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MOLECULAR CHARACTERIZATION OF *GIARDIA INTESTINALIS*, *ENTEROCYTOZOON*, AND *CRYPTOSPORIDIUM* SPECIES IN ZOO WORKERS AND FELINE SPECIES OF PUBLIC AND PRIVATE ZOO

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Abstract: Zoonoses are diseases, naturally transmitted from vertebrate animals to humans. Parasitic diseases are often caused by common gastrointestinal (GI) protist pathogens like *Giardia intestinalis*, *Enterocytozoon*, and *Cryptosporidium* species. Focusing on the potential zoonotic transmission of these protozoan parasites, a comprehensive study was conducted from 20-01-2023 to 10-01-2024. Fecal samples (n=212) from zoo personnel and feline inhabitants were collected and subjected to molecular analyses to identify and characterize *G. intestinalis*, *Enterocytozoon*, and *Cryptosporidium* isolates. The study aimed to identify and determine the molecular epidemiology of *G. intestinalis*, *Enterocytozoon* spp., and *Cryptosporidium* spp. in zoo workers and feline species, revealing risk factors for zoonotic transmission in zoos. Molecular techniques, including PCR and DNA sequencing, were employed to analyze and characterize the identified *Giardia*, *Enterocytozoon*, and *Cryptosporidium* isolates. Additionally, factors such as hygiene practices, animal interactions, and environmental conditions were assessed to elucidate potential sources and routes of transmission. This study sheds light on *G. intestinalis*, *Enterocytozoon* spp. and *Cryptosporidium* spp. epidemiology in zoos, stressing the need for preventive measures against zoonotic transmission. The study highlights the need for sanitation, health screenings, and awareness programs for zoo staff and visitors. This ensures safety for both humans and animals in zoological settings.

Keywords: *Cryptosporidium*, *Enterocytozoon*, *Giardia intestinalis*, feline, molecular characterization

Introduction

Zoonoses are diseases naturally transmitted from vertebrate animals to humans. Parasitic diseases, particularly those transmitted from pet animals to humans, are among the most critical infectious diseases [1]. Pet cat, *Felis catus* is one of the major sources of transmission of zoonotic pathogens from animals to humans [2]. Giardiasis, blastocystosis and cryptosporidiosis are the most significant

parasitic infections common which are transmitted by *Giardia* spp., *Enterocytozoon blastocyst* and *Cryptosporidium* spp., respectively [3].

Certain parasitic infections can cause severe clinical symptoms like diarrhea, malnutrition, weight loss and even death of their hosts [4]. These pathogens are commonly transmitted from fecal to oral and also from contaminated food and water into their host's body [5] or through direct contact [6]. *Enterocytozoon bieneusi* is considered a significant species of phylum Microsporidia [7].

Polymorphism analysis based on the ribosomal internal transcribed spacer (ITS) has identified more than 500 *E. bieneusi* genotypes [8], and phylogenetic tree analysis has placed these genotypes into 11 distinct groups. Currently, above 80 genotypes have been studied in rodents and of these S6, CZ3, Henan-II, EbpA, C, Peru8, and CHN4 can also infect humans [9]

Giardia, an obligate intestinal parasite, is associated with a common infection, diarrhea, with contaminated water and food being the primary transmission sources in both animals and humans [10]. The transmission of *Giardia* through feces poses a high risk to immunocompromised individuals [11]. The range of host specificities, genotyping and phenotyping changes classify the genus *Giardia* into nine species [6]. *Giardia intestinalis*, also known as *G. duodenalis*, holds great significance for global public health due to its wide host range, including humans [12].

Cats are mainly infected with intestinal parasites such as *Cryptosporidium* spp. *G. duodenalis*, and *Blastocystis* species. Having close association with cats, there are concerns about the possible transmission of these parasites from infected cats to humans. Hence, this study was performed to identify and determine the molecular epidemiology of these zoonotic parasites in stray/household cats.

