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Prevalence of *E. coli, Salmonella*, and *Clostridium* in Migratory Birds from Balochistan Province, Pakistan

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ABSTRACT

Migratory birds undertake vast long distance journeys each year in response to changes in food availability, habitat, and climate, with millions of individuals traversing the globe. Pakistan, situated on a significant Asian flyway, hosts a diverse array of migratory bird species, making it a key stopover destination during the winter season. This study aimed to assess the prevalence of pathogenic bacteria, including *Escherichia coli*, *Salmonella*, and *Clostridium*, in fecal samples collected from various species of migratory birds in Balochistan, Pakistan. A total of 216 fecal samples were screened for the presence of pathogenic bacteria using culture, biochemical tests, and molecular techniques. The study revealed a high prevalence of *E. coli* (65.80%), *Salmonella* (23.83%), and *Clostridium* (10.36%) in migratory birds. Bird species-wise prevalence revealed substantial variation, with waterfowl exhibiting 75.5% *E. coli*, 34.8% Salmonella, and 15.1% *Clostridium*. Cranes showed 64.2% *E. coli*, 16.1% *Salmonella*, and 8.9% *Clostridium* prevalence. Conversely, sand grouse and houbara bustard had 41.8% and 25.8% *E. coli*, 11.6% and 6.45% *Salmonella*, and 4.6% and 0% *Clostridium*, respectively. Sequencing and phylogenetic analysis of these bacteria demonstrated genetic diversity within the isolates, with varying degrees of similarity to strains from other countries. These findings indicate a potential risk of bacterial transmission from migratory birds to both livestock and public health. Continued research on the prevalence and transmission of pathogenic bacteria by migratory birds is necessary to develop effective strategies for mitigating the potential spread of these pathogens and safeguarding public health.

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ANM conducted the whole research project under the supervision of MHS and AZD. MO assisted in write up and AAA helped in lab studies. All authors read and approved the final manuscript.

Key words
Migratory Birds, Escherichia coli, Salmonella, Clostridium, Balochistan

INTRODUCTION

Bird migration is the routine seasonal journey undertaken by many species of birds. Birds movement occurs as a response to changes in food availability, habitat, or climate (Runge and Tulloch, 2018). Annually, the worldwide population of migratory birds soars to an astonishing five billion. This remarkable figure underscores the vast scale of these avian journeys across the globe (Guenther *et al.*, 2012). Pakistan offers attractive wetlands to a vast number of migratory bird species annually in the winter season. It serves as a middle Asian flying route for migratory birds (Umar *et al.*, 2018). Wetland areas from the northern mountains to the southern coast serve as a habitat for water birds arriving from Siberia (Bibi *et al.*, 2021). It has been estimated that 1 million birds migrate by using International Migratory Bird Route Number 4 covering around 2800 miles (4500 km). There has been a drastic decrease in the number of species making stopovers at water reservoirs in Pakistan (Sheikh and Kashif, 2006). The major species of birds that migrate from Siberia to Pakistan territory include houbara bustards *(Chlamydotis undulata),* cranes (*Grus grus*), teals *(Anas crecca*), pintails (*Anas acuta*), mallards *(Anas platyrhynchos*), geese (*Anser anser*), spoonbills *(Platalea leucorodia*), waders (*Calidris pusilla*), and pelicans *(Pelecanus occidentalis*) (Express Tribune, 2016).

Migratory birds are known for their ability to cover

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vast distances during their seasonal migrations and have been found to carry a range of gut-associated microbes with genetic modifications. Studies have shown that these birds can harbor resistant strains in their guts, feathers, and even respiratory systems. The bacteria can survive and remain viable during migration, facilitating their transportation to new geographical locations (Lin *et al*., 2020). Migratory birds have been identified as potential vectors for the spread of bacterial pathogens such as *E.coli*, *Salmonella*, and *Clostridium* (Islam *et al*., 2021). The prevalence of these bacteria in migratory birds varies depending on the species and location. For example, a study conducted in China found that the prevalence of *E. coli* and *Salmonella* in migratory birds was 18.8% and 2.6%, respectively (Yuan *et al*., 2021). Another study found that the prevalence of *Clostridium perfringens*, a bacterium belonging to the *Clostridium* genus, in migratory ducks was 4.8% (Grenda *et al*., 2023). The primary concern is the potential transmission of these bacteria to humans and animals, as migratory birds traverse diverse habitats and interact with different populations during their journeys. The primary objectives that guided this study involved first time conducting to find out the prevalence of *E. coli*, *Salmonella,* and *Clostridium* spp. within the population of migratory birds visiting Balochistan, Pakistan.

