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Detection of Campylobacter spp. in Healthy Pet Rabbits and Rodents Using Multiplex PCR

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Running title: Campylobacter detection in healthy pets

Abstract

Background: Campylobacter is an important and common cause of human gastroenteritis. These species can cause diarrhea, hematochezia, meningitis, septicemia, and human Guillain-Barre syndrome.

Objectives: This study determined the prevalence of Campylobacter and its species in healthy pet rabbits, guinea pigs, hamsters, and squirrels referred to the Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran**.** Food-producing animals are the most important source of infection in humans. While rabbit and guinea pig meat and fur consumption have increased in many countries, little is known about their role as a source of infection**.** The true incidence of Campylobacter may be underestimated.

Methods: Fecal samples from 92 rabbits, 4 guinea pigs, 2 hamsters, and 2 squirrels were acquired and assessed for the presence of campylobacter species by culture and Multiplex Polymerase Chain Reaction (PCR). Statistical analysis was done using SPSS version 26.0 software. Chi-square and Fisher's exact tests were used to analyze qualitative data

Results: Five samples from rabbits, one sample from hamsters, and one sample of squirrels were positive for campylobacter spp. no campylobacter spp. were detected in guinea pigs. All the species were *C. jejuni*.no campylobacter was isolated in culture.

Conclusion: According to the results of this study, Campylobacter spp. was detected in healthy rabbits, hamsters, and squirrels. In addition, age, gender, and sexual status did not have a significant effect on Campylobacter infection. Furthermore, rabbits and rodents kept as pets should be considered an important source of zoonotic pathogens for humans. They can be reservoirs of Campylobacter spp. and can infect people and other animals by shedding these organisms in their stools.

Keywords: guinea pig; hamster; multiplex PCR; rabbit; squirrel

Introduction

Campylobacter has been described as the most prevalent bacterial food-borne disease that causes gastroenteritis in humans around the world (Guo YT *et al*., 2023; Aboi *et al*., 2019; Kaakoush *et al*., 2015). The genus Campylobacter includes many species, and in most cases, *C.jejuni* and *C. coli* are common pathogens. Most human infections occur due to ingesting contaminated poultry products and direct contact with infected animals (Berthenet *et al*. 2019), unpasteurized milk, or contaminated water (Kenyon *et al*.,2016; Steinhauserova *et al*., 2000; Koene *et al*., 2009; Chaban *et al*., 2010; Parsons *et al*., 2010; Ansarifar *et al*., 2023).

These species can cause diarrhea, hematochezia, meningitis, septicemia, and human Guillain-Barre syndrome (Finsterer *et al*., 2022; Brooks *et al*., 2017). Food-producing animals may be the most significant source of infection for humans. Although the consumption of rabbit and guinea

pig meat and fur has increased in many countries, their role as a source of infection remains poorly understood (Tawab *et al*., 2017).

Campylobacter *jejuni* is one of the significant causes of gastroenteritis around the world. Additionally, *C. jejuni* contamination may lead to autoimmune conditions such as Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome. Many Campylobacter species are considered pathogens in humans and animals (Man SM. 2011). In human beings, Campylobacter species have been associated with some gastrointestinal situations, which include inflammatory bowel diseases (IBD), Barrett's esophagus, and colorectal cancers (Castaño-Rodríguez *et al*., 2017; Poosari *et al*., 2021). They have also been stated to be worried about extra gastrointestinal manifestations, including bacteremia, lung infections, mind abscesses, meningitis, and reactive arthritis, in individual cases and small cohorts of sufferer.

Nowadays, keeping rabbits and rodents as pets is a growing trend worldwide. In Iran, in particular, housing animals such as rabbits, guinea pigs, and hamsters as pets has increased due to cultural and religious issues. Meanwhile, children who have the most contact with these animals at home may be exposed to infections. It seems that these animals can be clinically asymptomatic and only be the agent of transmission to humans

Studies have demonstrated a correlation between Campylobacter infection in dogs and their owners by shedding bacteria in their stools (Damborg *et al*., 2004; Holmberg *et al*. 2015; Giacomelli *et al*., 2015; Karama *et al*.,2019). Reservoir dogs can infect their owners and other animals (Parsons et al., 2010; Fox, 2012; Iannino *et al*., 2022)

So far, there have been few descriptions of Campylobacter spp. isolation from rabbits, specially, C. *jejuni* (Prescott & Bruin-Mosch, 1981) and a Campylobacter-like organism (Revez *et al*., 2008)

Various methods, including direct microscopy, culture, serology, PCR, etc., have been developed to detect Campylobacter infection (Fox, 2012).

