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Original Article

Assessment of Antibiotic Resistance Profiles of Pathogenic Bacteria Isolates from Migratory Birds in the River Ravi Stopover Site

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ABSTRACT

Migratory birds are important vectors for the spread of zoonotic and antimicrobial-resistant (AMR) bacteria, posing a global public health risk. The River Ravi in Pakistan, is a crucial stopover for migratory birds, is seeing increased anthropogenic activity, which contributes to the spread of AMR bacteria. **Objective:** To assess the prevalence of specific antibiotic-resistant bacterial species among different migratory birds, and significance of migrating birds as possible reservoirs and disseminators of AMR bacteria. **Methods:** The study was carried out along the Ravi River in Lahore. Fecal samples from five migrating bird species were gathered at five distinct locations utilizing non-invasive mist netting and fresh fecal collection. The bacteria were isolated and identified using Gram staining and biochemical analysis. The Kirby-Bauer technique of disc diffusion was employed to assess the sensitivity of microorganisms. **Results:** All sites showed significant contamination from Gram-positive and Gram-negative bacteria. *Enterococcus spp.* and *Escherichia coli* were most common, indicating fecal contamination. It was found that *E. coli* and *S. aureus* are highly resistant to Azithromycin and Gentamycin, whereas *K. pneumoniae* is resistant to Norfloxacin. However, erythromycin and tetracycline were effective against *P. aeruginosa* and *C. septicum*, respectively. **Conclusions:** The research concluded that the River Ravi was significantly contaminated with germs from humans and the surroundings, along with distinct AMR patterns. The elevated levels of *S. aureus*, *E. coli*, *Enterococcus spp.* and *Pseudomonas spp.* point to serious health risks for the general public. AMR patterns emphasize the difficulty in controlling bacterial infections, emphasizing the importance of targeted antimicrobial treatments and ongoing surveillance.

INTRODUCTION

Migratory birds have been related to the carriage and transmission of antibiotic-resistant microbes that are important for both humans and animals. Every year, billions of birds migrate to practically every continent in the world. Migratory birds, due to their high movement, can acquire and spread antibiotic-resistant pathogens from one location to another [1]. Throughout the migration, they can interact with resident birds, come into encounters with feeds from various settings, and eventually pick up resistant bacteria from those regions. They can operate as reservoirs and propagators of resistant bacteria in aquatic habitats through fecal contamination [2]. *E. coli* [3, 4], *Salmonella spp.* [5], *Staphylococcus* [6], and *Campylobacter* [7, 8] act like the carriers and spreaders by migratory birds. They can be transmitted to people through the consumption of contaminated food and water [9]. Antibiotic use has gradually expanded since its initial use for the treatment of bacterial illnesses. Though antibiotics were first developed to treat humans, they are now widely employed in veterinary care and cattle production. The indiscriminate utilization of antibiotics has led to the development of antibiotic resistance or multidrug resistance in bacteria across several environments. Antibiotic-resistant bacteria obtained in a certain region are simple to spread to other environments, affecting natural ecosystems worldwide. Antibiotic exploitation and malfunctioning healthcare systems have been linked to the proliferation of resistant bacteria in a variety of ecological factors [10]. The level of antibiotic use influences the number of resistant bacteria in habitat [11]. Remarkably, although not directly linked to human exposures, pristine habitats are implicated in bacterial antibiotic resistance [12]. These findings imply that other factors are influencing the spread of resistant bacteria to such clean habitats. River Ravi is a transnational river that enters Pakistan at Shakkargarh (Sialkot) and falls into River Chenab at Shorkot. The Ravi River supports a variety of habitats, which makes it a significant wintering habitat for migrating birds [13]. However, the area has experienced rapid population growth, the Industrial Revolution, and expansion of agriculture during the last few decades. Human endeavors have increased the consumption of antibiotics while also releasing prescription waste, toxic metals, and other pollutants into the River Ravi ecosystem [14]. Antibiotics along with other selective factors in surroundings can promote the evolution and dissemination of antibiotic-resistant bacteria [15]. The quantity and characteristics of AMR bacteria associated with migrating birds in the River Ravi basin have not been adequately studied, despite the fact that there may be dangers. An effective strategy to curb the spread of drug- resistant germs in the region can only be developed when a thorough comprehension of the role these birds play in AMR transmission is achieved.