MATERIALS AND METHODS

Sampling and study area

This study was conducted on different species of migratory birds houbara bustard, sand grouse, cranes, and waterfowls irrespective of their age and sex in different districts (Noshki, Chaghi, Mustung, Pishin, Quetta, and Qilla Saifullah) of Balochistan, Pakistan in (Fig. 1). A total of 216 fecal samples from migratory birds were collected through nesting and hunting from different stopovers of migratory birds and studied.

Culturing and identification of E. coli, Salmonella, *and* Clostridium

Fecal samples were collected using sterilized cotton swabs containing phosphate-buffered saline (Thermo Fisher Scientific Inc., Massachusetts, USA) directly from cloacae and were immediately transferred to the Institute of Microbiology, UVAS, Lahore maintaining a cold chain (4°C). Samples were cultured on nutrient base agar media (Thermo Fisher Scientific Inc., Massachusetts, USA) according to the manufacturer's instructions labeled, and incubated at 37 ° C for 24 h.

Positive isolates of *E. coli, Salmonella, and clostridium* from fecal samples cultured on nutrient agar were inoculated on differential media MacConkey agar (Thermos Fisher Scientific Inc., Massachusetts, USA) were incubated at 37 °C. After 24 h suspected pink color colonies of *E. coli* and white colorless suspected colonies of *Salmonella* were inoculated on selective media eosin methylene blue (EMB) (Thermo Fisher Scientific Inc., Massachusetts, USA) and *Salmonella Shigella* (SS) agar (Thermo Fisher Scientific Inc., Massachusetts, USA) to confirm the growth of E. *coli* and *Salmonella*. For *Clostridium perferingens Clostridium* base agar supplemented with egg yolk emulsion (50ml/liter) and D-cycloserine (2 vials/liter) was inoculated with clostridium colonies and incubated at 37°C for 24 h. These purified isolates of *E. coli*, *Salmonella,* and *Clostridium* were further processed for identification considering colony characters, microscopic characters using Gram staining, and biochemical profile through catalase, oxidase, indole production test, methyl red test, Voges-Proskaur's test and citrate utilization tests following Bergey's manual of systematics of Archaea and bacteria.

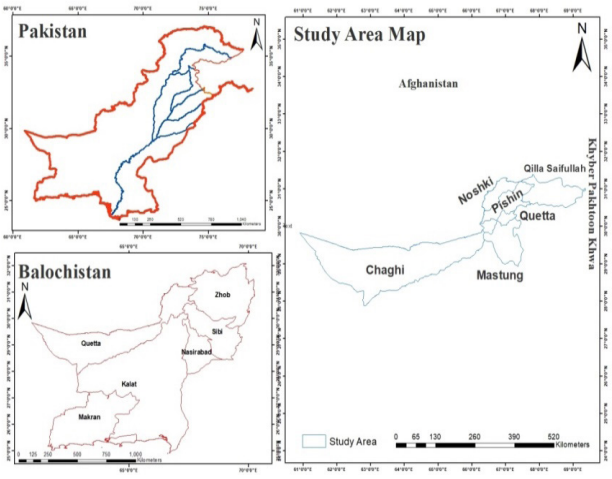

Fig. 1. Study area showing six districts Noshki, Chagai, Mastung, Pishin, Quetta, and Qilla Saifullah, which are stopovers for migratory birds.

Molecular detection of bacteria

DNA of positive isolates was extracted using DNA extraction kits (Wiz Prep gDNA Mini Kit) as per manufacturer's directions.Extracted DNA was amplified through PCR using master mix 12.5 µl, template DNA 2.5 µl, nuclease-free water 5 µl and reported forward/reverse primers 2.5 µl each (Table I) with initial denaturation at 95°C for 3 min, 35 cycles each of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 15 min (Au-Lorenz, 2012).

Table I. Primer sets and conditions of PCR used for amplification of *16Sr RNA* of different bateria.

