










Enterobacteriaceae Antibiotic Resistance Identification in Slender-billed Gull Species Migrating to Libya

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ABSTRACT

The Libyan coast has various types of wetlands that are passed by many migratory birds throughout the year, however, studies on bacterial isolation from these birds in Libya are scarce. The present study aimed to isolate enteropathogenic bacterial species from the seagulls that migrated to the Libyan coast as well as identification of the antibiotics that are resistant to these bacteria. A total of 50 fresh fecal samples were collected from slender-billed gulls in January 2023 at Farwa Island near the city of Zuwara, in Western Libya. Bacteria were isolated by conventional culturing method, identified using the Enterosystem 18R, and antibiotic susceptibility tests were conducted on the isolated bacteria. The results revealed the isolation of 46 bacteria, but only 32 of them were identified using biochemical tests. These identified bacteria belong to six species of Enterobacteriaceae, namely *Citrobacter (C.) freundii*, *Pantoea (P.) agglomerans*, *Escherichia (E.) coli*, *Enterobacter (En.) cloacae*, *Serratia liquifaciens*, and *Proteus mirabilis*, with percentages of 53.125%, 31.25%, 6.25%, 3.125%, 3.125%, and 3.125%, respectively. All isolated bacteria were 100% sensitive to gentamicin (10 µg) and ciprofloxacin (5 µg). The highest resistance result was observed against the antibiotic cefoxitin (30 µg), with both *C. freundii* (5 samples) and *P. agglomerans* (4 samples). Resistance was observed in 5 samples of *C. freundii* and 4 samples of *P. agglomerans* out of 11 samples. Resistance to antibiotics, such as azithromycin (15 µg), ceftriaxone (30 µg), and ampicillin (10 µg), was also noted in a few isolates. The results indicated that *C. freundii* was the most antibiotic-resistant bacterial species isolated in this study. The highest multiple antibiotic resistance index was demonstrated by bacteria *C. freundii*, *P. agglomerans*, and *En. cloacae*, with a value of 0.33 for each of them. In conclusion, slender-billed gulls carry multi-drug-resistant bacteria. The study recommends the implementation of a national program to survey antibiotic-resistant bacteria, determine their prevalence, and assess the presence of antibiotic residues in animal-derived food. Furthermore, the present study advises expanding scientific studies on risk analysis, and antibiotic alternatives in migratory birds.

Keywords: *Citrobacter freundii*, Enterobacteriaceae, Multiple antibiotic resistance, Prevalence, Slender-billed gull

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 (Brenner et al., 2005). They are responsible for various diseases, including intestinal inflammation, food poisoning, urinary tract infections, and other illnesses in

both humans and animals (Tilahun et al., 2021; Zaitsev et al., 2022). This family includes several genera, such as *Escherichia coli*, *Klebsiella*, *Shigella*, *Salmonella*, *Enterobacter*, and others. One of Enterobacteriaceae's 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 (Brenner et al., 2005).

Wild birds are among the most important reservoirs and potential carriers of antibiotic-resistant bacteria (Bonnedahl and Järhult, 2014). Many 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 (Bonnedahl and Järhult, 2014). 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 (Bonnedahl and Järhult, 2014). These birds can travel hundreds of kilometers and spread disease-causing agents and antibiotic resistance across different regions and continents (Martín-Maldonado et al., 2022).

The survival instinct 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 can lead to the transmission of antibiotic-resistant bacteria (Georgopoulou and Tsiouris, 2008). Approximately 5 billion migratory wild birds fly across continents twice every year, which may lead to a global transfer and spread of many diseases (Elsohaby et al., 2021).

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 (Etayeb et al., 2023). Antibiotic-resistant bacteria have been isolated and identified in both resident and migratory gulls worldwide, and some studies have suggested that gulls can serve as carriers of antibiotic-resistant bacteria, distributing them through aquatic environments (Zeballos-Gross et al., 2021). 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 and identify antibiotic resistance bacteria among these isolated enteric bacteria.

MATERIALS AND METHODS

Ethical approval

The Animal Care and Use Committee, Faculty of Veterinary Medicine, University of Tripoli (Lybia) has approved this research study following protocols of animal welfare.

