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Molecular Diagnosis of *Clostridium Piliforme*, the Causative Agent of Tyzzer's Disease in Mice and Rats*

Fare ve Ratlarda Tyzerr's Hastalığı'nın Etkeni *Clostridium piliforme*'nin Moleküler Teşhisi

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ABSTRACT

Tyzzer's disease, caused by *Clostridium piliforme* (*C. piliforme*), occurs in many species of mammals mainly in rodents, rabbits and foals is an acute, epizootic bacterial disease that can result in mortality. In our study, *C. piliforme* was investigated in feces samples from mice and rats in a laboratory experimental animals center by Polymerase Chain Reaction (PCR) technique. *C. piliforme* was detected in 44 (83.01%) of a total of 53 mouse feces of different ages. *C. piliforme* was detected in 40 (90.9%) of the feces of a total of 44 rats of different ages. *C. piliforme*, the causative agent of Tyzzer's disease, was detected at high rates in mice and rats examined by the classical PCR method. When evaluated in terms of gender, 28 (73.6%) of 40 positive mice for Tyzerr's disease were female and 16 (88.8%) were male; While 25 (96.1%) of the rats were female, 15 (83.3%) were male. When age was evaluated, the disease was found to be higher in mice at the ages of 4, 5, and 8, and in rats at the ages of 4, 5, 6, and 7. Due to the reliability of the analyses and the high risk of transmission to other animals in centers where laboratory animals are raised, necessary precautions must be taken to control Tyzzer's disease and prevent the disease.

Keywords: *Clostridium piliforme*, mice, PCR, rat, Tyzzer's disease.

ÖZ

Clostridium piliforme (*C. piliforme*)'nin neden olduğu Tyzzer hastalığı, başta kemirgenler, tavşanlar ve taylar olmak üzere birçok memeli türünde ortaya çıkan, ölümle sonuçlanabilen akut, epizootik bir bakteriyel hastalıktır. Çalışmamızda laboratuvar deney hayvanları merkezinde yetiştirilen fare ve ratlardan alınan dışkı örneklerinde, PCR ile *C. piliforme* araştırıldı. Farklı yaşlardaki toplam 53 fare dışkısının 44'ünde (%83,01) *C. piliforme* tespit edildi. Farklı yaşlardaki toplam 44 ratın dışkısının 40'ında (%90,9) *C. piliforme* tespit edildi. Tyzzer'z hastalığı'nın etkeni olan *C. piliforme*, klasik PCR yöntemiyle incelenen fare ve ratlarda yüksek oranda tespit edildi. Cinsiyet olarak değerlendirildiğinde, Tyzerr's Hastalığı 40 adet pozitif bulunan farelerin 28'i (%73.6) dişi, 16'sı (%88.8) erkek; ratların ise 25'i (%96.1) dişi iken, 15'i (%83.3) erkek idi. Yaş olarak değerlendirme yapıldığında hastalık farelerde 4, 5 ve 8. yaşlarda, ratlarda ise 4, 5, 6 ve 7. yaşlarda daha yüksek saptandı. Analizlerin güvenilirliği ve laboratuvar hayvanlarının yetiştirildiği merkezlerde, diğer hayvanlara bulaşma riskinin yüksek olması nedeniyle, Tyzzer's hastalığı'nın kontrol edilmesi ve hastalığın önlenmesi için gerekli önlemlerin alınması gerekmektedir.

Anahtar Kelimeler: *Clostridium piliforme*, fare, rat, PCR, Tyzzer's hastalığı.

Introduction

Tyzzer's Disease (TD), is an enterohepatic disease caused by *C. piliforme* first known as Bacillus piliformis. The disease can be seen generally in laboratory animals, some domestic animal species, and immunosuppressed individuals. *C. piliforme*, unlike other Clostridium species, is gram-negative and obligat intracellular spor-forming bacillus with flagella (Waner et al., 2005).

