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Original article

Nucleotide analysis and prevalence of *Escherichia coli* isolated from feces of some captive avian species

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ABSTRACT

The aim of the study was to check the prevalence of *Escherichia coli* in some captive avian species, seasonal effect on the *E.coli* prevalence and analysis of nucleotide sequences of *E.coli*. A total of 132 samples, 33 from Turkey (*Meleagris gallopavo)*, 33 form Pheasant (*Phasianus colchicus)*, 33 from Budgerigar (*Melopsittacus undulates)* and 33 from Chukar partridge (*Alectoris chukar)* were collected from Conservation and Research Center, UVAS, Ravi Campus, Pattoki. Colony forming units was quantified for each sample. *E. coli* confirmation was done by biochemical and molecular characterization. 16S rRNA was amplified and sequenced. 16S rRNA sequence was submitted to NCBI under the accession number MN841017, MN841018 and MN841019.Descriptive statistics showed the mean ± SEM value for *E. coli* CFU/ml of fecal sample from Turkey $1.91 \times 10^8 \pm 4.4 \times 10^7$, for Pheasants, the mean ± SEM was $1.55 \times 10^8 \pm 5.2 \times 10^7$ CFU/ml of fecal sample. The mean ± SEM of the fecal sample for Budgerigars and Chukar were $2.12 \times 10^8 \pm 3.3 \times 10^7$ CFU/ml and $1.6 \times 10^8 \pm 4.5 \times 10^7$ CFU/ml respectively. Inferential statistics showed that regardless of the bird species, there was almost a similar frequency of *E. coli* CFU/ml of fecal sample ($p = 0.74$). However, the incidence of *E. coli* fluctuates significantly depending on the season in the case of turkey and pheasants, and the impact was statistically significant ($p < 0.0005$). *E.coli* was most prevalent in Turkey during rainy summer and in Pheasants during cool dry winter. These findings show that accidental or direct contact with feces of these captive birds have possible risk of gastric illness to humans and animals. Furthermore, understanding the mechanisms driving the seasonality of this important zoonotic pathogen will allow for the execution of effective control strategies when it is most prevalent.

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1. Introduction

Captive avian species refers to those bird species that are kept in cages, aviary or in a confined environment. These avian species may be kept as pets (Dipineto et al. 2017), as source of income (Ombugadu et al. 2019), as a source of recreation for human especially for children or may be for captive breeding (Heinrichs et al. 2019). For captive breeding or conservation, the areas in use are zoos, private or government state agencies, private breeding farms, conservation foundations and research centers that exist inside or may be outside the universities (Ombugadu et al. 2019).

Zoo visitors and pet owners are more in danger of acquiring zoonotic diseases from cage birds and their companion birds (Conrad et al. 2017). The zoonotic disease transfer from diseased or carrier birds can either be direct or indirect. Direct mode of transmission includes direct bird to bird contact (Dipineto et al. 2017). While indirect transmission includes contact with their fecal material, saliva, nasal discharge, feathers (Miskiewicz et al. 2018) or by the fomites, such as bedding, panels and even the cages.

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The most common candidate of zoonotic disease transfer from cages to visitors is bacteria (Conrad et al. 2017; de Oliveira et al. 2018). In tropical countries, Psittacine birds have been proved as the potential source of diarrheagenic *Escherichia coli*. These pathogens are linked with mortality of children (Conrad et al. 2017).

Family Enterobacteriaceae includes six species and out of all *E. coli* is the most efficient and opportunistic candidate in captive animals (Walk et al. 2009). It was initially known as harmless commensal but with the passage of time *E.coli* afforded an alternate site through gene gain and loss and become a highly diverse and adapted pathogen (Croxen and Finlay, 2010). *E.coli* has following types of enteric pathotypes: Enteropathogenic *E.coli* (EPEC), Shiga toxin-producing *E.coli* (STEC), Enteroinvasive *E.coli* (EIEC), Enteroaggregative *E.coli* (EAEC), Enterotoxigenic *E.coli* (ETEC), Diffusely Adherent *E.coli* (DAEC) and Adherent-invasive *E.coli* (AIEC) (Croxen et al. 2013). All these pathotypes have different specific hosts and causes diarrhea in individuals of certain age groups (Croxen et al. 2013). External contact and ingestion of food contaminated with fecal bacteria is the source of zoonoses and illness (Mirsepasi-Lauridsen et al. 2019).