Methods

Site Selection

The proposed study was carried out in public and private zoos of Punjab, Pakistan (Figure 1). It is the second-largest province of Pakistan by land area and the largest by population. The latitude of this province is 31.1704° N, and longitude is. 72.7097° E. In the South, Punjab's elevation reaches 2,327 meters (7,635 ft) near the hill station of Fort Munro in Dera Ghazi Khan.

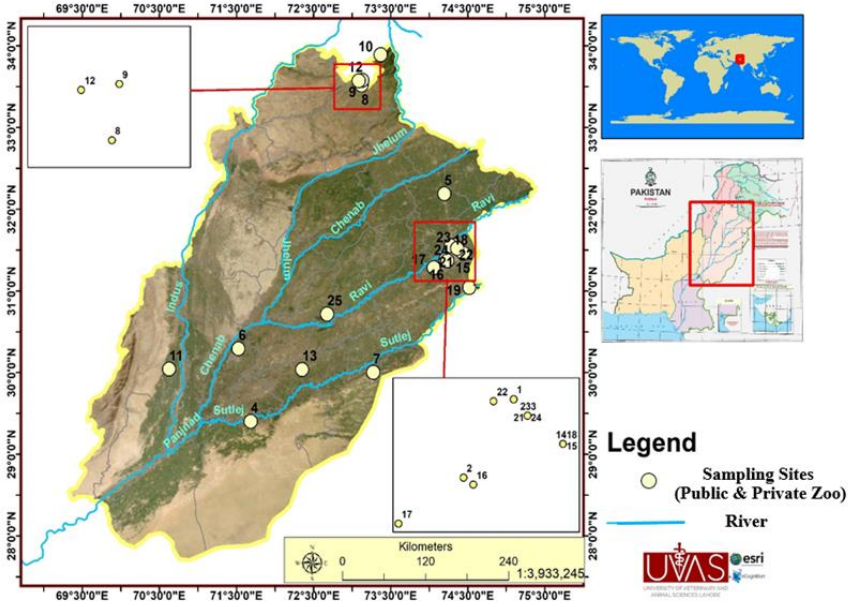

Figure 1: GIS map showing location of each selected zoo

Sample collection

Field surveys were conducted in the selected districts to locate and identify felid populations. Fresh fecal samples (n=212) were collected directly from felid species, including African lions, Bengal tiger, common leopard, Siberian tigers, and small wildcats, using sterile gloves and disposable containers from districts across Pakistan. Each sample was labeled with a unique identifier, including the species,

district, and individual identification. The collected fecal samples were immediately placed in a cool box with ice packs and transported to the laboratory within 24 hours. Fecal samples were homogenized and divided into smaller aliquots for different analyses. A portion of each fecal sample was examined under a microscope using standard techniques to detect the presence of *Giardia*, *Enterocytozoon*, and *Cryptosporidium* species.

Isolation and Identification

Direct Immunofluorescence Assay (IFA): Fecal samples were processed using an IFA kit specific for *Giardia* spp., following the manufacturer's instructions. Positive samples were recorded. Fecal smears were prepared and stained with modified trichrome stain or Gram-chromotrope stain to identify *Enterocytozoon* spp. Acid-Fast Staining: Fecal smears were subjected to acid-fast staining, such as Ziehl-Neelsen or modified Ziehl-Neelsen stain, to detect *Cryptosporidium* spp.

Molecular Characterization

Genomic DNA was extracted from the fecal samples using a commercial DNA extraction kit, following the manufacturer's instructions. PCR amplification of the small subunit ribosomal RNA (SSU rRNA) gene was performed using specific primers targeting *Giardia* species. PCR amplification of the SSU rRNA gene was carried out using primers specific to *Enterocytozoon* species. PCR amplification of the 18S rRNA gene or the *Cryptosporidium* oocyst wall protein (COWP) gene was conducted using *Cryptosporidium*-specific primers. A detail of PCR tube composition and conditions for amplification of 18S rRNA of targeted pathogens is given in Table 1 and Table 2, respectively.