Infection occurs by the ingestion of bacterial spores or contaminated materials by the fecal-oral route (Waner et al., 2005). The spores of the bacteria present in infected feces can contaminate soil and feedstuff and can be ingested by a living host (Banes et al., 2013). After oral contamination of bacterial spores and/or vegetative forms, replication takes place in the ileocaecal- colic region. Depending on the immune system of each animal, the organisms are absorbed in animals and they reach to liver and sometimes to the heart via systemic circulation. Typical lesions may be hepatitis, enterocolitis, and myocarditis, however, hepatic lesions are more frequent (Young et al., 1995; Poonacha KB, 1997). The most common clinical symptoms are diarrhea, abdominal tension, anorexia, and weight loss and although young animals are affected more frequently, infected animals die without any symptoms in general (Sasseville et al., 2007).

TD, widespread in laboratory animals all over the world, has been reported from wild animals in America and Australia, from muskrats (Ondatra zibethicus) in many countries of North America, and also from cotton tail rabbits (Sylvilagus floridanus) (Ganoe et al., 2020; Ganaway et al., 1976). Because *C. piliforme* grows on routine media, isolation and diagnosis are more difficult than other diseases (Niepceron and Licois, 2010). Molecular methods are preferred in the diagnosis of *C. piliforme*, which has difficulties in isolation, and identification using conventional methods (Weisbroth et al., 1998).

Smith et al. (1996), found in a study that people with weak immunity were susceptible to *C. piliforme* infection, and that caregivers who had contact with laboratory animals (85% positivity) as well as personnel who did not have contact (40.5% positivity) were also serologically positive for *C. piliforme*. For this reason, it has been stated that *C. piliforme* poses o threat to personnel in close contact with laboratory animals and other personnel.

With a development conducted in China, LAMP-LFD analysis, *C. piliforme* was detected 5.08% of clean-grade animals and 9.96% of specific pathogen-free animals. It is reported that *C. piliforme* is detected at a higher rate in experimental animals used in schools than in companies and research institutes (Tao et al., 2024).

In this study, the incidence of *C. piliforme* infections causative agent of Tyzzer's disease in rats and mice in an experimental animal center was investigated by PCR.

Materials and Methods

Feces Samples

In the experiments, 44 Wistar albino rat feces and 53 mouse feces samples in the laboratory experimental animals center were examined. Feces samples were kept at -20℃ till the study started. No clinical findings were observed in animals. Information about the age information of the animals is available in years, but there is no information in weeks. This study was approved by the Veterinary Control Central Research Institute Directorate, Local Ethical Committee Decision date and no. 08.11.2022/43.

DNA Isolation

Feces samples to be examined were left to melt before the extraction procedure. After melting, 1X PBS was added to all samples. After keeping tubes at 95°C for 10 minutes, they were centrifuged at 10.000 rpm for 2 minutes. The supernatants were transferred into the sterile tubes and the amounts of DNA obtained were estimated by nano-drop spectrophotometry (Dallenne et al., 2010).

Polymerase Chain Reaction (PCR)

Extracted patient DNA samples were used as template DNA in PCR to find *C. piliforme*. Forward 5' ACCATTGACAGCCTACGTAA-3' and Reverse 5' GTCTCGCTTCACTTTGTTGTA-3' primer sequences were used. The reaction was composed of 25 µl volume and 2,5 µl PCR buffer (10X), 0,5 µl dNTP mix (10 mm), 1 µl Primer F (10 pmol), 1 µl Primer R (10 pmol), 0,4 µl Taq polymerase,

13,6 µl H2O and 3 µl DNA complements. Termal circle; at 94°C 5 minutes first denaturation, then at 98°C 10 seconds denaturation, at 55°C, 35 seconds primer binding and at 72°C 1 minutes chain extending all contained 40 cycles and at 72°C 5 minutes final extension. The analysis of all PCR findings was run on %1,5 agarose prepared from 5 µl Safe DNA gel at 100 Volt for 40 min, and after that examination under UV light was carried out. In electrophoresis, 270 bp amplified products were accepted as *C. piliforme* gene (Aboellail et al., 2013).

