



Molecular Identification of *Eimeria tenella* in Migratory Birds, Quails, and *Coturnix japonica* in Balochistan, Pakistan

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Abstract

Introduction: Coccidiosis is a global health issue in the poultry industry. It causes serious economic losses and huge treatment costs to farmers. The current study was conducted to detect *Eimeria tenella* in migratory Japanese quail (*Coturnix japonica*) in Balochistan.

Methods: In this study, a total of 300 quail fecal samples were used. Quails from Zhob, Sibi, and Quetta districts were captured using nets in September 2017 and 2018. Oocysts were detected by direct microscopy in a saline solution (0.85%), which were further identified by morphology and polymerase chain reaction (PCR) targeting mitochondrial CO-1 gene.

Results: Of 300 fecal samples, 218 (72.6%) were positive for oocysts. The length, width, and shape index of oocysts of *E. tenella* were $21.7 \pm 1.29 \mu\text{m}$, $19.5 \pm 1.9 \mu\text{m}$, and 1.27 ± 0.12 , respectively. The oocysts of *E. tenella* were further confirmed by PCR targeting mitochondrial CO-1 gene (723 bp).

Conclusion: It can be concluded that the migratory Japanese quails may be infested with *E. tenella*, and these birds may play roles in the transmission of this protozoan to domestic poultry.

Keywords: Detection, Japanese quail, *Eimeria tenella*, Oocyst, Steel beads, PCR, Domestic poultry

Received: January 28, 2021, Accepted: December 12, 2022, ePublished: December 31, 2022

Introduction

Quail is a general term for several genera of medium-sized birds belonging to the family Phasianidae. There are two important species, the Japanese quails (*Coturnix japonica*) and Bobwhites quails (*Colinus virginianus*) which have been considered domestic birds since the 14th century (1). Quail production is a branch of the modern poultry industry (2). The breeding of Japanese quails has excelled in aviculture, due to increased consumption of exotic meats and eggs. Japanese quail farming can be a substitute for chicken production (3). The brown-colored Japanese quail is used for commercial breeding (4). The natural habitat of Japanese quail is East Asia (5). It is migratory in nature (5). The wild Japanese quails are also available in South Eastern Siberia Manchuria, and the Korean Peninsula (5). It is also found in some parts of the north eastern regions of India. Quails live in grasslands and cultivated fields (5).

Commercial chicken production is an important sub-

sector of agriculture in Pakistan and plays a pivotal role in rural economic development. The estimated number of commercial chickens in the country in 2017 was 1022 million birds with a production of 17083 million eggs and 1 270 000 tons of meat, providing direct and indirect employment to over 1.5 million people (6). The growth of the livestock industry in low- and middle-income countries is determined by a rise in the total number of livestock, whereas carcass weight is the key indicator in high-income countries (7). In 2015, Pakistan was the 11th largest chicken producer in the world on the basis of number of birds produced (8). Since then, investment by private and public sectors has increased, from PKR 200 billion (US\$ 1.28 billion) in 2015 to PKR 700 billion (US\$ 4.47 billion) in 2018.

Parasitic diseases in poultry, such as coccidiosis, pose major challenges to the poor farming community both in tropical and subtropical regions of Pakistan. Coccidian parasite is mainly divided into *Isospora* and *Eimeria* (9).



Species of *Eimeria*, including *Eimeria tenella* and *Eimeria maxima*, and *Isospora* cause coccidiosis in chickens, quails, and pigeons (10). *E. tenella* is the best known host-specific protozoan parasite of the genus *Eimeria* that causes coccidiosis in poultry (11). However, some experimental studies have shown that this host-specific coccidia can sexually develop in the intestine of Japanese quail (12). The predilection site of *Eimeria* is the caecum. It causes bloody diarrhea which results in severe morbidity and mortality with major economic loss (11). Birds infected with *Eimeria* are considered potential sources of infection for other birds as they excrete oocysts in their feces (13). Infection with *Eimeria* spp. has been reported to have a high mortality rate in poultry. *E. tenella* is the best known coccidian species causing huge economic losses in commercial broilers and mortality in layers (14). It is estimated that annually more than \$300 million are spent to control coccidiosis in poultry (15). In countries such as Pakistan, where farming is substandard, the disease is more serious and causes heavy economic losses. Although the exact losses due to coccidiosis in Pakistan are not known due to the lack of statistical indices, millions of rupees are lost every year.

