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Research Article

Possible role of companion birds in one health implication of multidrug-resistant *Pseudomonas aeruginosa* isolated from feces of captive wild birds

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Abstract

Many zoonotic infections affect wild birds in captivity, primarily because companion birds pose a serious threat to their conservation. The faecal-oral cycle typically spreads zoonotic diseases. *Pseudomonas aeruginosa*, a zoonotic pathogen, is responsible for many nosocomial infections in wild birds and is also a health risk to other birds and humans. The current research is to explore antibacterial resistance in *P. aeruginosa* and the significant role of companion birds as potential carriers of this pathogen. A total of 45 cloacal/fecal samples were collected from 20 bird species using swabs from Safari Zoo Lahore and Lahore Zoological Garden. The prevalence of *P. aeruginosa* at the aviary of Safari Zoo Lahore was 54%, and 67% at the Lake of Lahore Zoological Garden. The

prevalence of *P. aeruginosa* between two different captive sites was not significant ($P < 0.005$). The isolated strains of *P. aeruginosa* showed 100% resistance to six antibiotics, which suggests that *P. aeruginosa* is MDR (multidrug resistant). We must consider the recurring transmission of P. aeruginosa from companion birds to captive birds, which can result in zoonosis and potentially affect the health of both the keepers and visitors at these two captive locations. Controlling the interaction between free-living birds and captive birds through the implementation of a proper management plan is crucial.

Keywords: Zoonosis, *Pseudomonas aeruginosa*, Captive birds, One- health, Multidrug resistant

Introduction

Birds, animals, and humans all encounter the same bacterial species, including resistant strains. Therefore, we should conduct frequent epidemiological investigations of companion birds and captive wild birds to detect the presence of resistant strains. We should study wild animals, especially free-ranging wild birds, as potential reservoirs for the quick transfer of antibiotic-resistant bacteria (Shahid 2003). Pseudomonas, the most diverse and ecologically relevant bacterial genus, is widespread in nature and distinguished by its metabolic variability, which complicates the identification of non-pathogenic strains from pathogenic ones (Zhao 2020). The ecological relationships among birds and humans, particularly in shared habitats, intensify this problem. Considering their mobility and the possibility of zoonotic transmission, wild birds' position as possible transmitters of drug-resistant pathogens is especially alarming.

Pseudomonas aeruginosa is a zoonotic, multidrug-resistant, and opportunistic pathogen of significance (Ahmed et al., 2019) that can cause secondary infections in both people and animals (Saleem & Bokhari, 2020). It primarily affects patients who are immunocompromised and have cystic fibrosis; it is now recognized as a prevalent source of many population-acquired and nosocomial infections (Driscoll et al., 2007). The resistance in birds to many zoonotic pathogens is increasing due to the excessive use of antibiotics in clinical and agricultural settings, which is forcing resistant bacteria to become more prevalent. Wild birds are residents of the many novel bacterial pathogens (Varriale 2020). The studies have shown that wild or free-living birds could generate a probable threat to animal and human health by spreading MDR bacteria to the environment through their faeces. These findings have been reported by Kozak et al. (2009), Muehlenbein (2013), and Shobrak & Abo-Amer (2014). The outbreak of enteric infectious and zoonotic diseases in captivity can cause the mortality of the whole bird flock in the aviary of a zoo or safari (Hussain et al., 2021). The study took place in two distinct captive habitats, the Lahore Zoological Garden and the Safari Zoo Lahore. The

aim of this study was to acquire a comprehensive grasp of companion birds' potential role as carriers and the environmental spread of drug-resistant *P. aeruginosa* bacteria. We conducted this experiment with a focus on P. aeruginosa identification, molecular characterisation, and sensitivity profile to determine the presence of antimicrobial-resistant zoonotic P. aeruginosa in free-living birds.

Material and methods

Study site

The current study conducted in the duck pond of Safari Zoo Lahore's walk-through aviary, located at 31.3788477 North and 74.2196846 East (open to the public), Waterfowl Lake, and the walk-through aviary of Lahore Zoological Garden, with GPS coordinates of 31.55 North and 74.32 East (open to keepers only). The species that are more prone to infectious disease outbreaks are water birds (Keawcharoen et al., 2008) living both in water and on land (ducks). Arboreal birds, which graze on trees, visit the water to drink and deposit their droppings into it. In this way, the chances of transferring suspected bacteria are higher in water birds. The study selected water birds for sampling. A total of 11 species of birds from Safari Zoo Lahore were collected for this study. Crow (*Corvus splendens*), domestic geese (*Anser domesticus*), muscovy duck (*Cairina moschata*), common myna (*Acridotheres tristis*), demoiselle crane (*Grus virgo*), common peafowl (*Pavo cristatus*), mallard (*Anas platyrhynchos*), greater flamingo (*Phoenicopterus roseus*), spotted whistling duck (*Dendrocygna guttata*), ruddy shelduck (*Tadorna ferruginea*), Dalmatian pelican (*Pelecanus crispus*) . The eight species of birds from the Lahore Zoological Garden were selected for current study. Dalmatian pelican (*Pelecanus crispus*), greater flamingo (*Phoenicopterus roseus*), Muscovy duck (*Cairina moschata*), domestic goose (*Anser domesticus*), mallard drake (*Anas platyrhynchos),* and black swan (*Cygnus atratus*), sarus crane (*Grus antigone*), crow (*Corvus splendens*). The Punjab University Institutional Review Board granted bioethical approval before collecting bird faecal samples, and veterinarians from Safari Zoo Lahore and Lahore Zoological Garden provided a consent letter. This letter guaranteed that the birds would not suffer any harm during the cloacal fecal sampling process. We collected all the samples in accordance with international ethical standards and safety rules.