It seems that the true incidence of Campylobacter may be underestimated due to the limitations of routine culture methods. Molecular methods based on PCR can be an alternative to culture to detect Campylobacter.

This study was carried out to define more clearly the role of the mentioned animals as potential reservoirs and possible sources of infection in humans. The main role of this work was to determine Campylobacter's occurrence among healthy rabbits, guinea pigs, and hamsters.

Due to the authors' *knowledge*, this is the *first study* to estimate the frequency of campylobacter infection in healthy rabbits, guinea pigs, and *hamsters* in Iran.

Material and methods

Sample collection

From September 2022 to March 2023, the feces of 92 apparently healthy rabbits, four guinea pigs, two hamsters, and two squirrels were referred to the Small Animal Teaching Hospital of Faculty of Veterinary Medicine, University of Tehran were examined. All animals were apparently healthy and had a standard diet. Fresh feces were collected from each animal and

fecal samples were maintained in microtubes containing 1 ml of normal saline. Just one pet from each household was included in this study. Cases were selected between the ones with standard diets such as Hay and washed fresh green vegetables. Statistical analysis was done by using SPSS version 26 software. Chi-square and Fisher's exact tests were conducted to analyze qualitative data. $p \leq 0.05$ will be considered significant.

Culture

In this study, each sample was mixed for 1 minute using a rotator in order to obtain a homogeneous suspension. All samples were examined by direct culture less than 1 hour after sampling. Charcoal Cefoperozone Deoxycholate Agar (CCDA) the media were designated. These media were stored in the dark at 4°C in sealed bags for less than two weeks before inoculation. Samples were inoculated by streaking 10µl of each suspension directly onto media. After inoculation, all plates were incubated at 41°C under a microaerobic atmosphere for approximately 48 hours. Plates were checked for gray, flat, irregular, and spreading colonies typical of Campylobacter. From each sample 3 colonies showing the same morphotype referable to Gram-negative curved or spiral rod bacterial were cloned. All the selected colonies were subjected to genus-specific PCR for campylobacter. Finally, samples were stored at -20°C until DNA extraction.

DNA extraction

Extraction of bacterial DNA was performed using a commercial stool DNA extraction kit (SinaPure DNA**,** SinaClon, Iran) according to the manufacturer's instructions.

Stool samples from 92 rabbits, four guinea pigs, two hamsters, and two squirrels were collected and analyzed for the presence of *campylobacter* species. Thus, DNA extraction from stools was done, and specimens were analyzed by multiplex PCR for identification of the campylobacter genus. The first set of primers used in this study was specific for the genus, while the second pair was specific for campylobacter species. Three different samples subjected to sequencing analysis best matched with *C. jejuni* from the GenBank database and confirmed the precision of multiplex PCR assay.

Multiplex PCR

A multiplex PCR technique was used to detect Campylobacter spp. and identify the most common species (Yamazaki-Matsune *et al*., 2007; De Boer *et al*., 2015). For species identification, PCR was performed initially with the universal Campylobacter 16s rRNA((Linton et al., 1996)and Flagellin(Oyofo et al. 1992) All PCR positive samples were then subjected to a second PCR for differentiation of C. jejuni from C. coli (Table 1).Primers used in this study for PCR are listed in Table 1. Multiplex PCR was carried out in a final volume of 25 µl containing 2.5 µl of 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl, SinaClon, Iran), 0.5 µl of dNTP mix (10 mM, SinaClon, Iran), 2mM MgCl2 (50 mM, SinaClon, Iran), 2 µl template DNA, 0.2 µM of primers C412F, C1228R, C-1, C-3, CC18F, CC519R, CU61F, CU146R, MG3F, CF359R, CLF, CLR, HYO1F and HYOFET23SR; and 1U of SinnaGen Smar Taq DNA polymerase (SinaClon, Iran). Amplification of DNA was done in a Techne TC-512 Thermal Statistical analysis was done using SPSS version 26.0 software. Chi-square and Fisher's exact tests were used to analyze qualitative data Cycler (Techne TC-512, England). The PCR conditions were 95°C for 15 min

followed by 25 cycles of 95°C for 0.5 min, 58°C for 1.5 min 72°C for 1 min, and finally 72°C for 7 min. The PCR products were analyzed by electrophoresis in 2% (w/v) agarose gel in TBE buffer (0.5X), then stained with safe stain, and visualized under UV light.