Organism	Primers	Primer sequences 5`→3`	Annealing temp	Product size (bp)	Reference
Clostridium perfringens type A	CPAlpha	F GCTAATGTTACTGCCGTTGA R CCTCTGATACATCGTGTAAG	53°C	324	(Navarro *et al*., 2020)
Salmonella	8FLP XB4	F AGTTTGATCCTGGCTCAG R GTGTGTACAAGGCCCGGGAAC	55 °C	1500	(Ali *et al*., 2022)
E. coli	VTcom-u	F GAGCGAAATAATTTATATGTG R TGATGATGGCAATTCAGTAT	52 °C	518	(Murrad *et al*., 2020)

A total of 2µl PCR product was run through 1.5% agarose gel electrophoresis (Lee *et al*., 2012). The amplification bands were visualized under UV light using a gel documentation system (Bio-Rad, USA).

The PCR product was sequenced by the commercial Sanger di-deoxy sequencing analysis (ABI Genetics Lab, Lahore, Pakistan). The accession numbers were then submitted to the NCBI database. The obtained sequences were aligned with those from NCBI through the utilization of Bio-Edit and ClustalW software. Subsequently, the Neighbor-Joining tree for the 16S rRNA gene was constructed using MEGA 11 BLAST software. Phylogenetic analysis was performed using MEGA software (Tamura *et al*., 2013; Rajput *et al*., 2014) at Kumar Lab, Pennsylvania State University, USA.

Statistical analysis

Frequency tables were employed for the analysis of descriptive statistics to elucidate the proportions of prevalence. Moreover, a chi-square test was conducted to compare the prevalence of bacteria in migratory birds at a *p*-value (< 0.05). The analyses were carried out using IBM SPSS Statistics version 25 software, as outlined by (Kibret and Abera, 2011).

RESULTS

Prevalance of E. coli, Salmonella, *and* Clostridium *in migratory birds*

A total of 216 fecal samples were collected from 31 houbara bustard, 43 sand grouse, 56 cranes, and 86 waterfowls. All the fecal samples were screened for bacteria, and 193 isolates were found positive for bacteria of which 65.80% were of *E. coli*, 23.83% were of *Salmonella,* and 10.36% were of *Clostridium* (Table II).

Table III shows the accession number of DNA frequencies submitted to NCB1.

Phylogenetic analysis

Phylogenetic analysis of *E. coli* showed 94-89% similarity among our sequences and 80-71% similarity was recorded with other countries' strains. The *Clostridium* strains sequences of our study showed 90-87% similarity and 86-61% similarity with other countries. Sequences of the *Salmonella* strain showed 92% similarity, on the other hand, 93% similarity was observed with other sequences from the database in (Fig. 2).

Table II. Prevalence of *E. coli, Salmonella*, and *Clostridium* in different migratory birds in Balochistan, Pakistan.

Birds	Total samples	*E. coli* positive (%)	*Salmonella* positive (%)	*Clostridium* positive (%)
Houbara bustard	31	8 (25.8%)	2 (6.45%)	0 (0%)
Sand grouse	43	18 (41.8%)	5 (11.6%)	2 (4.6%)
Cranes	56	36 (64.2%)	9 (16.1%)	5 (8.9%)
Water fowls	86	65 (75.5%)	30 (34.8%)	13 (15.1%)
Total	216	127	46	20

Table III. Accession numbers of different genetic sequences of *E. coli, Salmonella*, and *Clostridium* submitted to NCBI.

Organism	ID	Accession number
E. coli	eco1	ON506255
E. coli	eco2	ON506256
E. coli	eco3	ON506257
Salmonella	sal1	ON506875
Salmonella	sal2	ON506876
Salmonella	sal3	ON506877
Clostridium	clos1	ON506258
Clostridium	clos2	ON506259
Clostridium	clos3	ON506260

Birds and district-wise prevalence of pathogens

Table II shows bird wise prevalence of *E. coli* (75.5%), *Salmonella* (34.8%), and *Clostridium* (15.1%) in waterfowl; and 64.2% of *E. coli*, 16.1% of *Salmonella*,

and 8.9% of *Clostridium* in cranes. On the other hand, sand grouse and houbara bustard carried 41.8%, 25.8% of *E. coli*, 11.6%, 6.45% of *Salmonella*, and 4.6%, 0% of *Clostridium*. The data was observed significant (P≤ 0.05).