Table 1: PCR Tube Composition.

No.	Ingredients	Volume per run
1.	Template DNA	1µl
2.	Forward Primer	1µl
3.	Reverse Primer	1µl
4.	Master Mix	12µl
5.	Distilled Water	10µl
Total volume of a PCR tube		**25µl**

Table 2: Conditions for amplification of 18S rRNA of targeted pathogens by PCR

Oligonucleotides (Sequences)	Product size (bp)	Uses	Annealing temp.	Reference
F 5′-TTCTAGAGCTAATACATGCG-3′ R 5′-CCCTAATCCTTCGAAACAGGA-3′	1325	Amplification of 18S of *Cryptosporidium* spp.	54°C	[13]
F 5-′TTCA GATGGTCATAGGGATG-3′ R 5-′ATTAGAG CATTCCGTGAGG-3′	465	Amplification of 18S fragment of rRNA gene of *Enterocytozoon* spp.	55°C	[14]
F 5′-AAG TGT GGT GCA GAC GGA CTC-3′ R 5′-CTG CTG CCG TCC TTG GAT GT-3′	499	Amplification of *Giardia* spp. 18S rRNA gene	53°C	[15]

Sequencing and phylogenetic tree analysis

The PCR amplicons were sent for DNA sequencing to determine the genotypes or subtypes of *Giardia* spp., *Enterocytozoon* spp., and *Cryptosporidium* species. The obtained sequences were compared with reference sequences available in online databases. Phylogenetic analysis was performed to determine the genetic relationships and to identify the specific genotypes or subtypes.

Statistical analysis

The obtained DNA sequences were analyzed using bioinformatics tools and software. Multiple sequence alignment and phylogenetic analysis were performed to determine the genotypes or subtypes of *Giardia* spp., *Enterocytozoon* spp., and *Cryptosporidium* spp. present in the samples. Geographic information system (GIS) software was used to create maps illustrating the distribution and occurrence of the parasites in different districts of Pakistan.

Results

Identification of *Cryptosporidium* spp. (18S rRNA gene)

In *Cryptosporidium* genus one specie, named *C. parvum*, was identified as shown in figure 2.