Sample Collection

The samples were collected form November 2021 to January 2022 under the supervision of trained staff and veterinarians, we collected 24 faecal samples of 11 bird species from the walk-through aviary of Safari Zoo Lahore and 21 faecal samples from the waterfowl lake and aviary of Lahore Zoological Garden using sterilised cotton swabs. Following collection, we transported the samples in a sterilised polythene zipper bag, exposed to UV light, within 24 hours for processing at the Conservation Biology Lab, University of the Punjab, Lahore.

Isolation, Identification of *Pseudomonas aeruginosa,* and Detection of haemolysis

The collected samples were enriched in buffer peptone water (BPW). We shifted the pre-enriched samples to nutrient broth and incubated them at 37C for 24 hoursWe streaked a loopful of growth from the overnight broth on the Pseudomonas agar base, which contained 0.1% cetramide, nutritional agar, and MacConkey agar. We plated one ml of each sample in duplicate, following I.C.M.S.F. (1998), and incubated it at 37°C for 24 hours under aseptic conditions. . Following LaBauve & Wargo's 2012 instructions, we streaked the isolates from Pseudomonas agar onto blood-agar plates and cultured them at 37°C for 24 hours. The presence of a transparent zone around the colonies indicated haemolysis. MorpholoGramme staining was used to identify the shapes of the bacteria. All the gram-negative rod-shaped isolates were then grown on MacConkey agar plates to separate lactose fermenters from non-lactose feWe further followed the isolates with biochemical confirmatory tests, including the catalase test, oxidase test, indole test, motility test, urease test, TSI (triple sugar iron) test, MR (methyl red), and gelatin hydrolysis test (Galushko and Kuever, 2020). . We made serial dilutions up to the 10th dilution. In the 10th dilution, 100 µl of the solution were taken and spread on the nutrient agar plate. Pathmanathan et al. (2003) counted the CFU of the *Pseudomonas aeruginosa*.

Drug susceptibility testing

According to the National Committee for Clinical Laboratory Standards (NCCLSI) (2012), we conducted antimicrobial sensitivity profiles of isolates using the Kirby-Bauer disc diffusion method. Table 2 displays the total number of antimicrobial discs used. Antimicrobial discs were placed on Muller Hinton plates and incubated at 37ºC for 24 hours. Following a 24-hour incubation period, we measured and interpreted the clear zones surrounding the discs into the resistant, intermediate, and sensitive categories using the NCCLSI (2012) interpretation table 1.

Table 1. Interpretation chart (given by NCCLS)

Resistant	Intermediate	Sensitive
≥ 12 mm	≥ 14 mm	≥ 17

Table 2. Antibiotic discs used to check the sensitivity profile of *P. aeruginosa*

Sensitive (≥ 17)		**Intermediate (≥ 14 mm)**		**Resistant (≥ 12 mm)**	
Antibiotics Disc	**Sensitivity Percentage**	**Antibiotics Disc**	**Intermediate Percentage**	**Antibiotics Disc**	**Resistance Percentage**
Ceftriaxone	98%	Lomefloxacin	32.20%	Streptomycin	100%
Ciprofloxacin	95%	Tobramycin	28%	Tetracycline	100%
Ofloxacin	93%	Gentamycin	20.20%	Doxycen	100%
Cephalexin	89%	Norfloxacin	9.10%	Erythromycin	100%
Azithromycin	60.50%			Ampicillin	100%
Enrofloxacin	49.50%			Trimethoprim	100%

