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Original Article

Morphological and molecular evidences of *Ascaridia galli* in migratory quail Coturnix *coturnix japonica* from Baluchistan Pakistan

Evidências morfológicas e moleculares de *Ascaridia galli* em codornas migratórias *Coturnix coturnix japonica*, do Baluchistão, Paquistão

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Abstract

The current study was conducted to examine the point prevalence of gastrointestinal parasites of migratory quails. Due to its economic importance, the control of ascaridiosis is critical. Migration of birds is considered to enhance the global spread and cross-species transmission of pathogens. The current study was aimed to detect *A.galli* in migratory quails, a potential contributory risk factor for transmission of this parasite to local birds. A total of 230 migratory quails were trapped using nets from migratory routes in Balochistan and examined under the compound microscope for the presence of *A. galli*. Conventionally, *A. galli* was identified by its morphology with the presence of three large lips and absence of posterior esophageal bulb. Results revealed that out of 230, 120 (52.17%) quails were positive for *A. galli* by targeting COX1 gene (533 bp) by using conventional PCR. Further, the amplicon was sequenced which showed 99% similarity with *A. galli* publically available in NCBI Gen Bank. Phylogenetic analysis of sequences of our isolated parasite indicated the close relationship with *A.galli* isolated from chickens. In conclusion migratory quails and other migratory birds may play a key role in spreading and transmission of these parasites and other pathogens to domestic chicken. Therefore, strict biosecurity measures should be adopted especially for commercial poultry farms.

Keywords: migratory quail, *A. galli*, morphology, molecular characterization.

Resumo

O presente estudo foi conduzido para examinar a prevalência pontual de parasitas gastrointestinais de codornas migratórias. Devido à sua importância econômica, o controle da ascaridiose é fundamental. Considera-se que a migração de aves aumenta a disseminação global e a transmissão entre espécies de patógenos. O presente estudo teve como objetivo detectar *A. galli* em codornas migratórias, um potencial fator de risco contributivo para a transmissão desse parasita para aves locais. Um total de 230 codornas migratórias foi capturado, usando redes de rotas migratórias no Baluchistão e examinadas sob o microscópio composto para a presença de *A. galli*. Convencionalmente, o *A. galli* foi identificado por sua morfologia com a presença de três grandes lábios e ausência de bulbo esofágico posterior. Os resultados revelaram que de 230, 120 (52,17%) codornas foram positivas para *A. galli* por direcionamento do gene COX1 (533 pb) usando PCR convencional. Além disso, o amplicon foi sequenciado, que mostrou 99% de similaridade com *A. galli* publicamente disponível no NCBI Gen Bank. A análise filogenética das sequências do nosso parasita isolado indicou a estreita relação com *A. galli* isolado de galinhas. Em conclusão, codornas migratórias e outras aves migratórias podem desempenhar papel fundamental na disseminação e transmissão desses parasitas e outros patógenos para as galinhas domésticas. Portanto, medidas rigorosas de biossegurança devem ser adotadas, especialmente para granjas comerciais.

Palavras-chave: codornas migratórias, *A. galli*, morfologia, caracterização molecular.

1. Introduction

During the winter season, a high diversity of birds migrate to wetlands in Pakistan, a middle Asian flying route for the migratory birds (Umar et al., 2018). Birds migrate due to seasonal changes, availability of food and to avoid threat of predation (Lank et al., 2003). Balochistan, with an area of 350,000 sq. km, is the largest province. The geographical coordinates include 24.32N and 60.70E. The mountainous ranges exist in

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the eastern and northern part of province. Mountains of about 7000 ft height are also found. While, Valleys are situated above 1500 m of sea level. The variety of biodiversity, province is quite rich in wildlife. (Syed Ali Ghalib et al., 2019). The diversity of migratory birds include houbara bustards (Chlamydotisundulate), cranes (Gruidae), teals (Anas crecca), pintails (Anaa), mallards (Anas platyrhynchos), geese (Anser), spoon bills (Platalea), waders (Cladorhynchus leucocephalus), pelicans (Pelecanus) and quails (Coturnix coturnix are included in migratory bird Faiza (2022) ilyas. Birds migrate mainly from Northern Arctic region towards Southern plains (Umar et al., 2018) and spend winter season in tropical areas (Baillie and Peach, 1992), where they breed too. However, birds also carry different pathogens like bacteria, virus, protozoa and parasitic helminths that can spread in areas of migration. (Mihaela and Marina, 2014).

