



## RESEARCH ARTICLE

### Molecular Prevalence and Genetic Characterization of *Eimeria tenella* in Backyard Chickens

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#### ABSTRACT

Coccidiosis, caused by *Eimeria (E.) tenella*, poses a significant threat to poultry farming. This study aimed to determine the prevalence, molecular characterization and to assess the epidemiological factors associated with *E. tenella* infection in backyard chickens in District Bannu, Khyber Pakhtunkhwa, Pakistan. Over six months (April to September 2022), faecal samples from 160 chickens of various breeds were microscopically screened for *E. tenella* oocysts, followed by DNA extraction and *ITS-1* marker-based molecular analysis. Phylogenetic analysis compared the obtained sequences with global *Eimeria* isolates. The overall prevalence of *E. tenella* infection was 34.37%, varying across tehsils, with the highest in Bannu tehsil (40%), which was statistically non-significant ( $P>0.05$ ). Age-related susceptibility was evident, with grower chickens exhibiting the highest prevalence (41.17%), which was statistically significant ( $P<0.05$ ). Females showed a higher prevalence (36.57%) than males (23.08%), with non-significant association ( $P>0.05$ ). Housing types revealed varying prevalence: cages (21.05%), night shelters (29.87%), and free-range chickens (43.75%), with no statistically significant difference ( $P>0.05$ ). Feed types also influenced prevalence, with commercial (11.11%), natural/household (40.54%), and mixed feeds (22.50%) with a statistically significant difference ( $P<0.05$ ). The highest prevalence at 44 and 42.86%, respectively, was observed in August and September, aligning with increased humidity, with no significant association ( $P>0.05$ ). Single nucleotide polymorphisms (SNPs) in the *ITS-1* region of *E. tenella* showed significant variations across samples, with distinct nucleotide substitutions at positions 108, 111, 123, 134, and 313. Phylogenetic analysis of 27 isolates, including 15 from this study, revealed a distinct cluster closely related to *E. tenella* from multiple countries. The tree also identified four separate clusters for *E. mitis*, *E. maxima*, *E. acervulina*, and *E. brunetti*, each grouping isolates from diverse geographic regions. This study provides novel insights into the prevalence of *E. tenella* in backyard chickens which may be helpful to have targeted control strategies for poultry farming.

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## INTRODUCTION

The term *backyard chicken* refers to the practice of small-scale poultry farming, typically conducted by households, especially in rural areas. Backyard poultry farming, particularly the raising of chickens (*Gallus gallus domesticus*), is widely practiced in rural regions across underdeveloped and developing countries. In Pakistan, the poultry sector, including backyard chickens, significantly contributes to the economy, accounting for 1.3 to 2.3% of the national GDP (Ahmed *et al.*, 2021; Begum *et al.*, 2024). Despite its economic importance, chickens are highly susceptible to various infections, including bacterial, fungal, viral, and parasitic diseases, which result in high mortality and morbidity rates (Mohsin *et al.*, 2021; Mehtab *et al.*, 2022; Raffie *et al.*, 2023). Among these diseases, coccidiosis, caused by various protozoan parasites, with *E. tenella* being one of the most prevalent species, is a common parasitic infection in chickens (Blake *et al.*, 2014; Begum *et al.*, 2024).

Backyard chickens are susceptible to nine recognized pathogenic *Eimeria* species, namely *E. maxima*, *E. brunetti*, *E. necatrix*, *E. tenella*, *E. acervulina*, *E. mitis*, *E. hagani*, *E. praecox*, and *E. mivati*, each of which predominantly colonizes distinct segments of the intestinal tract (Chen *et al.*, 2020; Mohsin *et al.*, 2021). Of these, *E. tenella* is most pathogenic, for it selectively infects the ceca and induces extensive hemorrhage, critical morbidity, and mortality due to its extensive asexual reproduction. The parasite transmission is mostly fecal-oral, wherein infection ensues following oral intake of sporulated oocysts (Fayer, 1980; Dalloul *et al.*, 2005). The infectious capacity of such oocysts is influenced by several factors, including the concentration of oocysts in the litter, the stocking rate of the birds, and immunological status of the host (Carvalho *et al.*, 2025).

Once ingested, the oocysts release sporozoites in the chicken's stomach, which then invade intestinal cells by binding to the cell membrane, going through various stages till completion of life cycle (Chen *et al.*, 2025). Coccidiosis due to *E. tenella* results in substantial economic losses to the global poultry industry, with estimated annual costs surpassing 3 billion USD (Dalloul & Lillehoj, 2006).

