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Antimicrobial Resistant Bacteria in Shrimp and Shrimp Farms of Bangladesh

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Abstract: The purpose of this study was to investigate the presence of pathogenic bacteria, specifically *Escherichia coli* and *Salmonella* and *Vibrio* species, and their antimicrobial resistance in shrimp aquaculture facilities of Bagerhat (Bangladesh). Sediment samples were collected from both *Penaeus monodon* and *Macrobrachium rosenbergii* farms and shrimp samples from the *Macrobrachium rosenbergii* facility. The abovementioned bacteria were not found, but five Enterobacterales (*Proteus penneri*, *Proteus alimentorum*, *Morganella morganii*, *Enterobacter hormaechei* subsp. *xiangfangensis* and *Plesiomonas shigelloides)* were detected. This is the first documented case of *Enterobacter hormaechei* subsp. *xiangfangensis* in a shrimp farm. Nine antibiotics—ampicillin, gentamicin, chloramphenicol, oxytetracycline, nitrofurantoin, levofloxacin, ciprofloxacin, azithromycin, and co-trimoxazole—were selected for antibiotic resistance testing, and the majority (88.9%) had at least one isolate that was resistant. Across sources, 78.0% of isolates were resistant to at least one antimicrobial, and multidrug resistance was also detected in 29.3% of all isolates. Despite the low number of samples analyzed, nine in total, the results of this experiment emphasize that shrimp farms in Bagerhat may have a problem with antimicrobial-resistant bacteria. This could have negative impacts on shrimp quality and consumers' health.

Keywords: shrimp industry; Giant tiger prawn; Giant river prawn; Freshwater prawn; pathogenic bacteria

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1. Introduction

Shrimp are one of the top internationally-traded seafood commodities across the globe with the majority of production coming from aquaculture [1,2]. One of the most important challenges facing the shrimp industry is antimicrobial resistance (AMR) due to the treatment of disease in shrimp culture [2,3]. AMR occurs when microbial organisms develop resistance to the antimicrobials that normally would kill them [2,4]. According to the World Health Organization, AMR is considered one of the most important problems for human health [5]. Some shrimp farms are of major concern as a source for AMR since traditionally antimicrobial drugs are used in prophylactic and therapeutic doses [2]. These treated shrimp are exported around the world, potentially spreading AMR organisms [2,6]. Moreover, the overuse and misuse of antimicrobials increases the likelihood of the antimicrobial or AMR bacteria spreading into the environment [7,8] and the likelihood of antimicrobial residues or AMR bacteria in seafood [9]. Thus, antimicrobial residues and AMR bacteria have been found in shrimp farm sediment and shrimp hatcheries, leading to mass mortalities in worldwide shrimp productions [10–12]. An additional concern is that climate change could increase global temperatures, which is expected to increase AMR [13].

Most of the shrimp production occurs in Asia (87% in 2017) [14], where AMR has been detected in effluents of shrimp farms and in the surrounding environment [6,15]. For instance, the Southeast Asian region has been identified for a high risk of development and spread of AMR for several reasons [16]: the shrimp production often involves the direct contact of workers with both the pond sediment and water. Additionally, along the supply line, processors and market workers come into direct contact with the shrimps, which facilitate the transmission of resistant bacteria [2,3,17]. On the other hand, antimicrobials used to target specific organisms, as well as AMR genes, can enter the surrounding environment through water discharge and cause harm to the ecosystem [18]. In order to qualify and quantify the problem of antimicrobial use in shrimp production in the regions adjacent to the Bay of Bengal (Bangladesh), Hinchliffe and colleagues reported that 23 antimicrobials have been found in shrimp hatcheries in those regions [3], and Shamsuzzaman and Biswas described 14 different branded antibiotics that were used in Bangladesh shrimp farms in the last decade [19]. The extensive use of antimicrobials for years increases the probability of AMR, and bacteria with AMR to ampicillin and tetracycline were found in water samples collected in some shrimp farms of Bangladesh [20]. In Bangladesh, pond waters of shrimp farms can also be contaminated with fecal coliforms during the rainy season, mainly due to poor waste management systems, poor sanitary conditions of rural areas, pets, as well as poultry from nearby farms [21,22].