Captive birds cause direct or indirect human exposure to avian microbes. Fecal microbes i.e, *E.coli* are the potential source of avian species mortality (Ewers et al. 2003; Kiliç, et al. 2007) and human illness (Mirsepasi-Lauridsen et al. 2019). Avian pathogenic *Escherichia coli* is economically dangerous and affects poultry worldwide. Septicemia, omphalitis, swollen head syndrome, cellulitis, pericarditis, perihepatitis, yolk sac infection, or a combination of these disorders can all be caused by avian colibacillosis (Kabir, 2010). Solà-Ginés et al. (2012) found that avian pathogenic *Escherichia coli* strains cause a 2–3 % decline in egg production and a 3–4 % increase in bird mortality on a farm. Some of the signs and symptoms include subacute pericarditis, acute fatal septicemia, salpingitis, airsacculitis, cellulitis and peritonitis. The present study has been designed to check the *E.coli* prevalence in fecal material of captive avian species, effect of seasonality on the prevalence and to analyze the nucleotide sequence of fecal *E.coli*.

2. Materials and methods

2.1. Collection of fecal samples

Fecal sample were collected within 1 h of deposition from healthy birds, thrice a month from July 2018 to June 2019, from the captive Pheasant (*Phasianus colchicus*), Chukar partridge (*Alectoris chukar*), Budgerigar (*Melopsittacus undulates)* and Turkey (*Meleagris gallopavo*) reared privately at Avian Conservation and Research Center, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Pakistan. The map of the study site shown in Fig. 1. Fecal sample was collected from the ground of cages following the method of Garcia-Mazcorro et al., (2017). During fecal sample collection no direct contact with the captive birds was made. Because birds are frequently reared together in captivity, an aggregate of feces per flock was collected.

Fecal samples (5 g) were collected in sterilized falcon tube (10 ml) from the metallic tray and were stored at −20 °C until processed (Table 1).

2.2. Processing of fecal samples

Fecal samples were dried (2 h at 40 °C) and ground into powder form. A 0.2 g of each powdered sample was mixed in 1 ml of PBS solution separately in proper labeled 1.5 ml Eppendorf tubes. Mixture was centrifuged at 5000 rpm for 5 min on Bio-Rad centrifuge machine (Murphy et al. 2005) and the supernatant of the centrifuged sample was serially diluted up to 10^{-6} folds (Jahan et al. 2018).

2.3. Prevalence, identification and molecular characterization of E.coli

The plate count method was used to determine the number of *E.coli* colonies per milliliter of the fecal sample using 200 µl of each dilution on MacConkey agar (Jahan et al. 2018). Culture plate with colonies within 30–300 was considered for the calculation of CFU (Sutton, 2011).

Number of cells/ml = colonies counted / volume plated × dilution.

For conformation of *E.coli* three putative *E. coli* colonies from each plate were individually identified by cultural characteristics and conventional biochemical tests following Cheesbrough (1985). QIAGEN amp Bacterial Genomic Extraction kit was used to extract DNA from pure culture of *E.coli*. DNA presence and concentration were checked through Agarose Gel electrophoresis and NanoDrop 2000 and 2000c Thermo Scientific™. A fragment of the 16S rRNA region of bacterial DNA was amplified using the predesigned universal primers FP 5′-AGAGTTTGATCCTGGCTCAG-3′ RP5′- CTTGTGCGGGCCCCCGTCAATTC-3′primers (Magray et al. 2011).