Molecular identification of isolates

DNA of bacterial culture grown on Pseudomonas agar base containing 0.1% cetramide media was extracted by the phenol-chloroform (organic) method (Shin, 2012). We added 1 l of template DNA to 20 l of the PCR reaction solution. 35 cWe carried out 35 cycles of amplification at 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s. V3 and V4 hyper-variable regions of 16s rRNA were amplified; approximately 1100 bps of DNA were amplified. Using the Montage PCR Clean-up kit, unincorporated PCR primers and dNTPs were eliminated from the PCR results (Millipore). We performed polymerase chain reactions on a Galaxy XP Thermal Cycler (BIOER, PRC). In this study, the 1492/27 primer set (GGTTACCTTGTTACGACTT and AGAGTTTGATCCTGGCTCA) was utilised as a barcode to amplify the 16S rRNA gene of the bacterial samples (Queipo-Ortuno et al. 2008). The chemicals and reagents used with their concentrations were template DNA, PCR buffer (Thermo-Scientific-01047431), Taq polymerase enzyme 5U/µL (Thermo-Scientific-01047431), PCR water (Invitrogen RT-PCR grade water—AM9935), dNTPs (Thermo-Scientific-01044193), and $MgCl_2$ (Thermo-Scientific-01047431) were utilised (Ahmed et al. 2009). The amplified PCR products (DNA) were sent to Macrogen Korea for 16S rRNA gene sequencing (Macrogen HQ 238, Teheran-ro, Gangnam-gu, Seoul, Republic of Korea).

Results

Isolation of *Pseudomonas aeruginosa* on pseudomonas cetrimide agar

Out of 24 faecal samples, 13 samples from the Aviary of Safari Zoo Lahore, including crow and starling, and 14 samples from the waterfowl lake of the Lahore zoological garden, including crow, tested positive for different serovars of *Pseudomonas aeruginosa* on the selective medium pseudomonas cetrimide agar with bluish-green color. Overall, the prevalence of *Pseudomonas aeruginosa* in safari was 54.1%, and in the Lahore zoological garden, it was 61.90% on the basis of selective medium (Fig. 1). The *Pseudomonas aeruginosa* colonies were gram negative and rod shaped under a 100X microscope. Catalase, oxidase, gelatin, and simon citrate were found in the isolates, but indole, urease, MR (methyl red), and H2S (hydrogen sulphide) were not. *Pseudomonas* aeruginosa demonstrated motility in semisolid nutrient agar/broth medium and demonstrated non-lactose fermentation on MacConkey agar. All the isolates of *Pseudomonas aeruginosa* showed pathogenicity on the blood agar.

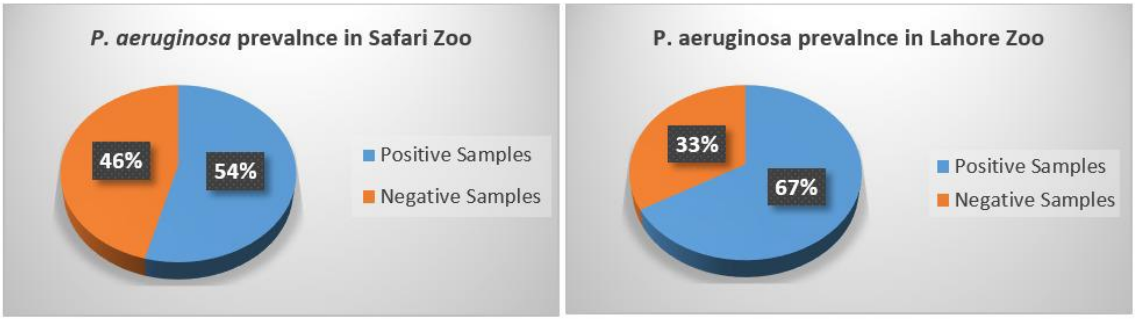


Figure 1. Showing the percentage frequency of *P. aeruginosa* isolated on selective agar medium

Two-sample t test was applied against the mean values of prevalence of *P. aeruginosa* in Lahore Zoo and Safari Zoo Lahore. The p-value of the prevalence was P=0.001, showing the significant difference in the prevalence of P. aeruginosa in two captive sites (Fig. 1).

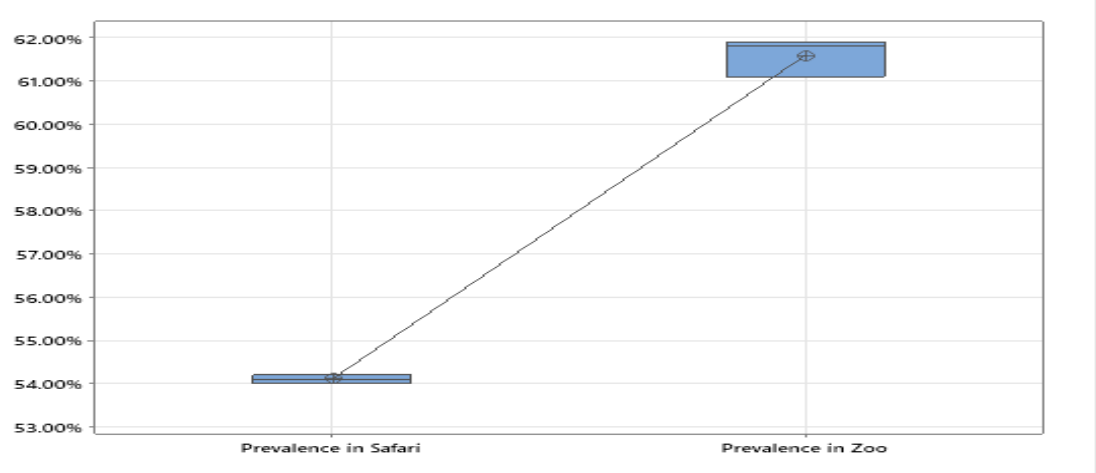


Figure 2. The boxplot compares the prevalence of P. aeruginosa in Safari Zoo Lahore and Lahore Zoo. Graphs show the Lahore Zoo has the highest prevalence of *P. aeruginosa* in different species of captive wild ducks.