Migratory quails may be reservoirs for a wide array of zoonotic pathogens, including bacterial, viral, mycotic and parasitic ones, they act as either healthy carrier or host for such pathogens (Benskin et al., 2009). Quail can also be a reservoir for many enteric pathogens like *Campylobacter* spp. and Shiga toxin-producing *Escherichia coli* (Dipineto et al., 2014). Threatening outbreaks like a recent opinion about the spread of lumpy skin disease (LSD) in various Asian countries where the ticks carried by the migratory birds are thought to be behind the recent spread of LSD in Asia. (Shahaan Azeem et al., 2022)

Nematodes are the most important group of helminth parasites in poultry that can pose a hazard for commercially raised birds. *A. galli* may inhabit in various organs such as intestinal lumen, esophagus, crop, gizzard, oviduct and body cavity (Thapa et al., 2015). Infection of *A. galli* in chicken is augmented by various clinical signs such as loss of body weight, drooped wings, stunted muscular and osteological development, anorexia and increased mortality (Ackert and Herrick, 1928; Dahl et al., 2002).

The prevalence of *A galli* as revealed by numrouse studies, ranges from (22-84%) of total parasite load (Martin-Pacho et al., 2005) parasitic infection may also favors various secondary bacterial infection (Permin et al., 2006) to different birds that may render the bird more vulnerable to more serious infection. They can act as vectors and lead to secondary infections, E.coli (Chadfield et al., 2001; Dahl et al., 2002). Understanding the host-parasite relationship is a necessary prerequisite for the development of parasite control strategies. Moreover, morphological and genetic identification of parasites may be useful for analyzing the transmission potential of parasitic infection to domestic birds. Migratory birds infested with *A. galli* are considered potential carriers and can contribute to the spread of verminosis, include *Ascaridia galli Heterakis gallinarum* and various *Capillaria* species (Leeson and Summer, 2009) However, no published data is available about *A. galli* infestation of migratory quails in Baluchistan. This study was designed to evaluate the prevalence of *A. galli* in migratory quails using morphological and molecular techniques.

2. Material and Methods

2.1. Study area and collection of birds

The present study was conducted in Quetta, Pishin, Zhob and Sibi districts of Balochistan focusing the migratory routes of quails. Where migratory quails were commonly trapped, captured. Using ball-chatri techniques these are traps designed to catch birds of prey or shrikes. The cage is constructed using mesh wire with nylon nooses on top. Inside the cage, a visible live rodent, small bird or pigeon is placed as bait. The bird of prey that attacks the bait will be snared by its legs Bird Life International (2020). The Districts are favorable for breeding of migratory birds in winter. These birds change their regions as well as the host thus dispersing the parasites in new region and play important role as a potential source of spreading ecto, endo and protozoan parasites to other birds, especially at the stopover places where the parasite attaches and detaches. A total of 230 migratory quails were trapped during migratory seasons. The first phase of Present study was conducted from March to April of the years 2019-20 while second was from September to October. The species were identified by using field guides (Ali and Ripley, 1978).

The birds were slaughtered and intestine along with the contents were collected in sterile polythene bags to recover *A. galli*. Fecal samples were grossly examined for the presence of parasitic infestation. To estimate the actual worm burden, each sample was counted separately.