BIO RAD PCR Gradient machine was used for the amplification. QIA-quick PCR purification kit (28704; Qiagen, West Sussex, UK) was used for the purification of amplicons. Purified products were sent for genomics and sequencing analysis to Novagene Bioengineering Company.

2.4. Statistical analysis

Two-way ANOVA was used to examine the effect of season and type of host species on the prevalence of *E.coli*.

The partial gene sequences of pathogenic strains were edited by using BioEdit 7.2. and compared using BLAST against the public database available in gene bank (https://www.ncbi.nlm.nih.gov/) and Mega 7.0.2. Software was used for analyzing the genomics and sequence data. Identification of strains was based on similarity and dissimilarity of nucleotides (Suardana, 2014). All sequences were submitted to NCBI GenBank for accession numbers.

3. Results

3.1. E.coli enumeration by viable count from collected sample

Descriptive statistics shows the mean ± SEM value $1.91 \times 10^8 \pm 4.4 \times 10^7$ for *E.coli* CFU/ml of fecal sample of Turkey. For Pheasants, the mean ± SEM is $1.55 \times 10^8 \pm 5.2 \times 10^7$ CFU/ml of fecal sample. Budgerigars and Chukar showed the mean ± SEM $2.12 \times 10^8 \pm 3.3 \times 10^7$ CFU/ml and $1.6 \times 10^8 \pm 4.5 \times 10^7$ CFU/ml of fecal sample respectively.

Two-way ANOVA showed that there is no statistically significant effect of type of specie on *E.coli* CFU/ml of fecal sample as $p = 0.73$ as shown in Table 2. However, CFU/ml of fecal sample varies greatly with respect to season and the effect was statistically significant $p < 0.0005$ only in turkey and pheasants.

3.2. Identification of E.coli

E.coli showed different cultural characteristics on different media as shown in Table 4. Biochemical tests were performed for the putative *E.coli* colonies isolated from fecal sample on MacConkey agar. Results are shown in Table 4.