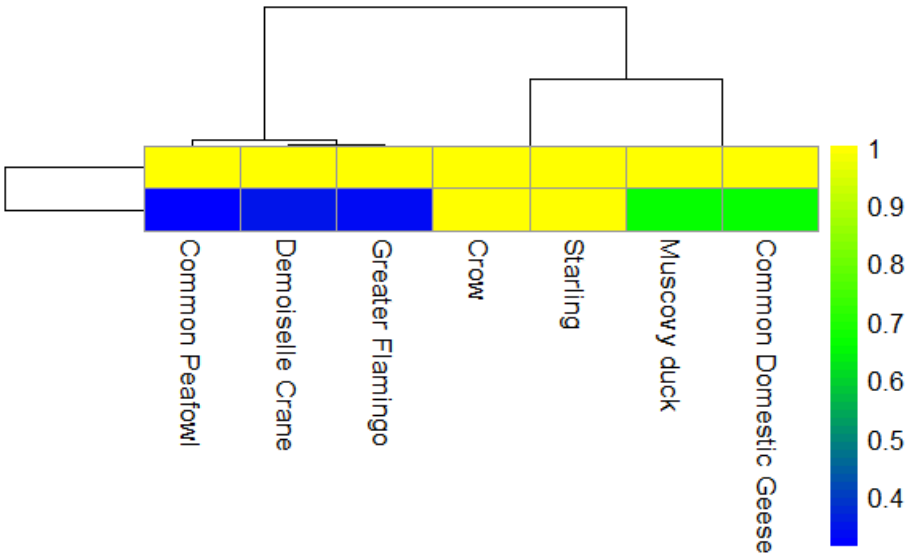


Figure 3. Heatmap showing prevalence of *P. aeruginosa* in the selected species of birds

CFU/ml (colony forming unit)

The CFU count of all the isolates showed the highest CFU in the crow (companion bird) among all collected fecal samples from both captive sites, which were 13.9×10^8 CFU/ml at Lahore Zoological Garden and 8.09×10^8 CFU/ml at the aviary of Safari Zoo Lahore. Afterward, the crow, the most infected bird with *Pseudomonas aeruginosa,* having CFU of 7.23 × 108 CFU/ml, was the Muscovy duck in the safari zoo in Lahore, and the Mallard with 5.7×10^8 CFU/ml in the Lahore zoological garden showed the CFU of isolates.

Antimicrobial Sensitivity Profile

Out of all the isolates, 13 were found to be sensitive to the following conventional antibiotics: ceftriaxone (98%), ciprofloxacin (95%), ofloxacin (93%), cephalexin (89%), azithromycin (60.5%), enrofloxacin (49.5%), lomefloxacin (32.2%), tobramycin (28%), gentamicin (20.2%), and norfloxacin (9.1%). Ten isolates displayed the MDR (multidrug resistance pattern) and demonstrated complete resistance to six antibiotics: streptomycin (100%), tetracycline (100%), doxycycline (100%), erythromycin (100%), ampicillin (100%), and trimethoprim (100%) (Table 1). Results of antimicrobial susceptibility were measured and interpreted according to the diameter limits given by the National Committee for Clinical Laboratory Standards (NCCLS), followed by the WHO (World Health Organization) as resistant: ≥ 12 mm; intermediate: ≥ 14 mm; and sensitive: ≥ 17 (Table 3).

Table 3. Antibiotic sensitivity profile of *P. aeruginosa* strains towards the conventional antibiotics

Sensitive (≥ 17)		**Intermediate (≥ 14 mm)**		**Resistant (≥ 12 mm)**	
Antibiotics Disc	Sensitivity Percentage	Antibiotics Disc	Intermediate Percentage	Antibiotics Disc	Resistance Percentage
Ceftriaxone	98%	Lomefloxacin	32.20%	Streptomycin	100%
Ciprofloxacin	95%	Tobramycin	28%	Tetracycline	100%
Ofloxacin	93%	Gentamycin	20.20%	Doxycen	100%
Cephalexin	89%	Norfloxacin	9.10%	Erythromycin	100%
Azithromycin	60.50%			Ampicillin	100%
Enrofloxacin	49.50%			Trimethoprim	100%

Molecular identification

The V3 and V4 hyper-variable regions of 16s rRNA were amplified. DNA extracted was of 1100 bps against the ladder of 50kb size that was run in the 1st and last well against the sample DNA.