Antimicrobial resistance is especially concerning in pathogenic bacteria, and several harmful bacteria are known to occur in shrimp aquaculture. An example is *Escherichia coli*, which is an indicator of fecal contamination and causes health problems [23]. Moreover, in 2021, shrimp imported into the US from multiple Asian countries were rejected due to the presence of *Salmonella* spp. [24], and source waters of shrimp farms and ready-to-eat shrimp were recently found to be contaminated with Salmonella [25,26]. *Vibrio* spp. constitute part of the natural microflora of aquatic organisms but also include human pathogens, and *Vibrio* spp. pathogens have been responsible for mass mortalities in shrimp ponds [27,28]. Thus, in order to understand AMR risks and threats to aquatic and human health, the present study aims to determine if resistant bacteria such as *E. coli, Salmonella,* and *Vibrio* are present in shrimp farms of Bangladesh and study AMR by testing the antimicrobial compounds ampicillin, gentamicin, chloramphenicol, oxytetracycline, nitrofurantoin, levofloxacin, ciprofloxacin, azithromycin, and co-trimoxazole in isolated bacteria species.

2. Materials and Methods

2.1. Sampling Area and Sample Collection

All the samples were collected from shrimp farms during spring 2018 in Bagerhat, Khulna, near the coast of Bangladesh, in the Ganges River delta. Sediment samples were collected from a *Penaeus monodon* farm (PM sediment) and a *Macrobrachium rosenbergii* farm (MR sediment), and shrimp samples were collected from the *M. rosenbergii* facility (MR shrimp). The total area of the *P. monodon* farm is 3.25 ha, comprising 10 ponds with pond size ranging from 0.2–0.4 ha. Three separate ponds, approximately of 0.2 ha in size, were randomly selected for sample collection. The total area of the *M. rosenbergii* farm is 1.5 ha, comprising four ponds with pond size ranging from 0.2–0.3 ha. Three ponds were randomly selected for sediment and shrimp collection.

In each selected pond, sediment (top 6 cm, 500 mg) was collected aseptically in glass jars from ponds with lowered water levels below the normal water line [29], and the sediment was homogenized. For MR shrimp, shrimps were individually placed in sterile bags (one shrimp was collected by pond). After collection, all the samples, in a total of nine, were stored at −20 °C. From each pond (n = 3), three separate samples of MR shrimp, MR sediment, and PM sediment were tested (n = 27 total samples).

2.2. Isolation Procedure for Escherichia coli, Salmonella spp. and Vibrio spp.

To perform the isolation procedure, samples were thawed at 2–5 °C. The head and shell of the shrimp were removed, and only the flesh was used to isolate the bacteria. The flesh was ground by a blender (Cuisinart food processor). Three sub-samples of each replicate were used, in a total of 27 sub-samples, and 1.0 g of sediment and 25 g of shrimp tissue were considered. The *E. coli* isolation followed the standard petrifilm method (3M™, Maplewood, MN, USA, Petrifilm™ *E. coli*). Aseptically, the sample was blended with 225 mL sterile phosphate-buffered saline, and solutions were prepared up to 10^{-4} dilutions. A 1 mL diluted sample was placed in the middle of the 3M petrifilm, and the lid of the film was closed. The incubation period was 24 h at 35 °C, and the blue gas-forming colonies were counted as an indicator of *E. coli*. The *Salmonella* isolation followed the standard Bacteriological Analytical Manual [30]. Aseptically, the samples were homogenized with 225 mL sterile lactose broth (Himedia, Mumbai, India) and then incubated at 35 °C for 24 h. Then, 0.1 mL of the pre-enriched sample was mixed with 10 mL of Rappaport-Vassiliadis (RV) broth, incubated at 42 °C for 24 h. After incubation, a 3 mm loopful of RV broth was streaked on xylose lysine deoxycholate (XLD) agar (Himedia, Mumbai, India) and then incubated for 24 h at 35 °C. The susceptive colonies (pink colonies with or without black centers) were streaked on nutrient agar (NA) to isolate a single colony and incubation was performed for 24 h at 35 °C. For the confirmation test, triple sugar iron agar, (Himedia-M021, Mumbai, India) and lysine iron agar (Himedia-M377, Mumbai, India) were used. The *Vibrio* isolation followed the Bacteriological Analytical Manual [31] for shrimp and sediment samples. Aseptically, 25 g of sample and 225 mL alkaline peptone water were blended for 60 s and incubated at 35 °C for 24 h. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Himedia, Mumbai, India) plates were used for streaking and incubated at 35 °C for 24 h. The isolates were routinely sub-cultured on NA plates and incubated at 37 °C for 24 h. To identify other bacteria than *E. coli*, *Salmonella* spp., and *Vibrio* spp., the red colonies from the petrifilm (considered as total coliform), the yellow colonies (oxidase positive) from XLD agar and oxidase negative yellow and green colonies from TCBS agar were selected. Those isolates were systematically sub-cultured on NA before molecular identification.

