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Antibiotic Resistant Enterobacteriaceae in Slender-Billed Gulls Species Migrating to Libya

Aya Mansour [1], Ehab Sharif [2,3], Abdulmajid Hamhoom [3,4], Khaled Etayeb [3,5], Abdunaser Dayhum [3,6], Ibrahim Eldaghayes [3,7,*] and Abdulwahab Kammon [2,3,*]

1 Department of Microbiology, Faculty of Science, Zintan University, Alzintan, Libya; myfirstgoal2020@gmail.com
2 Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya; eelshrif@hotmail.com
3 National Research Center for Tropical and Transboundary Diseases, Alzintan, Libya; a.kammon@nrcttd.ly
4 National Center for Animal Health, Tripoli, Libya; Abdulmajidhamhoum546@gmail.com
5 Department of Zoology, Faculty of Science, University of Tripoli, Tripoli, Libya; k.etayeb@uot.edu.ly
6 Department of Preventive Medicine, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya; adayhum@yahoo.com
7 Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya; ibrahim.eldaghayes@vetmed.edu.ly
* Correspondence: ibrahim.eldaghayes@vetmed.edu.ly (I.E.); a.kammon@nrcttd.ly (A.K.)

Simple Summary: The slender-billed gull is a mid-sized gull which breeds around the Mediterranean Sea and the northwestern part of the Indian Ocean on islands, lakes and coastal lagoons. This is the first study in Libya on the slender-billed gull where few fecal samples have been collected for bacterial isolation and antibiotic sensitivity. Few studies have focused on slender-billed gull, that can be one of sources for transmitting bacteria and pathogens at beaches. Migratory birds could play a significant role in the spread of antibiotic resistant bacteria between countries. There is also a risk of transmission of antibiotic-resistant bacteria by direct contact to domestic, wild birds and birds raised for commercial purposes.

Abstract: This study aimed to isolate, identify enteric bacterial species from the migratory seagulls migrated to Libyan cost and to assess the antibiotic resistance of these bacteria. A total of 50 fecal samples were collected from slender-billed gulls in January, 2023 at Farwa Island near the city of Zwara. Bacteria were isolated by conventional culturing method, identified by using the Enterosystem 18R, and antibiotic susceptibility testing was conducted on the isolated bacteria. Only 32 of 46 bacteria were identified using biochemical tests. These identified bacteria belong to six species of Enterobacteriacae namely *Citrobacter freundii*, *Pantoea agglomerans*, *E. coli*, *Enterobacter cloacae*, *Serratia liquifaciens*, and *Proteus mirabilis*, with percentages of (17) 53.125%, (10) 31.25%, (2) 6.25%, (1) 3.125%, (1) 3.125%, and (1) 3.125%, respectively. All isolated bacterial species in this study were 100% sensitive to gentamicin and ciprofloxacin. The highest resistance rate was observed against the antibiotic cefoxitin. The results indicated that *C. freundii* was the most antibiotic-resistant bacterial species isolated in this study. In conclusion, slender-billed gulls carry multi-drug resistant bacteria. The study recommends implementation of a national program to survey antibiotic-resistant bacteria and expanding scientific research on migratory birds.

Keywords: Enterobacteriaceae; bacteria; slender-billed gulls; MAR; Libya

1. Introduction

Enterobacteriaceae members are among the most important types of bacteria naturally found in the human and animal digestive tract. Some of them can also be pathogenic. They are responsible for various diseases, including intestinal inflammation, food poisoning, urinary tract infections, and other illnesses in both humans and animals. This family includes several genera, such as *Escherichia coli*, *Klebsiella*, *Shigella*, *Salmonella*, *Streptococcus*, *Enterobacter*, and others. One of their key characteristics is being Gram-negative rods, easy to grow on simple media, motile, and with noticeable biochemical activity. Some of them also produce endotoxins [1].

Wild birds are among the most important reservoirs and potential carriers of antibiotic-resistant bacteria. Many types of antibiotic-resistant bacteria have been isolated from wild birds, and several studies have shown that wild birds can spread antibiotic-resistant bacteria through long-distance migration to distant areas. These bacteria can also transfer from birds to humans and vice versa, posing a significant threat [2]. Even if these bacteria are not pathogenic themselves, they can transfer antibiotic resistance to pathogenic bacteria, which is a concerning issue. Therefore, the study of antibiotic resistance in wild birds, especially migratory ones, is of great importance. These birds can travel hundreds of kilometers and spread disease-causing agents and antibiotic resistance across different regions and continents [3].