Results and Statistical analysis

Five out of 92 (5.4%) samples were positive for *Campylobacter* spp. in rabbits, while no positive cases were detected in 4 samples taken from guinea pigs. However, and 1 out of 2 samples of hamsters (50%) and 1 out of 2 samples of squirrels (50%) were positive.

Overall, no sample was positive by culture.

Eventually, age, gender and lifestyle had no significant effect on Campylobacter infection. (A *p*-value less than 0.05 was statistically considered significant.)

Sequencing analysis was performed on two samples for checking *mapA* gene and *flagellin A* gene. (GenBank accession numbers: OR891686, OR891687)

Table 1. Primers used for identification genus and species of Campylobacter by polymerase chain and multiplex polymerase chain reaction.

Target specificity	primer name	sequence (5'-3')	PCR product size	reference

16S genus	CampF Wangroongsarb et al, 2011	AGTCTTGGCAGTAATGCACCTAACG	408
rRNA	CampR	ATATGCCATTGTAGCACGTGTGTCG	
Flagellin Oyofo et al.1992		ATGGGATTTCGTATTAAC GAACTTGAACCGATTTG	
MapA *C. jejuni*	MDmapA1 Ashrafi et al.2015	CTATTTTATTTTTGAGTGCTTGTG	589
	MDmapA2	GCTTTATTTGCCATTTGTTTTATTA	

Table 2: Frequency of *Campylobacter* species in rabbits, guinea pigs, hamsters and squirrels

Sample	No of samples	campylobacter spp. positive	*C.*jujeni	C.coli

Rabbit	**92**	**5**	**5**	**0**
Guinea pig	**4**	**0**	**0**	**0**
Hamster	**2**	**1**	**1**	**0**
Squirrel	**2**	**1**	**1**	**0**

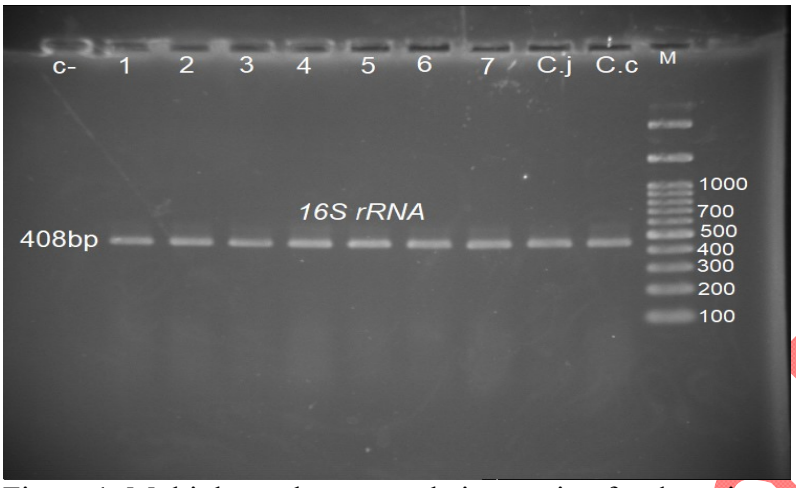

Figure 1. Multiplex polymerase chain reaction for detection of Campylobacter: C- :control negative, 1-7 samples, C.J: campylobacter jejuni, C.c: campylobacter coli, M: Gene Ruller, 408 bp: fragment represents Campylobacter genus

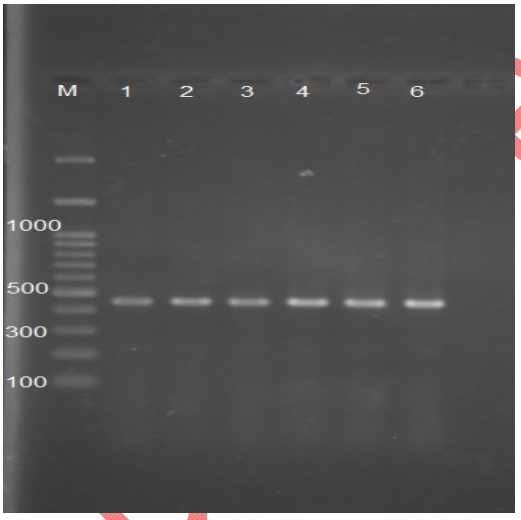

Figure 2: Multiplex polymerase chain reaction for detection of Campylobacter genus; M; Gene Ruller, flagellin :408 bp: fragment represents Campylobacter genus