Sampling

Fifty fresh fecal samples from 50 migratory Slender-billed gull species were collected during January 2023 at Farwa Island 40 Km west of Zwara City in Western Libya, close to the Libyan border with Tunisia. Following monitoring and identification of the migrated flock by a specific telescope (Kite spotting scope, Belgium), the place of the flock is determined and visited immediately for sampling of fresh feces. The samples were collected using sterilized cotton swabs with care (not to touch the soil), labeled from Z1 to Z50, and stored in a refrigerated container. On the same day, the samples were then transported to the Microbiology Laboratory at the Faculty of Science in Zintan University (Lybia) for bacterial isolation, identification, and antibiotic susceptibility testing.

Culture

Bacteria were isolated by culturing the samples in Peptone water (BD, USA), and incubated at 37°C for 24 hours. Since salmonella was highly expected, enrichment and selective media were used. 1 ml of peptone water was then transferred to 9 ml of Rappaport- Vassiliadis (RV) liquid medium (Park Scientific Limited, UK), and incubated at 42°C for 24 hours. A loopful of RV medium was then streaked on Salmonella-Shigella Agar (SSA) and for isolation of other enterobacteria, a loopful of cultured peptone water was streaked on MacConkey Agar (Bio Tec Diagnostics, UK) and incubated at 37°C for 24 hours. The isolated bacteria were Gram-stained and examined for morphological characteristics under a microscope (Optech, Germany). The Enterosystem 18R, which includes 20 biochemical tests was used for identifying the isolated bacteria (Liofilchem, Italy) as per the manufacturer's instructions.

Antibiotic susceptibility test

The Bauer-Kirby method was used to determine antibiotics' resistance. 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 24 hours. Muller-Hinton Agar

(Himedia, USA) was used to cultivate the bacteria following the disk diffusion technique. Antibiotic discs namely Gentamicin (10 µg), Ciprofloxacin (5 µg), Azithromycin (15 µg), Ceftriaxone (30 µg), Ampicillin (10 µg), and Cefoxitin (30 µg) were used. A single colony from each isolate was cultured in Nutrient broth (BD, USA) and then incubated at 37°C for 24 hours. The cultures were then streaked on Muller-Hinton Agar. By using sterile forceps, antibiotic discs were placed on the agar. Following the incubation time for 24 hours at 37°C (Bauer et al., 1966), the inhibition zones were measured and the results were recorded in millimeters. 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 (Krumperman, 1983).

RESULTS

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

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 (14 isolates were not identified using the Enterosystem 18R). These identified bacteria belong to six species of the family Enterobacteriaceae, namely *Citrobacter* (*C.*) *freundii*, *Pantoea* (*P.*) *agglomerans* (formerly known as *Enterobacter agglomerans*), *E. coli*, *Enterobacter* (*En.*) *cloacae*, *Serratia liquifaciens*, and *Proteus mirabilis*, at

levels of 53.125% (17), 31.25% (10), 6.25% (2), 3.125% (1), 3.125% (1), and 3.125% (1), respectively (Table 1).

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

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

Antibiotic susceptibility testing

The results in Table 2 indicated that all isolated bacterial species in this study were 100% sensitive to the antibiotics gentamicin (10 µg) and ciprofloxacin (5 µg).

Regarding bacterial resistance in this study, the highest resistance record was observed against the antibiotic cefoxitin (30 µg), 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 (15 µg), ceftriaxone (30 µg), and ampicillin (10 µg, *Enterobacter cloacae*, *Serratia liquifaciens*, and *Proteus mirabilis*). Table 3 shows the result of the 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 *En. cloacae*, with a value of 0.33 for each of them.

Table 2. Number and bacteria percentage regarding the antibiotics susceptibility tests in identified bacteria isolated from feces of Slender-billed gull species in Lybia

Antibiotic	Antibiotic Code	Sensitive	Intermediate	Resistant	
				No. and (percentage) 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%)	<i>Citrobacter freundii</i>
Ceftriaxone	CRO	27 (84.4%)	4 (12.5%)	1 (3.1%)	<i>Enterobacter cloacae</i>
Ampicillin	AMP	27 (84.4%)	3 (9.4%)	2 (6.3%)	<i>Pantoea agglomerans</i> <i>Proteus mirabilis</i>
Cefoxitin	FOX	18 (56.3%)	3 (9.4%)	11 (43.4%)	<i>Citrobacter freundii</i> <i>Pantoea agglomerans</i> <i>Enterobacter cloacae</i> <i>Serratia liquifaciens</i>

No: Number.