2.3. DNA Extraction and Quantitative PCR

Isolated bacteria from sediment and shrimp tissue samples were confirmed by Polymerase Chain Reaction (PCR). Only representative colonies were selected for molecular identification. For that, pure cultures were kept in nutrient broth with 10% glycerol and stored at −20 °C until molecular identification. The susceptive bacterial colonies were taken from pure culture stock, inoculated into a nutrient broth (Liofilchem, Roseto degli Abruzzi, Italy), and incubated in a shaker incubator (120 rpm) at 28 °C for 24–48 h. A GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for DNA extraction following manufacture methods. Aliquots (5 µL) of the extracted DNA were analyzed by gel electrophoresis on a 1% agarose gel compared to a 1 Kb plus DNA ladder marker (Thermo Fisher Scientific, USA).

The PCR with universal primer sets were used for amplification (Table 1). A 100 µL PCR mixture was prepared containing 10 µL of 1× PCR buffer, 6 µL of 1.5 mM $MgCl_2$, 1 µL of 0.05 U/µL Taq DNA polymerase, 2 µL of 200 µM dNTPs (all 4 Thermo Fisher Scientific), 3 µL of 0.1–1.0 µM each primer (F primer and R primer; Macrogen, Seoul, Korea), and 5 µL of 100 ng/100 µL DNA template. The thermal profile of PCR (2720 thermal cycler, Applied Biosystems, Waltham, MA, USA) consisted of an initial denaturation step at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min; annealing for 40 s at 57 °C; extension for 1 min at 72 °C; and final extension step for 10 min at 72 °C [32]. The PCR amplicons were verified with gel electrophoresis.

Table 1. Primer sequence used for PCR amplification.

Primers	Sequences (5′–3′)	Primer Size (bp)	GC Content (%)	PCR Amplification Size (bp)
8F	AGAGTTTGATCCTGGCTCAG	20	50.0%	1484
1492R	GGTTACCTTGTTACGACTT	19	42.1%	

A GeneJET PCR Purification Kit (Thermo Scientific #K0701, USA) was used to purify the sample according to manufacturer methods. The purified PCR product was stored at −20 °C for further use.

The purified PCR products with sequencing primer were sent to National Institute of Biotechnology, Savar, Dhaka, for sequencing of the 16S rRNA gene. The sequence data was extracted by using BIOAD software as FASTA format, and the sequences were analyzed using BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov/ accessed on 6 August 2019) [33].

2.4. Antibiotic Resistance Test

The selection of the nine antibiotics tested (ampicillin, gentamicin, chloramphenicol, oxytetracycline, nitrofurantoin, levofloxacin, ciprofloxacin, azithromycin, and co-trimoxazole) was based on its use both in human and veterinary medicine [18,34]. Non-brand antibiotics (generic compounds) were used with 6 mm discs: ampicillin (25 µg/disc), gentamicin (10 µg/disc), chloramphenicol (10 µg/disc), oxytetracycline (30 µg/disc), nitrofurantoin (300 µg/disc), levofloxacin (5 µg/disc), ciprofloxacin (5 µg/disc), azithromycin (30 µg/disc), and co-trimoxazole (25 mcg/disc).

The sensitivity of the isolated bacteria (n = 38) to different antibiotics was determined by the Kirby–Bauer disc diffusion method [35]. For each isolated bacteria, 30 µL of broth were spread on iso sensitive agar media (Micro Master, Camarillo, CA, USA). Then, the nine commercially prepared discs (Liofilchem, Italy and Himedia, India) were placed on the agar plate and incubated at 35 °C for 16 to 18 h. After incubation, the zone around disc was measured. An established measuring scale was used for the measurement of the diameter of the zone (Table 2) [36]. Each sample was run in duplicate. If any bacteria showed resistance to the antimicrobial, that batch was retested again for confirmation. When multiple colonies were tested, the high and low values for the zone diameter were taken. If only one colony was obtained, then the single value of the duplicate plate was considered.

Table 2. Antimicrobial sensitivity reference table [36].