Efficient control measures for *E. tenella* are good management practices, immunization, anticoccidial drug use, and cleanliness and dryness of poultry farms. Traditionally, diagnosis includes isolation of oocysts from droppings and examination of pathological lesions. Molecular diagnostics have now replaced these as more sensitive and effective for identification and control (Ogedengbe *et al.*, 2011). The prevalence of *Eimeria* infections among chickens is extremely variable between locations. Pakistan has repeatedly shown high levels of infection (24-44%) in various studies (Awais *et al.*, 2012; Jamil and Mansoor, 2016; Yousaf *et al.*, 2018). Globally, highest prevalence of 75% is reported in South Korea by Flores *et al.* (2022) primarily with *E. acervulina* and *E. tenella* infection. In contrast, the lowest prevalence was found in India by Yaqub *et al.* (2023), with a 10.6% infection rate in chickens, wherein the predominant species were *E. tenella*, *E. acervulina*, *E. maxima*, and *E. necatrix*. Moderate prevalence rates were also seen in other research, Adem *et al.* (2023) at 27.1% in Ethiopian

chickens, and Nana-Mariam *et al.* (2023) at 36.3% in Nigerian chickens, where the most common species were *E. acervulina*, *E. necatrix*, and *E. brunetti* that contributes to regional variation in the prevalence of *Eimeria* and species distribution, highlighting the disproportionate global burden of these infections.

Good management practices, vaccination, anticoccidial medication use, cleanliness and dryness of farm conditions are effective control measures. Conventional diagnosis has conventionally depended on the detection of oocysts in fecal smears and examination of distinctive lesions, though more accurate and reliable techniques like PCR are available (Ogedengbe *et al.*, 2011). Nuclear ribosomal DNA segments, particularly the 18S rDNA and internal transcribed spacer 1 (*ITS-1*), are good markers for genetic characterization and phylogenetic analysis of protozoan parasites such as *E. tenella* (Ogedengbe *et al.*, 2018). *ITS-1* is a good PCR-based diagnostic region because it has a high number of copies in the genome, thus a sensitive marker and useful tool for phylogenetic work (Hafeez *et al.*, 2015). Though *E. tenella* is having a high prevalence and economic loss, less information is available regarding its genetic diversity and epizootiology, especially in backyard chickens of Pakistan. Thus, the present study was conducted with the objectives to determine the prevalence and genetic diversity of *E. tenella* in backyard chickens of District Bannu, Pakistan, by using the *ITS-1* region as a molecular marker.

## MATERIALS AND METHODS

**Study area and ethical approval:** The study spanned a six-month period, from April to September 2022 and was conducted in district Bannu (Fig. 1), situated in the northeast part of the Khyber Pakhtunkhwa province of Pakistan. Bannu district encompasses five tehsils, namely Bannu, Domel, Miryan, Kakki, and Baka Khel. The climate in this region is characterized by harsh conditions, including short, cold winters and long hot summers. Ethical approval for this research study was obtained from the Ethical Approval Committee of Kohat University of Science and Technology (KUST), with approval number KUST/ZOO/338, ensuring compliance with ethical standards and guidelines.

**Sample collection:** A total of 160 fresh fecal samples were collected from backyard chickens across five administrative regions (Bannu, Domail, Kakki, Miryan, and Baka Khel) within District Bannu. Sample collection was performed aseptically using a slightly modified version of the method described by Kumar *et al.* (2014). Feces were either gathered directly from the ground or obtained from the intestines of freshly deceased birds. Each specimen was transferred into a 50mL Falcon tube preloaded with 5mL of 2% (w/v) potassium dichromate solution and adjusted to a total volume of 10mL. Chicken exhibiting visible signs of illness, such as feather ruffling, yellow discoloration of shanks, lethargy, stunted growth, or diarrhea, were randomly selected for sampling. All samples were immediately transported to the Molecular Parasitology and Virology Laboratory, Department of Zoology, Kohat University of Science and Technology, and stored at 4°C until further parasitological examination.

