Original Article

Detection of *Campylobacter* spp. in Healthy Pet Rabbits and Rodents Using Multiplex Polymerase Chain Reaction

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ABSTRACT

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

Objectives: This study aims to determine the prevalence of *Campylobacter* spp. in healthy pet rabbits, guinea pigs, hamsters and squirrels referred to Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran. Food-producing animals are vital sources 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. Therefore, the true incidence of *Campylobacter* infections may be underestimated.

Methods: Fecal samples from 92 rabbits, four guinea pigs, two hamsters and two squirrels were acquired and assessed for *Campylobacter* species by culture and multiplex polymerase chain reaction (PCR). Statistical analysis was performed using the SPSS software, version 26. 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 from squirrels were positive for *Campylobacter* spp. No *Campylobacter* spp. were detected in guinea pigs. All the species were *Campylobacter jejuni*. No *Campylobacter* was isolated in culture.

Conclusion: According to the results of this study, *Campylobacter* spp. were detected in healthy rabbits, hamsters, and squirrels. In addition, age, gender and sexual status did not significantly affect *Campylobacter* infection. Furthermore, rabbits and rodents kept as pets should be considered crucial sources of human zoonotic pathogens. They can be reservoirs of *Campylobacter* spp. and infect people and other animals by shedding these organisms in their stools.

Keywords: Guinea pig, Hamster, Multiplex polymerase chain reaction (PCR), Rabbit, Squirrel

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Introduction

Campylobacter is the most prevalent bacterial food-borne disease that causes gastroenteritis in humans worldwide (Guo et al., 2023; Aboi et al., 2019; Kaakoush et al., 2015). The genus *Campylobacter* includes many species; in most cases, *Campylobacter jejuni* and *Campylobacter coli* are common pathogens. Most human infections occur due to the ingestion of 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 Guillain-Barre syndrome in humans (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 sources of infection remains poorly understood (Tawab et al., 2017).

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

Currently, maintaining rabbits and rodents as pets is a growing trend worldwide. In Iran, housing animals, such as rabbits, guinea pigs, and hamsters as pets has increased due to cultual and religious issues. Children who have the most contact with these animals at home may be exposed to infections. These animals can be clinically asymptomatic and only be the agent of transmission to humans (Azami et al.,2024).

Studies have demonstrated a correlation between *Campylobacter* infections 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 and polymerase chain reaction (PCR), have been developed to detect *Campylobacter* infections (Fox, 2012).

Due to the limitations of routine culture methods, the true incidence of *Campylobacter* may be underestimated. Molecular methods based on PCR can be alternatives to culture to detect *Campylobacter* spp.

This study was conducted to clearly define the role of these animals as potential reservoirs and possible sources of infection in humans. The main aim of this study was to determine *Campylobacter*'s occurrence in healthy rabbits, guinea pigs and hamsters.

This is the first study to estimate the frequency of *Campylobacter* infections in healthy rabbits, guinea pigs and hamsters in Iran.

Material and Methods

Sample collection

From September 2022 to March 2023, the feces of 92 healthy rabbits, four guinea pigs, two hamsters, and two squirrels were referred to the Small Animal Teaching Hospital of the Faculty of Veterinary Medicine, University of Tehran. All animals were healthy and fed a standard diet. Fresh feces were collected from each animal, and fecal samples were maintained in microtubes containing 1 mL of normal saline. Only one pet from each household was included in this study. Cases were selected between those with standard diets, such as Hay, and those with washed fresh green vegetables. Statistical analysis was performed using SPSS software, version 26. Chi-square and Fisher's exact tests were used to analyze the qualitative data. $P \leq 0.05$ will be considered significant.

Culture

Each sample was mixed for one minute using a rotator to obtain a homogeneous suspension. All samples were examined by direct culture less than one hour after sampling. Charcoal Cefoperozone Deoxycholate Agar media was 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 10 µL of each suspension directly onto the media. After inoculation, all the plates were incubated at 41 ˚C in a microaerobic atmosphere for approximately 48 hours. Plates were checked for gray, flat, irregular and spreading colonies typical of *Campylobacter*. Three colonies showing the same morphotype from each sample, referable to gram-negative curved or spiral-rod bacterial, were cloned. All the selected colonies were subjected to genus-specific PCR for *Campylobacter* spp. Finally, the samples were stored at -20 ˚C until DNA extraction.

DNA extraction

Bacterial DNA was extracted 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 stool samples was performed, and the specimens were analyzed to identify multiplex PCR the *Campylobacter* genus. This study's first set of primers was specific to the genus, while the second pair was specific for *Campylobacter* species. Three samples subjected to sequencing analysis best matched *C. jejuni* from the GenBank database and confirmed the precision of the multiplex PCR assay.