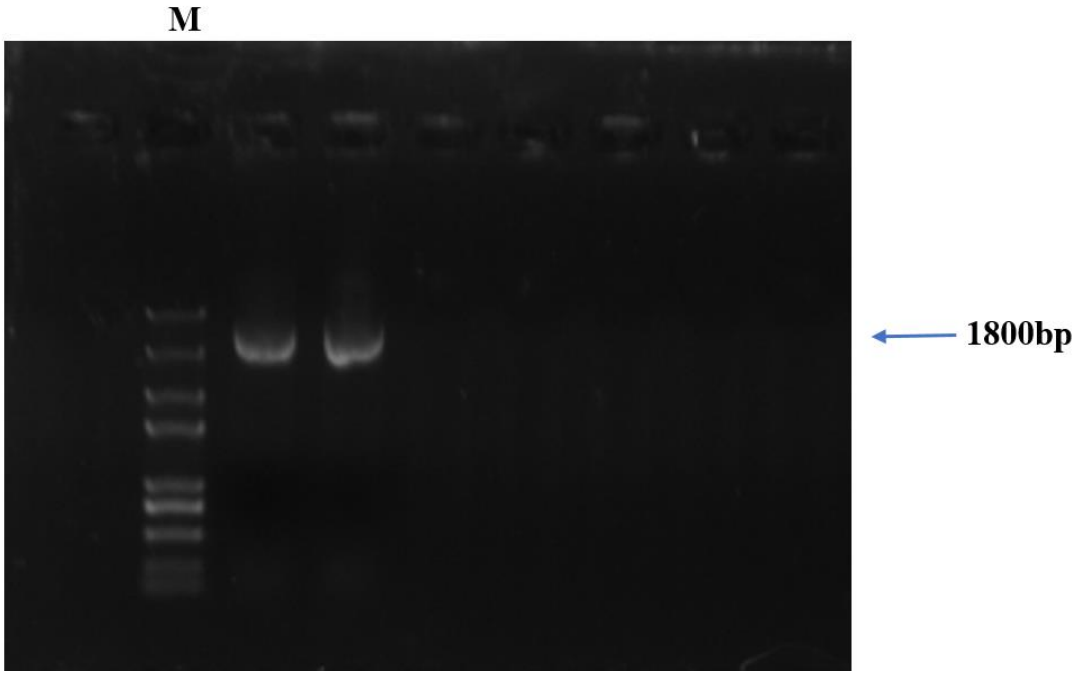

Figure 2: Amplification of 18S rRNA gene from *C. parvum*

Amplification of 18S rRNA gene from *G. duodenalis*

One *Giardia duodenalis* specie was amplified on the basis of 18S rRNA gene with universal primers as shown in figure 3.

Figure 3: Amplification of 18S rRNA gene from *G. duodenalis*

Amplification of 18S rRNA gene for the detection of *Enterocytozoon* spp.

A total of 20 genotypes of *Enterocytozoon* were subjected to amplify while one specie was amplified with 18S rRNA gene (Figure 4).