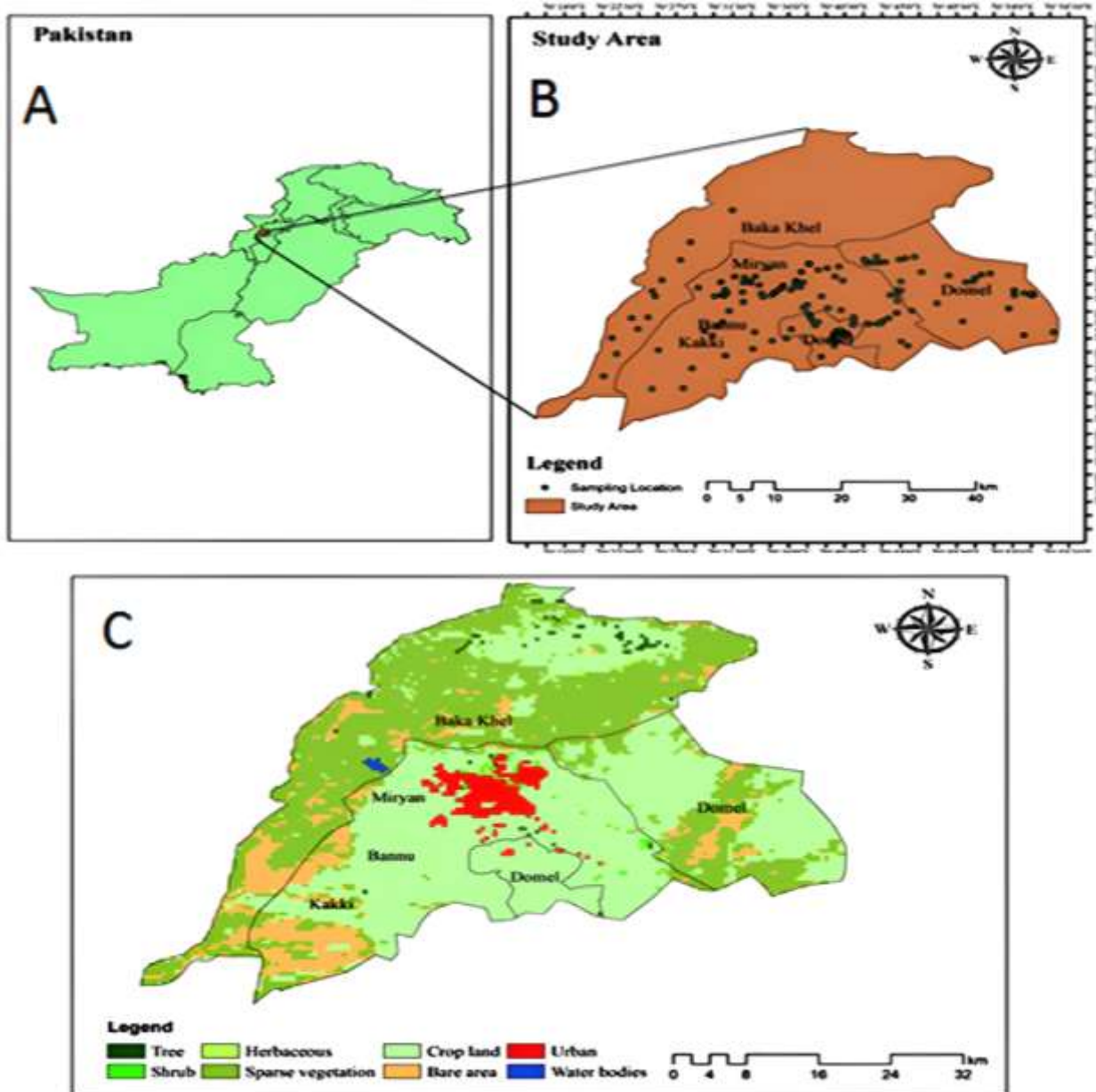


Fig. 1: Map of the study area (A) Pakistan (B) District Bannu and (C) District Bannu based on demography. Map generated using ArcGIS version 10.8.

**Isolation, sporulation, and morphological identification of *E. tenella* oocysts:** Identification of *E. tenella* oocysts was performed using basic fecal flotation and sedimentation as outlined by Ogedengbe *et al.* (2011). Briefly, saturated salt solution of sodium chloride was added to the fecal tubes and then centrifuged. Sediment was examined for oocysts with caution. Identification was by morphological differences in oocyst size based on criteria mentioned by Loo *et al.* (2022) to differentiate between *E. tenella* and other *Eimeria* species. Positive samples were sporulated and stored in 2% potassium dichromate solution for further analysis.

**DNA isolation from oocysts:** Genomic DNA was isolated from microscopy-positive and negative fecal samples stored in potassium dichromate with the QIAamp Stool DNA Extraction Kit (Qiagen), according to the manufacturer's instructions. DNA concentration was measured and extracts were kept at  $-4^{\circ}\text{C}$  until molecular examination (Carrisoza *et al.*, 2021).

**Molecular analysis:** The internal transcribed spacer 1 (ITS-1) region of genomic DNA was targeted for amplification. A 279-bp fragment specific to *E. tenella* was amplified using species-specific primers (ETF: 5'-AATTTAGTCCATCGCAACCCT-3' and ETR: 5'-CGAGCGCTCTGCATACGACA-3'), following the method described by Alam *et al.* (2022). The PCR reaction was carried out in a 30 $\mu\text{L}$  total volume containing 14 $\mu\text{L}$  of Blue Taq Master Mix (BioShop), 11 $\mu\text{L}$  of distilled water, 1 $\mu\text{L}$  each of forward and reverse primers, and 3 $\mu\text{L}$  of template DNA. The thermal cycling conditions began with an initial five cycles of denaturation at  $96^{\circ}\text{C}$ , annealing at an experimentally optimized  $60^{\circ}\text{C}$  and extension at  $72^{\circ}\text{C}$ , each for 1 minute followed by a final extension at  $72^{\circ}\text{C}$  for 10 minutes. This was followed by 38 additional cycles with the same parameters till the final extension at  $72^{\circ}\text{C}$  for 10 minutes. PCR products were resolved on a 2% agarose gel to confirm amplification of single bands, and positive

amplicons were submitted for commercial sequencing at Applied Biosystems (ABI), Singapore.