Antimicrobial	Disc Content	Zone Diameter (mm)		
		Susceptible (S)	Intermediate (I)	Resistant (R)
Ampicillin	10 µg	≥17	14–16	≤13
Ciprofloxacin	5 µg	≥21	16–20	≤15
Gentamicin	10 µg	≥15	13–14	≤12
Nitrofurantoin	300 µg	≥17	15–16	≤14
Levofloxacin	5 µg	≥17	14–16	≤13
Chloramphenicol	30 µg	≥18	13–17	≤12
Tetracycline	30 µg	≥15	12–14	≤11
Azithromycin	15 µg	≥13	-	≤12
Trimethoprim	5 µg	≥16	11–15	≤10

3. Results

The sediment and shrimp tissue samples collected in the two shrimp farms contain a total of 41 bacteria isolates, all Enterobacterales (Table 3): 12 from PM sediment, 14 from MR sediment, and 15 from MR shrimp. Surprisingly, E. coli, Salmonella spp., and Vibrio

spp. are not present. A total of five bacteria species were identified from the isolates, which are Proteus penneri, Proteus alimentorum, Morganella morganii, Enterobacter hormaechei subsp. xiangfangensis, and Plesiomonas shigelloides. Proteus penneri, Morganella morganii, and Plesiomonas shigelloides is present in MR sediment. P. penneri is present in all sediment and shrimp tissue samples analyzed (Table 3).

Table 3. Number and source of species isolated from farm sediment and shrimp.

Source	Species	Isolates	Batches Found (Out of 3)	Accession Number *
MR sediment	*P. penneri*	7	3	MN262212
	M. morganii	6	1	MN262231
	P. shigelloides	1	1	MN262458
MR shrimp	*P. penneri*	10	3	MN262211, MN262213, MN262230, MN262440
	P. alimentorum	2	1	MN262480
	E. hormaechei subsp. *xiangfangensis*	3	1	MN262441, MN262459
PM sediment	*P. penneri*	12	3	MN262192, MN262439, MN262469

Note: * Only representative colonies were tested for molecular identification and sequencing.

Regarding antimicrobial resistance, P. penneri from PM sediment, MR sediment, and MR shrimp are resistant (0 to 13 mm) to 10 µg of ampicillin (Table 4). However, in this study, P. penneri isolates are susceptible (MR sediment = 18–31 mm, MR shrimp = 28 mm, PM sediment = 30 mm), intermediate (MR shrimp = 15mm), and resistant (PM sediment = 0 mm) to nitrofurantoin (300 µg) (Table 4). In this study, P. penneri (all three sources, MR shrimp, MR sediment, and PM sediment) are very sensitive (15 mm to 37 mm) to oxytetracycline (30 µg). For ciprofloxacin (5 µg), two isolates of P. penneri are in the intermediate range of resistance (MR shrimp = 18 mm, PM sediment = 20 mm), and the rest of the isolates are susceptible to ciprofloxacin. All P. penneri from MR sediment are sensitive (15 mm to 21 mm) to gentamicin, but some P. penneri from MR shrimp and PM sediment are both sensitive (MR shrimp = 17 mm, PM sediment = 20 mm, respectively) and resistant (MR shrimp = 0 mm and PM sediment = 8 mm, respectively). All isolates (all three sources, MR shrimp, MR sediment, and PM sediment) are sensitive (19 mm to 45 mm) to levofloxacin (5 µg). For chloramphenicol (30 µg), one isolate of P. penneri from MR shrimp is resistant (0 mm), and one isolate from PM sediment is intermediate resistant (13 mm). The other isolates are very sensitive (19 mm to 39 mm). Azithromycin (15 µg) has mixed effects on the P. penneri bacterial strains. P. penneri isolates (all three sources, MR shrimp, MR sediment, and PM sediment) are both resistant (all three sources = 0 mm) and sensitive (MR sediment = 33 mm, MR shrimp = 26 mm, PM sediment = 17 mm) to azithromycin (15 µg). Co-trimethoprim (5 µg) is effective (28 mm to 42 mm) for P. penneri from the sediment and shrimp samples. P. penneri is intrinsically resistant to nitrofurantoin, but most of the isolates tested show some sensitivity. Similarly, chloramphenicol and oxytetracyclines also have sensitivity.

Table 4. The zone diameter (mm) of the bacteria isolated from sediment or shrimp. Bold values represent resistant levels and underlined values represent intermediate levels. ^ denotes intrinsic resistance.