2.2. Morphological identification of A. galli

Adult worms were collected and washed in phosphate buffer saline (PBS).The washed worms were preserved in 90% ethanol. Olympus stereomicroscope SD30 (Germany), was used to collect and identify the worms according to their morphological characteristics. The permanent slide was prepared by adding glycerol, followed by fixation, staining dehydration, clearing and mounting. Recovered parasite were identified as *A. galli* according to the keys given by Soulsby (1982). Light microscopy was conducted under Olympus compound microscope model no. BX 41TF (Tokyo Japan) with the lens of 7X.10X and 15X in eye pieces and 4X,10X,20X and 40X objectives. The drawing for identification were made to scale with help the of prism camera Lucida (Dariya Ganj, New Delhi India) The photographs was taken with the help of Sony digital SLR Camera model no. DSLR-model no. Sony A 200 (USA). Photomicrography were conducted with digital camera model PK-5 part 1507 (FCC China) attached with Olympus microscope (Tokyo Japan) in the Department of Parasitology CASVAB Quetta.Taxonomic keys, showing morphological points such as mouth including 3 strong lips (a dorsal and two sub-ventral), club shaped esophagus with no posterior bulb were used (Rahman et al., 2009; Rahman and Manap. 2014). Worms were preserved at -80 for further DNA extraction. The prevalence of roundworms was recorded as per formulae described by Margolis et al. (1982) (Equation 1).

$$\text{Prevelence} = \frac{\text{Total number of hosts infected}}{\text{Total number of hosts examined}} \times 100 \quad (1)$$

2.3. Statistics analysis

Data was entered in Microsoft Excel (version 13.0) and SPSS (version 20.0) was used for data analysis. Descriptive data was presented as frequency tables and percentages. Sizes were presented as mean and standard deviation.

2.4. DNA extraction and quantification

Small pieces of adult worms were made, washed twice in distilled water, centrifuged sigma 1-44 (Germany) at 8000 g for 5 minutes and incubated overnight at -80. The specimen were then treated with liquid nitrogen and grinded in a sterile pestle and mortar. The grinded material was collected in 1.5 ml Eppendorf tubes, added lysis buffer solution, and incubated overnight at 56°C in rotatory shaker water bath D.91126 (Germany). The genomic DNA was extracted using Gene All Exgene™, tissue SV minikit (biotechnology South Korea) following manufacturer's instructions. Final elution of the genomic DNA was made by using 50 µl of elution buffer and finally quantified by Nanodrop c (thermo Scientific) spectrophotometer.

2.5. PCR amplification

DNA was amplified using the following pair of primers previously designed; Forward primer; F-5' ATT ATT ACT GCT CAT GCT ATT TGA TG-3', Reverse primer; R- 5' CAA AC AAA GTG TTA AATCAAAGG-3 (Katakam et al., 2010) using PCR Model 2720 | Foster City, CA 94404 (USA) thermo cycler. Reaction volume of 25 µl was standardized with master mix 11.5 µl, each primer 1 µl, DNA template 5 µl and RNAse free water 6.5 µl. The cycling conditions were adjusted at 95 °C for 15 min as initial activation of Taq polymerase followed by 35 cycles of 95 °C for 30 sec as denaturation. Annealing temperature was adjusted at 55 °C for 40 sec, extension was made at 7 2°C for 1 min and final extension of 10 min at 72 °C (Katakam et al., 2010). The PCR products were observed by 1.5% agarose gel with electrophoresis for 35 min at 120 V.

2.6. Purification of PCR products

PCR product was placed in 1.5 ml tube and purified using kit (Gene All Expain™ Gel SV, 200 P, and Cat No. (102-202 South Korea). Added 300 µl buffer solution and finally added 30 µl elution buffer to obtain concentrated DNA according to the kit protocol.

2.7. Sequencing and BLAST

The purified PCR product was sent to ABI for sequencing the sequences were analyised by using BLAST software where we used Maximum Likelihood method, Phylogenetic tree was constructed using the BLAST results.

3. Result

3.1. Morphological identification of A. galli

A.galli was found in the gastrointestinal tract of 52.17% of the 230 quails collected. Morphological points such as mouth, 3 strong lips (a single dorsal and two sub-ventral), and.club shaped esophagus without posterior bulb and lateral alae were observed. In males Caudal alae was either poorly developed or absent. Spicules were almost equal in size, caudal papillae were relatively large and Pre-anal sucker was also present. The females were 60-116 mm long and 90 µm 1.8 mm wide the vulva is the interior part of the body. Worms were compared for further morphological identification (Table 1).