Sequence Data Analysis

The 16s rRNA sequences were normalized to 10,000 followed by the Blast on NCBI by 97% similarity read, confirmed the presence of *Pseudomonas aeruginosa* in sample (Table 4).

Table 4. Prevalence of *P. aeruginosa* strains in captive Birds of Lahore Zoo and Safari Zoo Lahore.

Birds	**Sample Site**	**Isolate**	**Strain**	**Accession Number**
Black Swan	Lahore Zoo	*P. stutzeri*	ATCC17588	LC055836.1
Greater Flamingo	Lahore Zoo	*P. oleovorans*	PI 11	MT560339.1
Muscovy Duck	Lahore Zoo	*P. aeruginosa*	JCM5962	LC066145.1
Pelican	Lahore Zoo	*P. aeruginosa*	JCM5962	JX085622.1
Demoiselle Crane	Safari Zoo Lahore	*P. aeruginosa*	DSM30120	OK052600.1
Crow	Safari Zoo	*P. aeruginosa*	MF105	LC066145.1
Muscovy Duck	Safari Zoo	*P. aeruginosa*	MF106	LC066146.1

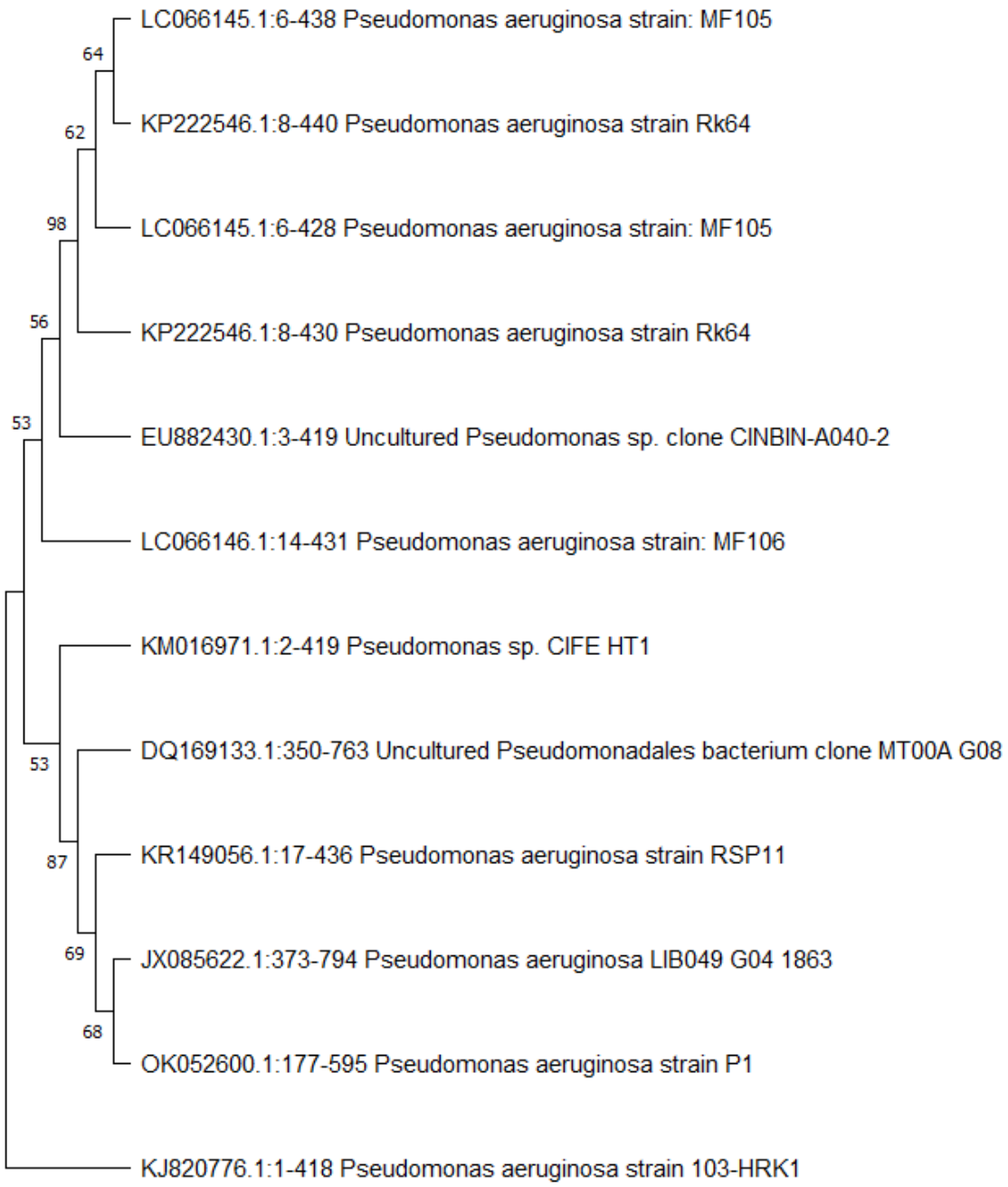


Figure 4. The phylogenetic tree based on the 16s rRNA of the multidrug-resistant *P. aeruginosa* by neighbor-joining tree method was constructed on Mega11. The out-group in the phylogenetic tree is the *Pseudomonas aeruginosa* strain (103-HRK1) with accession no (KJ820776.1). The genetic distance is shown by the scale bar. Each node has a number that shows the bootstrap percentage value of 1000 replicates. The accession number of each bacteria is given in the parenthesis generated by GenBank.