Source	Species (n)	Ampicillin	Ciprofloxacin	Gentamicin	Nitrofurantoin	Levoflox	Chloram	Oxytetracycline	Azithromycin	Co–Trimethoprim
MR sediment	*P. penneri* (7)	**0 ^–11 ^**	34–46	15–21	18 ^–31 ^	29–45	19–37	15 ^–37 ^	**0**–33	31–42
	P. shigelloides (1)	21	54	**12**	21	49	33	35	26	40
	M. morganii (6)	**0 ^–8 ^**	36–43	16–19	16 ^–29 ^	26–39	25–35	17–31	**0**–22	29–32
MR shrimp	*P. alimentorum* (2)	**0 ^–0 ^**	**15**–19	**8**–14	15–17	**9**–17	**0–10**	**11**–19	**0 ^–14 ^**	21–26
	P. penneri (10)	**0–13 ^**	18–42	**0**–17	15 ^–28 ^	19–38	0–39	15 ^–30 ^	**0**–26	28–32
	E. h. subsp. *xiangfangensis* (3)	**0 ^–0 ^**	27–30	15–17	**0**–20	22–28	**0–10**	**0**–24	**12**–22	**10**–13
PM sediment	*P. penneri* (12)	**0 ^–13 ^**	20–47	**8**–20	**0 ^**–30 ^	19–38	13–36	15 ^–36 ^	**0**–17	30–36
Total species (per source) resistant *		**0/1**	**1/7**	**4/7**	**1/3**	**1/7**	**3/7**	**2/4**	**5/6**	**1/7**

Note: * excludes those intrinsically resistant; out of total known susceptible.

The P. shigelloides species is susceptible (21 mm) to ampicillin (10 µg) (Table 4). P. shigelloides from MR sediment is resistant (12 mm) to gentamicin (10 µg) (Table 4). Except gentamicin, P. shigelloides were susceptible to all antibiotics (Table 4). On the other hand, P. alimentorum is resistant (MR shrimp = 0 mm) to ampicillin (10 µg) (Table 4). P. alimentorum isolates test both resistant (0) and sensitive (14 mm) to azithromycin (Table 4). P. alimentorum shows resistance to ciprofloxacin (15 mm), gentamicin (8 mm), levofloxacin (9 mm), chloramphenicol (0–10 mm), and oxytetracycline (11 mm). Similarly, E. hormaechei subsp. xiangfangensis from MR shrimp is resistant (0 mm) to ampicillin (10 µg) (Table 4). E. hormaechei subsp. xiangfangensis is susceptible to gentamicin (10 µg, 15–17 mm), levofloxacin (5 µg, 22–28 mm), and ciprofloxacin (5 µg, 27–30 mm). One isolate of E. hormaechei subsp. xiangfangensis shows resistance (0 mm), and one isolate is sensitive (20 mm) to nitrofurantoin (300 µg). All strains of E. hormaechei subsp. xiangfangensis are resistant (0–10 mm) for chloramphenicol (30 µg). For oxytetracycline (30 µg) and azithromycin (15 µg), E. hormaechei subsp. xiangfangensis is both sensitive (24 mm, 22 mm) and resistant (0 mm, 12 mm), respectively. For co-trimoxazole (5 µg), E. hormaechei subsp. xiangfangensis shows both intermediate (13 mm) and full resistance (10 mm).

M. morganii was resistant to ampicillin (10 µg) (Table 4). However, M. morganii from MR sediment is susceptible (29 mm) to nitrofurantoin (Table 4). M. morganii isolates are sensitive to chloramphenicol (25–35 mm), oxytetracycline (17–31 mm), levofloxacin (26–39 mm), ciprofloxacin (36–43 mm), gentamicin (16–19 mm), and co-trimethoprim (29–32 mm). For azithromycin, M. morganii is both resistant (0 mm) and sensitive (22 mm).

Altogether, there were 41 isolates of the five species tested for AMR, and 31 isolates show resistance to antimicrobials with no known intrinsic resistance (78.04%) (Table 5). Additionally, most of the isolates show resistance to at least one antimicrobial tested, and 29.26% of them show resistance to multiple antimicrobials (Table 5).

Table 5. Isolates with antimicrobial resistance and multidrug resistance.