The instinct for survival encourages many birds to migrate, taking routes such as coasts, mountain ranges, valleys, rivers, beaches, and other paths. Although bird migration is a fascinating natural phenomenon, it poses inevitable risks to public and animal health due to direct or indirect contact between birds and humans or animals can lead to the transmission of antibiotic-resistant bacteria [4]. Approximately 5 billion migratory wild birds fly across continents twice a year, facilitating the global transfer of many disease-causing agents [5].

The Libyan coast, in particular, has various types of wetlands, such as saline marshes, lagoons, lakes, and islands, with approximately 50 observatories for migratory birds in Libyan territory. These areas provide good shelters and feeding sites for migratory birds during their journeys between Eurasia and Africa. The winter census of migratory birds in Libya for the year 2022 revealed the presence of 2,169 individuals of the Slender-billed Gull species [6]. Antibiotic-resistant bacteria have been discovered in both resident and migratory gulls worldwide for over a decade, and some studies have suggested that gulls can serve as carriers of antibiotic-resistant bacteria, distributing them through aquatic environments [7]. However, confirming this remains challenging and incomplete, requiring further studies, and there is a lack, or perhaps an absence, of studies targeting the isolation of bacteria from migratory birds in wetlands in Libya. Therefore, this study was conducted to isolate and identify enteric bacterial species from the Slender-billed Gull, a migratory bird to Farwa Island and to investigate the extent of antibiotic resistance in these isolated enteric species.

2. Materials and Methods

2.1. Sampling

Fifty fecal samples from migratory Slender-billed gull species were collected during January 2023 at Farwa Island 40 Km west to Zwara City near to the Libyan and Tunisian borders. The samples were collected using sterilized cotton swabs with careful not to touch the soil, labeled from Z1 to Z50, and stored in a refrigerated container. They were then transported to the Microbiology Laboratory at the Faculty of Science in Zintan University for bacterial isolation, identification, and antibiotic susceptibility testing.

2.2. Culture

Bacteria were isolated by culturing the samples in Peptone water (BD, USA), Vassiliadis Rappaport liquid (Park Scientific Limited, UK) medium, and Salmonella-Shigella Agar (SSA) medium (Bio Tec Diagnostics, UK). The isolated bacteria were Gram-stained and examined for morphological characteristics under a microscope. The Enterosystem 18R, which includes 20 biochemical tests was used for identifying the isolated bacteria (Liofilchem, Italy) as per the manufacturer instructions.

2.3. Antibiotic susceptibility test

The Bauer-Kirby method was used to determine antibiotics' resistance. effectively affected the isolated bacteria and which had no effect. A swab from the isolated bacterial colonies was transferred to sterilized glass tubes containing 5 ml of peptone water and incubated in an incubator at 37°C for

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24 hours. Muller-Hinton Agar (Himedia, USA) was used to cultivate the bacteria and antibiotic discs namely Gentamicin, Ciprofloxacin, Azithromycin, Ceftriaxone, Ampicillin, and Cefoxitin were used. The plates were then incubated at 37°C for 24 hours [8]. The inhibition zones were measured in millimeters using a ruler, and the results were recorded. The Multiple Antibiotic Resistance (MAR) index was calculated using the formula: "A/B," where "A" is the number of antibiotics resisted by the isolated bacteria, and "B" is the total number of antibiotics tested in this study [9].

3. Results

In this study, 50 fecal samples were collected from migratory seagulls in Libyan territories, specifically at Farwa Island near the city of Zwara, during January 2023. These seagulls belong to the Slender-billed gull species.

3.1. Bacterial Isolation and Identification

Following the culturing of the samples on various media, a total of 46 bacteria were isolated, and only 32 of them were identified using biochemical tests. These identified bacteria belong to six species of the family Enterobacteriacae namely; *Citrobacter freundii*, *Pantoea agglomerans* (formerly known as *Enterobacter agglomerans*), *E. coli*, *Enterobacter cloacae*, *Serratia liquifaciens*, and *Proteus mirabilis*, with percentages of (17) 53.125%, (10) 31.25%, (2) 6.25%, (1) 3.125%, (1) 3.125%, and (1) 3.125%, respectively (Table 1).