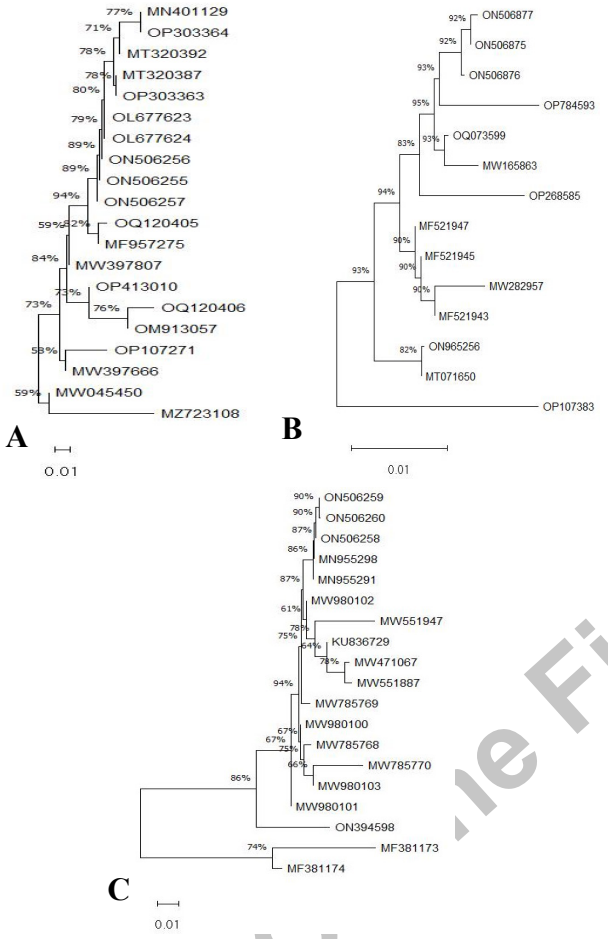

Fig. 2. Phylogenetic analysis of 3 different isolates of *E. coli* (A), *salmonella* (B) and *Clostridium* (C) from migratory birds.

Figure 3 and Table IV show district wise prevalence of *E. coli, Salmonella,* and *Clostridium* which varied across different regions. In Noshki the prevulance was 22.8%, 28.2%, and 35%, respectively. In Chaghi, the prevalence for *E. coli*, *Salmonella*, and *Clostridium* was 14.9%, 21.7%, and 25%, respectively. For Mustung and Pishin, the prevalence was 15.7% for *E. coli*, 14.9% for *Salmonella*, 13.04% for *Salmonella*, and 19.5% for *Clostridium*. Lower prevalence was observed in birds from Qilla Saifullah, with rates of 10.2% for *E. coli*, 8.6% for *Salmonella*, and 0% for *Clostridium*. Statically the data was observed non-significant (P ≤ 0.05) in (Table IV, Fig. 3).

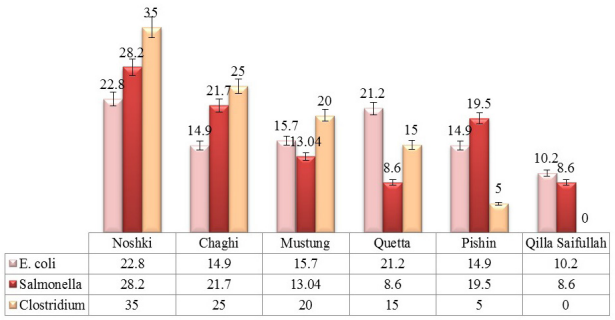

Fig. 3. Districts wise prevelance of *E. coli, Salmonella*, and *Clostridium* in migratory birds in Balochistan.

DISCUSSION

Migratory birds have been identified as potential vectors for the spread of bacterial pathogens such as *E. coli, Salmonella, and Clostridium* (Benskin *et al*., 2009). In this study the prevalence of *E. coli*, *Salmonella*, and *Clostridium* in migratory birds was found to be 65.80%, 23.83% and 10.36%, respectively.