A. galli are large thick, yellowish white worms their head has three large lips, may be found in the lumen of the intestine. These parasites were isolated from small intestines of quail. Important taxonomic pointes of *A. galli* are shown in Figure 1.

3.2. Molecular characterization

PCR targeting COX1 gene, 500 bp, identified as *A. galli* in migratory quails (Figure 2). The PCR amplicon was sequenced and BLAST analysis revealed 90% similarity with *Anisakidae* and *Ascarididae* families that fall in order *Ascaridida*. In current study the sequence obtained depicted 99% matching with that of *A.galli* recovered from poultry.

3.3. Nucleotide sequencing and phylogenetic analysis of A. galli

BLAST analysis revealed 88%-90% genetic identity with *Anisakidae* and *Ascarididae* families that also fall in the order *Ascaridida*. In the current study the sequence obtained depicted 90% matching with that of *A. galli* of poultry. The phylogenetic tree was generated through Maximum Likelihood (ML) algorithm with the KP-2 model.

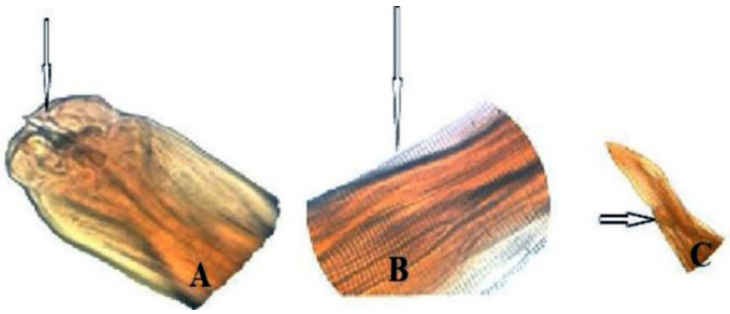

Figure 1. (A) Male mouth parts of *A. galli* (arrow showing the two lips) one lip is behind; (B) Mid portion of male; (C) Preanal sucker in tail region.

Table 1. Measurement of *A. galli* comparative characteristics.

	Year	Body length (Milli Meter)		Body width (mm)		Esophagus length (mm)		Esophagus width (mm)		Tail length (mm)		Spicule length	Pharynx length (µm)	
-	-	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	-	Male	Female
Kates and Co lglazier	1970	6o-65	80-100	-	-	-	-	-	-	-	-	1.5-2.4	-	-
Tanveer	1989	10/dez	15-17	0-4-0.7	0.7-0.9	-	-	1.5-2.4	0.7-0.9	0.32-0.53	01/mai	1.2.7	-	-
Aziz	1992	45.3-68.4	80-109	0.79-1.18	1.38-1.50	-	0.45-0.55	3.88-4.34	1.38-1.50	0.68-0.86	1.35-1.4	2.20-2.52	-	-
Ramadan	1992	42-76	72-108	0.56-91	0.90-1.80	0.28-0.59	0.38-0.49	2.48-5.32	0.90-1.80	0.57-0.78	-	-	-	-
present study	2020	45-48	59-90	0.5-0.7	0.7-0.9	-	-	1.95-2.1	0.7-0.9	0.90-0.99	220 320	3.50	140-190	1250

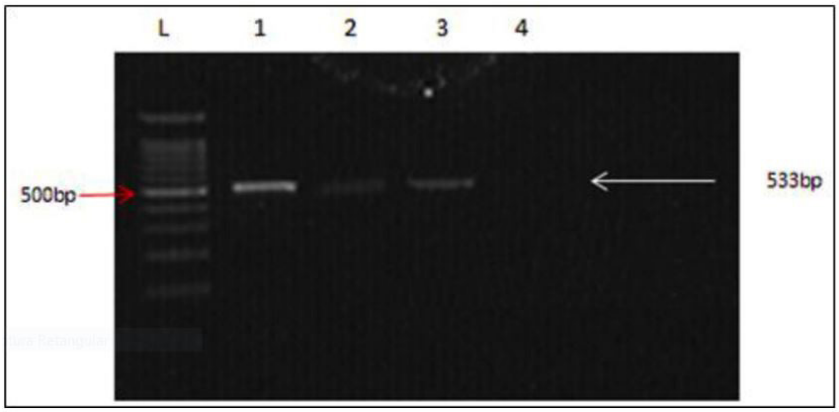

Figure 2. PCR (COX1) product of *A. galli*. L: 100 bp molecular marker; Lane-1: positive control; Lanes-2 and 3 partial COX1 amplified products; Lane-4: negative control Phylogenetic Tree.