Multiplex PCR

Multiplex PCR 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 initially performed with the universal *Campylobacter* 16s ribosomal ribonucleic acid (rRNA) (Linton et al., 1996) and flagellin (Oyofo et al., 1992). All PCR-positive samples were then subjected to a second PCR to differentiate *C. jejuni* from *C. coli* (Table 1). Table 1 lists primers used in this study. Multiplex PCR was performed 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 deoxyribonucleotide triphosphate (dNTP) mix (10 mM, SinaClon, Iran), 2 mM magnesium chloride ($MgCl_2$) (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). DNA amplification was performed using Techne TC-512. Thermal statistical analysis was performed using SPSS software, version 26. Chi-square and Fisher's exact tests were used to analyze the qualitative data cycler (Techne TC-512, England). The PCR conditions were 95 ˚C for 15 minutes followed by 25 cycles of 95 ˚C for 0.5 minutes, 58 ˚C for 1.5 minutes 72 ˚C for one minute, and finally 72 ˚C for 7 minutes. The PCR products were analyzed by electrophoresis in 2% (w/v) agarose gel in TBE buffer (0.5 X), stained with safe stain, and visualized under UV light.

Results

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

Overall, none of the samples tested positive in culture.

Eventually, age, sex and lifestyle had no significant effect on *Campylobacter* infection. (A $P<0.05$ was statistically considered significant).

Sequencing analysis was performed on two samples to check the *mapA* and *flagellin A* genes (GenBank accession numbers: OR891686, OR891687) (Figures 1, 2 and 3).

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 study focused on the occurrence of *Campylobacter* spp. in healthy rabbits, guinea pigs, hamsters, and squirrels referred to the Small Animal Teaching Hospital of the Faculty of Veterinary Medicine at the University of Tehran. To date, little data are available on the role of rabbit meat as a potential source of *Campylobacter* spp. in humans (Piccirillo et al., 2011) and the role of these animals as reservoirs, and few studies have been conducted in some countries, but not in Iran. Therefore, more information regarding the epidemiology of this bacterium is crucial.

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

Organism	Target	Primer Name	Sequence (5'-3')	PCR Product	Reference	Accession Number
Rattus rattus	*Hsd3b1*	F	CCCTGCTCTACTGGCTTGC	189 bp	Ji et al. (2021)	XM_032897634.1
		R	TCTGCTTGGCTTCCTCCC			
R. rattus	*GAPDH*	F	ATGACTCTACCCACGGCAAG	89 bp	Kunst et al. (2012)	NM_017008
		R	ATGACTCTACCCACGGCAAG			

Abbreviations: PCR: Polymerase chain reaction; rRNA: Ribosomal ribonucleic acid.

This zoonotic disease has gained importance due to there has been increasing interest in these pets for many cultural and religious reasons in Iran. Because some of these animals are not kept in cages, are moving freely in the home, and are in direct contact with humans, the risk of human infection, especially children, increases. Some studies have shown that owners of companion animals, such as dogs and cats, are at an 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 can also be bred for fur or meat production. Their meat can be considered a source of human campylobacteriosis (Tawab et al., 2017).

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

The age of the studied patients 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 of 124 samples). More recently, Kohler et al. (2008) reported a carriage rate of 0.04% in a slaughterhouse in Switzerland. Comparable results were also obtained in studies conducted in Spain (Rodriguez-Calleja et al., 2004; Rodriguez-Calleja et al., 2006) and Italy (Cerrone et al., 2004). However, there have been several reports on *Campylobacter* spp. in rabbits at the farm level in Italy, with contradictory results. In contrast to this research, Piccirillo et al. (2011) and Marin et al. (2016) suggested that this pathogen appeared absent in rabbits. At the same time, Revez et al. (2008) reported a carriage rate for *Campylobacter* spp. of 92.3% (36 positives of 39 samples). The differences between these data in various studies may be related to variations in the evaluated population, geographical, and environmental conditions.

A study conducted in Iran by Rahimi et al. (2011) revealed that one of fifteen squirrel samples tested positive for *C. jejuni*. Similarly, using a PCR assay, 8.3% of 60 samples in Southern Italy were positive for *C. jejuni* (Dipineto et al., 2009). Consistent with the results of this study, both mentioned studies identified the strain as *C. jejuni*. 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. (1989) reported that high levels of *Campylobacter* were isolated from 54(75%) of 72 healthy hamsters in a study in the