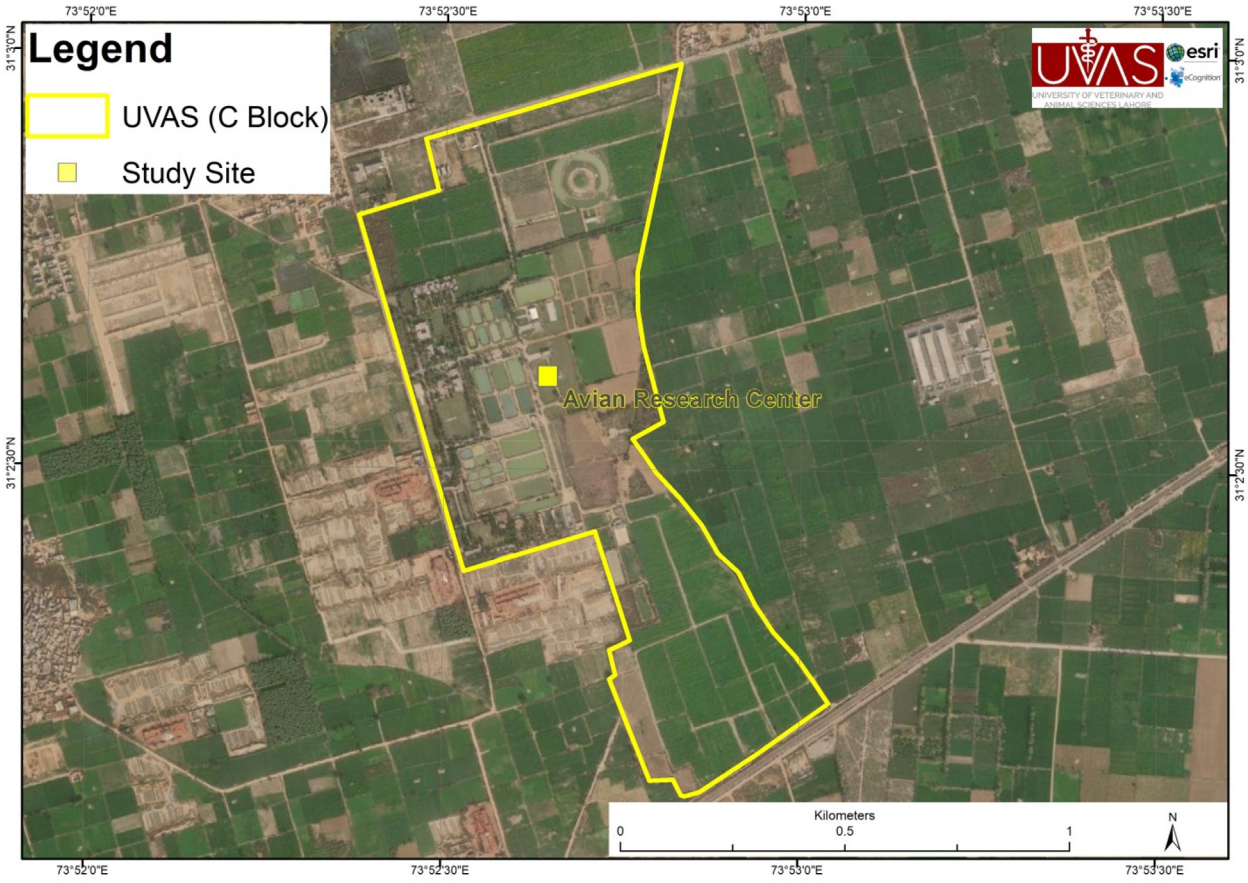

Fig. 1. Farm location in which the samples were collected.

Table 1
Data of the sampled avian species.

Sample ID	Bird species	No. of birds	Male: Female	Feeding
RP1	Ring-necked Pheasant (*Phasianus colchicus*)	4	1:3	Seeds and grains
CP1	Chukar partridge (*Alectoris chukar*)	3	1:2	Seeds and grains
BR1	Budgerigar (*Melopsittacus undulates*)	12	5:7	Mix of seeds and fresh fruits
TR1	Turkey (*Meleagris gallopavo*)	4	1:3	Seeds and grasses

Table 2
Comparison of *E.coli* prevalence in fecal sample of Turkey, Pheasants, Budgerigars and Chukar.

Species	Season	CFU ± SEM	P value	CFU ± SEM	P value
Turkey	Rainy summer	$4.1 \times 10^8 \pm 6.8 \times 10^7$	0.0001*	**$1.91 \times 10^8 \pm 4.4 \times 10^7$**	0.739
	Monsoon	$9.3 \times 10^7 \pm 9.2 \times 10^7$			
	Cool Dry winter	$1.2 \times 10^8 \pm 6.5 \times 10^7$			
	Hot Dry summer	$3.9 \times 10^7 \pm 7.3 \times 10^7$			
Pheasant	Rainy summer	$3.7 \times 10^8 \pm 7.3 \times 10^7$	0.0001*	**$1.55 \times 10^8 \pm 5.2 \times 10^7$**	
	Monsoon	$3.3 \times 10^8 \pm 8.4 \times 10^7$			
	Cool Dry winter	$9.5 \times 10^8 \pm 6.5 \times 10^7$			
	Hot Dry summer	$7.3 \times 10^7 \pm 7.3 \times 10^7$			
Budgerigars	Rainy summer	$2.8 \times 10^8 \pm 7.3 \times 10^7$	0.118	**$2.12 \times 10^8 \pm 3.3 \times 10^7$**	
	Monsoon	$1.8 \times 10^8 \pm 8.4 \times 10^7$			
	Cool Dry winter	$9.4 \times 10^6 \pm 6.5 \times 10^7$			
	Hot Dry summer	$1.5 \times 10^8 \pm 7.3 \times 10^7$			
Chukar	Rainy summer	$3.1 \times 10^8 \pm 6.8 \times 10^7$	0.137	**$1.6 \times 10^8 \pm 4.5 \times 10^7$**	
	Monsoon	$9.0 \times 10^7 \pm 8.4 \times 10^7$			
	Cool Dry winter	$5.3 \times 10^7 \pm 6.5 \times 10^7$			
	Hot Dry summer	$1.8 \times 10^8 \pm 7.3 \times 10^7$			

Note: "*" shows significant difference at ($p < 0.001$).

