

Research Article

Molecular Detection and Multidrug Resistance of *Shigella* spp. Isolated from Wild Waterfowl and Migratory Birds in Bangladesh

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Birds, especially wild waterfowl and migratory birds have the potential to carry antibiotic-resistant bacteria, but their role in the dissemination of these resistant pathogens is still neglected in Bangladesh. To the best of our knowledge, this study was carried out for the first time in Bangladesh to isolate and determine the occurrence of multidrug-resistant (MDR) Shigella spp. from fecal materials of wild waterfowl and migratory birds. A total of 80 fecal materials from wild waterfowl (n = 50) and migratory birds (n = 30) were screened to detect MDR Shigella isolates. Shigella spp. were isolated and identified by culturing, staining, and biochemical tests followed by polymerase chain reaction (PCR). A disk diffusion assay was employed to investigate antibiotic phenotypes, while the resistance genes were detected by PCR. Among the 80 samples, 15 (18.75%) were found positive for Shigella spp. by PCR, among which the occurrence rate of Shigella spp. was higher in migratory birds (20%, 6/30) than in wild waterfowl (18%, 9/50). By the disk diffusion test, 86.67% (13/ 15) of Shigella spp. isolates were found to be MDR in nature, including 93.33% of isolates resistant to imipenem. Moreover, frequent and moderate resistance was also observed against tetracycline (86.67%), azithromycin (80%), ampicillin (66.67%), ciprofloxacin and cotrimoxazole (40%), meropenem (26.67%), and streptomycin (13.33%). The bivariate analysis revealed a positive correlation between the resistance profiles of ciprofloxacin and cotrimoxazole, imipenem and tetracycline, tetracycline and ampicillin, and imipenem and azithromycin. Furthermore, the isolates had a multiple antibiotic resistance index of up to 0.47. Antibiotic resistance genes tetA and SHV were found in 69.23% and 50% of relevant antibiotic-resistant Shigella spp. isolates, respectively. The present study suggests that wild waterfowl and migratory birds are reservoirs of MDR Shigella spp., which may have detrimental impacts on One Health components. We suggest keeping these birds under an AMR monitoring program to avoid the possibility of AMR contamination of the environment and its consequences in all health settings.

1. Introduction

Every year, thousands of migratory birds travel worldwide for suitable weather and food. Bangladesh hosts millions of migratory birds because of its suitable weather and vast water lands during the winter [1]. They are apt reservoirs for spreading pathogenic and antimicrobial-resistant bacteria to the aquatic environment, including wild waterfowl. Moreover, poor sanitation and water management systems help bacterial agents to spread through the fecal and environmental samples into the water bodies. Rural people often come close to these wild waterfowl and migratory bird habitats as they are intimately associated with the water lands for fishing, farming, or bathing. The same water used by the farmer or fisherman and wild waterfowl and migratory birds enhances the chances of spreading antimicrobial-resistant bacteria to people and poses health risks to humans and animals [2].

Antimicrobial resistance (AMR) may be the next catastrophe of the twenty-first century that global health will face in the coming days [3]. It has already become a global threat to humans, animals, and the environment. Bacterial pathogens have developed AMR or multidrug resistance due to the selective pressure caused by the abuse and overuse of antibiotics [4]. AMR poses a significant risk to both the wellbeing of humans and the growth of the economy. Low- and middle-income countries (LMICs) in Africa and Asia, such as Bangladesh, would be affected the most strongly. They spread in numerous ways, mainly through environmental sources, food chains, and human and animal waste [5].