**Sequencing and phylogenetic analysis:** The PCR-amplified products were commercially sequenced using the Sanger method at Applied Biosystems (ABI), Singapore. The resulting sequence data were analyzed using BioEdit version 9 for chromatogram inspection, MEGA version 11 for phylogenetic analysis, and the NCBI BLAST tool for sequence similarity searches. The sequences were compared with those available in GenBank isolated from different species of *Eimeria* genus and from various *E. tenella* isolates. One *Toxoplasma gondii* (EU025025) isolate was used as an out-group. A total of 15 nucleotide sequences were submitted to the NCBI GenBank database under accession numbers PV844770 to PV844784. By using Mega11 software (Tamura *et al.*, 2013), the tree displayed exhibits the highest log likelihood (-2745.20). The initial trees for the heuristic search were generated by applying the Maximum Likelihood method to a matrix of pairwise distances. The phylogenetic tree was constructed using the topology that yielded the highest log likelihood score. Branch lengths represent the number of substitutions per site, and the tree is displayed to scale. Bootstrap support values ( $\geq 50\%$ ) are indicated at internal nodes, representing the proportion of sites with at least one unambiguous nucleotide present in at least one sequence within each descendant clade. A total of 58 nucleotide sequences were included in the analysis, incorporating all codon positions (1st, 2nd, 3rd) as well as non-coding regions, resulting in a final alignment of 1,395 nucleotide positions.

**Statistical analysis:** Data related to various risk factors was tabulated and analyzed for percentage prevalence using Statistix software version 9. Statistical analyses included One-Way ANOVA, Chi-square tests, and independent sample t-tests. A p-value of less than 0.05 was considered indicative of statistical significance.

## RESULTS

**Overall prevalence of *E. tenella* infection in different tehsils of Bannu district:** Our study revealed the overall prevalence of *E. tenella* infection in district Bannu is 34.37% (55/160). Analyzing different tehsils, the highest prevalence was estimated in Bannu 40% (16/40), followed

by Kakki 37.78% (17/45), Domel 28% (7/25), and Baka Khel 25% (5/20) (Table 1).

**Prevalence of *E. tenella* infection across chicken breeds in Tehsils of Bannu district:** In Tehsil Bannu, the prevalence rate was 40% (16/40), with Naked Neck chickens exhibiting the highest prevalence of 55.56% (5/9) and Australorp the lowest of 20% (1/5). In Tehsil Kakki, the overall prevalence was 37.78% (17/45 samples), with Aseel chickens showing the highest prevalence of 45.45% (5/11) and Australorp the lowest of 25% (1/4). In Tehsil Miryan, the overall prevalence of infection was 33.33% (10/30), with Naked Neck chickens exhibiting the highest prevalence of 50% (3/6), while Australorp chickens showed no infections. In Tehsil Domel, the overall prevalence was 28% (7/25), with Aseel and Naked Neck chickens having the highest prevalence of 33.33% (2/6), and Desi chickens showing the lowest of 20% (1/5). In Tehsil Baka Khel, the overall prevalence was 25% (5/20), with Golden Misri and Desi chickens demonstrating the highest prevalence at 33.33% (2/6), while Aseel and Australorp chickens were free of infections (Table 1).

**Prevalence of *E. tenella* in backyard chicken according to months:** Between April and September 2022, the prevalence of *E. tenella* showed noticeable variation throughout the six-month observation period. The peak prevalence was observed in August and September 2022, reaching 44 and 42.86%, respectively. This coincided with an average rainfall of 66mm (marked by flood conditions in the region), an average relative humidity of 64%, and a mean temperature of 88.9°F. In contrast, the lowest prevalence of 20% was documented in May 2022. During this month, the average rainfall was 21mm, the average relative humidity was 42%, and the mean temperature was 84.4°F (Table 1).

**Prevalence of *E. tenella* in Backyard Chicken according to different biotic risk factors:** Age-wise analysis revealed a prevalence of 13.63% (3/22) in chicks (0–8 weeks), 41.17% (35/85) in growers (8–16 weeks), and 32.07% (17/53) in adult birds (>16 weeks). A statistically significant difference in prevalence across age groups was observed ( $P=0.048$ ). The prevalence was 23.07% (6/26) in males and 36.56% (49/134) in females, with no statistically significant difference ( $P=0.186$ ) between genders (Table 2).

