti, Şerit 1: 100 bp DNA marker, Şerit 2 ve 3: negatif kontrol, Şerit 4: pozitif kontrol, Şerit 5-9: DNA örnekleri

Fig 4. PCR detection of 16S rRNA in gastric biopsy specimens. Lane 1: positive control, Lane 2-4: DNA samples, Lane 5: Negative control, Lane 6: 100 bp DNA marker

Şekil 4. Mide biyopsi örneklerinde 16S rRNA PCR tespiti. Şerit 1: pozitif kontrol, Şerit 2-4: DNA örnekleri, Şerit 5: negatif kontrol, Şerit 6: 100 bp DNA marker



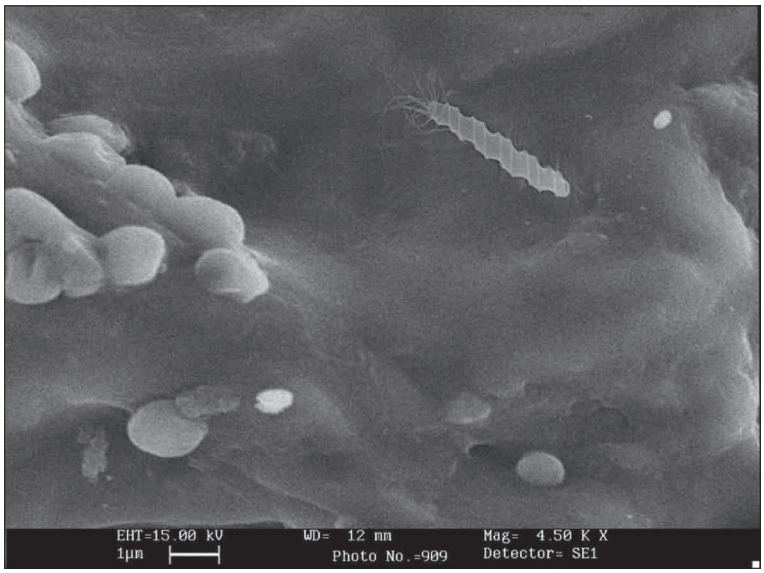


Fig 5. Electron microscopy scanning of *Helicobacter felis* in canine gastric mucosa

Şekil 5. Köpek mide mukozasında *Helicobacter felis*'in elektron mikroskobu taraması

of human chronic gastritis and gastric malignancy, the exact role of gastric helicobacters in dogs has not been established yet [2,7,8]. Six species of helicobacters including *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. bilis*, *H. rappini* (*Flexispira rappini*) and *H. cynogastricus* were cultivated in dogs [2,4,8,25-27]. It is unknown whether these species are representative of all canine gastric helicobacters or not [14]. Furthermore, some studies showed new cultivable *Helicobacter* strains in canine stomach [19,20].