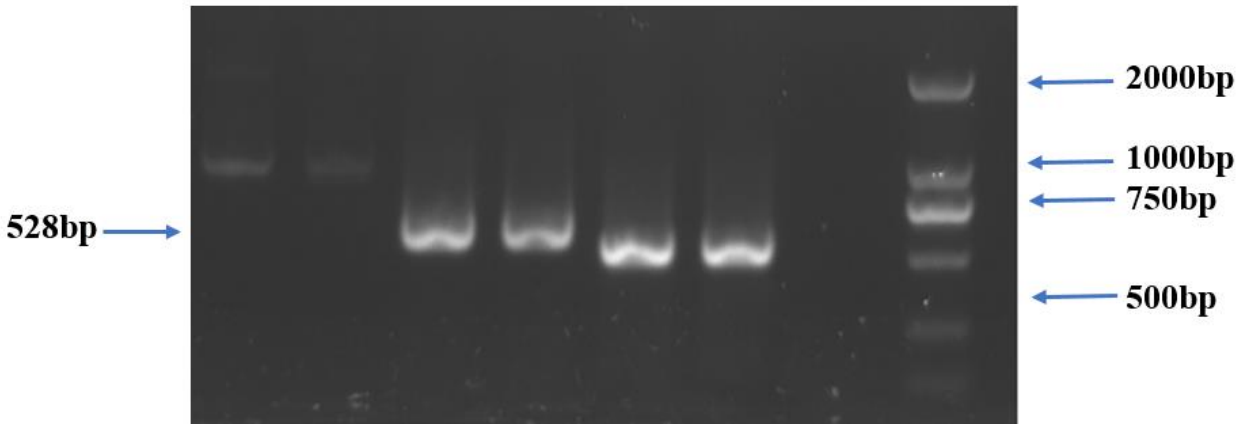

Figure 4: Amplification of 18S rRNA gene from *Enterocytozoon bieneusi*

Phylogenetic tree analysis of 18S rRNA gene of *Cryptosporidium parvum*

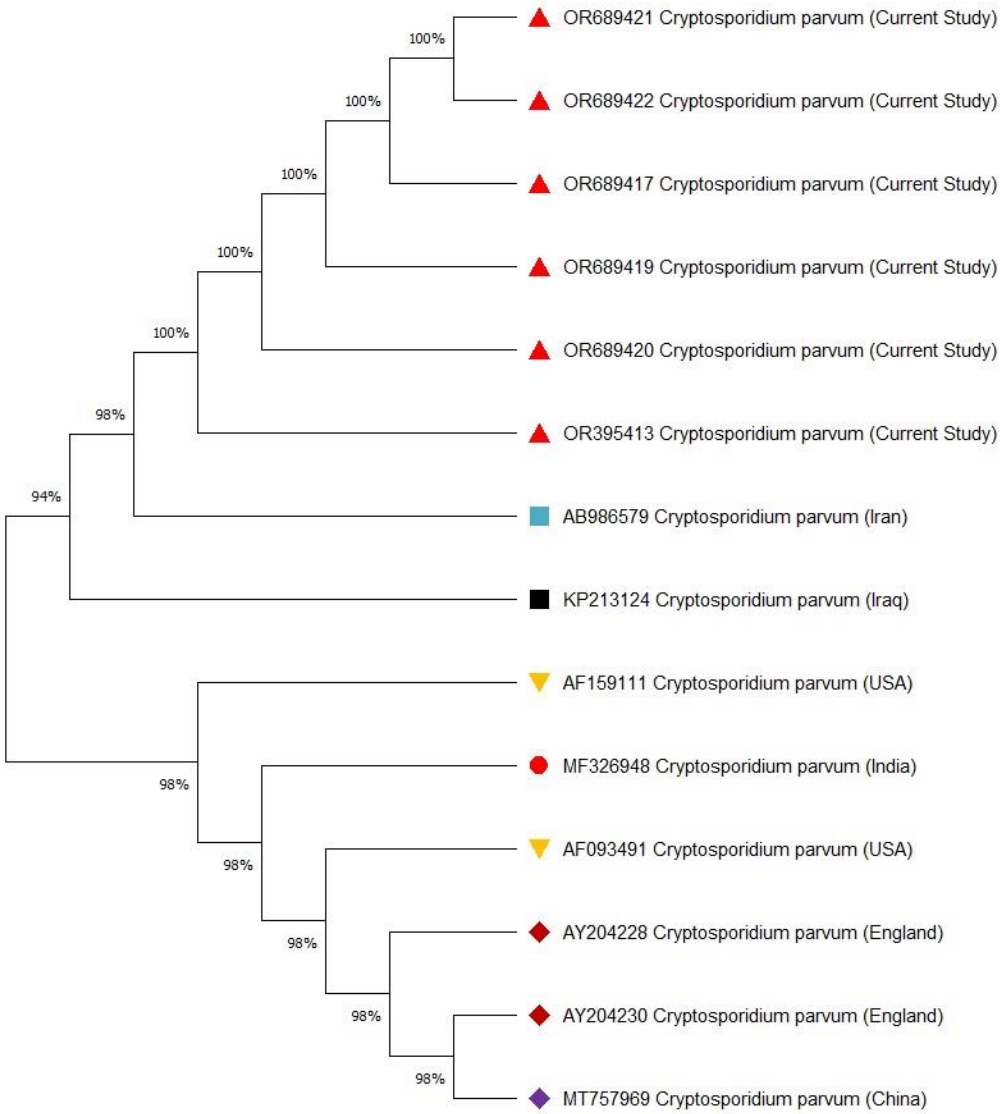

Figure: Phylogenetic tree analysis of 18S rRNA gene of *Cryptosporidium parvum*

Phylogenetic tree analysis of the 18S rRNA gene of Cryptosporidium parvum revealed 100% similarity among all the isolates in the current study, as well as 100% similarity with the isolate (AB986579) from Iran, and 98% similarity with isolates from England (AY204230), China (MT757969), India (MF326948), and the USA (AF093491). However, isolates of the current study were distantly similar to isolates from China (MT757969), while closely related to isolates from Iran (AB986579) within the current study.