**Table 1:** Prevalence of *E. tenella* infection in different tehsils and months according to breeds

Factor	Classes	Breed (Positive/total, rate (%±SD))					Total (Infected/Total, %)	P value <sup>2</sup>
		Naked Neck	Golden Misri	Aseel	Desi	Australorp		
Tehsils	Bannu	5/9(55.56±0.325)	2/6 (33.33±0.376)	4/9 (44.44±0.325)	4/11 (36.36±0.284)	1/5 (20±0.350)	16/40 (40±0.150)	0.738
	Kakki	2/5 (40±0.429)	2/7 (28.57±0.335)	5/11 (45.45±0.294)	5/13 (38.46±0.264)	1/4 (25±0.425)	17/45 (37.78±0.141)	0.935
	Miryan	3/6 (50±0.399)	3/8 (37.5±0.335)	2/7 (28.57±0.335)	2/6 (33.33±0.376)	0/3 (0)	10/30 (33.33±0.168)	0.665
	Domel	2/6 (33.33±0.376)	1/4 (25±0.425)	2/6 (33.33±0.376)	1/5 (20±0.350)	1/4 (25±0.425)	7/25 (28±0.176)	0.985
	Baka Khel	1/4 (25±0.425)	2/6 (33.33±0.376)	0/5 (0)	2/6 (33.33±0.376)	0/1 (0)	5/20 (25±0.190)	0.636
	P value <sup>3</sup>	0.831	0.992	0.429	0.965	0.778	0.729	-
Months	April	1/4 (25±0.425)	3/5 (60±0.429)	1/3 (33.33±0.533)	2/5 (40±0.429)	1/3 (33.33±0.533)	8/20 (40±0.215)	0.858
	May	1/5 (20±0.350)	1/4 (25±0.425)	1/7 (14.29±0.258)	1/5 (20±0.350)	1/4 (25±0.425)	5/25 (20±0.156)	0.991
	June	2/6 (33.33±0.376)	1/6 (16.67±0.297)	1/4 (25±0.425)	1/4 (25±0.425)	2/5 (40±0.429)	7/25 (28±0.176)	0.930
	July	2/5 (40±0.429)	3/7 (42.86±0.366)	1/5 (20±0.350)	1/6 (16.67±0.297)	2/7 (28.57±0.335)	9/30 (30±0.164)	0.819
	August	1/4 (25±0.425)	1/5 (20±0.350)	1/5 (20±0.350)	1/7 (14.29±0.258)	1/4 (25±0.425)	11/25 (44±0.194)	0.991
	September	3/6 (50±0.399)	5/9 (55.56±0.325)	3/8 (37.5±0.335)	2/7 (28.57±0.335)	2/5 (40±0.429)	15/35 (42.86±0.164)	0.842
P value <sup>4</sup>	0.913	0.499	0.930	0.926	0.991	0.366	-	

**Abbreviations:** SD<sup>1</sup>: Standard Deviation; P-value<sup>2</sup> calculated between different breeds for each tehsil or month; P-value<sup>3</sup> calculated between different tehsils overall and for each breed; P-value<sup>4</sup> calculated between different months overall and for each breed.

**Prevalence of *E. tenella* based on abiotic factors:** The study examined *E. tenella* prevalence across different housing types (cages, night shelters, and free-range). The prevalence was 21.05% for caged chickens, 29.87% for night-sheltered chickens, and 43.75% for free-range chickens. The p-value of 0.096 indicated no statistically significant difference in prevalence between the housing types. Prevalence by feed type was also analysed with commercially fed chickens showing a prevalence of 11.11%, naturally or household-fed chickens displaying a higher prevalence of 40.54% and mixed-fed chickens exhibiting a prevalence of 22.50%. A statistically significant difference in prevalence ( $P=0.038$ ) across feed types was found (Table 2).

**Table 2:** Prevalence of *E. tenella* in backyard chicken according to different risk factors

Factors	Classes	Total	Positive Rate (%±SD) <sup>1</sup>	P value	
Age (weeks)	Chick (≤ 8)	22	3	13.63±0.143	0.048*
	Grower (> 8 and ≤ 16)	85	35	41.17±0.103	
	Adult (>16)	53	17	32.07±0.125	
Gender	Male	26	6	23.07±0.162	0.186
	Female	134	49	36.56±0.082	
Housing type	Cages	19	4	21.05±0.184	0.096
	Night shelter	77	23	29.87±0.101	
	Free range	64	28	43.75±0.121	
Type of feed	Commercial	9	1	11.11±0.205	0.038*
	Natural/household	111	45	40.54±0.092	
	Mixed	40	9	22.50±0.129	

Abbreviations: SD<sup>1</sup>: Standard Deviation; Chi-square test – \* $P<0.05$  significant.

**Breed-specific prevalence of *E. tenella* according to sexes of backyard chicken:** Among females, the Naked Neck breed had the highest infection rate at 43.75%, followed by Desi (37.50%), Aseel and Golden Misri (36.66 and 36.00%, respectively). The Australorp breed had the lowest female prevalence at 20%. For males, the Naked Neck breed again had the highest prevalence at 33.33%, followed by Golden Misri and Aseel (both 25%) and Desi (22.22%). Notably, no Australorp male chickens were found infected (Table 3).