The conventional microscopic method is less specific and less reliable to differentiate the species of *Eimeria* due to similar morphology (16,17). Molecular techniques are more reliable, rapid, and cost-effective for the identification of species (18). 18S rRNA gene has been used for species identification of *Eimeria*. Furthermore, partial sequences of 18S rRNA gene used for phylogenetic analysis can further characterize *E. tenella* (18).

Pakistan needs more animal protein to feed its growing human population; hence, the demand for poultry products is at its peak to fulfill the requirements (19). Among other obstacles to increased commercial poultry products, parasitic diseases are of great importance (15). Coccidiosis results in huge economic losses to the poultry industry annually (20). Published data shows that coccidiosis is a threat to quails reared commercially (21). However, there is no data available about the occurrence of coccidiosis caused by *E. tenella* in migratory quails in Balochistan. This study aimed to determine the occurrence of *E. tenella* in migratory quails captured from areas in the migratory route of quails.

Materials and Methods

Animal Ethics

This experiment was approved by the Balochistan Forest and Wildlife Department, Quetta. All birds were trapped following the protocols of the Wildlife Department of Balochistan from 2017 to 2018. The permission code was 127.

Sampling Method

The present study was conducted in September 2017 and

2018. Quails were captured using Ball chatri traps (22) from Pishin, Zhob, and Sibi districts of Balochistan. The trapped quails were transported to CASVAB, University of Balochistan, Quetta, and kept in individual cages. The birds were fed and watered in the cage. No anticoccidial agent was given to the birds during the study. Fresh fecal samples were collected in the morning in sterile polythene bags and stored at 4 °C.

Microscopy for Detection of Oocysts

Fresh fecal samples were examined by direct smear method (23) and fecal flotation techniques (24). Positive samples were centrifuged for 8 minutes at 13 000 g in a 15 mL tube followed by washing with distilled water. Then, the oocysts were purified by salt flotation technique as described in a previous study (25). The purified oocysts were sporulated in 2.5% potassium dichromate solution and kept at room temperature for one week. The sporulated oocysts were washed with distilled water prior to morphological analysis. The rest of the oocysts were stored at 4 °C for further studies and DNA extraction.

Morphology of Oocysts

Oocysts were examined microscopically as described previously (26). A compound microscope (MC 30, at 20X lens Poland) with an apochromatic oil immersion objective lens and an ocular micrometer (K-15X PZO) was used for morphological observations and measurements Figure 1. For measurement of oocyst size, an Olympus BX 51 microscope equipped with an Olympus DP71 camera and software Image-Pro Express 6.0 were used (27).

Oocyst and its Shape Index

The oocyst shape index was determined by measuring its length and width using a calibrated ocular micrometer at 20X magnification (28). The size of the oocysts was compared with established standards described

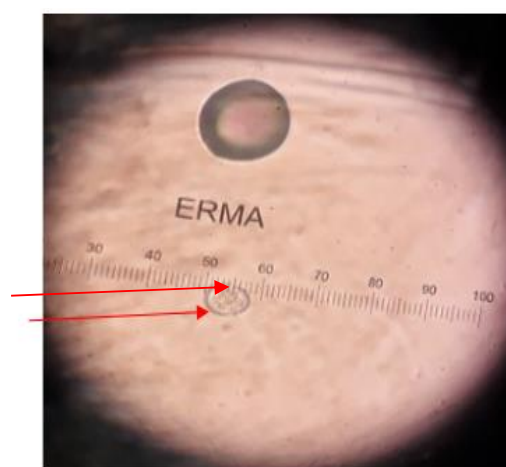


Figure 1. Size of the Oocyst (21.7 µm)

previously (29).