The study aimed to assess the prevalence of specific antibiotic-resistant bacterial species among different migratory birds, and significance of migrating birds as possible reservoirs and disseminators of AMR bacteria

METHODS

This study focused on the Ravi river belt in the city of Lahore. Migratory avian species inhabit the vicinity of the Ravi River during winter seasons. The study was conducted between December 2022 and February 2023. The fecal samples were collected from five different locations of the border of River Ravi belt (Talwara) surrounding Lahore [16].

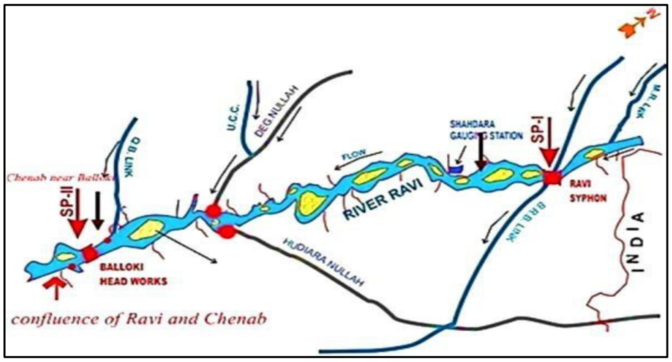

Figure 1: Map of River Ravi to Depict Fecal Sample Collection Sites

A pilot survey is a small-scale preliminary study aimed at assessing feasibility, with Concerned Department. The meeting was done with Hunter and the Local Community They Consulted with hunters using GPS and other tools for bird tracking and trapping and interacted with local community members including fishermen and residents to facilitate the sampling process. Fresh fecal samples were collected from birds using non-invasive mist nets and fresh fecal sample collection from five sites. The birds were released at their capture sites within 30 minutes to minimize stress and environmental impact. A total of 05 wild bird species (Gad Wall, Pouchard, Teal, Mallard, and Shoveller) were selected for sampling on the basis of availability. In order to detect bacteria from certain bird species, fifty fecal samples were gathered from five different places along the River Ravi belt (Talwara). The fecal matter was collected in sterilized swabs from the birds by mist nets method through cloacal swabbing. It was tried to not the harm or disturbs the birds during sampling. Samples were stored in ice boxes and shifted to the Conservation Biology Lab, Institute of Zoology-University of Punjab of isolation and identification of bacteria. Each swab was streaked on MacConkey agar and incubated aerobically at 35°C for 18-20 hours. Distinct colonies were isolated, purified, and cultured in soft nutrient agar at room temperature for subsequent analysis. The fecal samples, each weighing 1 gram, were diluted in 9 milliliters of normal saline solution, resulting in a series of dilutions ranging from 10^-1 to 10^-10. A 20µl aliquot from each dilution was evenly distributed on nutritional agar plate and subjected to a 24-hour incubation period. Colonies were counted using the formula [17].

CFU/ml= Number of Bacteria colony × Dilution Factor/ Volume of Culture

A sterilized slide was prepared with a saline drop and a single bacterial colony. After drying and heat fixing, slides were stained with crystal violet, iodine, decolorized with acetone alcohol, and counterstained with safranine. Observations under a microscope identified the bacteria as Gram-positive or Gram-negative. The biochemical identification of tests was done through Gram-positive bacteria were tested using the Mannitol Salt Agar (MSA), Catalase and DNase tests. Gram-negative bacteria were detected by employing MacConkey agar and conducting various, tests, including Urease, Motility, Oxidase, Indole, KIA, and TSI assays [18]. The Kirby-Bauer method of disc diffusion was used to measure sensitivity on Muller Hinton agar. The bacterial colonies that were identified were collected using a swab and transferred onto plates. Then, sixteen different antibiotic discs were placed on the plates. These disc included (Cip) ciprofloxacin (5ug), (Nor) norfloxacin (5ug), (Lom) lomefloxacin (5ug), (Tob) tobromycin (10ug), (Cef) ceftriaxone (10ug), (Ste) streptomycine (10ug), (Gen) gentamycin (10ug), (Tri) trimethoprim (25ug), (Tet) tetracycline (30ug), (Dox) doxycen (20ug), (Ery) erythromycine (15ug), (Amp) ampicillin (30ug), (Enr) enrofloxacin (5ug), (Azi) azithromycin (15 µg), (Ofl) Ofloxacin (5µg), (Cep) Cephalexin (10 µg). The plates were then kept in an incubator overnight