One study conducted in Norway found that *Salmonella* was present in 11.6% of migratory birds tested. Another study in the United States witnessed a prevalence of 16.7% for *Salmonella* in migratory birds (Refsum *et al*., 2002). The prevalence of *Salmonella* in migratory birds in previous studies (13.5%) is less than the current study (23.83%). The intermittent shedding of *Salmonella* makes it difficult to identify carriers; *Salmonella* has been isolated from wild mammals and birds (Tizard, 2004). Although *Salmonella* may survive for long periods in the environment, it is the carrier state that provides the major source of infection for animals and humans, and various carrier states are recognized (Gunn *et al*., 2014). In this case, the spread of the infection was restricted or limited as different species of migratory birds have varying prevalence of different bacteria. Several studies support the finding that *Salmonella* is present in a significant percentage of migratory birds. Fu *et al*. (2022) found that 25% of migratory birds sampled in the United States had *Salmonella* infections coherent with this study. Another study by Smith *et al*. (2020) reported that 37% of migratory shorebirds in Australia were positive for *Salmonella* much greater than our study. The presence of *Salmonella* in migratory birds has implications for the spread of this bacterial disease. Migratory birds can travel long distances and encounter a wide range of habitats and populations, potentially spreading *Salmonella* to new areas. This is a concern for both human and animal health, as *Salmonella* infections can cause serious illness in both humans and animals (Bengtsson and Greko, 2014).

Overall, these findings highlight the need for further study

Table IV. District-wise prevalence of *E. coli, Salmonella,* and *Clostridium* in migratory birds in Balochistan, Pakistan.

Sampling area	Birds species	*E. coli* (%)	*Salmonella* (%)	*Clostridium* (%)	P-value
Noshki	Houbara bustard	3/29 (10.34)	1/13 (7.69)	0/7 (0)	0.48
	Sand grouse	6/29 (20.68)	3/13 (23.07)	1/7 (14.28)	
	Cranes	7/29 (24.13)	4/13 (30.76)	2/7 (28.57)	
	Water fowls	13/29 (44.82)	5/13 (38.46)	4/7 (57.14)	
Chaghi	Houbara bustard	2/19 (10.52)	1/10 (10)	0/5 (0)	0.403
	Sand grouse	3/19 (15.78)	2/10 (20)	1/5 (20)	
	Cranes	5/19 (26.31)	1/10 (10)	1/5 (20)	
	Water fowls	9/19 (47.36)	6/10 (60)	3/5 (60)	
Mustung	Houbara bustard	1/20 (5)	0/6 (0)	0/4 (0)	0.791
	Sand grouse	4/20 (20)	0/6 (0)	0/4 (0)	
	Cranes	8/20 (40)	2/6 (33.33)	3/4 (75)	
	Water fowls	7/20 (35)	4/6 (66.66)	1/4 (25)	
Quetta	Houbara bustard	0/27 (0)	0/4 (0)	0/3 (0)	0.144
	Sand grouse	2/27 (7.40)	0/4 (0)	0/3 (0)	
	Cranes	11/27 (40.74)	1/4 (25)	0/3 (0)	
	Water fowls	14/27 (51.85)	3/4 (75)	3/3 (100)	
Pishin	Houbara bustard	2/19 (10.52)	0/9 (0)	0/1 (0)	0.591
	Sand grouse	1/19 (5.26)	0/9 (0)	0/1 (0)	
	Cranes	2/19 (10.52)	1/9 (11.11)	0/1 (0)	
	Water fowls	14/19 (73.68)	8/9 (88.88)	1/1 (100)	
Qilla Saifullah	Houbara bustard	0/13 (0)	0/4 (0)	0/0 (0)	0.365
	Sand grouse	2/13 (15.38)	0/4 (0)	0/0 (0)	
	Cranes	3/13 (23.07)	0/4 (0)	0/0 (0)	
	Water fowls	8/13 (61.53)	4/4 (100)	0/0 (0)	

on the prevalence and transmission of *Salmonella* in migratory birds. Further understanding of this issue could inform the development of strategies to mitigate the spread of *Salmonella* and protect public health.