DNA Extraction

Step 1: Oocyst Lysis

Twenty to thirty oocysts were washed three times with phosphate-buffered saline (PBS) in a 1.5 mL centrifuge tube and centrifuged at 8000 rpm for 5 minutes (Sigma Aldrich Model 1-14). The supernatant was discarded and the pellet was resuspended in 40 μ L of sodium hypochlorite (NaOCl, 7%). After a gentle vortex and incubation for 2 hours at 4 °C, the mixture was mixed with 30 μ L of saturated salt solution (SSS) and incubated at 55 °C for 1 hour. Finally, 300 μ L of TE buffer (10 mM Tris HCL, 0.1Mm EDTA, pH 8), 3 μ L of 10% SDS, 10 μ L of proteinase K (Sigma Aldrich), and 2 steel beads (a weight of 26.14 mg and a size of 3 mm) were added. The mixture was vortexed at 800 rpm for 1 minute and examined microscopically for lysis of the oocyst wall. In case of no lysis of the oocyst wall, the mixture was further incubated at 37 °C for 1 hour and vortexed at 800 rpm for 1 minute.

Step 2: DNA Extraction

DNA was extracted using a tissue kit following the manufacturer's instructions (GeneAll Exgene mini cat No.104-101 Korea). DNA was purified using kits following the manufacturer's instruction (GeneAll Exgene Tissue Kit mini cat No.104-101 Korea). The quality and concentration of DNA were measured by a NanoDrop spectrophotometer.

PCR and Sequencing for Species Identification

A PCR fragment of the mitochondrial CO-1 gene was amplified for identification of *E. tenella* using *Eimeria*-specific primers. The set of primers used to amplify a product of 723 bp were: forward primer 5'GGT TCA GGT GTT GGT TGG AC'3 and reverse primer 5'ATT CCA ATA ACC GCA CCA AG'3. A thermal cycler (Model GS 5482) was used for DNA amplification. PCR reaction cocktail with a final volume of 25 μ L contained 11.5 μ L of master mix, 1 μ L of each primer, 5 μ L of DNA, and 6.5 μ L of ddH₂O. PCR conditions previously optimized consisted of an initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of 30 seconds at 94 °C, annealing at 62 °C for 30 seconds, extension at 72 °C for 1 minute, and final extension at 72 °C for 10 minutes (30). The amplified PCR products were electrophoresed using 1.5% agarose gel stained with ethidium bromide, visualized through transillumination device, and photographed. Gel electrophoresis was performed at 120 V for 30 minutes. A 100 bp DNA ladder was used to estimate DNA fragment size.

PCR product band was cut using a blade, put in a 1.5 mL tube, and purified using a purification kit (Gene All Exgen™, Gel SV, 100P Cat No; 102-101) following the

manufacturer's instructions. PCR amplicons of CO-1 were sent to Advance Bioscience International based in Singapore for partial sequencing. The sequence data were compared with *Eimeria* sequences publically available on NCBI GenBank using BLAST. Percentage of similarity with the reference sequences and query coverage were recorded. Multiple sequence alignment was performed using BioEdit version 7.2.5. Mega software (Mega 6) was used to reconstruct the phylogenetic tree using maximum likelihood estimation (31).

Statistical Analysis

Data were entered in Microsoft Excel version 13.0 and SPSS version 20.0 was used for data analysis. Descriptive data was presented as frequencies and percentages. The size of the oocysts was presented as mean and standard deviation.