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at a temperature of 37°C. The measurement of zones of inhibition was conducted to ascertain the sensitivity of the microorganisms to antimicrobial agents. Data were entered and analyzed by Graph Pad Prism. To create the graphs, OriginPro 2022 was used.

RESULTS

From River Ravi, five different migratory birds' fecal samples were collected through non-invasive method. Gram Positive and Gram Negative bacteria were isolated and identified by a variety of different biochemical tests as described in figure 2.

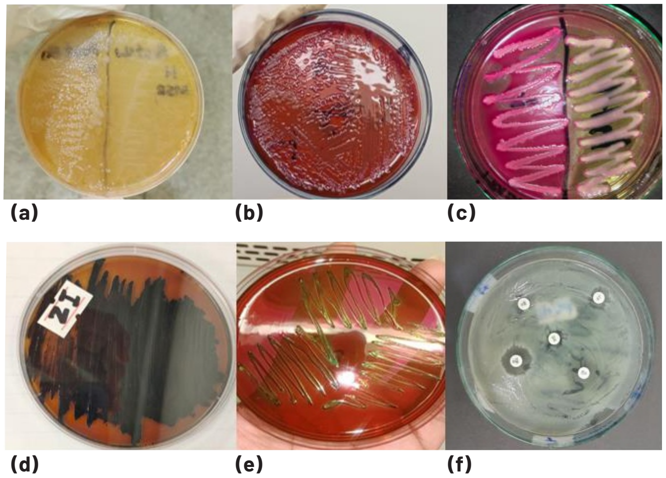

Figure 2: (a) Non-lactose fermenting colony for Gram Negative bacteria grown on MacConkey agar, (b) Gram Negative bacteria growth (fermenter colonies). (c) Mannitol Salt Agar (MSA) for Staphylococcus aureus (left side of the plate) and *Staphylococcus epidermidis* (right side of the plate). (d) *Salmonella* spp. on SS Agar (e) *E. coli* on EMB Agar (f) AMR Bacteria Drug Sensitivity Test

Gram-positive and Gram-negative bacteria found in the samples are included with the findings of the first sampling site in table 1. The data reflects the microbial variety at the initial sampling site, as some species host numerous types of bacteria.

Table 1: Gram Negative and Gram Positive Bacteria Isolated from Birds at First Sampling Site

Name of Specie	Gram Negative Bacteria	Gram Positive Bacteria
Gadwall	*Salmonella spp.*	*Staptococcus spp.*
Pouchard	*E. coli spp.*	*Enterococcus spp.*
Teal	*E. coli spp.*	*Enterococcus spp.*
Mallard	*Pseudomonas spp.*	*Enterococcus spp.*
Shoveller	*Pseudomonas spp.*	*Enterococcus spp.*
Gadwall	*E. coli spp.*	*Staphylococcus spp.*
Pouchard	*E. coli spp.*	*Staphylococcus aureus spp.*
Teal	*E. coli Pseudomonas spp*	*Enterococcus spp.*
Mallard	*Pseudomonas spp.*	*Enterococcus spp.*
Shoveller	*E. coli spp.*	*Enterococcus spp.*

Different bird species and the kinds of Gram-positive and Gram-negative bacteria found in each, along with the results from the second sampling site have been enlisted in table 2. Gadwall, Pouchard, Teal, Mallard, and Shoveller are among the species that are included. *Pseudomonas species*, *E. coli species*, *Klebsiella*, *Salmonella species*, and *Coliform species* are among the Gram-negative bacteria that were discovered; *Clostridium species*, *Enterococcus species*, *Staphylococcus species*, and *Staphylococcus aureus* are among the Gram-positive bacteria that were found. The data demonstrates the microbial diversity found at the second sample site by showing that numerous kinds of bacteria are hosted by them.