3.3. Molecular characterization of E.coli

E.coli Genome obtained as shown in Fig. 2 was used as a template for the identification and amplification of 16S gene (930 bp fragment) by using universal pair of primers. The PCR amplification product was run on gel for the confirmation of PCR shown in Fig. 3. Sequenced data was edited, BLAST and submitted to the NCBI for attaining accession numbers shown in Table 3.

4. Discussion

E.coli isolates from fecal samples of the apparently healthy avian species (*Phasianus colchicus*, *Alectoris chukar*, *Melopsittacus undulates* and *Meleagris gallopavo*) were grown on previously recommended (Buxton and Fraser, 1977; Cowan, 1985; Cheesbrough, 1985) media. *E.coli* were isolated and identified based on colony morphology by using MacCokey agar and EMB agar shown in Table 4. Parallel findings were stated by former workers (Boro et al. 2018; Kar et al. 2017). Biochemical characteristics exhibited by the *E.coli* coincides with the discoveries of other researchers (Boro et al. 2018; Elafify et al. 2016).

Present study was an attempt to compare the *E.coli* prevalence in four important captive avian species and seasonal effect on *E.coli* prevalence. All the fecal samples analyzed were positive for *E.coli*. and it was concluded that *E.coli* prevalence doesn't depend upon the type of the species because the difference between mean of CFU/ml of the *E.coli* was non-significantly different from others showing *p*-value > 0.05. *E.coli* prevailed in species as budgerigars > turkey > Chukar > Pheasants. The prevalence of *E.coli* was higher in budgerigar's fecal material than other species.

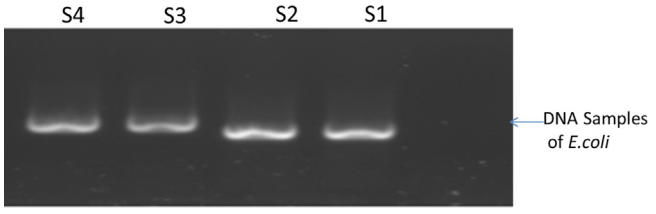

Fig. 2. Analysis of target DNA, extracted from pure culture of *E.coli* by QIAGEN amp Bacterial Genomic Extraction Kit.

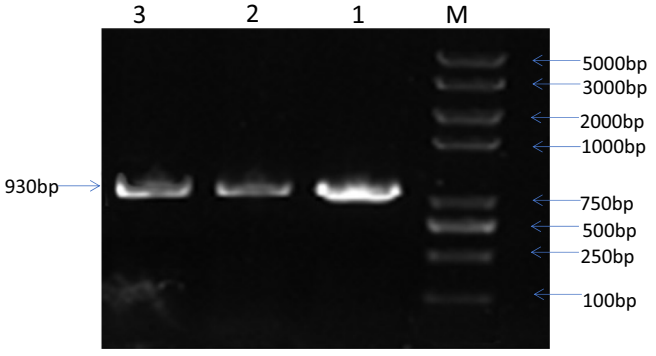

Fig. 3. Lane 1–3 confirmation of 16S rRNAgene by PCR amplification with FP 5′-AGAGTTTGATCCTGGCTCAG-3′RP5′- CTTGTGCGGGCCCCCGTCAATTC-3′primers. M: DNA marker Trans 2 K.

