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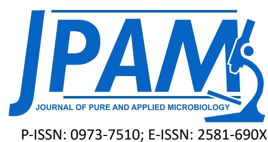

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Detection of *Clostridium piliforme* by Nested PCR in Faecal Samples of Rabbits

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Abstract

Clostridium piliforme **is an anaerobic, spore-forming, obligate intracellular bacterial pathogen that causes Tyzzer's disease in laboratory, wild, and domestic animals. *C. piliforme* has significant economic implications for laboratory and commercial animal facilities due to its impact on research integrity, increased veterinary and management costs, and the need for enhanced biosecurity measures. In the present study, 100 rabbit faecal samples were collected, and nested PCR was performed using *C. piliforme* specific 16S rRNA primers. *C. piliforme* was detected in all five rabbit farms, with an overall prevalence of 40%. A statistically significant difference in prevalence was observed among farms. Male faecal samples accounted for only 25% of the total, and although more females (45.33%) were found to be infected, this difference was not statistically significant. Rabbits older than 5 months (42.05%) were more frequently infected than those younger than 5 months (25%), though this was not statistically significant. Breed, weight, rearing system, and feeding type did not influence prevalence. Because 16S rRNA primers may yield nonspecific amplicons, their use in detecting *C. piliforme* in samples, particularly faeces, should be interpreted in conjunction with clinical signs or gross lesions. Identification of *C. piliforme* specific gene target primers is urgently needed for effective screening of susceptible populations.**

Keywords: *Clostridium piliforme*, Rabbit, Nested PCR, Molecular Detection

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INTRODUCTION

Rabbits, valued for their roles in agriculture, research, and as companion animals, face numerous health challenges. Among them is Tyzzer's disease, caused by *C. piliforme*. This elusive and often fatal pathogen poses a significant threat to the health and productivity of domestic rabbit populations. *C. piliforme* is an anaerobic, spore-forming, obligate intracellular bacterium that causes disease in laboratory, wild, and domestic animals.[1,2] It has also been reported in an HIV-positive human patient.[3]

C. piliforme primarily targets the liver and intestinal tissues of rabbits. The severity of Tyzzer's disease lies not only in its acute presentation but also in its ability to cause sudden, devastating outbreaks, leading to substantial economic losses and compromised animal welfare. The bacterium's environmental persistence further complicates disease management.

Culturing *C. piliforme* is difficult, as it does not grow on routine bacteriological media. It can, however, be propagated in embryonated hen's eggs or mammalian cell culture.[1] Diagnosis is therefore generally based on histopathological examination, immunological techniques, or PCR. Histopathology allows identification of the bacterium but is laborious and time-consuming. Immunological techniques are widely used for colony surveillance because they are relatively rapid and inexpensive. However, false-positive or false-negative results due to antigen cross-reactivity limit their utility for confirmatory diagnosis in field cases. Molecular diagnosis of *C. piliforme* is highly specific and comparatively cost-effective.[4-6] Niepceron and Licois[7] developed nested PCR (nPCR) with improved sensitivity and specificity.

Despite the economic and animal welfare consequences of Tyzzer's disease, knowledge of its prevalence, epidemiology, and risk factors remains incomplete. The present study therefore aimed to determine the prevalence, epidemiology, and associated risk factors of *C. piliforme* infection in rabbits in India.

MATERIALS AND METHODS

Sample collection

A total of 100 fresh faecal samples were collected from both organized farms and backyard rabbitries in two locations in Tamil Nadu and three in Uttar Pradesh. Data on age, sex, feeding pattern, and other characteristics were collected and are summarized in Table 1.

Fresh faecal samples were collected directly from the rectum or from freshly voided pellets. Samples were hygienically collected with blunt thumb forceps, placed in airtight plastic containers with proper labelling, transported to the laboratory on ice, and stored at -20 °C until processing.