Table 2: Gram Negative and Gram Positive Bacteria Isolated from Birds at Second Sampling Site

Name of Specie	Gram Negative Bacteria	Gram Positive Bacteria
Gadwall	*Pseudomonas spp.*	*Clostridium spp.*
Pouchard	*Pseudomonas spp.*	*Enterococcus spp.*
Teal	*E. coli spp.*	*Enterococcus spp.*
Mallard	*E. coli spp.*	*Enterococcus spp.*
Shoveller	*E. coli spp.*	*Enterococcus spp.*
Gadwall	*E. coli Klebsiella Coliform spp.*	*Enterococcus* *Staphylococcus spp.*
Pouchard	*E. coli Klebsiella Coliform spp.*	*Enterococcus* *Staphylococcus spp.*
Teal	*E. coli Salmonella spp.*	*Staphylococcus aureus*
Mallard	*E. coli Salmonella spp.*	*Staphylococcus aureus*
Shoveller	*E. coli Salmonella spp.*	*Staphylococcus aureus*

The third sampling site yielded a variety of bird species in addition to different kinds of Gram-positive and Gram-negative bacteria, as shown in table 3. The third sampling site's bacterial diversity and prevalence frequency among various bird species are highlighted by this data.

Table 3: Gram Negative and Gram Positive Bacteria Isolated from Birds at Third Sampling Site

Name of Specie	Gram Negative Bacteria	Gram Positive Bacteria
Gadwall	*Salmonella* *Klebsiella spp.*	*Enterococcus,* *Staphylococcus spp.*
Pouchard	*E.coli*	*Staphylococcus spp.*
Teal	*E. coli*	*Staphylococcus aureus*
Mallard	*E. coli*	*Enterococcus spp.*
Shoveller	*Pseudomonas spp.*	*Staphylococcus aureus*
Gadwall	*Pseudomonas spp.*	*Staphylococcus aureus*
Pouchard	*E. coli spp.*	*Enterococcus spp.*
Teal	*E. coli spp.*	*Enterococcus spp.*
Mallard	*E. coli spp.*	*Enterococcus spp.*
Shoveller	*Salmonella spp.*	*Staphylococcus epidermis*

The different bird species and the kinds of Gram-positive and Gram-negative bacteria found at the fourth sampling site are shown in table 4. The data presented here illustrates the prevalence frequency and diversity of bacteria across several bird species at the fourth sample site.

Table 4: Gram Negative and Gram Positive Bacteria Isolated from Birds at Forth Sampling Site

Name of Specie	Gram Negative Bacteria	Gram Positive Bacteria
Gadwall	*Salmonella Klebsiella spp.*	*Enterococcus, Staphylococcus spp*
Pouchard	*E.coli*	*Staphylococcus aureus*
Teal	*E. coli*	*Staphylococcus aureus*
Mallard	*E. coli* *Pseudomonas spp.*	*Enterococcus spp.*
Shoveller	*Pseudomonas spp.*	*Enterococcus spp.*
Gadwall	*E.coli*	*Enterococcus spp.*
Pouchard	*Pseudomonas spp.*	*Clostridium spp.*
Teal	*Pseudomonas spp.*	*Clostridium spp.*
Mallard	*Salmonella, Pseudomonas spp.*	*Salmonella, Pseudomonas spp.*
Shoveller	*Pseudomonas spp.*	*Enterococcus spp.*
Gadwall	*E.coli, Pseudomonas spp.*	*Staphylococcus aureus*
Pouchard	*E.coli*	*Staphylococcus penumonia*
Teal	*E.coli*	*Staphylococcus penumonia*
Mallard	*E.coli*	*Staphylococcus*
Shoveller	*E.coli*	*Staphylococcus*

The Gram-positive and Gram-negative bacteria found at the fifth sampling site are listed in table 5 along with a variety of bird species. The data presented here illustrates the prevalence frequency and diversity of bacteria across several bird species at the fifth sample site.