Table 1. Name, number and percentage of isolated and identified bacteria from Slender-billed gull species.

Name of bacteria	No. of identified bacteria	Percentage (%)
Citrobacter freundii	17	53.125
Pantoea agglomerans	10	31.25
E. coli	2	6.25
Enterobacter cloacae	1	3.125
Serratia liquifaciens	1	3.125
Proteus mirabilis	1	3.125
Total	**32**	**100**

3.2. Antibiotic Susceptibility Testing

The results showed in Table 2 indicate that all isolated bacterial species in this study were 100% sensitive to the antibiotics gentamicin and ciprofloxacin.

Regarding bacterial resistance in this study, the highest resistance rate was observed against the antibiotic cefoxitin, with both *C. freundii* and *P. agglomerans* being the most resistant. Resistance was observed in 5 samples of *C. freundii* and 4 samples of *P. agglomerans* out of 11 samples. Resistance was also noted in a few isolates to antibiotics such as azithromycin, ceftriaxone, and ampicillin.

Table 3 shows the result of multiple antibiotic resistance (MAR) index in which the calculation reveals indicators of multiple antibiotic resistance. The highest recorded indicator was demonstrated by bacteria *C. freundii*, *P. agglomerans*, and *E. cloacae*, with a value of 0.33 for each of them.

Table 2. Results (number and % of bacteria) of antibiotics susceptibility testing.

Antibiotic	Code	Sensitive	Intermediate	Resistant	
				No. of bacteria (%)	Bacterial species
Gentamicin	CN	32 (100%)	0	0	None
Ciprofloxacin	CIP	32 (100%)	0	0	None
Azithromycin	AZM	27 (84.4%)	3 (9.4%)	2 (6.3%)	*Citrobacter freundii*
Ceftriaxone	CRO	27 (84.4%)	4 (12.5%)	1 (3.1%)	*Enterobacter cloacae*
Ampicillin	AMP	27 (84.4%)	3 (9.4%)	2 (6.3%)	*Enterobacter agglomerans* *Proteus mirabilis*

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Cefoxitin	FOX	18 (56.3%)	3 (9.4%)	11 (43.4%)	*Citrobacter freundii* *Enterobacter agglomerans* *Enterobacter cloacae* *Serratia liquifaciens*

Table 3. Result of multiple antibiotic resistance (MAR) index.

No.	Bacterial species	Resistance to antibiotics	MAR Index
1	*Citrobacter freundii*	AZM, FOX	0.33
2	*Enterobacter agglomerans*	AMP, FOX	0.33
3	*Enterobacter cloacae*	CRO, FOX	0.33
4	*Serratia liquifaciens*	FOX	0.17
5	*Proteus mirabilis*	AMP	0.17

4. Discussion

In the current study, a total of 46 bacteria were isolated, and only 32 of them were identified using biochemical tests. The Enterosystem 18R system, consisting of 20 tests, was employed for identification. Previous studies that utilized this system for identifying intestinal bacterial species have shown success rates of 90% or higher [10,11].

In this study, 17 bacteria of the *Citrobacter freundii* species were isolated, accounting for 53.125% of the total isolates. This finding aligns with a study conducted in Europe [12], as well as studies in Egypt [13] and Portugal [14]. However, it differs from the results of studies conducted in Italy [15] and South America [16], where the prevalence of these bacteria was lower. *Citrobacter freundii* is known to play a significant role in opportunistic infections and is associated with neonatal meningitis, where mortality rates in neonates can reach 25-50% [17]. It also causes urinary tract infections and respiratory infections in humans [18]. *C. freundii* was also isolated from diseased domestic ducks in Bangladesh [19]. This bacterium showed multiple drug resistance to some antibiotics such as gentamicin and ciprofloxacin and the index of multiple antibiotic resistance ranged from 0.07 to 0.79.