Isolated Species	Total Isolates	Isolates Resistant	Isolates Multidrug Resistant
P. pennari	29	24	8
M. morganii	6	2	0
P. shigelloides	1	1	0
P. alimentorum	2	2	2
E. h. subsp. *xiangfangensis*	3	2	2
	41	31 (78.04%)	12 (29.26%)

Note: excludes any species with intrinsic resistance.

4. Discussion

The prevalence of *Salmonella* on shrimp aquaculture farms worldwide is well known and was recently reported in farm water and sediment samples in India [26]. However, possibly due to the low number of samples, *E. coli*, *Salmonella* spp., and *Vibrio* spp. were not detected in our sediment and shrimp tissue samples. Nevertheless, the present study identified five species of Enterobacterales, with *P. penneri* being present in the three types of samples. *P. penneri* is considered an invasive pathogen [37], and a destructive agent of farmed shrimp [38,39]. This species is responsible for causing red body disease outbreaks in several Southeast and East Asian countries resulting in large economic losses due to red body disease [38,39]. In addition to causing disease in shrimp, it is also a known pathogen in fish [40] and humans [37]. In this research, it was the most widespread bacteria species, indicating that this microorganism might be very common in shrimp farms in the region of Bagerhat. This is a matter of great concern for natural shrimp and fish populations, shrimp aquaculture, and human health. *P. alimentorum* was recently characterized as a facultative anaerobic, short rod gram-negative bacterium, responsible for causing food poisoning [41], and was, thus, found in the present study in shrimp tissues. *M. morganii* is a gram-negative, facultative anaerobic bacterium in the Morganellaceae family and found in the gastrointestinal tract of humans and vertebrate animals [42]. *M. morganii* possesses histamine decarboxylase and is able to produce histamine when fish are stored above 4 °C [43]. *M. morganii* is responsible for shrimp spoilage, and *M. morganii* has been isolated from several species of shrimp [44–46]. Known to be present in multiple countries, the presence of *M. morganii* in Bangladesh was not unusual. However, this microorganism is a concern for consumers and farmers as it is responsible for shrimp spoilage and human diseases often with fatal consequences [42]. Moreover, for the first time, *E. hormaechei* subsp. *xiangfangensis* was identified in a shrimp farm, but only in MR shrimp samples. *E. hormaechei* subsp. *xiangfangensis* is a gram-negative bacteria which can tolerate 9% salinity in nutrient broth culture and was first isolated from sourdough bread [47]. The presence of this species in shrimp is a concern as it is a nosocomial and communicable pathogen that can infect hospitalized vulnerable patients [48]. *P. shigelloides* is in the Enterobacteriaceae family and is responsible for gastroenteritis, eye infection, septicemia, and central nervous system disease in humans [49]. While previously detected in farmed and wild shrimp [50], our study found *P. shigelloides* was only found in MR sediment. There is concern that these bacteria could transfer from the sediment to the shrimp and pond water, which would be a concern for human health and organisms in the surrounding aquatic environment. Shrimp farmed in Bangladesh tolerate a wide salinity range (*P. monodon*, 1 to 57 ppt [51]; *M. rosenbergii*, 0 to 25 ppt [52]. Most of these five bacteria can also tolerate a wide range in salinity; *P. shigelloides*, *P. penneri*, and *M. morganii* can easily grow in freshwater and brackish water [53–55]. This wide salinity range increases the chances of cross-contamination between ponds, species, and into the marine environment with regular water discharge into the Ganges Delta. Despite the small number of facilities studied, these results highlight the implications for environmental and human health since the procedures of aquaculture in Bangladesh directly expose the workers and nearby

environment to the water and sediment of the shrimp farms. Finally, Bangladesh shrimp trade travels across the country and crosses the world.

When these five species were tested for AMR, 78% of isolates show resistance to antimicrobials with no known intrinsic resistance. Additionally, most of the isolates showed resistance to at least one antimicrobial tested, and 29.26% of them showed resistance to multiple antimicrobials (Table 5). The mechanisms of resistance are the limitation about up taking a drug, drug target modification, drug inactiveness, and the drug's active efflux. Additionally, based on the mechanism, antimicrobial agents divided into groups: agents hinder cell wall synthesis, cell membrane depolarization, inhibit protein and nuclei acid synthesis, and hamper metabolic pathways in bacteria [56]. There are two types of resistance; natural or intrinsic resistance and acquired resistance. It is possible for bacteria to acquire genetic material that imparts resistance by transformation, transposition, and conjugation (horizontal gene transfer, or HGT), as well as through alterations to their own chromosomal DNA. The acquisition could be either temporary or permanent [56].