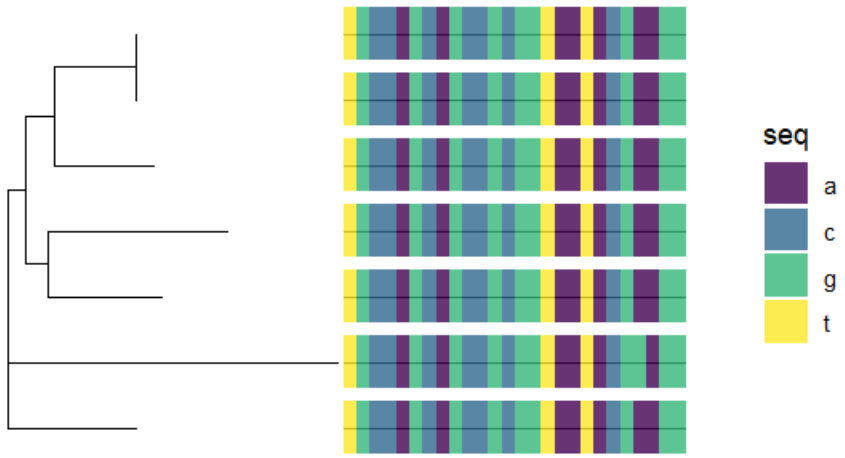


Figure 5. MSA plot showing the changes in sequences forming the basis of variation in the genomes of isolated strains.

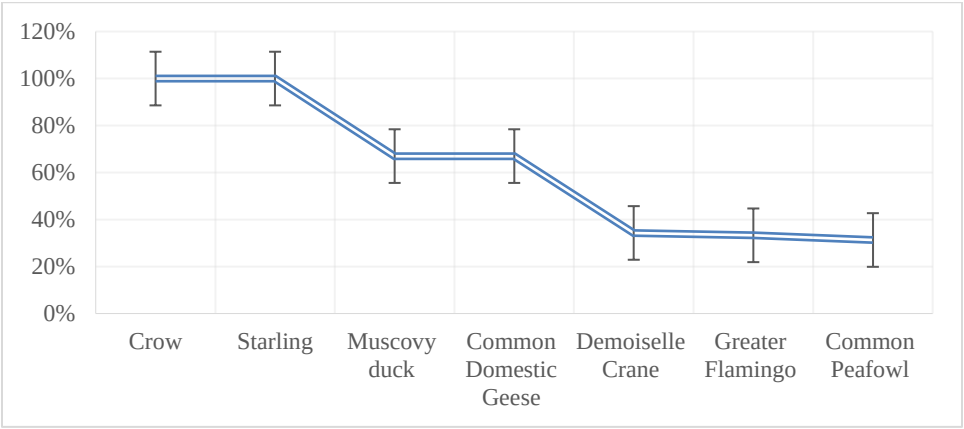


Figure 6. The prevalence of *P. aeruginosa* among the birds of the aviary of the Safari Zoo Lahore. The trend line shows a significant difference in the prevalence of *P. aeruginosa* in different species of Safari Zoo Lahore.