Statistical Analysis

The chi-square test was used as a statistical method. P values between the two genders were investigated. Data were analyzed with Statistical Package for the Social Sciences (SPSS) 25.0 software statistics program.

Results

Of the 44 rats feces samples from different ages, 40 (90.9%) samples were found to have *C. piliforme*. 20 female rats and 24 male rats positive results revealed that 80% of females and 88.8% of males had *C. piliforme* agent.

C. piliforme was detected in 44 (83%) of a total of 53 mice feces of different ages. When age was evaluated, the disease was found to be higher in mice at the ages of 4, 5, and 8, and in rats at the ages of 4, 5, 6, and 7. Of the mice found positive, 28 were female and 16 were male. *C. piliforme* was detected in 80% of females and 88.8% of males. *C. piliforme* PCR positivity is given in Table 1 and Table 2 according to the ages of mice and rats. Considering the ages of the mice examined, it was observed that the agent was detected mostly in young animals. According to the genders of mice and rats *C. piliforme* PCR positivity is given in Tablo 3.

Table 1. *C. piliforme* PCR positivity according to the ages of mice.

Mice Ages	<1	2	3	4	5	6	7	8	9	10	11
Positive (%)	4 (80)	1 (50)	4 (80)	9 (90)	6 (85.7)	4 (100)	3 (100)	6 (100)	1 (50)	2 (50)	0 (0)
Negative (%)	1 (20)	1 (50)	1 (20)	1 (10)	1 (14.2)	0 (0)	0 (0)	0 (0)	1 (50)	2 (50)	1 (100)
Total (53)	5	2	5	10	7	4	3	6	2	4	1
Total Positivity (%)	53/4 (7.5)	53/1 (1.8)	53/4 (7.5)	53/9 (16.9)	53/6 (11.3)	53/4 (7.5)	53/3 (5.6)	53/6 (11.3)	53/1 (1.8)	53/2 (3.7)	53/0 (0)

Table 2. *C. piliforme* PCR positivity according to the ages of rats.

Rat Ages	<1	2	3	4	5	6	7	8	9
Positive (%)	1 (100)	2 (100)	10 (90.9)	11 (100)	6 (75)	9 (100)	4 (80)	0 (0)	1 (100)
Negative (%)	0 (0)	0 (0)	1 (9.09)	0 (0)	2 (25)	0 (0)	1 (20)	0 (0)	0 (0)
Total (44)	1	2	11	11	8	9	5	0	1
Total Positivity (%)	44/1 (2.2)	44/2 (4.5)	44/10 (4.4)	44/11 (25)	44/6 (13.6)	44/9 (20.4)	44/4 (9.09)	44/0 (0)	44/1 (2.2)

Table 3. According to the genders of mice and rats *C. piliforme* PCR positivity.

Gender	Mice Number	Positive (%)	Negative (%)	Rat Number	Positive (%)	Negative (%)
Female	35	28 (73.6)	7 (20)	26	25 (96.1)	1 (3.8)
Male	18	16 (88.8)	2 (11.1)	18	15 (83.3)	3 (16.6)
Total	53	44 (83.01)	9 (16,9)	44	40 (90.9)	4 (9.09)

C. piliforme, the causative agent of Tyzzer's disease, was detected at a high rate in mice and rats examined by the classical PCR method. When the percentage of *C. piliforme* PCR positivity of mice and rats is compared according to their genders, the height is significantly observed in males.

C. piliforme PCR positivity is given in Table 1 according to the genders of mice and rats. *C. piliforme* PCR positivity of mice and according to the genders rats is given in graphical form in Figure 1.