**Table 3:** Breed-specific prevalence of *E. tenella* in overall and according to each sex of backyard chicken

Breed	Positive/total (rate, %± SD) <sup>1</sup>			P value
	Male	Female	Overall	
Naked Neck	1/3 (33.33±0.533)	14/32 (43.75±0.172)	15/35 (42.85±0.164)	0.731
Golden Misri	1/4 (25.00±0.425)	9/25 (36.00±0.188)	10/29 (34.48±0.172)	0.672
Aseel	2/8 (25.00±0.299)	11/30 (36.66±0.172)	13/38 (34.21±0.150)	0.541
Desi	2/9 (22.22±0.272)	12/32 (37.50±0.168)	14/41 (34.14±0.145)	0.398
Australorp	0/2 (0)	3/15 (20.00±0.201)	3/17 (17.64±0.180)	0.498
Total	6/26 (23.08±0.162)	49/134 (36.57±0.082)	55/160 (34.38±0.074)	0.186
P value	0.937	0.644	0.520	-

Abbreviations: SD<sup>1</sup>: Standard Deviation; One-sample t-test –  $P<0.05$  significant.

**Genetic analysis and phylogenetic study:** The observed SNPs in the *ITS-1* region of *E. tenella* revealed significant nucleotide variations across different samples (Table 4). Specifically, variations were noted at positions 108, 111, 123, 134, and 313, with distinct patterns of SNP presence. For instance, sample ETBFGFM 52 (PV844773) showed

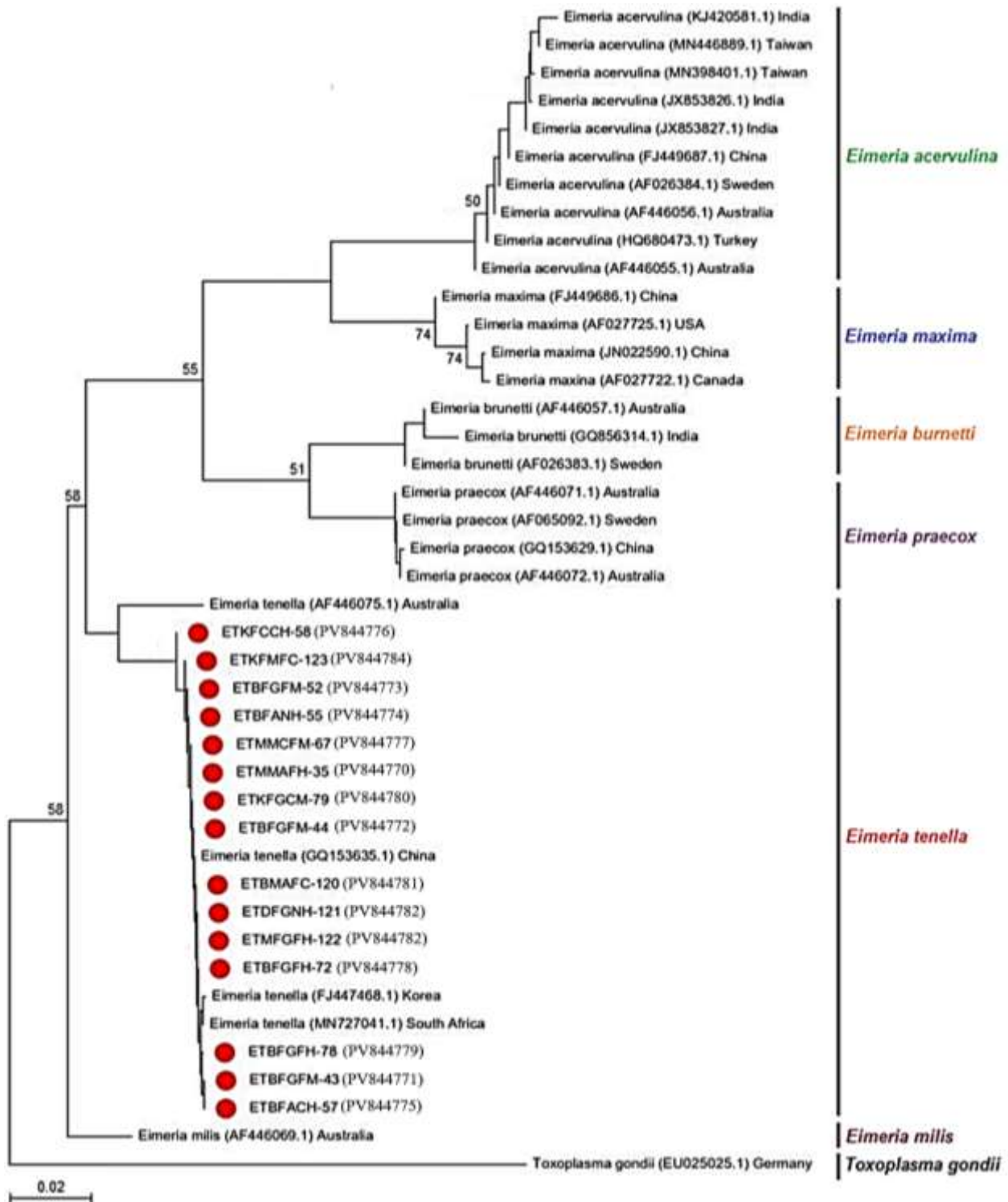
an adenine (A) at position 313, while ETBFANH 55 (PV844774) exhibited a thymine (T) at position 111. Other samples, such as ETMMAFH 35 (PV844770) and ETDFGNH 121 (PV844782), display guanine (G) and cytosine (C) at positions 134 and 111, respectively (Table 4). The phylogenetic tree incorporated a total of 27 isolates, where 15 originated from the present study and were highlighted by blue dots. These sequences formed a distinct cluster, closely related to *E. tenella* isolates from various countries, including Pakistan, India, China, Korea, Australia, the United States, and South Africa. Likewise, the phylogenetic tree included, in the first cluster, sequences isolated from *E. mitis* located in the USA, Australia, India, Sweden, and China. Partial sequences isolated from *E. maxima* were observed in the second cluster, representing different countries such as China, the USA, and Canada. The third cluster featured *E. acervulina*, identified in China, India, Taiwan, Australia, and Sweden. The fourth cluster included *E. brunetti*, found in Sweden, Australia, and India (Fig. 2).