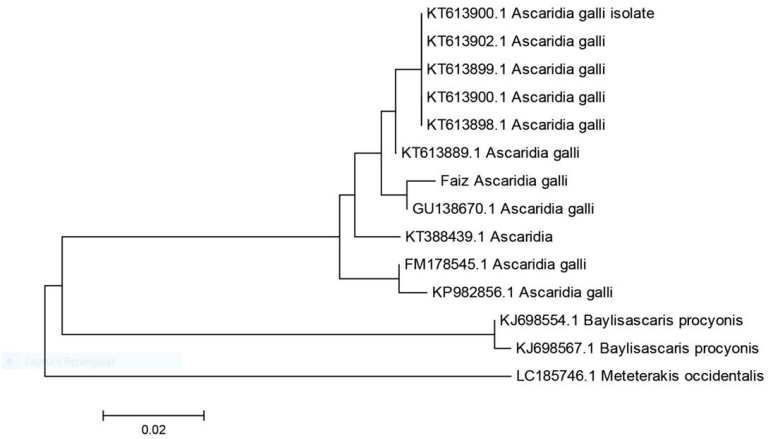

Figure 3. The maximum likelihood tree inferred from COX1 sequence (533 bp) of *A. galli* haplotypes and other *Ascaridia* species. Evolutionary analysis were conducted in MEGA 7. Scale bar shows genetic variation.

Intra-specific and inter-specific pair wise distances of *A. galli* was analyzed by using complete deletion in the ML. Phylogenetic relationship of selected native *A. galli* was based on the maximum likelihood tree method (Figure 3). The nearest strain to Faiz *A. galli* was highly homologous with *A. galli* GU 138670.199% from Australia poultry strain. Over all negligible distance was found between currently isolated sequence and other sequences of *A. galli* when analyzed (Tamura et al., 2013).

4. Discussion

Migratory birds are considered important reservoir for a variety of pathogens as well as being potential factor of their dissemination (Hahn et al., 2009). These spread pathogens in the areas where they stay during their migratory period. Baluchistan is one of the most important migratory stopovers for a diversity of migratory birds where they feed and breed. Circulation of parasite in different bird gatherings contributes to a better understanding of the epidemiology of these parasitic diseases, responsible for changing in host population dynamics (Fuller et al., 2012).

As there is a big knowledge gap regarding diversity, prevalence, intensity and risk factors of gastrointestinal helminthes in Balochistan, studies on helminthes parasites in migratory birds are important from multiple prospective like better understanding like parasite ecology, parasitic spread across diverse regions falling in the migratory routs of these birds economic losses incurred in poultry and zoonosis by them.. *A. galli* infection causes weight loss Ikeme (1971) and in severe conditions, intestinal blockage can occur. Birds with severe infection suffer from anemia, low protein level, low packed cell volume, reduced blood sugar content, increased urates, shrunken thymus, retarded growth and increased mortality Ikeme (1971).

The parasitic infection caused by helminthes in the Japanese quail has been reported from different areas like Japan and China Uchida (1985). Migratory quails are carrier of different diseases. *A.galli*, a nematode parasite infests domestic and wild birds Soulsby (1982). High prevalence of this caecal worms indirectly affects digestive tract as

well as the survivability of the quails (Dunham et al., 2017). *A. gall* can also synergize the effects of other diseases like coccidiosis and infectious bronchitis. *A.galli* may also transmit avian reoviruses (Reid et al., 1973). These gastrointestinal parasites have a disturbing effect on growth, egg production and overall health status. We compared some body particulars of the parasite mentioned in our study with the study ofsome other scientists (Table 1), here we noted that the study results of Aziz (1992) about body length was near to our results, the body width results of Tanveer (1989) were very near to our results, esophagus width in the results of Ramadan et al. (1992) was non-significant when compared with our results while on the other side (Kates and Colglazier, 1970) who studied the parasite in 1970 an old study was different in every particular when compared with our results.