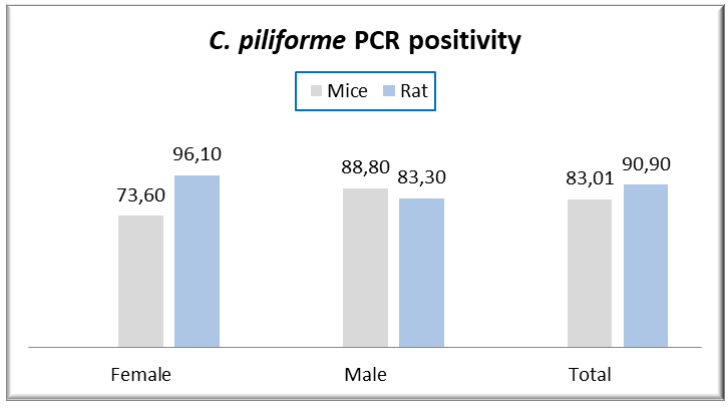

Figure 1. According to the genders of mice and rats *C. piliforme* PCR positivity in graphical form.

When statistically evaluated, no significant relationship was detected between gender-related positivity rates in mice and rats. The p values determined between the genders are given in Table 4.

Table 4. The p values between two genders.

Significance value	Mice-Rat Female	Mice-Rat Male
p	0.6943	0.6818
p	> 0.05	> 0.05

Discussion

C. piliforme is a pathogen that can cause mortality and is known as the cause of enteric, hepatic, and cardiac diseases in some animal species like as mouse, rats, and hamsters. Young animals are more susceptible to this pathogen and stress factors such as extreme crowding, transportation, and poor sanitation are predisposing factors (Ganaway et al., 1971). In 4 different studies conducted in America and Canada on muskrats in 1966, 1971, 1977-79, and 2019, the mortality rate was found to be 67% (Ganoe et al., 2020). In 2022, systemic Clostridium tarantellae infection, whose pathological findings resemble Tyzzer's disease, was detected in the wild Korean raccoon (Nyctereutes procyonoides koreensis) (Rho et al., 2022). Between 2000 and 2021, Tyzzer's disease was confirmed in at least one organ in 19 (51%) of 37 kittens under 6 months of age at the Veterinary Medical Teaching Hospital, University of California-Davis. Tyzzer's disease has been reported to be more common than thought in orphaned kittens and is associated with colitis and/or hepatitis (Fingerhood et al., 2023).

Naturally occurring Tyzzer's disease was detected in a calf in 1999 (Ikegami et al., 1999). A study conducted at the University of California Davis reported that PCR can be used for the early and specific diagnosis of Tyzzer's disease in foals (Borchers et al., 2006). It was reported that necrotic hepatitis was the hallmark of 25 horses diagnosed with Tyzzer's disease in the United States in 2022 (Garcia et al., 2022). In a study conducted in America in 2022, Tyzzer's disease was detected in 2 canine littermates and 1 gray fox and was confirmed by PCR (Jacobson et al., 2022).

Researchers from Iran have reported that they had found less than 10% positivity by ELISA for *C. piliforme* in 82 rats randomly chosen among laboratory animals (Fallahi, R., & Mansouri, M. A., 2017). Our study, found *C. piliforme*, the causative agent of Tyzzer's Disease, at high rates in rats and mice.

In a cat dying after upper respiratory tract infection and diarrhea symptoms in necropsy, *C. piliforme* was found and it was noted the third kitten in the same region dying with similar clinical findings with a similar course (Neto et al., 2015). In a study, *C. piliforme* positive 150 laboratory rabbits were used and immunofluorescence assay (IFA) and multiplexed fluorometric immunoassay (MFIA) techniques were used to assess the results and *C. piliforme* was found to be 73% and 34.7% in order (Pritt et al., 2010).

Conclusion

Without neglecting the subclinical nature of Tyzzer's disease that can be frequently determined, it is important to control laboratory animal breeding centers and that protective and preventive studies be carried out for the accuracy of the analyses and because of the high transmission risk to other animals. In particular, *C. piliforme* pathogen can cause mortality and is highly contagious, additional studies should be carried out in terms of method and efficiency.

Ethics Committee Approval: This study was approved by the Veterinary Control Central Research Institute Directorate, Local Ethical Committee Decision. (Date: 08.11.2022, number 2022/43).
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