Table 4
Cultural Characteristics of isolated *E.coli.*

Serial no	Tests and Media used	Results
1	**Nutrient agar**	Big, mucoid colonies of off-white/ milky white color appeared
2	**MacConkey agar**	Large mucoid rose-pink colored colonies with somehow darker center
3	**EMB agar**	Dark colored circular colonies showing metallic sheen of green color
4	**LB Broth**	Equally and smoothly dispersed growth
5	**Gram staining**	Gram negative, short bacilli
6	**Motility**	+
7	**Lactose Fermentation**	+
8	**Endospore staining**	–
9	**Catalase test**	+
10	**Oxidase**	–
11	**MR (Methyl Red)**	+
12	**VP (Voges–Proskauer)**	–
13	**Glucose Fermentation**	+
14	**Fructose fermentation**	–

Effect of seasonal variation on the prevalence of *E.coli* was checked through examine the fecal sample from the same population thrice per month from July-2018 to May- 2019. In Pakistan there are total four seasons and the division of months were made according to (Blood, 1996) climate of Pakistan. Statistical analysis showed that season effected *E.coli* prevalence in each species differently. Seasonal effect on the prevalence of *E.coli* in captive avian species has not been reported before and it was observed in present study that *E.coli* prevalence is more in rainy summer than the winter. The reason might be temperature, humidity, and the type of food.

Neher et al. 2016 also isolated the *E.coli* from the 16/25 samples of fecal, oral, and gut of healthy bird species and concluded that even the healthy avian species could be a source of *E.coli*. Present findings are also supported by Sarker et al. 2012 who isolated the different pathogenic species from the fecal sample of healthy water birds and found *E.coli* as the most prevalent bacteria. *E.coli* from the healthy turkey (Kar et al. 2017) and psittacine (Gioia et al., 2016) has been reported also and it was concluded that these healthy birds are the carrier of vast range of *E.coli* either pathogenic or nonpathogenic and can be a risk to the sanitary condition and potential source of zoonoses. These findings are consistent with Bukhari et al. 2022 findings for next generation sequencing analysis of fecal material from two pheasant species, where the second most abundant phylum identified from fecal material was *Proteobacteria*, and *E.coli* belongs to this phylum.

E.coli inhibits the most animal intestine as natural intestinal flora, and it does not vary significantly from species to species. Though, the sampling population used in this study was inhibiting the same area throughout the study duration, this can also be the reason for having approximately same prevalence rate of *E.coli* in

Table 3
GenBank accession numbers for 16S rRNA nucleotide sequences.

Sequence_ID	Organism	strain	Collection Date	Isolation source	Accession number
nimkp01-19	*E. coli*	nimkp01-19	29-Jan-2019	fecal sample of a captive *Phasianus colchicus*	MN841017
nimkb02-19	*E. coli*	nimkb02-19	03-April-2019	fecal sample of a captive *Melopsittacus undulatus*	MN841018
nimkt03-19	*E. coli*	nimkt03-19	06-Feb-2019	fecal sample of a captive *Meleagris gallopavo*	MN841019

fecal samples. As stated above all the samples were positive for *E.-coli* these findings contradict the findings of previous studies where *E.coli* was found only in 16 % (Gioia et al., 2016), 13.6 % (Graham and Graham, 1978), 14 % from private collected sample, 63 % from sample collected from zoo and 19 % samples collected from pet shop (Medani et al. 2008).

From the finding it can be stated that the captive avian species could be responsible for the pathogenic *E.coli*. Proper protective measure in captivating these avian species should be applied, and further research is needed to determine the strain type and virulence gene factors linked with these *E.coli* in order to develop better control strategies and preventive measures. *E. coli* ecology should also be investigated to establish whether they are actually part of the natural flora of birds or more reflective of the environment in which the birds reside.

5. Conclusion

It was concluded that captive birds are a reservoir of pathogenic *E.coli* that can be transmitted to humans and other animals. *E.coli* cause severe gastric illness individuals that consumed these birds. Furthermore, understanding the mechanisms driving the seasonality of this important zoonotic pathogen could be beneficial for the execution of effective control strategies when it is most prevalent.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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