Table 3. Multiple antibiotic resistance index in identified bacteria isolated from feces of Slender-billed gull species in Lybia

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

AZM: Azithromycin (15 µg), FOX: Cefoxitin (30 µg), AMP: Ampicillin (10 µg), CRO: Ceftriaxone (30 µg), MAR: Multiple antibiotic resistance

DISCUSSION

In the current study, a total of 46 bacteria were isolated from fecal samples of slender-billed gull birds, and only 32 of them were identified using biochemical tests namely, the Enterosystem 18R, consisting of 20 tests, was employed for the identification of enteropathogenic bacterial species. Previous studies that utilized this system for identifying enteropathogenic bacterial species have shown success rates of at least 90% (Piccolomini et al., 1991; Bissong et al., 2017).

In this study, 17 bacteria of the *C. freundii* species were isolated, accounting for 53.125% of the total isolates. This finding aligns with a study conducted in France (Vittecoq et al., 2022), as well as studies in Egypt (Nabil et al., 2020) and Portugal (Fournier et al., 2022). However, it differs from the results of studies conducted in Italy (Russo et al., 2021) and South America (Liakopoulos et al., 2016), where the prevalence of these species was at a low level. *C. 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% (Badger et al., 1999). It also causes urinary tract and respiratory infections in humans (Wanger et al., 2017). *C. freundii* was also isolated from diseased domestic ducks in Bangladesh (Ahmed et al., 2023), in which the isolated bacteria 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%) *P. agglomerans* bacteria were isolated from slender-billed gull in the current study, which is a gram-negative aerobic bacillus and a member of the family Enterobacteriaceae. The bacterial genus *Pantoea* can be isolated from the environment including indoor dusts of animal sheds, plants, and soil (Andersson et al., 1999; Monier and Lindow, 2005). This bacterium can be either pathogen or commensal causing secondary infections. The *P. agglomerans* is the most common species of the genus *Pantoea* isolated from humans, which

may cause soft tissue or bone/joint infections following penetrating trauma by vegetation (Ulloa-Gutierrez et al., 2004). Cruz et al. (2007) reported the isolation of *P. agglomerans* from the urine and urinary tract of 4 children. The *P. agglomerans* was most associated with penetrating trauma by vegetative material and catheter-related bacteremia (Cruz et al., 2007). In a study conducted by Giorgio et al. (2018), *P. agglomerans* was isolated from *Muscicapa striata* migratory birds. However, there is a lack of information on the isolation of *P. agglomerans* from cloacal swabs or fecal samples of slender-billed gull species.

In the current study, two isolates (6.25%) of *E. coli* were identified. A recent study conducted in Poland on strains of wild birds (including Mallards, white-tailed eagles, common buzzards, Eurasian sparrow hawks, Eurasian tawny owls, mute swans, little bitterns, little owls, short-eared owl, great spotted woodpecker, lesser spotted woodpecker, European green woodpecker, bohemian waxwing, western capercaillie, grey heron, and Eurasian golden oriole) yielded important results, as *E. coli* bacteria were isolated from 32 samples out of 34, and the results reached were as follows including 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 (Nowaczek et al., 2021). In Bangladesh, *E. coli* were isolated and identified from a total of 66 fecal matter samples from migratory birds (Islam et al., 2021). The diseases caused by *E. coli* in humans include urinary tract infections, hospital-acquired pneumonia, gastrointestinal infection, meningitis, and sepsis (Sarowska et al., 2019). However, migratory birds were found as reservoirs of multi-drug resistant (MDR) *E. coli* isolates that can carry virulence genes of avian pathogenic *E. coli* (APEC-associated), which can contribute to developing human and animal diseases (Islam et al., 2021).

Enterobacter cloacae was also isolated and identified in the current study. It is a Gram-negative bacterium, that

can be aerobic or anaerobic, and under the microscope, they are rod-shaped with rounded ends (Buckle, 2016). It is a common pathogen in hospitals, capable of producing a variety of infections, such as pneumonia, urinary tract infections, and septicemia (Annavaiah et al., 2019). The *En. cloacae* have shown resistance to multiple drugs, such as aminoglycosides, fluoroquinolones, third-generation cephalosporins, and carbapenems (Liu et al., 2021). In a study conducted on common wild birds in Europe, bacterial species, including *En. cloacae*, showed significant frequent resistance to antibiotics, and multiple resistance to three or more groups of antibiotics (Giacopello et al., 2016).