Shigella is a Gram-negative facultative rod-shaped anaerobe with four species responsible for shigellosis which causes watery diarrhea, vomiting, and dysentery with mucoid stool, cramps, and abdominal pain [6]. Moreover, bacillary dysentery caused by Shigella spp. is the most abundant disease for children in middle- and low-income countries worldwide, resulting in thousands of deaths every year [7]. Shigella was first found in chickens in 2004, and 3day-old chicks showed signs of human dysentery [8]. Recently, Shigella exhibited AMR in the newer or atypical stains by harboring integrons that are more significant than any other enteric bacteria [9]. It carries several AMR genes, showing resistance to multiple antimicrobial classes, including beta-lactams, tetracyclines, fluoroquinolones, folate pathway antagonists, and others [10]. The ability to horizontally transfer genes through mobile genetic materials is the essential factor that makes these bacteria develop antimicrobial-resistant strains [11]. The fact that Shigella has developed resistance to multiple antimicrobial agents in the modern period highlights how critical it is to maintain constant surveillance of the pathogen. Shigella causes epidemics by contaminating food and water supplies [12]. Wild bird species, such as wild waterfowl and migratory birds have been recognized as major transport modes of pathogens, highlighting the importance of unrestrained animals in the natural setting as a key driver in the spread of infections [13].

Humans, animals, and poultry have all been subjected to considerable research into AMR. Despite the prevalence of AMR, there is still a lot we don't know about it when it occurs in nontypical hosts like wild waterfowl and migratory birds. Previously, several studies identified bacterial isolates from wild waterfowl and migratory birds in Bangladesh [14–19], but, to our knowledge, none focused on the detection of *Shigella* spp. from wild waterfowl and migratory birds in Bangladesh. Therefore, we conducted this study to detect multidrug-resistant (MDR) *Shigella* spp. from fecal materials of wild waterfowl and migratory birds in Bangladesh.

2. Materials and Methods

2.1. Ethical Approval. All the methods and procedures followed in this study were approved by the institutional ethical committee (AWEEC/BAU/2019(14)).

2.2. Sample Collection and Processing. Eighty freshly dropped wet fecal materials of wild waterfowl (n = 50) and migratory birds (n = 30) were collected from Jahangir Nagar University (23.8796°N, 90.2726°E), Savar, Bangladesh

(Figure 1), from December 2020 to March 2021. In this study, two types of wild waterfowl (Asian Openbill Stork: *Anastomus oscitans*, n = 30; Oriental Darter: *Anhinga melanogaster*, n = 20) and one type of migratory birds (White Stork: *Ciconia ciconia*, n = 30). Fecal materials were collected by swirling a sterilized cotton bud into the wet fecal materials [1] and shifted to sterilized zip-locked bags with unique identification name badges. All the samples were then transferred to the laboratory by maintaining a proper cooling chain. Immediately after transferring them to the laboratory, a sample containing a sterile cotton bud was taken into 5 ml of sterile nutrient broth (HiMedia, India) and left overnight in a shaker incubator at 37° C.

2.3. Isolation of Bacteria. After overnight enrichment, a loopful of broth was transferred and streaked on a Salmonella-Shigella (SS) Agar (HiMedia, India) plate. Subsequently, the streaked plates were transferred to the incubator and left for 18-24 hours at 37° C. Serial subcultures were performed to acquire pure colonies of the target bacteria. Large, circular, convex, and transparent colonies on SS agar plates were suspected to be *Shigella* spp. Culture-positive samples were then subjected to Gram staining and different biochemical tests (oxidase, urease, carbohydrate fermentation test or mannitol, H_2 S, methyl red, motility, sucrose, citrate utilization, lysine decarboxylase, and indole tests) [20].

2.4. Molecular Detection of Shigella spp. Finally, the suspected isolates were confirmed as Shigella by polymerase chain reaction (PCR) targeting the genus-specific gene, as mentioned in Table 1.

Before performing PCR, the genomic DNA of isolated bacteria was extracted by the boiling and chilling method [24]. In brief, a pure colony of Shigella was inoculated in one ml of sterile nutrient broth contained in an Eppendorf tube and placed in a shaker incubator for overnight growth at 37°C. Overnight incubated culture was centrifuged at 5,000 rpm for 5 min and the supernatant was discarded. Then, one ml of phosphate buffer solution (PBS) was added in the same Eppendorf tube, and the same centrifugation procedure was followed. After the subsequent centrifugation, the supernatant was discarded, and $250\,\mu$ l of PBS was added and mixed with the vortex. The suspension was then boiled for 10 minutes and chilled for another 10 minutes. After cooling, the tube was centrifuged at 10,000 rpm for 10 minutes, followed by the collection of the supernatant as genomic DNA. The final product was stored at -20°C until subsequent use.