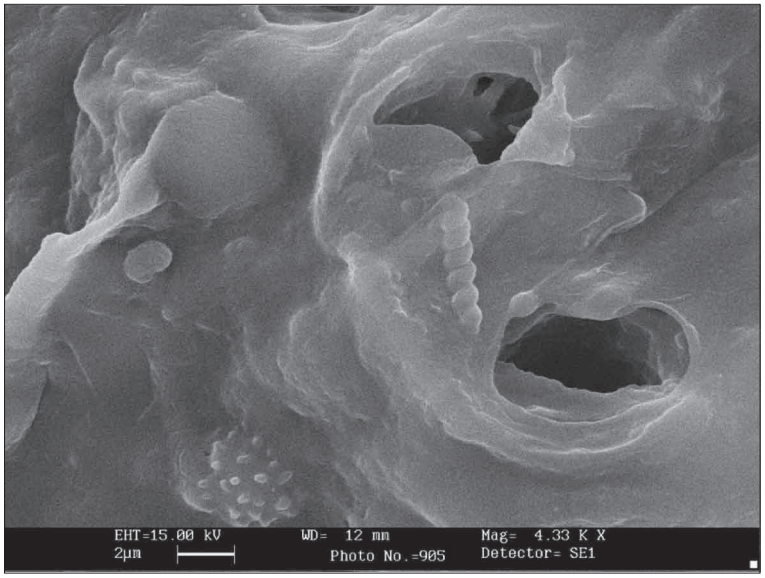
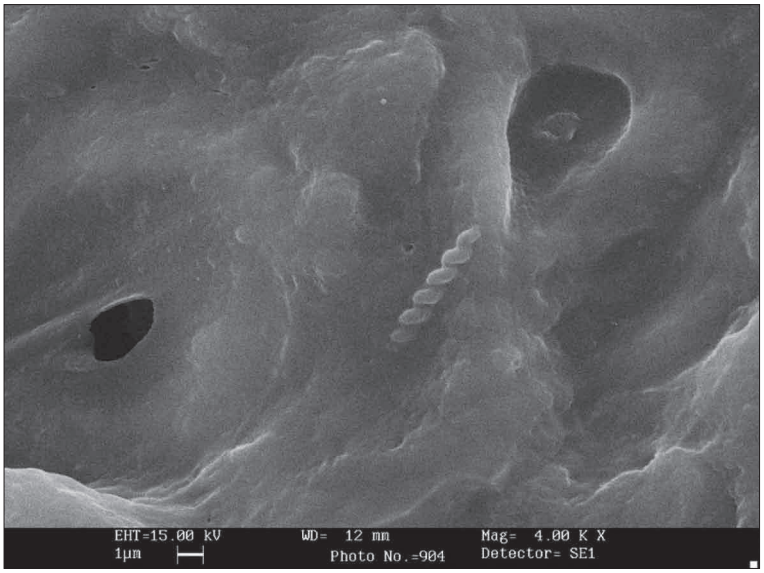


Fig 6. Electron microscopy scanning of candidatus *Helicobacter heilmannii* in canine gastric mucosa

Şekil 6. Köpek mide mukozasında candidatus *Helicobacter heilmannii*'in elektron mikroskobu taraması

Fig 7. Electron microscopy scanning of *Helicobacter bizzozeronii* in canine gastric mucosa

Şekil 7. Köpek mide mukozasında *Helicobacter bizzozeronii*'nin elektron mikroskobu taraması



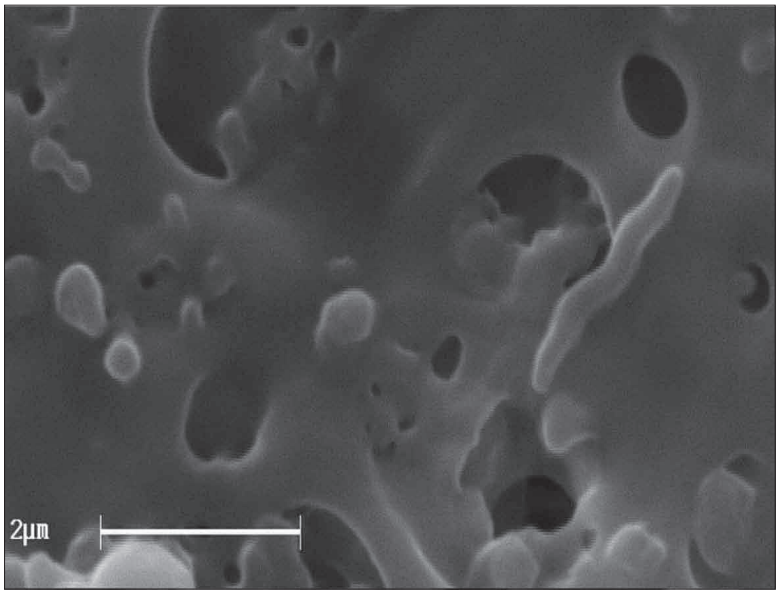


Fig 8. Electron microscopy scanning of Atypical *Helicobacter* in canine gastric mucosa

Şekil 8. Köpek mide mukozasında atipik *Helicobacter*'in elektron mikroskobu taraması