**Table 4:** SNPs in different positions of *E. tenella ITS-1* region

Accession Number	Sample code	Nucleotide position of SNP				
		108	111	123	134	313
		T	A	C	G	T
PV844773	ETBFGFM 52	-	-	-	-	A
PV844779	ETBFGFH 78	-	-	-	-	-
PV844774	ETBFANH 55	-	T	-	-	-
PV844784	ETKFMFC 123	-	-	-	-	A
PV844772	ETBFGFM 44	-	-	-	-	-
PV844770	ETMMAFH 35	-	-	G	C	-
PV844780	ETKFGCM 79	-	-	-	-	-
PV844782	ETDFGNH 121	-	-	-	C	-
PV844771	ETBFGFM 43	-	-	-	-	-
PV844776	ETKFCCH 58	-	-	-	-	A
PV844781	ETBMAFC 120	-	T	-	-	A
PV844783	ETMFGFH 122	-	-	G	-	-
PV844775	ETBFACH 57	A	-	G	-	-
PV844778	ETBFGFH 72	-	-	-	-	-
PV844777	ETMMCFM 67	-	-	-	-	-

## DISCUSSION

Chickens are indispensable in ensuring food security at the world level, and yet the ubiquitous nature of coccidiosis remains an impending threat to poultry production systems globally (Mohsin *et al.*, 2021; Jamil *et al.*, 2022). The current study was conducted to evaluate the prevalence of *E. tenella* infection and to conduct phylogenetic analysis based on the partial *ITS-1* sequence of *E. tenella* in backyard chickens from District Bannu, Khyber Pakhtunkhwa, Pakistan. The results indicated the overall prevalence of 34.37% for *E. tenella*, and statistically significant differences were found to exist among various Tehsils. The prevalence is similar to that noted by Ahmed *et al.* (2025) in Malegaon poultry, India (35%). Other reports have reported prevalence rates of between 27.04% among Faisalabad broilers (Awais *et al.*, 2012) and 40.92% among Southern Punjab (Bachaya *et al.*, 2015). Rashid and Shanawa (2024) also reported a 25% prevalence among Soran City backyard chickens, Erbil, Iraq. Increased prevalence rates have been noted in broiler farms in multiple regions at 61% for Romania (Györke *et al.*, 2013), 46% for Hubei and Henan provinces, China (Geng *et al.*, 2021), 48% for Vojvodina province, Serbia (Pajić *et al.*, 2023), and 82.8% for Korea (Flores *et al.*, 2022). Differences in prevalence are expected due to



**Fig. 2:** Phylogenetic tree based on Maximum Likelihood method after alignment of partial sequences of *ITS-1* region isolated from different species of *Eimeria* genus.

differences in geography, sampling times, climatic environments, management systems, and diagnostic techniques.

Age-specific trends in infection were pronounced in this investigation, with growers having greater prevalence than adults and chicks. This finding is consistent with results from Gari *et al.* (2008) and Ashenafi *et al.* (2004), who indicated greater infection rates among growers (11.6 and 23.2%, respectively) than among adults (22.3 and 35.3%, respectively). Increased susceptibility in growers is most likely attributable to declining maternal immunity

prior to full adaptive immune establishment, making them more susceptible to infection. Age effect on prevalence of coccidiosis captures the dynamic nature of immune system development in poultry, with chicks being vulnerable owing to immature immunity but adults developing protective immunity through prior exposure (Györke *et al.*, 2013). This is corroborated by Muazu *et al.* (2008), who recorded greater infection rates in growers and chicks (52.9%) than adults (36.7%). On the other hand, research by Wondimu *et al.* (2019) found higher prevalence among young chickens. Together, these results

emphasize the necessity for age-specific control measures, especially for chicks and growers, which are prime windows of vulnerability.