DNA extraction

DNA extraction followed the method of Wang et al.[8] and Osmundson et al.[9] who used NaOH to extract DNA from plant and fungal tissues, respectively, for PCR templates. The protocol was modified as follows: 250 mg of faecal sample was homogenized in 1 ml of Milli-Q water and centrifuged at 3000 rpm for 5 min at 20 °C. The supernatant was collected and adjusted to 1.25 ml with Milli-Q water, then centrifuged at 8000 rpm for 3 min at 20 °C. The supernatant was discarded, 10 µl of 0.5 M NaOH was added, and the mixture was mixed thoroughly. Then, 490 µl of Milli-Q water was added, followed by centrifugation at

Table 1. Details of rabbit faecal samples collection

Place	No. of samples	Male	Female	Breed*	Average weight (kg)	Age group	Farming system
Chengalpattu	26	4	22	NZW and SC	2.49	4 months to 6.2 years	Organised
Chennai	20	8	12	SC	5.71	3 months to 3.1 years	Organised
Fatehgarh	14	5	9	NZW	2.25	7 months to 6.2 years	Backyard
Shamsabad	20	4	16	NZW	2.25	5 months to 6.2 years	Backyard
Mohammadabad	20	4	16	NZW	2.24	6 months to 6.6 years	Backyard

*NWZ - New Zealand White; SC - Soviet Chinchilla

Table 2. Prevalence of *Clostridium piliforme* in rabbits with associated risk factors

Variables	No. positive/ No. tested (%)	Statistical test used	p-value
Farm			
Chennai	10/20 (50)	Chi-square	0.0074*
Chengalpattu	5/26 (19.23)		
Fatehgarh	14/20 (70)		
Shamsabad	7/20 (35)		
Mohammadabad	4/14 (28.57)		
Sex			
Male	6/25 (24)	Two-sided Fisher's exact test	0.0649
Female	34/75 (45.33)		
Age			
<5 months	3/12 (25)	Two-sided Fisher's exact test	0.3525
>5 months	37/88 (42.05)		
Breed			
New Zealand White	28/71 (39.44)	Two-sided Fisher's exact test	1.0000
Soviet Chinchilla	12/29 (41.38)		
Weight			
<3 Kg	25/58 (43.10)	Two-sided Fisher's exact test	1.0000
>3 Kg	15/42 (35.71)		
Farm Management			
Organized farm	15/46 (32.60)	Two-sided Fisher's exact test	0.2194
Backyard farming	25/54 (46.30)		
Type of feeding			
Dry Powder form with grasses	10/20 (50)	Chi-square	0.0694
Wet Mash form with grasses	5/26 (19.23)		
Vegetables with grasses	25/54 (46.30)		

*p-value <0.01 - Significant

8000 rpm for 3 min at 20 °C. The extracted DNA was stored at -20 °C. The 260/280 absorbance ratio of the DNA ranged from 1.57 to 1.85, with concentrations of 52-107 ng/µL.

Detection of *C. piliforme* by nested PCR

First-round PCR was performed in a 10 µl reaction volume containing 100-150 ng of DNA, 0.5 µM universal 16S rRNA primers (Forward: 5'-AGAGTTTGATCCTGGCTCAG-3', Reverse: 5' -TACGGYTACCTTGTTACGACTT-3'),[10] and 2X PCR master mix (Ampliqon, Denmark). Cycling conditions were 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 45 sec, with a final extension at 72 °C for 5 min.

The second round of nPCR was performed using 1 µl of a 5-fold diluted first-round product and 0.5 µM *C. piliforme*-specific primers: PiliF (5'-TGGGATAACATCGAGAAATC-3') and PiliR (5'-ACGTAGYCTGTCAATGGT-3').[7] Nuclease-free water was used as the no-template control. PCR cycling was as above, except annealing was at 58 °C for 30 cycles. Amplified products were electrophoresed in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and run at 100 V for 30-40 min (Medox Biotech India Pvt. Ltd.). Gels were visualized and documented using a gel documentation system (Bio-Rad, Inc., Laboratories, USA). To prevent cross-contamination, PCR reactions without template were prepared in a separate facility from DNA extraction and electrophoresis areas.