Results

Morphological Identification of *Eimeria Tenella* Oocysts

Unsporulated coccidian oocysts were seen in microscopic examination of the feces. A total of 100 oocysts were randomly photographed and it was revealed that oocysts of *Eimeria tenella* were ovoid, smooth, and colorless. There was no micropyle or residuum but a polar granule was present. The sporocyst had a Stieda body (Figure 2). *E. tenella* had a length of $21.7 \pm 1.29 \mu\text{m}$, a width of $19.5 \pm 1.9 \mu\text{m}$, and a shape index of 1.27 ± 0.12 . Oocysts of other parasites including *Isospora* were also seen in this study. However, further identification was not made because other parasites were not included in this study. Oocysts of *E. tenella* were further characterized by PCR targeting mitochondrial CO-1 gene (727 bp in size) (Figure 3).

Nucleotide Sequencing

BLAST results obtained in the current study showed similarity (98%–99%) to *E. tenella* (JX853830.1) available publicly in NCBI GenBank. The BLAST results indicated that the sequence obtained in the current study had high similarity with the sequence of *E. tenella* isolated from chicken (accession no. KX094951.1). The Phylogenetic analysis showed that the sequence of *E. tenella* obtained in the current study was closely related to *E. tenella* isolates (accession no. KX094951.1). Phylogenetic analysis revealed that *E. tenella* isolated from Japanese quail was closely related to *E. tenella* isolated from chicken (Figure 4).

Discussion

Coccidiosis caused by *Eimeria* species is a global issue in the poultry industry (15). Coccidiosis, as one of the most prevalent infections in poultry, is transmitted mechanically through the movement of infected birds, as well as personnel moving between pens, houses, or farms (15). The role of migratory birds in the spread of pathogens (viruses, bacteria, and parasites) is well

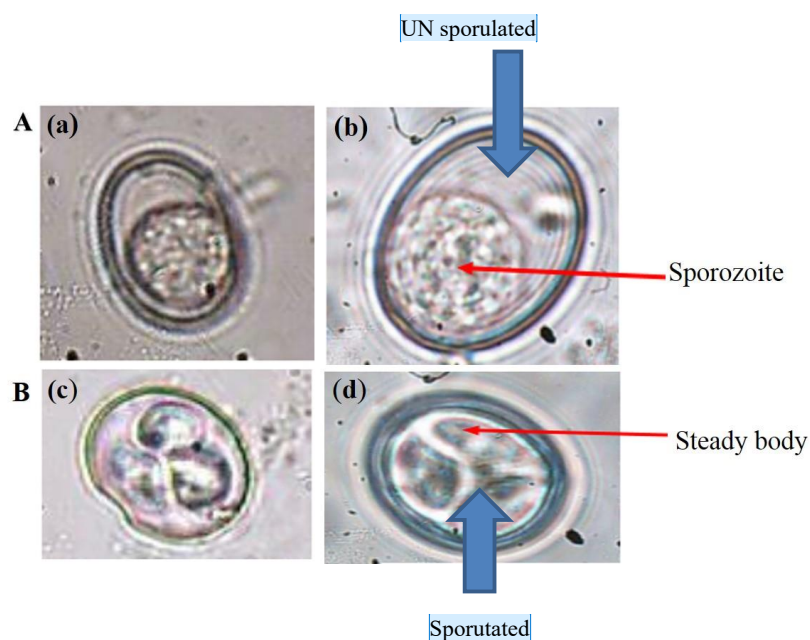


Figure 2. Morphology of *E. tenella* Oocysts. Stages of the Oocysts: A. Unsporulated Oocyst: (a) Microscopic View of Unsporulated Oocyst at 20X Magnification, (b) Microscopic View of Unsporulated Oocyst at 40X Magnification; B. Fully Sporulated Oocyst with Four Sporocysts each with Two Sporozoites, (c) Microscopic View of Sporulated Oocyst at 20X Magnification, (d) Microscopic View of Sporulated Oocyst at 40X Magnification