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 (Harvey et al., 2015). 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 prevalence level of 2.3% (Othman et al., 2023). *Proteus mirabilis* is a Gram-negative intestinal bacterium, a motile bacillus, positive for the urease test, negative for lactose and indole, and produces hydrogen sulfide (Schaffer and Pearson, 2015). It is the second most common cause of urinary tract infections after *E. coli*, especially in patients with kidney stones (Mo et al., 2022). 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 antibiotic resistance, and multiple resistance to three or more groups of antibiotics (Giacopello et al., 2016). Machado et al. (2018) have studied free-living grey-breasted parakeets and isolated many bacteria of the genera *Escherichia*, *Proteus*, *Citrobacter*, *Pantoea*, *Klebsiella*, *Enterobacter*, *Morganella*, *Hafnia*, *Enterobacter*, and *Serratia* in which the most common isolated bacteria were *E. coli*, *Proteus mirabilis*, and *Proteus vulgaris*, with percentages of 36.1%, 26.4%, and 8.3%, respectively. They found that these bacteria were resistant to azithromycin and tetracycline, whereas *E. coli* was presenting multidrug resistance.

Isolated bacterial species in this study were 100% sensitive to 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 (Chaves

and Tadi, 2023). Ciprofloxacin is a well-known broad-spectrum antibiotic that is used to treat many bacterial infections, such as uncomplicated urinary tract infections, respiratory infections (including pneumonia), skin infections, and bone infections (Thai et al., 2023). These results provide some reassurance and are consistent with a study by Young et al. (2018), that suggested a low gentamicin consumption is associated with a low resistance level, 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 (Darwich et al., 2019).

Regarding bacterial resistance in this study, the highest resistance record 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 that *C. freundii* is 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 (Ahmed et al., 2023). The *P. agglomerans* ranked second in antibiotic resistance in the current study. This bacterium can carry multiple resistance genes on its plasmids, including ESBL genes (Raphael and Riley, 2017). 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 (Dutkiewicz et al., 2016). 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 (Tardón et al., 2021).

Calculation of the multiple antibiotic resistance (MAR) index revealed that the highest recorded indicator was demonstrated by bacteria *C. freundii*, *P. agglomerans*, and *En. 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 (Tao et al., 2022). There are many mechanisms that bacteria may develop in order to resist antibiotics including changes in drug targets, prevention of cell entry, elimination through efflux pumps, or drug inactivation. In order to select the most effective antibiotics to treat multidrug-resistant bacteria would be to understand and predict the patterns of resistance (Chiş et al., 2022).

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 including 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 (Bundy, 1976; Isenmann et al., 2016). 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 usually in the spring (Lehikoinen et al., 2019).

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, Libya, 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 (Etayeb et al., 2012). In March 2014, the team monitored and counted birds in the navigation area, which is a salt marsh 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 (Benyezza et al., 2017). 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 (Etayeb et al., 2023).

CONCLUSION

In light of these results, Slender-billed gulls play a role in the spread of potentially pathogenic and antibiotic-resistant agents. 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. The study recommends the implementation of a national program to survey antibiotic-resistant bacteria, determine their prevalence, and assess the presence of antibiotic residues in animal-derived food, ensuring they comply with acceptable levels. Furthermore, the study advises expanding scientific studies on risk factors and possible antibiotic alternatives used in wild and commercial birds.

DECLARATION

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Authors' contributions

Abdulmajid Hamhoom, Ehab Sharif, Ibrahim Eldaghayes, Khaled Etayeb, and Abdunaser Dayhum contributed to the sampling, data collection, and revision of the manuscript. Aya Mansour and Abdulwahab Kammon did the laboratory analysis and writing up the manuscript. All authors read and approved the final edition of the manuscript.

Availability of data and materials

All data are available in the manuscript. Any extra data needed can be provided by the corresponding author upon reasonable request.

Competing interests

The authors have declared that there are no competing interests in this study.

Ethical considerations

All the authors had checked and confirmed for ethical issues such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

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