Statistical analysis

PCR results and associated metadata were analysed using appropriate statistical tests to assess the prevalence of *C. piliforme* in farm and laboratory rabbits. Analyses were performed

using GraphPad Prism 5.0. Associations of age, sex, weight, farming type, and feeding with infection were evaluated using Chi-square or Fisher's exact tests. Significant parameters were further compared with Fisher's exact test and Bonferroni correction. P-values were interpreted as follows: $p < 0.05$ = least significant, $p < 0.01$ = significant, and $p < 0.001$ = highly significant.

RESULTS

In total, 100 rabbit faecal samples were collected from two farms located in two districts of Tamil Nadu (Chennai and Chengalpattu) and three farms (Fatehgarh, Shamsabad, and Mohammadabad) in the Farrukhabad district of Uttar Pradesh.

Detection of *C. piliforme* by nested-PCR

The first-round PCR was performed using universal 16S rRNA gene primers, which yielded a 1500 bp amplification product. Nested PCR was then carried out using *C. piliforme* specific primers, and positive faecal samples produced an 850 bp amplicon (Figure). A faint band at 1500 bp was observed, probably due to the presence of primers and products from the first-round PCR. In addition, some non-specific amplification products were detected along with specific products in certain rabbit faecal samples.

Table 3. Post-hoc pairwise comparisons between places (Fisher) with Bonferroni adjustment

Comparison	Odds Ratio (A vs B)	Unadjusted	Bonferroni	Significant (Bonferroni 0.05)
Fatehgarh vs Chengalpattu	1.68	0.69	1	False
Fatehgarh vs Chennai	0.4	0.30	1	False
Fatehgarh vs Mohammadabad	0.17	0.03	0.35	False
Fatehgarh vs Shamsabad	0.74	1	1	False
Chengalpattu vs Chennai	0.24	0.06	0.55	False
Chengalpattu vs Mohammadabad	0.10	0.00	0.01	True
Chengalpattu vs Shamsabad	0.44	0.31	1	False
Chennai vs Mohammadabad	0.43	0.33	1	False
Chennai vs Shamsabad	1.86	0.52	1	False
Mohammadabad vs Shamsabad	4.33	0.06	0.56	False

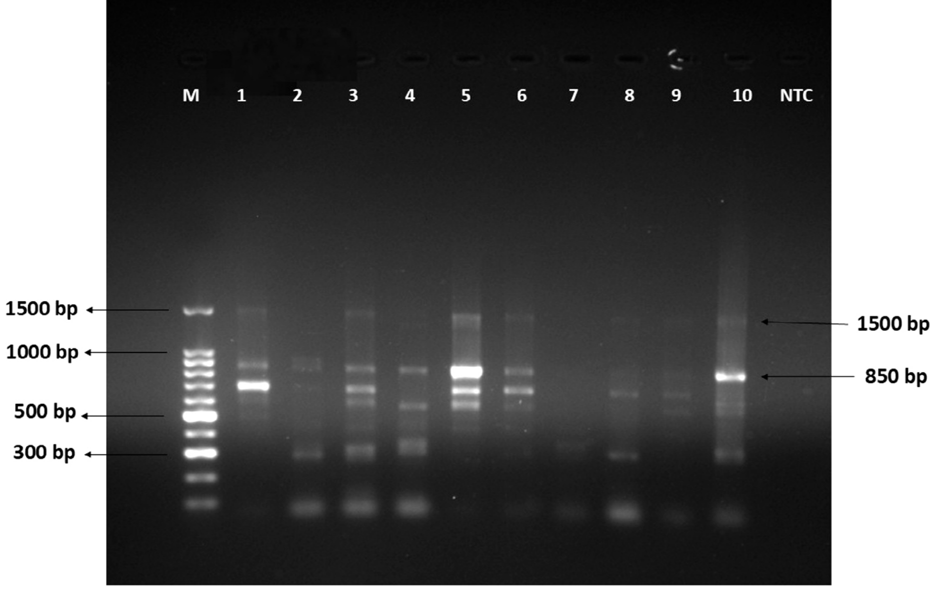

Figure. Agarose gel electrophoresis showing the results of nested PCR amplified products of *Clostridium piliforme*