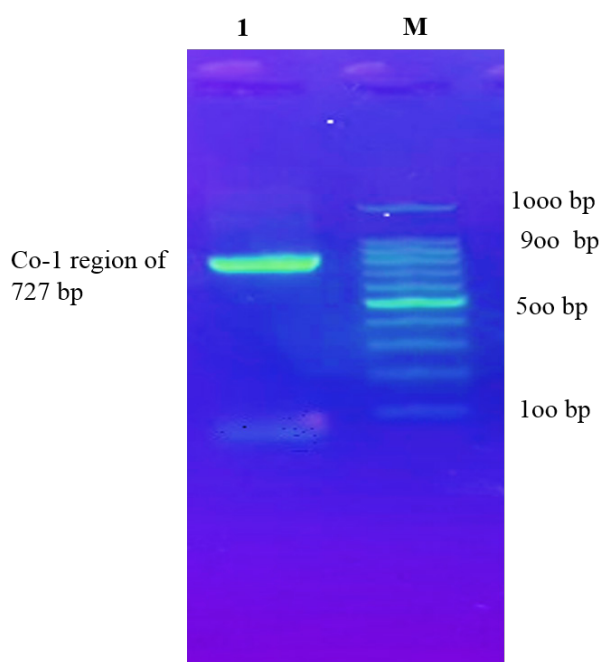


Figure 3. PCR Image of *Emiria tenella*. Lane 1 shows the amplification of CO-1 727 bp region of *Emiria tenella*. M shows the DNA marker 1000 bp

established (32). However, migratory strategies and behaviors potentially affecting the transmission of infections are difficult to study (33). The current study examined the dynamics of infection with the protozoan parasite *Eimeria* (coccidian) through the annual cycle of a long-distance migratory wild quail dispersing pathogen (34). Results of the current study showed that oocysts of *Eimeria* spp. were present in 72.2% of fecal samples

from migratory Japanese quails. The findings of this study are in line with those of a previous study (25), which reported the identification of *E. tenella* in 49.9% of Japanese quails in Mosul, Iraq. Another study also reported the presence of *E. tenella* in 52% of Japanese quails in Azerbaijan. The difference in the percentages might be due to managerial conditions, seasonal fluctuations in biotic factors, and exposure of migratory birds to anticoccidial drugs (35). The findings of the current study show that migratory quails may play a potential role in the dispersion of *Eimeria* spp. It is thought that this study may be the first study reporting natural coccidial infestation in quails.

Coccidia and *Isopora* in poultry are host-specific (9). However, the current study was successful in isolating *E. tenella* from migratory quails. Isolation of *E. tenella* from quails is in line with previous studies (12,36,37), which reported that *E. tenella* can sexually develop in the embryo of Japanese quail. They isolated *E. tenella* from Japanese quail embryos and mature oocysts were seen in the quail embryo after 7 days. Oocysts isolated from embryos were also sporulated. Furthermore, in another study (36), *E. tenella* isolated from diseased chickens and *E. kofidi* isolated from diseased young quails were studied. Both *Eimeria* spp are host specific to chicken and quails, respectively. The oocysts of *E. tenella* and *E. kofidi* were introduced to quails and chicken, respectively. The authors reported that sporulated oocysts of both species of *Eimeria* developed to shizogone in nonspecific hosts. In another study (12), it was reported that *E. tenella* equally infect chicken and quails. Chicken and quails