The gender disparities were also evident, with females having greater infection rates than males. This concurs with Oljira *et al.* (2012), who found greater prevalence of coccidiosis in females (21.43%) compared to males (19.38%). Females' higher risk of exposure can be attributed to their eating habits, which pose a greater risk of ingesting oocysts that have sporulated in dirty water or dung. Moreover, female social interaction during feeding, in which the females would react to male calls, may expose them more. Nevertheless, other research (Alemayehu *et al.*, 2012; Amare, 2012; Gebretensae *et al.*, 2014; Garbi *et al.*, 2015) has documented male preponderance in prevalence, whose causes may be breed-specific behavior, environmental factors, or gender-sensitive management. Male chickens' searching or fighting behavior could increase their exposure in specific situations, whereas local husbandry and reproductive physiology may be factors in gender differences in susceptibility.

Housing conditions also contributed significantly towards infection rates. Free-range birds had the highest prevalence, whereas caged chickens had the lowest prevalence. This may be due to improved sanitation, controlled feeding, and lower environmental contamination in cage systems (Abbas *et al.*, 2011). In contrast, poor management practices in free-range and backyard systems including use of untreated water, feeding on the ground, inadequate cleaning of feeders and drinkers, and placement of water near perches that are likely to increase the risk of oocyst transmission (Silva *et al.*, 2022). Birds rose for market generally had lower prevalence due to better nutrition and care, whereas family-fed chickens were more exposed to contaminated, diverse diets. These findings highlight the need to enhance management and housing techniques to manage coccidiosis in backyard systems.

Seasonal patterns showed the highest prevalence in August and September, which can be attributed to good environmental conditions that facilitate oocyst sporulation and survival, thereby increasing transmission potential during these months. This seasonal pattern is also reflected by observations of Khan *et al.* (2006) and Pant *et al.* (2018), who also identified most coccidiosis occurrences in August and September. Weather impacts on coccidiosis prevalence are also seen in the findings of Awais *et al.* (2012), where autumn was identified as having increased disease occurrences in District Faisalabad, Pakistan. Together, the studies affirm the profound effect of climatic factors such as moisture and heat on the epidemiology of coccidiosis. Comparative molecular analysis in the present research involved diverged research work carried out worldwide. Notably, our findings reported a direct similarity with that done at UVAS Pakistan, as observed from shared accession numbers (MN830381 and MN830382). Although the small nucleotide variations were observed (Fig 2), overall similarity highlights the consistency of our findings with those that are being obtained in a different geographical region.

This study employed the Qiagen QIAamp DNA Mini Kit, a commercially available extraction kit recognized for producing high-quality DNA free from PCR inhibitors. The careful selection of target genes, specifically nuclear ribosomal DNA (DNA) was critical for ensuring the reliability of downstream genetic analyses. For phylogenetic reconstruction, the internal transcribed spacer 1 (*ITS-1*) region was selected as the molecular marker due to its widespread use and effectiveness in genetic and molecular studies (Alam *et al.*, 2022).

The investigation delved into the morphological characteristics of oocysts and life cycle phases, commonly utilized for studying diversity within *Eimeria* spp. Despite the prevalence of studies focusing on morphological aspects, limited research exists on population genetics and molecular diversity of *Eimeria* species (Gao *et al.*, 2024). There remains a critical need for fundamental molecular investigations within the poultry industry to comprehensively understand the population diversity of *Eimeria* species. Notably, much of the current research is based on a narrow range of well-characterized laboratory strains, many of which are over three decades old and primarily derived from European and North American populations, resulting in limited global representation (Becket *et al.*, 2009). Recent initiatives, such as field isolates from Taiwan (Lien *et al.*, 2007) and Australia (Lew *et al.*, 2003), contribute valuable ITS sequencing data on chicken *Eimeria*, addressing the need for broader molecular insights into the genetic diversity of this pathogen.

**Conclusions:** This research examined the prevalence of infection of *E. tenella* in backyard poultry of the Bannu district of Khyber Pakhtunkhwa, Pakistan and reported a high infection rate affected by the environment, including humidity and rainfall. Phylogenetic analysis of the *ITS-1* region showed genetic homogeneity of *E. tenella* isolates at the global scale. The prevalence varied across different tehsils, age groups, genders, housing types and feed categories, highlighting the diverse nature of coccidiosis and the heightened susceptibility of chickens, particularly among growers. Seasonal trends were observed, with peak incidence occurring in August and September, coinciding with elevated humidity levels. These findings enhance the understanding of *E. tenella* dynamics and provide valuable insights for developing control measures in Pakistani poultry farming.

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