To amplify the specific gene of *Shigella*, a total of $20 \,\mu$ l of PCR volume was prepared with $10 \,\mu$ l of PCR master mix 2X (Promega, USA), $1 \,\mu$ l of each forward and reverse primer (100 pmol), $4 \,\mu$ l of nuclease-free water, and $4 \,\mu$ l of genomic DNA. The amplified product was analyzed through gel electrophoresis (100 volts) in 1.5% agarose gel (HiMedia, India) and visualized under an ultraviolet transilluminator (Biometra, Germany). A 100 bp DNA ladder (Promega, Madison, WI, USA) was utilized to distinguish the target amplicon size of the amplified gene.



FIGURE 1: Study area of this study created with ArcGIS Pro (version 3.0.3, ESRI, Redlands, CA, USA).

2.5. Antimicrobial Susceptibility Test. Antibiotic susceptibility testing (AST) of Shigella isolates was performed according to the Kirby-Bauer disc diffusion method [25]. Overnight-grown freshly cultured bacteria adjusted with the 0.5 McFarland standard were spread on Muller Hilton agar (HiMedia, India) plate. The most frequently used 15 antibiotics from nine classes were used in this study: phosphonic acid (fosfomycin-200 μ g) aminoglycosides (gentamicin-10 μ g, streptomycin-10 μ g), tetracyclines (tetracycline-30 μ g), carbapenems (ertapenem-10, meropenem-10 μ g,

imipenem-10 μ g, doripenem-10 μ g), penicillins (ampicillin-10 μ g), macrolides (azithromycin-30 μ g), folate pathway antagonists (cotrimoxazole-25 μ g), and fluoroquinolones (ciprofloxacin-5 μ g, enrofloxacin-5 μ g). Antibiotic susceptibility profiles (resistant, intermediate, and sensitive) of *Shigella* isolates were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute [26]. Isolates showing resistance against three or more classes of antibiotics were considered MDR [27]. The following formula [28] was utilized to determine multiple antibiotic resistance (MAR) of *Shigella* isolates:

MAD -	The number of antimicrobial agents to which a particular Shigella isolate shows resistance	(1)
WIAK =	The total number of antibiotics that were utilized in treating an isolate	. (1)

2.6. Detection of Antibiotic-Resistant Genes. A simplex PCR was used to detect resistance genes of *Shigella* isolates associated with tetracycline (*tetA* and *tetB*) and imipenem or meropenem (*SHV*) (Table 1).

2.7. Statistical Analysis. All the data obtained from this study was initially entered into Microsoft Excel 2019 (Los Angeles, CA, USA) and subsequently transferred into the Statistical Package for the Social Sciences software (IBM SPSS.v.25, Chicago, IL, USA) and GraphPad Prism 8.4.2 (GraphPad

Software, Inc., San Diego, CA, USA) for further analysis. In SPPS, a chi-square test for relatedness was performed to check the variation between the occurrence of *Shigella* spp. in wild waterfowl and migratory birds. The significant p value was set at ≤ 0.05 . Moreover, any correlation between any of the two antibiotics found resistant to the isolates was calculated by the bivariate analysis in SPSS. The result with a p value less than or equal to 0.05 ($p \leq 0.05$) was considered statistically significant. In GraphPad Prism, the Wilson/Brown Hybrid technique [29] was utilized to calculate the binomial 95% confidence interval (CI).