Ten (31.25%) of *Pantoea agglomerans* bacteria were isolated from slender-billed gull in the current study. *P. agglomerans*, is a gram-negative aerobic bacillus belongs to the family Enterobacteriaceae. All species of the genus *Pantoea* can be isolated from indoor dusts of animal sheds, plants and soil [20,21]. This bacterium can be either pathogen or commensal causing secondary infections. Within the genus, *P. agglomerans* is the most commonly isolated species in humans, resulting in soft tissue or bone/joint infections following penetrating trauma by vegetation [22]. Another study reported isolation of *P. agglomerans* from urine and urinary tract of 4 children. *P. agglomerans* was most associated with penetrating trauma by vegetative material and catheter-related bacteremia [23]. In a study conducted by Giorgio et al. [24], *P. agglomerans* was isolated from Muscicapa striata migratory birds. The spotted flycatcher (Muscicapa striata) is a small passerine bird in the Old-World flycatcher family. It breeds in most of Europe and in the Palearctic to Siberia, and is migratory, wintering in Africa and south western Asia. However, there is lack of information on the isolation of *P. agglomerans* from cloacal swabs or fecal samples of slender-billed gull species.

In the current study, 2 isolates (6.25%) of *E. coli* were identified. A recent study conducted in Poland on strains of wild birds also yielded important results, as *E. coli* bacteria were isolated from 32 samples out of 34, and the results reached were as follows: Resistance to tetracycline (50%), ciprofloxacin (46.8%), gentamicin (34.3%), and ampicillin (28.1%) was frequently demonstrated, and approximately 31.2% of *E. coli* showed a multidrug resistance phenotype [25]. In Bangladesh, *E. coli* were isolated and identified from a total of 66 fecal matter samples of migratory birds [26]. The diseases caused by *E. coli* in humans include urinary tract infection, hospital-acquired pneumonia, gastrointestinal infection, meningitis, and sepsis [27]. However, migratory birds were found as reservoirs of MDR *E. coli* isolates carrying APEC-associated virulence genes, which can seriously contribute to the development of human and animal diseases [26].

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Enterobacter cloacae was also isolated and identified. It is a Gram-negative bacterium, it can be aerobic or anaerobic, and under the microscope they are rod-shaped with rounded ends [28]. It is a common pathogen in hospitals, capable of producing a variety of infections such as pneumonia, urinary tract infections, and septicemia [29]. *E. cloacae* has shown resistance to multiple drugs, such as aminoglycosides, fluoroquinolones, third-generation cephalosporins, and carbapenems [30]. In a study conducted on common wild birds in Europe, bacterial species, including *E. cloacae*, showed significant frequent resistance to antibiotics, and multiple resistance to three or more groups of antibiotics [31].

Serratia liquifaciens and *Proteus mirabilis* were also isolated and identified. *S. liquifaciens* is a bacillus bacterium with rounded ends, Gram-negative, facultatively anaerobic, motile, and positive for the catalase test. One of the common types of infections they cause is blood-borne infections caused by contaminated red blood cells [32]. In a study conducted in Egypt on 20 quails, the results showed the presence of many types of intestinal bacteria, including S. liquefaciens, at a rate of 2.3% [33]. *P. mirabilis* is a Gram-negative intestinal bacterium, a motile bacillus, positive for the urease test, negative for lactose and indole, and produces hydrogen sulfide [34]. It is the second most common cause of urinary tract infections after *E. coli*, especially in patients with kidney stones [35]. In a study conducted in the Messina region in Italy on common European wild birds, 83 strains of intestinal bacteria were isolated, including the genus *Proteus mirabilis*. The isolates showed frequent resistance to antibiotics, and multiple resistance to three or more groups of antibiotics occurred [31]. Machado et al. [36] isolated bacteria of the genus *Escherichia*, *Proteus*, *Citrobacter*, *Pantoea*, *Klebsiella*, *Enterobacter*, *Morganella*, *Hafnia*, *Enterobacter*, and *Serratia* from free-living grey-breasted parakeets. The most frequently isolated species were *Escherichia coli*, *Proteus mirabilis* and *Proteus vulgaris*, corresponding to 36.1%, 26.4%, and 8.3%, respectively. Isolates were more frequently resistant to azithromycin and tetracycline, while *Escherichia coli* was the main species presenting multidrug resistance.

Isolated bacterial species in this study were 100% sensitive to the gentamicin and ciprofloxacin. Gentamicin injections are used to treat severe bacterial infections such as meningitis, bloodstream infections, abdominal infections, pneumonia, skin and bone infections, joint infections, and urinary tract infections in humans. Ciprofloxacin is also a broad-spectrum antibiotic used to treat various bacterial infections, including uncomplicated urinary tract infections, respiratory infections (including pneumonia), skin infections, and bone infections. These results provide some reassurance and are consistent with a study by Young et al. [37], which suggested that lower gentamicin consumption is associated with lower resistance levels, emphasizing the need for national antibiotic rotation strategies since antibiotic susceptibility test is not routinely used and broad-spectrum antibiotics are being prescribed. However, a study in Catalonia on a group of wild birds revealed that *C. freundii* bacteria exhibited multidrug resistance, including resistance to fluoroquinolones, tetracyclines, sulfonamides, and aminoglycosides, including gentamicin [38].