Prevalence of *C. piliforme* in rabbits

Of the 100 rabbit faecal samples, 40 tested positive for *C. piliforme,* resulting in an overall prevalence of 40%. Details of *C. piliforme* prevalence with respect to other variables are shown in Table 2. The prevalence varied significantly between farms, with a P value of 0.0074 (<0.05). The highest prevalence (70%) was observed in Mohammadabad, whereas the lowest prevalence (19.23%) was recorded in Chengalpattu. Because a significant overall association was detected (p = 0.0074), a post-hoc Fisher's exact test with Bonferroni correction was performed. Although a significant association was found between Fatehgarh and Mohammadabad in the unadjusted test (p = 0.03), this was not significant after Bonferroni adjustment (p = 0.35), as shown in Table 3. The strongest association between farms was observed for Mohammadabad versus Chengalpattu, both in the unadjusted (p = 0.00) and adjusted (p = 0.01) analyses.

From the two organized farms in Tamil Nadu, 46 rabbit faecal samples were collected, of which 15 tested positive by nPCR, giving a prevalence of 32.60%. All three rabbit farms in Uttar Pradesh were backyard farms, from which 54 samples were collected; 25 of these tested positive by nPCR, resulting in a prevalence of 46.30%. However, the prevalence of *C. piliforme* infection was not statistically significant when compared between organized and backyard farms.

Faecal samples from male rabbits accounted for only 25% of the sample size. Although a higher proportion of females (45.33%) were infected with *C. piliforme,* this was not statistically significant. Similarly, rabbits older than five months (42.05%) showed a higher prevalence of infection than those younger than five months (25%), but this difference was also not statistically significant.

In this study, two rabbit breeds—Soviet Chinchilla and New Zealand White were examined for *C. piliforme* prevalence. The majority of samples (71%) were from New Zealand White rabbits. Neither breed nor body weight had a significant effect on the occurrence of infection. Rabbits fed wet mash were less commonly infected (19.23%) than those fed dry powder (50%) or vegetables (46.30%), but these differences were not statistically significant.

DISCUSSION

In this study, an overall *C. piliforme* prevalence of 40% was recorded in the faeces of clinically normal rabbits. Very high prevalence rates have been reported in healthy mice (83.01%) and rats (90.9%) by PCR in a laboratory animal facility in Turkey.[11] In contrast, a study in three provinces of China detected *C. piliforme* antibodies in only 5.08% of clean-grade animals and 9.96% of specific pathogen-free animals using LAMP-LFD analysis.[12]

Fingerhood et al.[13] detected *C. piliforme* by PCR in colon (5/10), liver (5/10), and heart (1/10) samples from pre-weaned orphaned kittens in the USA. Oliveira et al.[14] detected *C. piliforme* by PCR in skin, liver, heart, intestines, and brain samples from a kitten that died after exhibiting nervous signs and diarrhoea. Similarly, Brooks et al.[15] demonstrated the *C. piliforme* genome by PCR in liver samples of two farm-raised white-tailed deer fawns (*Odocoileus virginianus*), suggesting a probable association between copper toxicity and *C. piliforme* infection. Garcia et al.[16] detected *C. piliforme* in all 24 liver samples (100%), 20 of 25 colon samples (83.33%), and 5 of 25 heart samples (20%) from foals suspected to have died of Tyzzer's disease by PCR. These findings suggest that the sensitivity of PCR depends on the type of sample collected from infected animals.