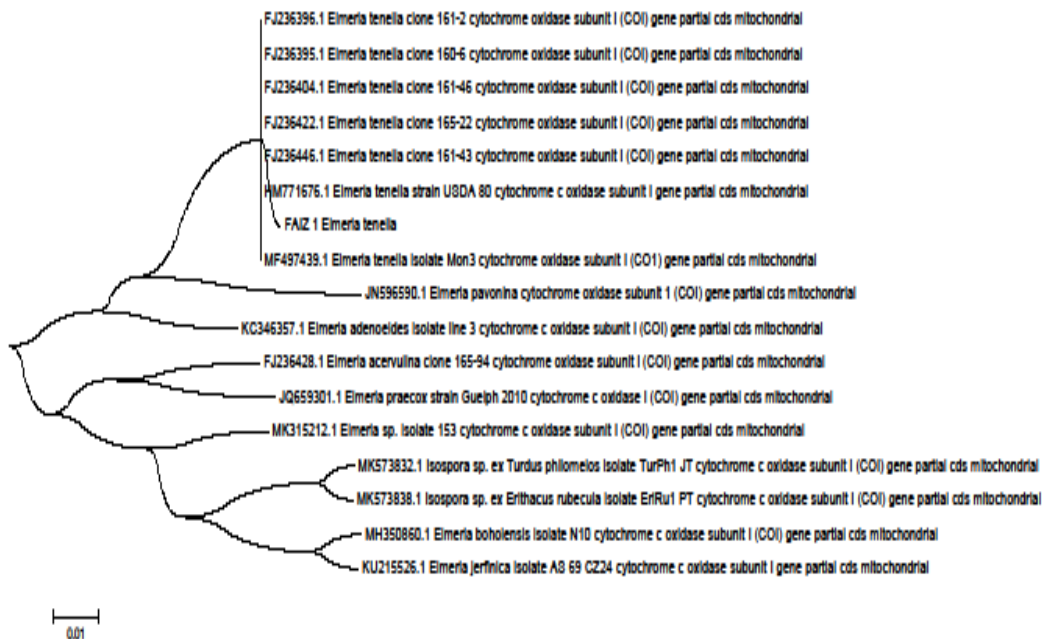


Figure 4. Phylogenetic Analysis of *Eimeria Tenella*

hybrid were orally inoculated with chicken coccidia like *E. tenella*, *E. acervulina*, and *E. maxima* and quail coccidia *E. bateri*. The chicken and quails hybrid were infected with chicken and quail coccidia passing oocysts in feces during normal latent period and control chicken and quails were severely infected.

Interestingly, a novel method of DNA extraction was optimized in the current study. DNA extraction from oocysts of *E. tenella* has been a challenge due to its thick and resistant oocyst wall. Several methods including sonication (11), phenol chloroform method, enzyme digestion through a high pressure cell (38), freezing and thawing, grinding in liquid nitrogen (39), and grinding by glass beads (17) have not shown satisfactory results. In the current study, the glass bead beating method was modified by replacing glass beads with steel beads which showed satisfactory results. It may be due to the weight of the steel beads which successfully ruptured the resistant and thick oocyst wall. However, further evaluation of this DNA extraction method may be required.

Furthermore, CO-1 genes were targeted for the identification of *Eimeria* species in the current study. Mitochondrial CO-I gene has been the most widely used genetic target in animal barcoding and has been found to be useful in species identification on a large scale (40,41). The ITS regions can be used to determine the species of *Eimeria*; however, there are multiple copies of these regions in the *Eimeria* genome with varying sequence lengths in a single oocyst. A notable strength of the current study is the use of mitochondrial CO-I as a marker in the epidemiological study of vector–host interactions. Our observations also suggest that the mitochondrial CO-1 gene is useful for species identification and phylogenetic

investigations of coccidia.

Conclusion

This study concludes that *E. tenella* may not be host-specific. It can parasitize other species of birds such as Japanese quail (*Coturnix coturnix japonica*). Molecular methods are best used for species identification of *Eimeria*. Furthermore, migratory quails may be potential risk factors for the transmission of *E. tenella* to local and commercial chickens along the migratory route.

Acknowledgements

The authors are thankful to the staff of Molecular Parasitology Laboratory, Department of Parasitology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan, for the laboratory facilities and their technical help during the study.

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Supervision: Faizullah Khan, Muhammad Masood Tariq, Asma Zafar.

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Competing Interests

The authors declare that they have no competing interests.

Funding

This study was supported personally by Faizullah, PhD candidate.

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