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

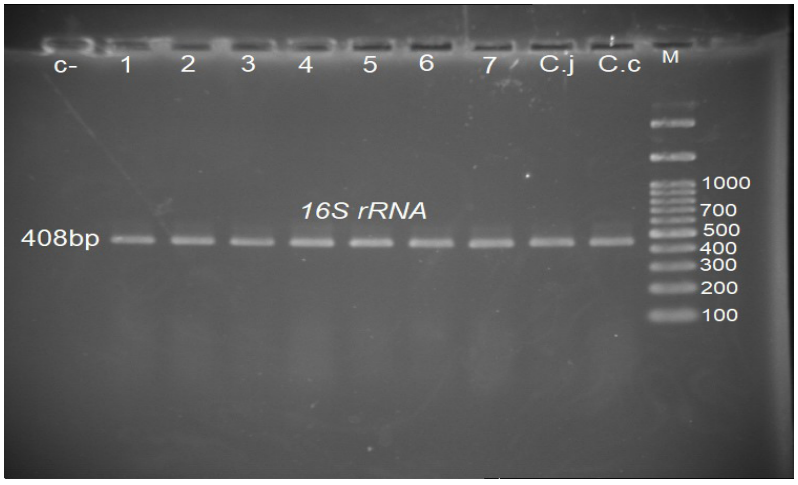

Figure 1. Multiplex PCR for detection of campylobacter: C-: Control negative, 1-7 samples, C.J: *Campylobacter jejuni*, C. c: *Campylobacter coli*, M: Gene Ruller, 408 bp: Fragment Representing *Campylobacter* genus

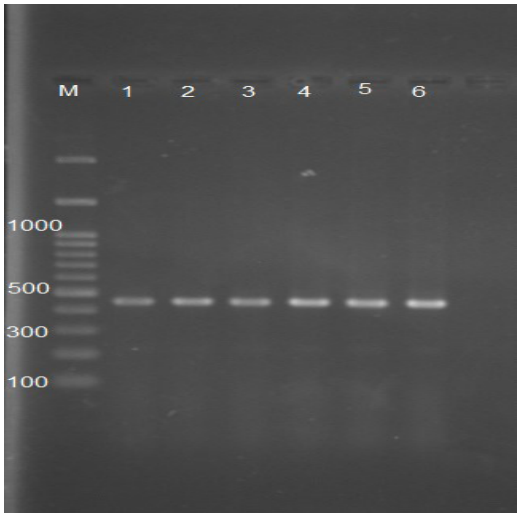

Figure 2. Multiplex PCR for detection of *Campylobacter* genus; M; Gene ruller, flagellin: 408 bp: Fragment representing *Campylobacter* genus

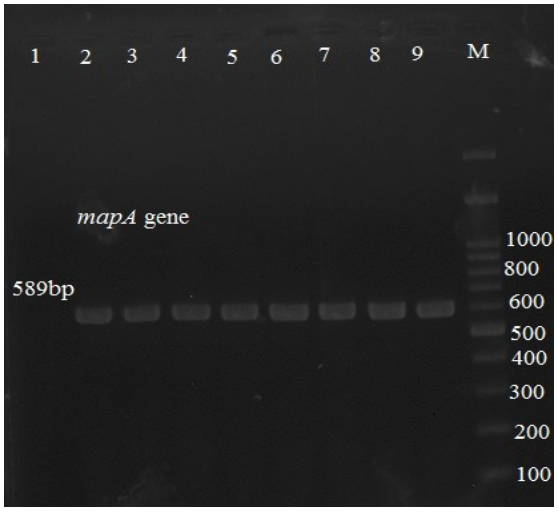

Figure 3. 589 bp: Fragment corresponding to *C. jejuni* species; 1: Control negative; 2-8 samples; 9: Control positive, M: Gene ruller

U.S. (Gebhart et al., 1989). Nagamine et al. (2015) revealed the first report of co-infection of *Helicobacter* spp. and *Campylobacter* sp. in asymptomatic Siberian hamsters in 2015. However, in Meanger and Marshall's study, rats took second place regarding *Campylobacter* infection rate; further research is required to ascertain the significance of hamsters in the epidemiology of *Campylobacter* (Meanger & Marshall, 1989).

In contrast to the current study's results, Graham conducted research in 2016 that identified high levels of *Campylobacter* spp. were identified in guinea pigs raised for food in Ecuador (Graham et al., 2016). In support of this research, Marshall's study found a prevalence of 7%; these data show that the prevalence of *Campylobacter* in guinea pigs can be low or undetectable.

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

Conclusion

According to the results of this study, *Campylobacter* spp. was shed in rabbits, hamsters, and squirrels. *Campylobacter* is a critical cause of enteric disease in humans, and these companion animals can be the source of infection. The fact that a few hundred bacteria can lead to clinical diseases in humans shows the importance of this issue. Therefore, veterinarians should warn pet owners of the zoonotic potential of this organism, especially in children.

Considering that *Campylobacter* testing programs cannot diagnose the disease in the first visit, the vaccine can be an effective preventative measure to prevent the transmission of infection to humans.

To obtain more precise conclusions, it is recommended that this study be conducted on a broader range of species and a larger number of animals, including sick animals with diarrhea.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

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Authors' contributions

Conceptualization, methodology, supervision, funding acquisition and resources: Amir Rostami and Bahar Nayeri Fasaei; Data collection: Tara Nazari, Amir Rostami, Bahar Nayeri Fasaei, and Iraj Ashrafi Tamaei; Data analysis: Hesameddin Akbarein and Iraj Ashrafi Tamaei; Investigation and writing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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