Furukawa et al.[6] detected *C. piliforme* DNA by PCR in 3 of 5 (60%) faecal samples collected from naturally infected rabbits. The authors explained that bile salts and bilirubin in faecal extracts reduced the sensitivity of PCR, and the detection limit of their assay was 10 bacteria per reaction.

Pritt et al.[17] were unable to detect *C. piliforme* genomic DNA by PCR in caecum samples from 20 randomly selected seropositive rabbits without clinical signs. This indicates that serological tests cannot be considered confirmatory for the presence of *C. piliforme* in laboratory or commercial farm animals, as antigens used in these tests may cross-react with antibodies produced against non-pathogenic bacteria.[18] Kirtland et al.[19] evaluated the utility of plasma sorbitol dehydrogenase measurement for identifying subclinical or early Tyzzer's disease in

farm populations and potential carrier animals but concluded that it lacked sensitivity and specificity.

Disparity in the prevalence of *C. piliforme* reported in previous studies may be attributed to the health status of the animals (diseased vs. healthy) and the type of samples analyzed (affected organs vs. faeces). These studies also demonstrated that *C. piliforme* infections are often subclinical and cleared by immunocompetent hosts following seroconversion.[20]

The significant prevalence difference observed between two farms: Mohammadabad (70%) and Chengalpattu (19.2%), even after Bonferroni correction, may be due to environmental and management-related differences such as farming system (backyard vs. organized) and feeding practices (wet mash vs. vegetables).

Garcia et al.[16] reported that all but one foal suspected to have died of *C. piliforme* were younger than 45 days. Artukovic et al.[21] also observed that weaned rabbits were more predisposed to *C. piliforme* infection than adult rabbits. Tyzzer's disease was confirmed in a 45-day-old kitten and in 4 to 5-day-old white-tailed deer fawns by Oliveira et al.[14] and Brooks et al.,[15] respectively. Tyzzer's disease primarily affects well-nourished young animals, particularly those on protein-rich diets.[22] In contrast, in the present study, although not statistically significant, *C. piliforme* was detected by nPCR in 42% of faecal samples collected from rabbits older than 5 months, while only 25% of samples from rabbits younger than 5 months were positive. Similarly, Ulker et al.[11] reported a higher prevalence of *C. piliforme* in adult mice and rats compared with young animals. The differences in prevalence rates between age groups in various studies may be due to sample selection from healthy versus diseased animals.

Garcia et al.,[16] Fingerhood et al.,[13] and Brooks et al.[15] studied foals, kittens, and deer fawns, respectively, that were suspected to have died of *C. piliforme*. In contrast, Ulker et al.[11] and the present study examined faecal samples collected from clinically normal animals. Brooks et al.[15] suggested that *C. piliforme* infection in deer fawns was probably associated with liver damage caused by copper toxicity. Co-infections of *C. piliforme* with canine distemper virus were also reported in two domestic dog puppies and a grey fox kit.[23] Fingerhood et al.[13] observed that 15 pre-weaned kittens that died of Tyzzer's disease had co-morbidities such as sepsis, coccidiosis, and feline parvovirus (FPV). Oliveira et al.[14] similarly reported co-infection with FPV in the ileum of a kitten that died of Tyzzer's disease. In their studies, adult animals (deer and cats) were clinically healthy, suggesting that *C. piliforme* infection causes mortality in animals that are either co-infected or immunosuppressed. The high positivity rate in healthy adult rabbits in the present study suggests that they may act as carriers, as Tyzzer's disease is generally transmitted through the faeco-oral route[24] and *C. piliforme* spores can survive in the environment for at least 5 years.[25]

In the present study, breed, sex, weight, and type of rearing or feeding did not have a statistically significant association with *C. piliforme* infection in rabbits. It has been recorded that Tyzzer's disease in animals is mainly predisposed by immunosuppression, stress, high environmental temperatures, overcrowding, poor sanitation, dietary changes, and the administration of sulfonamides or corticosteroids.[25,26]

In this study, *C. piliforme* DNA was amplified as an 850 bp amplicon by nested PCR. A larger-sized band of around 1500 bp was also observed. A similar larger band was reported by Niepceron and Licois,[7] who explained that this was probably due to the presence of outer primers from the first-round PCR. They attempted the second-round PCR with a higher annealing temperature to achieve a single 850 bp band without by-products, but this resulted in a tenfold reduction in sensitivity in caecal samples.