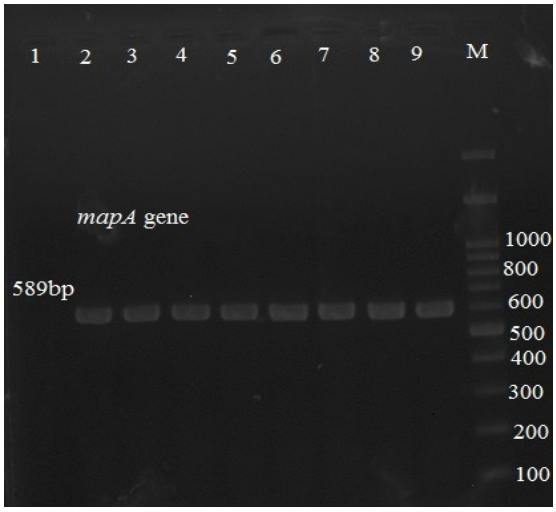

Figure 3: 589 bp: fragment corresponds to C. jejuni species; 1:control negative; 2-8 samples; 9:control positive, M: Gene Ruller

Discussion

Campylobacter is one of the main causes of bacterial gastroenteritis in humans (Aboi *et al*., 2019; Kaakoush *et al*., 2015; Yamazaki-Matsune *et al*., 2007; Chaban *et al*., 2010; Parsons *et al*., 2010). Based on the importance of this infection in humans in developed countries, this paper focused on the occurrence of Campylobacter spp. in healthy rabbits, guinea pigs, hamsters, and

squirrels referred to the Small Animal Teaching Hospital of Faculty of Veterinary Medicine, University of Tehran. Until now, few data are available on the role of rabbit meat as a potential source of Campylobacter spp. in humans (Piccirillo et al., 2011) and also the role of these animals as a reservoir.A few studies have been carried out in some countries but not in Iran. Therefore, more information on the epidemiology of this bacterium is important.

This zoonotic disease has gained importance due to there has been increasing interest in these pets owing to many cultural and religious reasons in Iran. Considering the fact that some of these animals are not kept in cages and are moving freely in the home and they are in direct contact with humans; the risk of human infection, especially children, increases. Some researches have shown that the owners of other companion animals such as dogs and cats are at increased risk of campylobacter transmission (Hald & Madsen, 1997; Steinhauserova *et al*., 2000; Koene *et al*., 2009; Chaban *et al*., 2010; Parsons *et al*., 2010).

Rabbits also can be bred for the production of fur or meat. Their meat can be considered as a source of human campylobacteriosis (Tawab *et al*., 2017).

Based on the results of this work, the overall prevalence of Campylobacter spp. was 7% (7 out of 100 samples). Campylobacter isolates were 5 out of 92 (5.4%) in rabbits, 1 out of 2 in hamsters (50%), and one out of two squirrels (50%), and no campylobacter was isolated from guinea pig samples. All the isolated campylobacters were *C. jejuni*. Similar results were shown that *C. jejuni* was the most prevalent species 26 (11.30%) in samples taken from rabbits in Tawab's study in Egypt (Tawab *et al.*, 2017).

The age of studied cases was between 1 month to 8 years.

In another study, Prescott and Bruin-Mosch (1981) identified Campylobacter in healthy rabbits and reported a carriage rate for *C. jejuni* of 11.3% (14 positives out of 124 samples). More recently, Kohler *et al*. (2008) reported a carriage rate of 0.04% at a slaughterhouse in Switzerland. Comparable results were also obtained in studies in Spain (Rodriguez-Calleja *et al*., 2004; 2006) and in Italy (Cerrone *et al.,* 2004). However, there have been several reports on the occurrence of Campylobacter spp. in rabbits at farm level in Italy with somewhat contradictory results. In contrast to this research Piccirillo *et al.* (2011) and Marin *et al.* (2003) suggested that this pathogen appeared to be absent in rabbits, while Revez *et al.* (2008) reported a carriage rate for Campylobacter spp. of 92.3% (36 positives out of 39 samples). It seems that differences between these data in various researches may be related to variation between evaluated population, geographical and environmental conditions.

A study conducted in Iran by Rahimi et al. (2011) revealed that one out of fifteen squirrel samples tested positive for *C. jejuni*. Similarly, in Southern Italy, 8.3% of 60 samples were positive for *C. jejuni* through a PCR assay (Dipineto *et al.* 2009). In agreement with the results of this study, both mentioned studies just identified the strain as *C. jejuni*. Needless to say, the role of this rodent in the epidemiology of campylobacter is not yet fully understood, and further research is needed. In addition, Gebhart *et al*. reported that high level of Campylobacter was isolated from 54(75%) out of 72 healthy hamsters in one study in the U.S. (Gebhart *et al*., 1989). Nagamine and colleagues revealed the first report of co-infection of Helicobacter spp. and Campylobacter sp. in asymptomatic Siberian hamsters in 2015. However, in Marshall's study,

rats took second place for campylobacter infection rate; further research is required to ascertain the significance of hamsters in the epidemiology of campylobacter.