Table 5: Gram Negative and Gram Positive Bacteria Isolated from Birds at Fifth Sampling Site

Species of Birds	Gram Negative Bacteria	Gram Positive Bacteria
Gadwall	*E.coli, Klebsiella Coliform spp.*	*Enterococcus, Staphylococcus spp.*
Pouchard	*E.coli, Klebsiella Coliform spp.*	*Enterococcus, Staphylococcus spp.*
Teal	*E.coli, Salmonella spp.*	*Staphylococcus aureus*
Mallard	*E.coli, Salmonella spp.*	*Staphylococcus aureus*
Shoveller	*E.coli, Salmonella spp.*	*Staphylococcus aureus*

The distribution of several bacterial species among the five sampling sites is depicted in the heat map (Figure 3). Darker hues indicate higher percentages of bacteria, and the color gradient shows the number of bacteria present. This graphic illustrates how the presence of bacteria varies in various places.

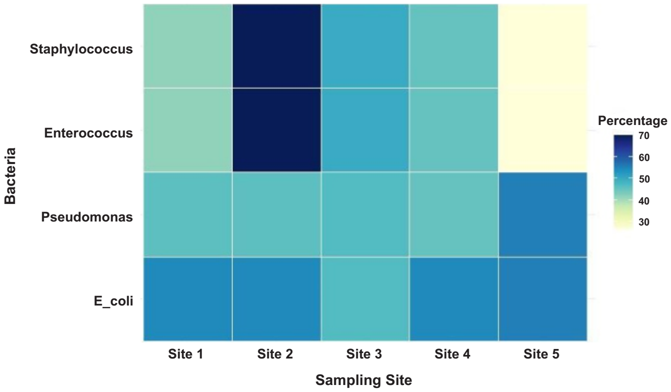

Figure 3: Heat Map for Bacterial Distribution at Each Sampling Site, Showing the Prevalence Frequency of Different Bacterial Genera

A total of 16 antimicrobial discs were used and the results were recorded (Table 6).

Table 6: Inhibition Zones of Different Bacteria Against Different Antibiotics

Bacterial Species	Cip	Nor	Lom	Tob	Cef	Ste	Gen	Tri	Tet	Dox	Ery	Amp	Enr	Azi	Ofl	Cep
E. coli.	R	R	R	S	R	I	R	R	S	R	S	R	I	S	R	S
Klebsiella pneumoniae	R	S	R	S	R	I	S	R	S	R	I	S	R	S	R	S
Enterococcus	R	R	R	S	R	I	I	S	R	I	S	R	S	R	I	S
Salmonella ssp.	R	R	R	S	R	I	S	R	S	R	R	R	I	R	R	S
Staphylococcus aureus	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Pseudomonas arignosa	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	I
Clostridium septicum	I	R	R	I	R	R	R	R	R	S	S	R	R	R	R	R

The antibiotic susceptibility of different bacterial species is shown in the heat map, where a colour gradient indicates the susceptibility levels (blue for high susceptibility, red for low susceptibility). Patterns of antibiotic efficaciousness and bacterial resistance are highlighted by hierarchical clustering. As an illustration, Clostridium septicum exhibits low susceptibility to doxycycline (Dox) and high susceptibility to ciprofloxacin (Cip). Salmonella species have significant resistance to Amoxicillin (Amp) and Azithromycin (Azi). The diverse ways that bacteria react to different antibiotics are well-illustrated (Figure 4).