	TABLE 1: Primers used to detect Shigella spp. and the	neir resistance genes from wild wate	rfowl and migratory birds.	
Target genes	Primer sequence $(5'-3')$	Amplicon size (bp)	Annealing temperature (°C)	References
Shigellagenus-specific gene	F: AACTGGTTACCTGCCGTGAG R: TGGTGATGGTGGTGGTAATG	154	56	[21]
tetA	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	577	57	[22]
tetB	F: CCTCAGCTTCTCAACGCGTG R: GCACCTTGCTGATGACTCTT	634	56	[22]
SHV	F: TCGCCTGTGTGTATTATCTCCC R: CGCAGATAAATCACCACAATG	768	53	[23]

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3. Results

3.1. Occurrence of Shigella spp. Out of 80 samples, 22 (27.5%, 95% CI: 18.92%–38.14%) were identified as Shigella after performing the morphological and biochemical tests, and 15 (18.75%, 95% CI: 11.71%–28.66%) were confirmed positive by PCR. The highest occurrence of Shigella spp. was detected in Asian Openbill Stork (*Anastomus oscitans*) (23.33%, 7/30), followed by White Stork (*Ciconia ciconia*) (20%, 6/30), and Oriental Darter (*Anhinga melanogaster*) (10%, 2/20) (Figure 2). Moreover, migratory birds (20%, 95% CI: 9.51%–37.31%) had higher occurrence rate of Shigella spp. than wild waterfowl (18%, 95% CI: 9.77%–30.79%), but no significant variation was observed (p > 0.05).

3.2. Antibiotic Susceptibility Test of Shigella spp. The AST revealed that 93.33% of isolated Shigella spp. showed resistance to imipenem (95% CI: 70.18%–99.66%), followed by tetracycline (86.67%, 95% CI: 62.12%–97.63%), azi-thromycin (80%, 95% CI: 54.82%–92.95%), ampicillin (66.67%, 95% CI: 41.71%–84.82%), cotrimoxazole and ciprofloxacin (40%, 95% CI: 19.82%–64.25%), meropenem (26.67%, 95% CI: 2.37%–37.88%). In addition, Shigella isolates were highly sensitive to cefotaxime, ceftazidime, ertapenem, doripenem, gentamicin, enrofloxacin, and fosfomycin (Figure 3).

Moreover, the bivariate analysis of antibiotic resistance profiles showed a positive correlation between cotrimoxazole and ciprofloxacin (Pearson's correlation coefficient, $\rho = 1.000$, p < 0.001), tetracycline and imipenem ($\rho = 0.681$, p < 0.001), tetracycline and ampicillin ($\rho = 0.555$, p = 0.032), and imipenem, and azithromycin ($\rho = 0.535$, p = 0.040).

3.3. MDR and MAR Profiles of Shigella spp. Thirteen (86.67%; 95% CI: 62.12%–97.63%) Shigella isolates showed phenotypically MDR characteristics. Eleven resistance patterns were found among the isolates, and three isolates were found to be resistant to seven antibiotics of six different classes. Moreover, 80% (12/15; 95% CI: 54.82%–92.95%) of the isolates had more than 0.2 of the MAR indices (Table 2).

3.4. Occurrence of Antibiotic Resistance Genes in Shigella Isolates. In PCR, the resistance genes tetA and SHV were detected in 69.23% (9/13, 95% CI: 42.37%-87.32%) of tetracycline-resistant and 50% (7/14, 95% CI: 26.80%-73.20%) of imipenem- and/or meropenem-resistant Shigella isolates, respectively. All the tetracycline-resistant isolates were negative for the tetB gene (Figure 4).

4. Discussion

Migratory birds can act as carriage and spreader of antibiotic-resistant *Shigella* spp. during migration, but their resistant bacteria disseminating activities are still frequently neglected. They have the potential to disseminate bacterial



FIGURE 2: Occurrence of *Shigella* spp. in fecal materials of wild waterfowl and migratory birds in Bangladesh.

pathogens to other waterfowl in Bangladesh. Therefore, we screened fecal materials of wild waterfowl and migratory birds in Bangladesh in this study to find MDR *Shigella* spp.