In contrast to the results of current work, in one research in 2016 done by Graham, high levels of Campylobacter *Spp*. were identified in guinea pigs raised for food in Ecuador. (Graham *et al*., 2016). In favor of this research, in Marshall's study (1989), the prevalence of campylobacter was 7%; this data shows that the prevalence of campylobacter in guinea pigs can be low or undetectable.

Finally, it can be said that this study found no correlation between age, gender, or sexual status and bacterial shedding in any of these animals.

Conclusions and suggestion

According to the results of this study, it was determined that Campylobacter spp. shedding in rabbits, hamsters, and squirrels. Campylobacter is an important cause of enteric disease in humans, and these companion animals can serve as the source of infection. The fact that a few hundred bacteria can lead to clinical disease in humans shows the importance of this issue. So, veterinarians should warn pet owners regarding the zoonotic potential of this organism, especially in children.

Considering that the vaccine is unavailable for Campylobacter testing programs for the diagnosis in first visit can be effective preventative way to prevent the transmission of infection to humans.

It is recommended to conduct this investigation on a broader range of species and a larger number of animals and also sick animals with diarrhea in order to obtain more precise

conclusion.

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Figure 1. Polymerase chain reaction for detection of *Campylobacters genus.* C- :(Control Negative), 1-7: (Positive Samples), C.j and C.c: (Control Positive for *Campylobacters jejuni* and *coli*) M: Gene Ruler, 408 bp.

Figure 2: Polymerase chain reaction for detection of *Campylobacters Jejuni.* 1 :(Control Negative), 2-8: (Positive Samples), 9: (Control Positive for *Campylobacters jejuni*) M: Gene Ruler, 589 bp

Figure 3: Amplification of Flagellin gene (458 bp) of *Campylobacter jejuni*

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چکیده

زمینه مطالعه: امروزه در ایران به دلایل مختلف فرهنگی، اعتقادی و همچنین سهولت نگهداری از پستانداران کوچک و جوندگان مانند خرگوش، خوکچه هندی و همستر در فضای سکونت کنونی شاهد افزایش علاقه به نگهداری از این گونه ها هستیم.با توجه به انکه این حیوانات بعضاً به غلط داخل قفس نگهداری نمی شوند و در فضای منزل آزادانه در حرکت هستند و در تماس مستقیم با انسان و به ویژه کودکان هستند ریسک ابتلای انسان از طریق این گونه ها می تواند افزایش یابد.

هدف: هدف از این مطالعه بررسی آلودگی مدفوعی رگوش ها و جوندگان به ظاهر سالم ارجاعی به بیمارستان دامپزشکی دانشگاه تهران می باشد.

روش کار: از مهر ماه 1401 طی شش ماه تعداد صد نمونه ی مدفوع از خرگوش ها،خوکچه ها، همسترها و نجاب های بالغ که در معاینه بالینی سالم بودند گرفته شد و حضور گونه های کمپیلوباکتر به وسیله ی واکنش زنجیره ای پلیمراز مورد سنجش قرار گرفت.ژن های MapA،16S rRNA به ترتیب برای تشخیص جنس و گونه کمپیلوباکتر ژوژنی انتخاب شدند.

نتایج: از میان صد نمونه مورد بررسی در ایم مطالعه، هفت مورد(7٪) واجد کمپیلوباکتر تشخیص داده شدند که از این تعداد پنج نمونه مثبت متعلق به خرگوش، یک نمونه متعلق به همستر و یک نمونه متعلق به سنجاب بود. هیچ کمیلوباکتری از نمونه های مربوط به خوکچه جداسازی نشد. همه ی کمپیلوباکتر های جدا شده کمپیلوباکتر ژوژنی بودند.

نتیجه گیری نهایی: نتایج این مطالعه نشان می دهد که کمپیلوباکتر می تواند در مدفوع خرگوش ها، همستر ها و سنجاب هایی که فاقد علائم بالینی مشخص هستند وجود داشته باشد و می تواند به عنوان منبع آلودگی موجب ابتلای انسان شود.

کلید واژه: جوندگان ،خرگوش ،کمپیلوباکتر ،واکنش زنجیره ای پلیمراز