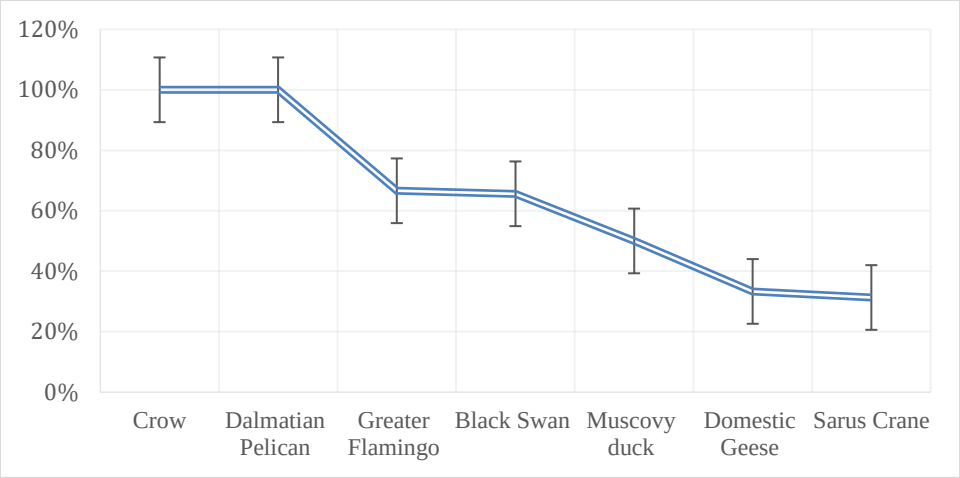
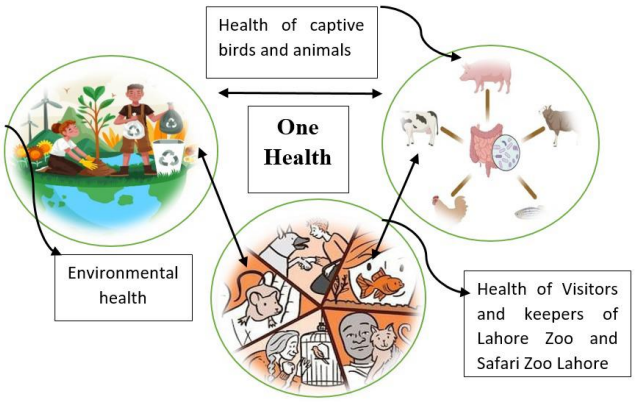


Figure 7. The prevalence of *P. aeruginosa* among the birds of waterfowl-lake of Lahore Zoo. The trend line shows a significant difference in the prevalence of *P. aeruginosa* in different species of Safari Zoo Lahore.



Zoonosis cycle

Figure 8. Shows the three components of one health concept that can be affected by zoonosis due to the transfer of *P. aeruginosa*

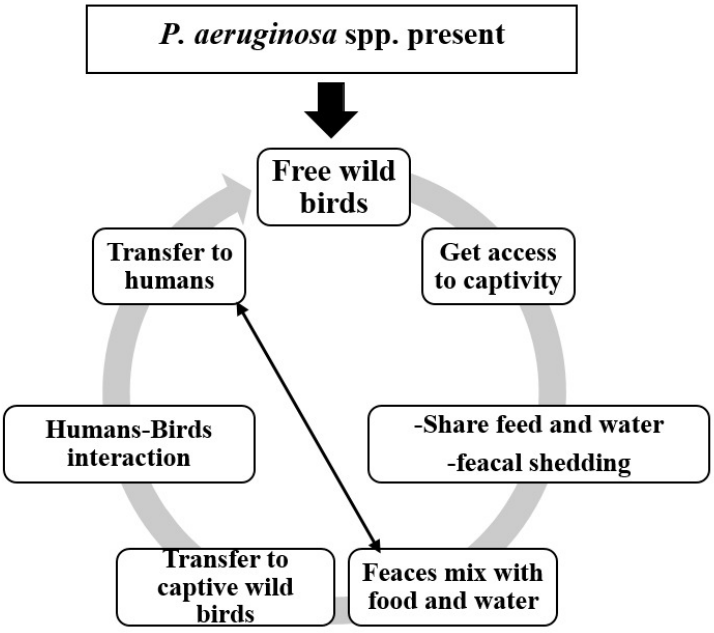


Figure 9. Zoonosis cycle. This figure shows the cyclic transfer of *P. aeruginosa* into captive birds and humans.

Discussion

In this study a total of 45 bird faecal samples were collected from two different captive sites in Lahore. We want to find out which antibiotics susceptibility test on *P. aeruginosa* and what role companion birds play in spreading zoonotic and drug-resistant *P. aeruginosa* to captive birds (Elsohaby et al., 2021). In the environment, companion birds are present in a high density (Fuller et al., 2009). Companion birds may seriously threaten the conservation of many unique species of birds and animals kept in zoos and safaris. In this study, the most prevalent bacteria were *P. aeruginosa*, isolated from the companion birds (crow and myna) and captive birds of the aviary of Safari Zoo Lahore and the waterfowl lake of Lahore Zoo. The phylogenetic tree *P. aeruginosa* is given in fig 4. In addition to *P. aeruginosa*, the study also isolated numerous pathogenic Pseudomonas species, including *P. stutzeri, P. sediminis, and P. oleovorans* (Table 4). The fig 5 is providing MSA plot showing the changes in sequences forming the basis of variation in the genomes of isolated strains as well. In 2020, a similar study in Italy discovered the prevalence of *P. aeruginosa* in companion birds at 7.8%. The present study finds that the prevalence (67%) in Lahore Zoo and Safari Zoo (54%) is higher than that in Varriale et al., 2020.