In this study, to our knowledge, we detected Shigella spp. in wild waterfowl and migratory birds for the first time in Bangladesh, showing an occurrence rate of 18.75%. The high detection rate of Shigella spp. indicates that wild waterfowl and migratory birds are vastly associated with the dissemination of Shigella spp. in the environment and from environmental sources. As wild waterfowl and migratory birds are directly linked to environmental sources, they have the potential to contaminate the environment with Shigella species. During the various types of migration, migrating birds typically occupy a diversity of ecological niches and develop a range of distinct feeding behaviors. In the course of these migrations, these birds can become hosts for Shigella spp. and aid in the pathogen's spread from one location to another. Moreover, Shigella spp. are increasingly isolated in migratory birds, and the possibility of their movement and transmission by wild birds is a growing public health concern. Because of this, it is even more crucial to keep an eye on migrating bird populations so that we may anticipate an epizootic state of the *Shigella* to one-health components. Previously, Alam et al. [13] detected Shigella spp. (36% of the samples) from watering sites of migratory birds in Pakistan, which is higher than the present study. However, a lower detection rate (4.1%) was also recorded in Ghana by Modupe et al. [30]. Moreover, Zhao et al. [31] reported Shigella spp. (11.02%) in duck-type waterfowl. The disparities in results might be related to variances in the climate and environment of locations, particularly temperature variations that affect bacterial development, as well as the types of wild waterfowl and migratory species, sample types, and sample size. Furthermore, migrating birds may have a change in their



FIGURE 3: Antibiogram profiles of *Shigella* spp. isolated from fecal materials of wild waterfowl and migratory birds in Bangladesh. AM = ampicillin, AZM = azithromycin, COT = cotrimoxazole, CIP = ciprofloxacin, EX = enrofloxacin, ETP = ertapenem, MEM = meropenem, DOR = doripenem, IMP = imipenem, CTX = cefotaxime, CAZ = ceftazidime, GN = gentamicin, TE = tetracycline; FO = fosfomycin, S = streptomycin.

rate of bacterial shedding owing to the stressful situations they endure throughout migration [32].

Resistance to antimicrobial agents is an urgent public health concern. In the present study, *Shigella* isolates exhibited high to moderate resistance to imipenem, tetracycline, azithromycin, ampicillin, cotrimoxazole, ciprofloxacin, meropenem, and streptomycin. Surprisingly, a high percentage of resistance was observed in *Shigella* isolates against imipenem (93.33%) and meropenem (26.67%), showing an alarming situation for public health. Carbapenem antibiotics, of which imipenem and meropenem are

members, are reserved for the direst of human medical emergencies [33, 34]. However, further confirmation of this resistance using MIC and molecular approaches is required before any definitive conclusion can be drawn. Moreover, it is noted that Shigella isolates showed a higher resistance to azithromycin (80%) and ciprofloxacin (40%), indicating a treatment limitation in humans. The Centers for Disease Control and Prevention (CDC) has recommended that ciprofloxacin (for adults) and azithromycin (for children) be considered first-line antibiotics for the treatment of shigellosis [35]. Moreover, as a member of the quinolone antimicrobial class, the use of ciprofloxacin in animals has been severely limited since the 1990s, when fluoroquinolone resistance began to spread rapidly [36]. Therefore, the presence of ciprofloxacin resistant Shigella spp. in wild waterfowl and migratory birds suggests a serious issue for human health. Furthermore, statistical analysis using a bivariate analysis demonstrated a significant positive correlation existed between the resistance patterns of ciprofloxacin and cotrimoxazole, tetracycline and imipenem, tetracycline and ampicillin, and tetracycline and imipenem. Possible causes for the strong antimicrobial connections discovered include the indiscriminate use of antibiotics in livestock and poultry in regions frequented by wild waterfowl and migrating birds. Possibly related factors include environmental contamination, particularly in water sources.