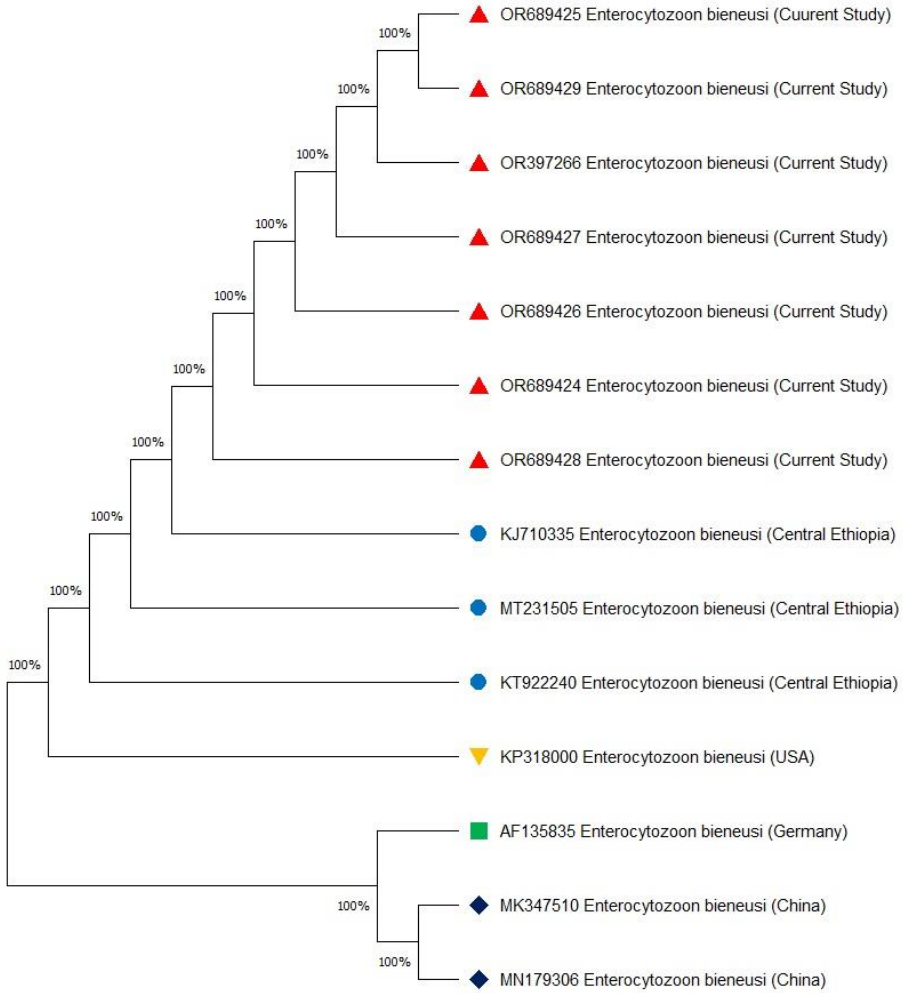

Figure: Phylogenetic tree analysis of 18S rRNA gene of *Enterocytozoon bieneusi*

Phylogenetic tree analysis of 18S rRNA/ITS of Enterocytozoon bieneusi revealed 100% similarity among the isolates in the current study and also with those previously isolated in China (MK347510), Germany (AF135835), the USA (KP318000), and Central Ethiopia (KT922240). However, isolates from Central Ethiopia (KJ710335 and MT231505) showed greater closeness and were distantly similar to isolates from China (MN179306) and Germany (AF135835).