Studies on prevalence of avian helminthes parasite are important both from economic, zoonotic and parasitic point of view. In our study, *A. galli* was isolated from 52.17% quails. Findings of this study are in line with the previous studies (Youssef and Mansour., 2014) who reported 28.7% intestinal infection with *Ascarida* species in quails. Another study (Ola-Fadunsin et al., 2019) detected *A. galli* in ducks (72.73%) and *A. columbae* in pigeons (88.24%). A study conducted by (Khan et al., 2018) reported 6.66% *Ascarida* spp in pigeon. This might be due to difference in bird species, different environmental factors like temperature and humidity, duration of migration period etc. Similar kind of study was also conducted Mouricio Silva Rosa et al. (2017) in Brazil in which the prevalence of *A. galli* in quail was 20%. Monte et al. (2018) observed that 16.1% had mixed infection of *A. galli*, in Japanese quails in Amazon region among 31 quails. The results (52.17%) of our study are similar to those of Movsessia and Pkhrikian (1994) who reported prevalence of *A. galli* as 64.7% in quails in Hungary. The differences in the number of helminthes species detected in this study vis-à-vis those of other studies could be attributed to environmental and climatic differences and dispersal of anti-helminthic drugs from the livestock in to the environment and active form.

Morphological characteristics of *A.galli* include whole length, body width, esophagus length, esophagus width, length of tail and spicule. Findings of the current study were compared with the morphological characteristics of *A. galli* reported in different studies. There were negligible variations in these morphological characteristics.

Molecular characterization including DNA amplification, sequencing and phylogenetic analysis are keys in confirmation identification of *A. galli*. Same kind of studies on *A. galli* were also conducted by cohorts (Katakam et al., 2010) who extracted DNA from larva of *A.galli* of chicken and amplified by polymerase chain reaction-linked restriction fragment length polymorphism (PCR-RFLP) assay. In the current study 500-bp long region of the cytochrome C oxidase subunit 1 gene of mitochondrial DNA was targeted which was extracted from *A.galli* isolated from the GIT of quail. Present study revealed that partial sequencing of *A. galli* COX1 region provide worthy information on taxonomy, lifecycle and epidemiology that might aid to moderate parasites in wild quail in Baluchistan Pakistan .Taxonomically *A. galli* is included in class *Secernetea,* order *Ascaridida and* family Ascaridiidae Anderson (2009). The order *Ascaridida* include several families parasitic round worms with three lips on the anterior end. The results of the current study are the use of mitochondrial COX1 as an ideal marker in the epidemiological study of vector–host interactions as well as observations mt DNA are passed almost exclusively from mother to offspring through the eggs cell. It strongly suggests the mitochondrial COXI for species identification and phylogenetic investigations in helminthes parasite of avian and other livestock.

5. Conclusion

This study reports that a high percentage of migratory quails are infested with gastrointestinal nematode *A. galli*. These migratory quails may play important role in spreading these parasites during their migrations to vast areas across the continents (trans-continental). Such a parasitic co-migration with their host, looks to be an excellent example of a species ensuring its eco-evolutionary survival through vast expansion of its habitat- thus greatly enhancing its opportunity to find hosts. Given the potential spread of these pathogens whereas at the same time, keeping in view the conservation efforts of the wild birds, a balanced strategy may be developed for control of pathogen-spread through migratory birds while ensuring natural habitat of wild birds. A comprehensive strategy for control of potential spill-overs from the migratory birds needs detailed deliberations involving wider list of stakeholders particularly the experts shouldering the conservations of ecosystems as well as of the fauna and flora. A moderate level of anthelmintic medication during the time these birds spend in these areas seems a good starting point.

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