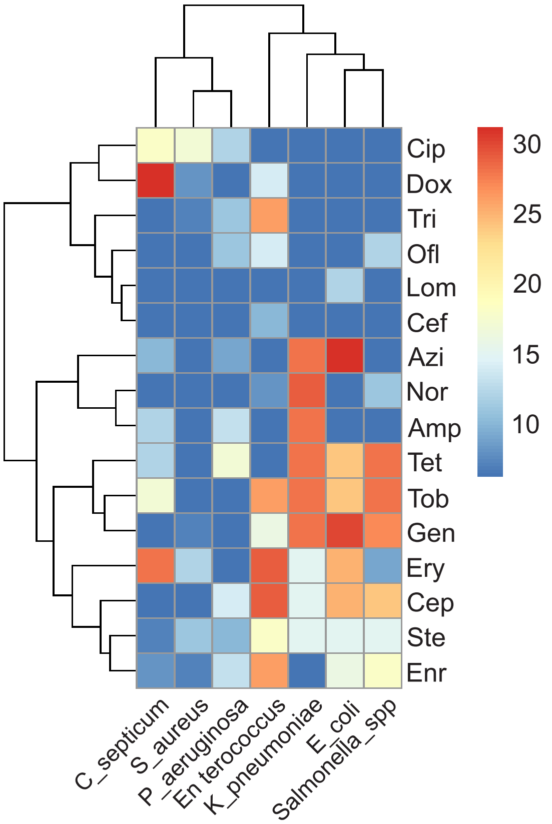

Figure 4: Inhibition Zones of Different Bacteria Against Different Antibiotics

DISCUSSION

The River Ravi basin in Pakistan is a significant stopover for many migratory waterfowl species, such as gadwall, common Pouchard, common teal, mallard, and northern Shoveler. These avian species migrate to the area from their breeding habitats in Central Asia and Siberia, in search of milder climates and adequate food supplies over the winter season.[19]. Although migratory wild birds aren't directly linked with the emergence of resistance to antibiotics due to their lack of exposure to antimicrobial drugs, they can act as reservoirs, facilitating the mixing and dissemination of antimicrobial resistance. They also function as a significant measure for assessing the influence of human actions, such as the inappropriate utilization of antimicrobials, on the ecosystem [20-22]. At the first sampling location, *Enterococcus* had the largest occurrence rate among Gram Positive bacteria, accounting for 41.7%, whereas Pseudomonas was the most prevalent Gram Negative bacterium, with a prevalence of 46.2%. The significance of these results was confirmed by the ANOVA analysis, which showed a significant change ($p < 0.05$) in the prevalence rates of various bacterial species. Animal and human intestines frequently contain Enterococcus species, and their presence in the environment may be a sign of fecal contamination. The adaptability and capacity of *Pseudomonas* species to thrive in a variety of settings, including contaminated locations, is well documented. Their high frequency could indicate the existence of contaminants or particular environmental factors that support their proliferation.[23, 24]. In the following sample location, *Enterococcus* made up 70% of Gram-Positive bacteria, whereas *E. coli* was present in 54.5%. Significant differences across bacterial species were confirmed by statistical analysis ($p < 0.05$), highlighting the significance of keeping an eye on the sources of contamination. Location, Gram-positive *Enterococcus* bacteria represented half the sample specimens, while Gram Negative *E. coli* bacteria made up 47.1%. The elevated *E. coli* and *Enterococcus* levels at this location indicate fecal contamination. This consistent observation from numerous areas highlights the broad problem of water contamination in the River Ravi. With a significant P value ($p < 0.05$), the fourth sample location showed the highest number of Gram-positive bacteria, namely *S. aureus*, at 45.5%. *E. coli* was present in 54.5% of Gram Negative bacteria. *S. aureus* is ubiquitous on human skin and mucous membranes, so its presence in this sample raises concerns about possible contamination caused by people [25]. The theme of fecal contamination is highlighted by the persistence of *E. coli* [26]. In the fifth place where samples were taken, Gram Positive bacteria had a predominance of 26.7% *S. aureus* and Gram Negative bacteria 55.6% *E. coli*, with both species showing significant differences in prevalence ($p < 0.05$). The findings from the fifth site corroborate those from the fourth site, suggesting that human-caused contamination and the presence of feces are ongoing issues.

CONCLUSIONS

It was concluded that the migratory birds contain many Gram-Positive and Gram-Negative pathogenic bacteria. These pathogens have antimicrobial resistance. It's troubling that many infectious bacteria like *Salmonella spp.*, *E. coli*, and *S. aureus* have been isolated from samples. The findings also suggest the fact that many medications have distinct effects on different types of bacteria opens up potential pathways for guided antimicrobial treatment, in addition to the required constant monitoring and individualized treatment plans. Protecting human and animal health necessitates better management and monitoring of environmental contamination with bird feces. The migratory birds could play a vital role in the dispersal of AMR bacteria.

Authors Contribution

Conceptualization: BNK
Methodology: BNK, ST
Formal analysis: BNK, YA
Writing-review and editing: BNK, ST, YA, AM, MAH, MF, SG, AT

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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