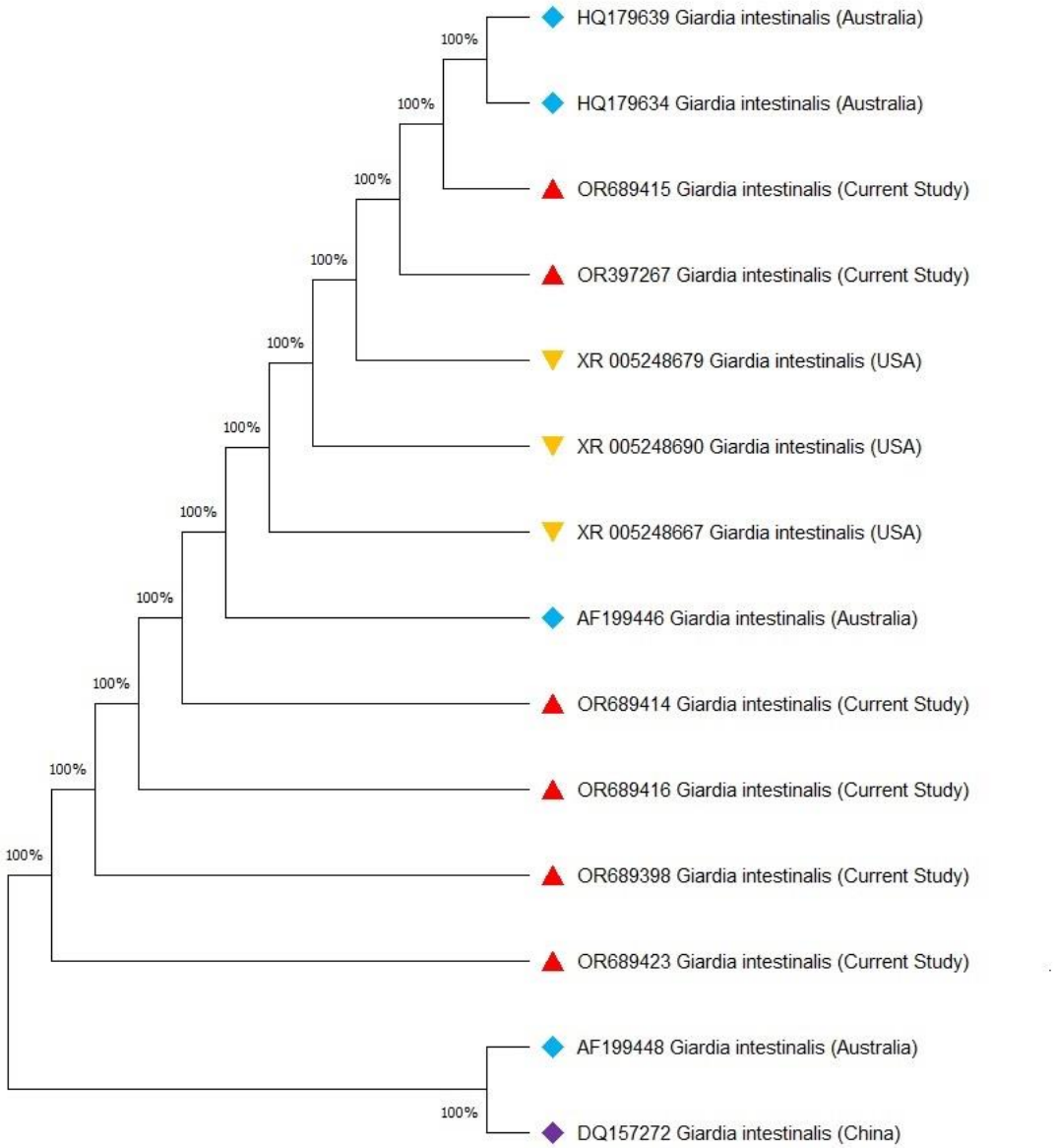

Figure: Phylogenetic tree analysis of 18S rRNA gene of *Giardia intestinalis*

Phylogenetic tree analysis of 18S rRNA of Giardia intestinalis revealed 100% similarity among the isolates in the current study and also with those previously isolated in Australia (HQ179639), the USA (XR 005248667), and China (DQ157272). However, isolates from Australia (HQ179634 and AF199448) and the USA (XR 005248679) showed a closer similarity with isolates of the current study. Conversely, isolates from China (DQ157272) and Australia (AF199448) exhibited a more distant similarity with the isolates of the current study.

Discussion

The potential role of wild and captive small mammals, felid species as a reservoir of these pathogenic parasites, namely *Giardia*, *Cryptosporidium* spp., and *Enterocytozoon* remains unknown in Punjab, Pakistan. To fill this knowledge gap, this study provides the first molecular-based evidence on the occurrence, and genetic diversity of *Giardia* and *Cryptosporidium* in wild rodents and shrews in Pakistan.