Lahore Zoo recorded a higher prevalence of *P. aeruginosa* (Figs. 6 & 7) compared to Safari Zoo, possibly due to its proximity to the busy Mall Road and Bagh e Jinnah in Lahore. Crow carries the contaminations (manure, sewage, food items, and clinical waste) of the entire Mall Road, Bagh-e-Jinnah, and nearby areas to the captive birds and other animals of Lahore Zoo. The Lahore Zoo's waterfowl lake lacks a net enclosure, allowing companion birds to freely enter and share their food and water sources. This open access to the companion birds leads to the spread of numerous pathogens among the captive birds through faecal shedding. This could potentially lead to further contamination of the birds in the waterfowl lake. The literature on the prevalence of different diseases in Lahore Zoo also supports the idea that it is more contaminated (Hussain et al., 2021).

The study found that the companion birds, crows (100%) and starlings (common mynas) (100%) in both captive sites, had the highest contamination of *P. aeruginosa.* There is evidence that all of the companion birds tested positive for *P. aeruginosa,* which supports the study's main idea that these birds may carry or be infected by new zoonotic pathogens (Vidal et al. 2017). The current study correlates with the findings of Wang et al., 2021 that companion birds are responsible for the spreading of zoonotic and antimicrobial-resistant pathogens to the environment.

Besides the companion birds, there are many other sources of transfer of *P. aeruginosa:* keepers and visitors. The keepers of both captive sites handle all the birds in their respective cages. Therefore, they have the potential to contract *Pseudomonas aeruginosa* and subsequently spread this pathogen to other animals. Similarly, visitors who have direct contact with the birds in the aviary at Safari Zoo Lahore have the potential to either acquire or transfer the bacteria to the birds in captivity, thereby contributing to the spread of zoonosis (the concept of one-health). Contaminated food and water may also be a lead factor in spreading this pathogen in birds, according to Lambertini et al., 2010. Figures 8 and 9 provide a better understanding of the concept of zoonosis and its impact on health. The current study's findings align with a 2017 American study by Cunningham & Tsiouris on the emergence of zoonotic disease from wildlife.

The current study reveals that Pseudomonas aeruginosa poses a risk of enteric infection to water birds in the duck pond of Safari Zoo Lahore and the waterfowl lake of Lahore Zoo. The current study's findings align with Georgopoulou and Tsiouris' 2008 assertion that zoonotic pathogens are more likely to infect ducks and water birds. In the current study, Dalmatian pelicans in the waterfowl lake of Lahore Zoo have shown 100% prevalence. In contrast, the prevalence of Muscovy ducks and common domestic geese in the duck pond of the aviary of Safari Zoo Lahore was 67%, a situation

that warrants the attention of both the Lahore Zoo and Safari Lahore authorities to implement preventive measures.

The antimicrobial resistance against *P. aeruginosa* is increasing in wild and captive birds worldwide due to excessive and irregular use of antibiotics. The present investigation mirrors the Wicklow study by Carroll et al., 2015, which found that *P. aeruginosa's* daily resistance to common antibiotics escalates, posing a challenge for treatment and raising concerns for both public and animal health. In this study, the isolated *P. aeruginosa* strains showed 37% resistance to 6 antibiotics, identical to the findings of Ahmed 2016, and 48% sensitivity to the 10 antibiotics; some antibiotics showed intermediate sensitivity (15%) against *P. aeruginosa.* Studies conducted in Pakistan by Khan et al., 2014, and Ijaz et al., 2019 support the current findings of the study. MDR bacteria exhibit resistance to at least two distinct representatives of at least two classes of antibiotics, according to the definition of the term MDR (Magiorakos et al., 2012; Rodrigues et al., 2021). In the current study, antibiotic resistance to 6 commonly used antibiotics suggested that *P. aeruginosa* is multidrug-resistant (MDR) (Table 3). The findings of this study correlate with the findings of Benskin et al., 2009. The sensitivity of antibiotics against aeruginosa was arranged as follows. Ceftriaxone > Ciprofloxacin > Ofloxacin > Cephalexin > Azithromycin > Enrofloxacin > Lomefloxacin > Tobromycin > Gentamycin > Norfloxacin**.** In this study, ceftriaxone (98%), ciprofloxacin (95%), ofloxacin (93%), and cephalexin (89%) showed the maximum sensitivity against *P. aeruginosa* in vitro. Present findings suggest these antibiotics for treating zoonotic *P. aeruginosa* infections in birds. To minimise the resistance, antibiotic treatment should be regular and avoid the excessive use of antibiotics without the proper knowledge of bacteria species.

Conclusion

The results of the present study verified the presence of *P. aeruginosa* in captive birds from both captive sites and companion birds, namely starlings and crows. Through a variety of routes, these companion birds contribute to the spread of *P. aeruginosa* among confined birds. If the captive birds contract a zoonotic infection, it could lead to the extinction of those rare bird species, as there would be no other means to preserve them. If wild birds harbor potentially zoonotic bacteria, it could impact public health. Birds are becoming increasingly resistant to antibiotics, and in addition to zoonotic infections, companion birds are also a source of antimicrobial resistance. Physicians, veterinarians, and public health specialists must work together due to the rise in antibiotic resistance.

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