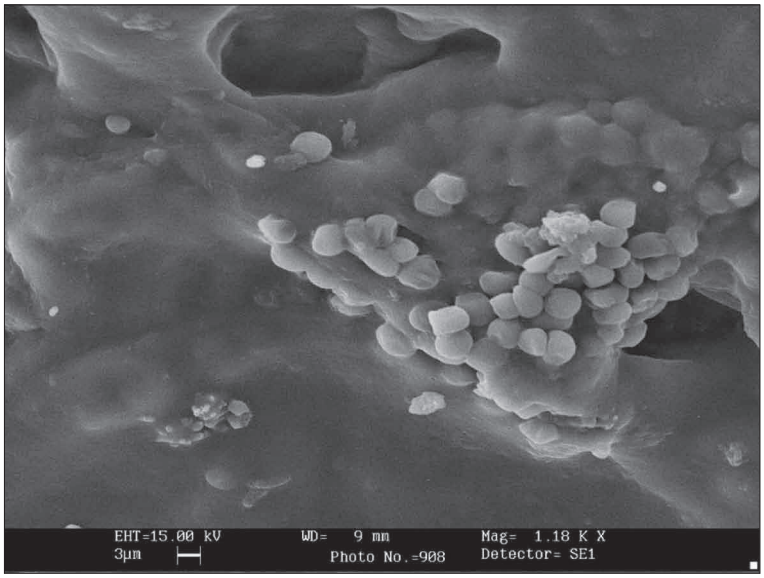


Fig 9. Electron microscopy scanning of canine gastric mucosa and infiltrations of immune cells

Şekil 9. Köpek mide mukozası ve bağışıklık hücreleri sızmasının elektron mikroskobu taraması

In spite of possibility to culture of canine gastric helicobacters, some of GHLO's are not cultivable [8,18]. The rates of success cultures of these organisms are quite low except *H. pylori* [18,21]. The culture of these organisms is important for better differential diagnosis of *Helicobacter* strains, phenotype description and whole cell protein profiles [27]. Additionally, culture of these organisms is essential for assessments of their sensitivities against different antibiotics; especially in recurrent infections. Because of difficulty in culture of these bacteria, PCR and DNA sequencing are being used for detection of various helicobacters [21].

SEM and Transmission Electron Microscopy (TEM) are useful methods for structural analysis of different gastric helicobacters. TEM can reveal better information about germs' ultra- structures; but it needs to consume more time and progressive method. There are a few researchers which are concerned with the morphological study of *Helicobacter* spp. in gastro- enteric specimens [21]. In addition, some studies showed the presence of various cultivable canine gastric helicobacters [19,25-28]. In some studies, obvious differences reported between dissimilar *Helicobacter* organisms [21,27]; but some researchers believe the differences are indistinguishable [26]. The studies indicated a need for accurate investigation about canine gastric helicobacters.

Cytological examination is a fast, cheap and available method only for identification of *Helicobacter* presences in gastric samples. The motility of helicobacters at the fixation time and similarity of *Helicobacter* strains (especially canine GHLO's) caused a hard and accurate diagnosis of these bacteria simply by light microscopy. Therefore, accurate detection of different *Helicobacter* strains can be achieved by combination of impression smears with other diagnostic methods.

Our SEM investigation on fresh gastric specimens showed the presence of four different strains of GHLO's which were distinguishable because of their apparent morphological differences. The ultra structural morphology of these bacteria indicated that *H. felis* was quite distinguishable because of its unique morphology and fibrils *(Fig. 5)*. Candidatus *H. heilmanii* and *H. bizzozeronii* are quite similar. These bacteria are distinguishable because of tight, bluntly and fatty helical structure. Morphologically, *H. bizzozeronii* has more space between its helices *(Fig. 7)*; meanwhile candidatus *H. heilmanii* is more compressed with closed helices *(Fig. 6)*. Based on our results, morphological comparison of different *Helicobacter* species can be an indicator for an accurate detection of different types of gastric helicobacters. It seems that SEM is a fast, available and cheap method for determination of these organisms in the fresh gastric samples.

Difficulties in isolation of some helicobacters can be a reason of not recall for culturing of all gastric helicobacters. Therefore, there is a need for diagnosis of all canine GHLOs' and also their effects and pathogenesis in canine and feline gastric mucosa [25]. It is recommended that large-scale studies with fast and simple methods for recognition and confirmation that would differentiate between dissimilar species of helicobacters (especially in fresh samples of naturally infected animals) are recommended.

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