Regarding bacterial resistance in this study, the highest resistance rate was observed against the antibiotic cefoxitin, with both *C. freundii* and *P. agglomerans* being the most resistant. Resistance was observed in 5 samples of *C. freundii* and 4 samples of *P. agglomerans* out of 11 samples. Resistance was also noted in a few isolates to antibiotics such as azithromycin, ceftriaxone, and ampicillin. The results indicate that *C. freundii* was the most antibiotic-resistant bacterial species isolated in this study. It is known to be capable of transferring antibiotic resistance genes between its strains, and studies suggest that the acquisition of resistance genes, such as beta-lactamase genes or sul1 and sul2 genes, from external sources such as the environment or other bacteria can lead to resistance to multiple drugs [19].

P. agglomerans ranked second in antibiotic resistance. This bacterium has the ability to carry multiple resistance genes on its plasmids, including ESBL genes [39] (Table 3). These bacteria are associated with plants and are not a common human pathogen. However, they can cause opportunistic infections resulting from injuries from plant materials or as healthcare-acquired infections, mainly affecting individuals with compromised immune systems [40]. A study conducted in Spain on wild birds showed that all the strains isolated, including *P. agglomerans*, exhibited resistance to at least one of the antibiotics used [41].

Calculation of multiple antibiotic resistance (MAR) index revealed that the highest recorded indicator was demonstrated by bacteria *C. freundii*, *P. agglomerans*, and *E. cloacae*, with a value of 0.33 for each of them. The acquisition of antibiotic resistance among bacterial species is a possible occurrence, and it happens through various methods, including vertical and horizontal gene transfer. However, the conjugative transfer of plasmids carrying resistance genes among bacterial species is considered one of the most important mechanisms for resistance transfer in bacteria [42]. The main mechanisms through which bacteria develop resistance to antibiotics include changes in drug targets, prevention of cell entry, elimination through efflux pumps, or drug inactivation. Better understanding and predicting patterns of resistance will lead to the selection of more effective antibiotics for treating multidrug-resistant infections [43].

Libya is characterized by diverse natural landscapes that lead to a wide variety of ecosystems. Based on this, it has been classified into two environmental regions: a northern region consisting of two parts (the coastal plain and mountainous regions in the north, and the central region, which is a pre-desert area); and a southern region representing the desert with some oases and mountains [44,45]. Most of these areas host migratory birds in varying numbers, especially the areas along the Mediterranean Sea coast, where the diversity of wetlands, water bodies, and the Mediterranean climate create favorable conditions that attract migratory birds from Europe to Africa in the early winter and vice versa when they return to their habitats in the spring.

The team responsible for monitoring and census of migratory birds, affiliated with the Department of Zoology at the Faculty of Science at the University of Tripoli, conducts annual monitoring of migratory bird species in collaboration with the Libyan Bird Society. In the winter of 2012, a total of 29,314 birds belonging to 69 species of water birds were counted. Relatively, the number of sites surveyed in 2012 was fewer than in previous survey years. Most of the birds belonged to seven species of gulls [46].

In March 2014, the team monitored and counted birds in the navigation area, which is a salt marsh fed by a channel from the sea throughout the year and is characterized by rainfall during the winter. This area was classified as nationally important for birds such as the Black-winged Stilt, Great Cormorant, Dunlin, Greater Flamingo, Shoveler, and Teal. The monitoring and census resulted in the observation of 47 species, with a total of 1,966 birds of all species recorded during this study [47]. The winter census of migratory birds in Libya for the year 2022 showed the presence of 2,169 birds of the Slender-billed Gull species [6].

5. Conclusions

In conclusion, wild migratory birds play a significant and potentially dangerous role in the global transmission of antibiotic resistance in bacteria, with borders between countries and strict crossing measures not preventing it. Libyan citizens may be at risk of antibiotic-resistant bacteria through direct contact, especially during migratory wild bird hunting seasons. Resistance can also be transferred to domestic wild birds and birds raised for commercial purposes.

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