The zoonotic diseases cryptosporidiosis, giardiasis, and microsporidiosis have previously been reported in a number of animal species worldwide [16, 17]. The potential spread of these diseases and parasites increases under the high-density feedings condition and the frequent contact between

populations in a zoo. In this study, we identified two *Cryptosporidium* species (*C. baileyi* and *C. felis*) and one genotype (*Cryptosporidium* genotype III) in three specimens (3/203, 1.5%). However, slightly lower infection rates were reported in Lisbon Zoo, Portugal (1.1%, 3/274) [18], and Osaka Zoo, Japan (0.4%, 1/284) [19]. *Cryptosporidium baileyi* was identified in the red-crowned crane in this study, but has previously been isolated predominantly from selected zoo felids.

Cryptosporidium felis is usually found in felidae animals (AF108862) and humans (HQ149021), but was detected in the manure in this study. *Cryptosporidium* genotype III isolated from lion is usually isolated from lion (HM116385), but has also been detected in wastewater (FJ205700). *G. duodenalis* is common in wild and captive nonhuman primates [20]. In this study, we identified two assemblages of *G. duodenalis*, B and F, in five specimens (5/203, 2.5%). This prevalence was much lower than the 29% (38/131) reported in Zagreb Zoo, Croatia [21], but much higher than the 1.1% (3/284) reported in Osaka Zoo, Japan [19].

Giardia duodenalis assemblage B was detected in the white-cheeked gibbon and beaver, and is zoonotic. A similar infection was also found by [20]. Assemblage F was identified in the Chinese leopard and Siberian tiger, which is consistent with data from cats [22], whereas isolates detected in the snow leopard, cheetah, serval, and lynx were assemblages A1 and C/D at the ITS1–ITS2 and tpi loci, respectively [21]. *E. bieneusi* has been reported in humans and a broad range of domestic, wild, and companion animals [17].

In this study, 15.8% (32/203) of specimens were positive for *E. bieneusi*, suggesting that this is a common parasite in the wildlife in Zhengzhou Zoo, although this incidence was lower than the rates of 29.8% (148/496) [20] and 28.2% (116/411) [23] reported for nonhuman primates in zoos and public parks in China. It is also lower than the rate of 32.5% (26/80) reported in the wild white-tailed deer in America [17]. In this study, we identified a high degree of genetic diversity in *E. bieneusi* in the wildlife at the zoo.

A total of 13 explicit ITS genotypes (seven known and six new) were observed in 32 positive specimens. The zoonotic genotypes D, Peru8, and type IV were previously found in humans, dogs, swine, goats, etc. Genotypes J, I, and CHG1 were previously thought to be cattle-specific. However, genotype J has also been reported in pigeons, deer, goats, etc. The new genotypes CHALT1, CHP1, CHY1, CHB1, CHK1, and CHK2 were first reported in this study.

A phylogenetic analysis of the genotypes detected in this study and other known *E. bieneusi* ITS genotypes revealed that two of the six new *E. bieneusi* genotypes (CHALW1 and CHP1) are genetically related to the humanpathogenic group designated “group 1” [20]. The remaining new genotypes formed a new cluster, confirming the high genetic diversity of the detected genotypes.

Conclusions

Molecular characterization studies have shed light on the prevalence, distribution, and genetic diversity of *Giardia, Enterocytozoon*, and *Cryptosporidium* species in felids across different districts of Pakistan. These parasites pose a potential zoonotic risk, emphasizing the importance of understanding their epidemiology and implementing appropriate control measures. The identification of these parasites as potential zoonotic risks underscores the urgency of comprehending their epidemiology to protect both animal and human populations. To effectively mitigate the transmission and impact of these infections, it is crucial to prioritize and implement appropriate control measures.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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Authorship credit

Muhammad Waris: Concept and design of study
Syed Mohsin Bukhari: Acquisition of data
Ali Hussain: Analysis and interpretation of data
Muhammad Hafeez-ur-Rehman: Drafting the article or revising it critically for intellectual content

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