In our current study, the *tetA* (69.23%) and *SHV* (50%) genes responsible for resistance to their corresponding antibiotics were found in Shigella spp. isolates. The tetB gene was not detected in any of the isolates. There is a possibility that mobile genetic elements are to blame for the existence of a variety of resistance genes in Shigella spp. isolates [37]. Moreover, the existence of these resistance genes in Shigella spp. can be attributed to many mechanisms, including a decrease in cellular permeability, the extrusion of drugs by active efflux pumps, the overexpression of drug-modifying and inactivating enzymes, or target modification by mutation [10]. The detection of resistant Shigella spp. in wild waterfowl and migratory birds might be associated with the transmission of antibiotic resistance genes from environmental sources. Water contaminated with feces of wild waterfowl and migratory birds might be deemed a significant risk factor for the dissemination of resistant Shigella spp. pathogens and their resistance genes.

The effects of infections caused by MDR bacteria are extremely serious for human health and may even be fatal. In this study, 86.67% of the *Shigella* isolates were phenotypically MDR in nature, showing resistance to up to seven different classes of antibiotics. Previously, Alam et al. [13] reported that all the *Shigella* isolates from migratory birds in Pakistan showed multidrug resistance, which is higher than this study. These differences might be due to variations in geographical locations, types of birds, sample sizes, detection methods, and others. Moreover, 12 out of 15 isolates had more than 0.2 MAR indices. Sources with a MAR value greater than 0.2 indicate heavy antibiotic usage, suggesting the presence of MDR-prone *Shigella* spp. [28]. The detection of high levels of MDR *Shigella* spp. along with their high MAR indices in wild waterfowl and migratory birds revealed an emerging situation. This might be

Pattern no.	Antibiotic resistance patterns	No. of isolates	Overall MDR isolates	MAR index
1	AM, AZM, COT, CIP, EX, IMP, TE	1	13/15 (86.67%)	0.47
2	AM, AZM, COT, CIP, IMP, TE, S	1		0.47
3	AM, AZM, COT, CIP, MEM, IMP, TE	1		0.47
4	AM, AZM, COT, CIP, IMP, TE	1		0.4
5	TE, IMP, CIP, COT, AM	1		0.33
6	AZM, COT, CIP, IMP, TE	1		0.33
7	AM, AZM, MEM, IMP, TE	2		0.33
8	AZM, MEM, IMP, TE, FO	1		0.33
9	AM, AZM, IMP, TE	3		0.27
10	AZM, IMP, S	1		0.2
11 ^a	IMP, TE	1		0.13
12 ^a	_	1		_

TABLE 2: Multidrug resistance profile of the isolated Shigella spp. from fecal materials of wild waterfowl and migratory birds in Bangladesh.

MDR = multidrug-resistant, MAR = multiple antibiotic resistance, ^a = not multidrug-resistant.



FIGURE 4: Occurrence of antibiotic resistance genes in *Shigella* spp. isolated from fecal materials of wild waterfowl and migratory birds in Bangladesh.

due to the impairment of wildlife habitats and stretches of urban areas that increase contact with contaminated environmental sources. Our results raise serious concerns about the potential for long-distance transmissions of MDR bacteria from their native habitats to distant sites, especially in regions where people are not well-versed in the need for infection prevention and control [14, 38]. Water is thought to be a major conduit for the spread of MDR bacteria. MDR bacteria can spread through human activities and contaminate natural water sources. Inadequate wastewater treatment in places like factory farms, human settlements, healthcare institutions, and pharmaceutical firms might endanger migrating bird populations by polluting rivers and other water sources. Wild waterfowl and migratory birds on the move can acquire these MDR bacteria from water sources, disperse them to other aquatic environments, and ultimately affect all health settings [16].

5. Conclusion

To the best of our knowledge, this is the first study in Bangladesh to isolate and identify *Shigella* spp. from fecal materials of wild waterfowl and migratory birds. The results of this study revealed the frequent occurrence of MDR *Shigella* spp. and their resistance genes in wild waterfowl and migratory birds in Bangladesh. The identification of MDR *Shigella* spp. in wild waterfowl and migrating birds raises significant concerns for the general public's health due to the potential of these pathogens to contaminate ecosystems and spread to One Health components. These birds therefore need to be kept under active strict surveillance with a One Health approach as a crucial step in combating the transmission of zoonotic potential *Shigella* spp. and their associated AMR hazards.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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