A few non-specific amplification products were also observed in the second-round PCR. Such non-specific amplicons have been reported in mouse, rat, and hamster faeces,[6] in rabbit caecum,[7] in deer fawn liver samples,[15] and in DNA extracted from cell-culture–propagated *C. piliforme* isolates.[18] This may be due to the use of 16S rRNA primers. Because faeces contain diverse bacteria, cross-reactions between primers and the 16S rRNA regions of closely related clostridial organisms in the gastrointestinal tract may generate erroneous products. Furukawa et al.[6] noted that diagnosis becomes challenging if obscure bands appear at the specific region when using faecal samples. Thus, although detection of

C. piliforme in faeces by PCR is simple, rapid, and non-invasive, the lack of sensitivity and specificity cautions against its use as a sole confirmatory test in live animals.

Primers targeting specific genes of *C. piliforme* could not be designed because no complete genome sequence is currently available. Confirmation of *C. piliforme* infection in suspected cases is therefore achieved not by a single test but by a combination of methods. Recent studies have used clinical signs, gross lesions, histopathological changes, detection of clostridia in tissues, immunohistochemistry, and 16S rRNA gene PCR for confirmation in dead animals.[13,14,23] However, no foolproof test exists for screening or diagnosis of *C. piliforme* in live animals, and only a combination of serological testing and PCR is recommended. Hence, the presence of *C. piliforme* in the present study should be further confirmed by sequencing the PCR products, and results should always be interpreted in correlation with serology and clinical signs/gross lesions.

Recently, Uprety et al.[4] used shotgun metagenomics to obtain partial genome sequences of *C. piliforme* from the liver of a foal that died of Tyzzer's disease. They identified partial sequences of virulence factor genes such as alveolysin, exo-α-sialidase, and those involved in flagellar and spore formation, thereby providing the first genetic evidence of virulence factors for *C. piliforme*. They also showed that flagellin protein sequences had low identity with those of *C. colinum*, the next closest phylogenetic species, indicating their potential utility in serological assays and vaccine development.

Immunocompromised humans are susceptible to *C. piliforme* infection, as demonstrated by a case detected in an HIV patient in the USA.[3] A serological study detected antibodies against *C. piliforme* in 85.7% of laboratory animal handlers, 40.5% of personnel involved in laboratory animal care, and 22.0% of unrelated personnel.[27] The drastic decline of North American muskrats over the last 50 years may be attributed to Tyzzer's disease,[28] and the susceptibility of a wide range of mammals to *C. piliforme* infection has been documented, especially in animals with co-morbidities.[13-15,23] These studies highlight the potential public health risk of *C. piliforme* infection for laboratory animal handlers and immunocompromised individuals.

CONCLUSION

In conclusion, the presence of *C. piliforme* in rabbits could affect the health and research outcomes of these animals, as well as the health of caretakers and research personnel. Therefore, proper monitoring, preventive measures, and potential treatments should be implemented to maintain animal well-being and research integrity. Use of 16S rRNA-based PCR for detecting *C. piliforme* in samples, particularly faeces, should always be correlated with serological tests and clinical or pathological findings. The identification of *C. piliforme* specific gene target primers is urgently needed for screening susceptible animal populations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

ACM conceptualized, designed the study and consolidated the data. KGT acquired funding and performed administrative work. SR, SJ, PJ, PR and MRS performed sample collection. PR carried out laboratory work. PJ performed statistical analysis. PR drafted the original manuscript. ACM, SR, SJ, PJ, and MRS wrote the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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