E. coli was found to be prevalent in 65.80% of the total migratory birds in our study. This doesn't agree with the percentages (22.8% and 20.4%) reported by (Islam *et al*., 2021; Shobrak and Abo-Amer, 2014), respectively. The prevalence rates of pathogenic *E. coli* (20%) and *Salmonella* spp. (6.4%) were relatively reduced in wild migratory birds (Smith *et al*., 2020). In another study *E. coli* (9%), *Salmonella* spp. (1%) were detected from wild birds (Al-Atfeehy *et al*., 2019). The high prevalence in this study may be due to unhygienic water sources, anthropogenic activities, and poor processing systems. These aspects align with the outcomes of all the cited studies consistent with the observation of the present study. The transmission of *E. coli* from migratory birds to humans can occur through a variety of pathways. For example, migratory birds may contaminate water sources with their feces, leading to the spread of *E. coli* to humans who consume the contaminated water. Migratory birds may also transmit *E. coli* to humans through the contamination of crops or other food sources. It is important for people to be aware of the potential risk of *E. coli* infections from migratory birds and to take steps to prevent transmission (Lagerstrom and Hadly, 2021).

The *Clostridium* was found to be 10.36% in migratory birds in our study aligning with the results (10.3%) of Pennycott (2016) contrary to Martin and Smyth (2009), and Bandelj *et al*. (2014) who reported 7.5% and 4.6%, respectively, whereas Forti *et al*. (2020) reported higher positive percentage (15.5%). In contrast, a study in Sweden found a much lower prevalence of *Clostridium perfringens* in migratory birds, with only 1.4% of birds testing positive for the bacterium (Engstrom *et al*., 2003).

This suggests that the prevalence of *C. perfringens* in migratory birds may vary depending on the region and the specific migratory bird species. It is important to note that the presence of *C. perfringens* in migratory birds does not necessarily mean that the bacteria will be transmitted to humans. However, the risk of transmission may increase if proper food handling and hygiene practices are not followed (Todd, 2020).

In the present study, 16S rRNA gene has been used to find the genetic diversity of *E. coli* isolates from wild birds. Our findings were compared with a previous study that used the *16S rRNA* gene that constitutes a remarkably conserved element within the transcriptional apparatus of all life forms based on DNA (Cox *et al*., 2013) and experiences only minimal impact from horizontal gene transfer (Daubin *et al*., 2003). Nevertheless, discrepancies persist within specific variable regions. A preceding study highlighted that, aside from these variations, the quantity of 16S rRNA copies could differ among distinct *E. coli* strains, offering a valuable means to characterize genomic diversity (Vetrovsky and Baldrian, 2013).

However, we confirmed *Salmonella* using 16S rRNA and compared our results to recent studies. The results of this study were comparable to a similar stduy (Milton *et al*., 2018), that confirmed the presence of *Salmonella* spp with 16S rRNA. Choi *et al*. (2021) also confirmed the *Salmonella* isolated from wild birds using 16S rRNA. In a study performed by Elsohaby *et al*. (2018) in Saudi Arabia; the author isolated *Salmonella* from migratory birds and confirmed with *16S rRNA* gene. *Salmonella* chatartiztion from human samples, and *16S rRNA* gene library preparation was performed for the typing and phylogenetic analysis (Fadlalla *et al*., 2021; Hellberg *et al*., 2012). Similarly, 16S rRNA was used to confirm *Clostridium* spp. as performed by Scupham *et al*. (2008) in domestic and wild turkeys. Some other studies also used this gene to confirm clostridia in different fish species (Clements *et al*., 2007), meat (beef, lamb, and venison) and from skin and fecal samples of wild boars (Dorn-In *et al*., 2018), and from the gut of domestic and wild mallards (He *et al*., 2023).

CONCLUSION

These findings suggest that *Salmonella, E. coli,* and *Clostridium* are common in migratory birds and may pose a risk to livestock and public health. The potential for migratory birds to transmit bacterial pathogens to other animals or humans highlights the importance of monitoring and studying these pathogens in migratory birds. This information can help veterinarians and public health officials to identify potential risks and implement measures to prevent the spread of these diseases. For example, migratory birds may be tested for bacterial pathogens to identify and isolate infected birds, or measures may be taken to prevent contact between migratory birds and domestic animals to reduce the risk of disease transmission.

DECLARATIONS

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Ethical approval and IRB approval

Ethical approval to work with animals was taken from the Ethical Review Committee, University of Veterinary and Animal Sciences Lahore, Pakistan with No. DR/894 dated 22-8-2017 and no animal was harmed during the study.

Generative AI and AI-assisted technology statement

The authors declare that they have not used generative AI or AI-assisted technologies in this manuscript.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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