All bacteria isolates were intrinsically resistance for ampicillin except *P. shigelloides*, which has been found to have 72–92% resistance to ampicillin [49]. The differences in the sensitivity could be due to different strains. Therefore, use of ampicillin in shrimp culture would not be effective for many infections, and specific diagnoses should be performed before use. Most of the bacteria were susceptible to ciprofloxacin; only *P. alimentorum* was weakly resistant. Ciprofloxacin is widely used in shrimp farming for the control of gram-negative bacteria including enteric pathogens such as *Pseudomonas* and in some cases gram-positive bacteria [12]. Overall, the results are positive from a human and animal health perspective that most of the bacteria would be susceptible to ciprofloxacin, but the two isolates showing AMR or intermediate resistance are a concern. For gentamicin, 57.1% of bacteria isolates were resistant: *P. shigelloides* from MR sediment, *P. penneri* from PM sediment, and *P. penneri* and *P. alimentorum* from MR shrimp. Another study reported that *P. alimentorum* is resistant to gentamycin [41]. In our research, *P. shigelloides* was very much susceptible to other antibiotics. For the partial control of *Proteus* infections, farmers used gentamicin [57]. Resistance to gentamicin is problematic for disease control. Of the bacteria known to be susceptible, only *E. h.* subsp. *xiangfangensis* was resistant to nitrofurantoin. However, nitrofurantoin is banned for use as a carcinogen, but it is still used in shrimp farming illegally. The observed sensitivity of *P. penneri* to nitrofurantoin could depend on characteristics of strains. In the past, consignments of shrimp from Bangladesh were rejected by USA and European Commission because of the presence of nitrofuran drugs [19]. The continued use is a serious concern for the potential for nitrofurantoin and AMR bacteria to pollute local waters. In this research, most of the bacteria were susceptible to levofloxacin; only *P. alimentorum* was resistant. Levofloxacin is used in both aquaculture and for treating human disease [58]. This increases the importance of levofloxacin as an antimicrobial with limited resistance, and care needs to be taken to avoid increased resistance in the future. All three species isolated from *M. rosenbergii* shrimp (*P. penneri*, *E. h.* subsp. *xiangfangensis*, and *P. alimentorum*) were resistant to chloramphenicol (42.9% of isolates known to be susceptible). As *P. penneri* isolates from other sources were susceptible, horizontal gene transfer between bacteria could be occurring in the shrimp. While the sediment contained susceptible bacteria, water transfers or escaped shrimp between the ponds and coastal environment could spread this AMR. Although there is a strict regulation against using chloramphenicol, overuse in Bangladesh shrimp farming may have caused bacteria to become resistant. *E. h.* subsp. *xiangfangensis* and *P. alimentorum* from MR shrimp showed resistance to oxytetracycline (50% of bacteria). However, intrinsically resistant *P. penneri* was sensitive to tetracyclines. The observed sensitivity of *P. penneri* to oxytetracycline could depend on the characteristics of strains. Oxytetracycline is a widely used antibiotic for the treatment of bacterial infections in aquaculture [59]. The incidence of resistance to oxytetracycline is increasing [45]. In 2017, use of oxytetracycline as a growth promotor was banned by the FDA [60]. However, in the last few years, shrimp imported into the US have tested positive for residual oxytetracycline [61]. While no resistance would be the

best for animal human health as well as the surrounding environment, limited resistance to such a widely used antibiotic implies it may be better than other antibiotics. *P. penneri* from all three sources, *E. h.* subsp. *xiangfangensis* from MR shrimp, and *M. morganii* from PM sediment showed resistance to azithromycin (83.3%). Overall, the results are concerning that most of the bacteria would be resistant to azithromycin. *E. h.* subsp. *xiangfangensis* from MR shrimp were resistant to co-trimethoprim (14.3%). Co-trimethoprim was effective for isolated bacteria from the sediment and shrimp samples. In South Asia, bacteria, including *Salmonella* and *V. cholerae*, showed resistant for trimethoprim [10,62,63].

There are several possible reasons for AMR to occur in Bagerhat shrimp farms. Disease outbreaks in shrimp farming are very common in Bangladesh. However, most farmers cannot recognize the disease, and accurate diagnosis facilities are not easily available. Encouragement for use from antimicrobial sales agents and feed stores is common, who have a financial gain if more is sold [2]. If a Bangladesh farmer uses antibiotics targeting *Vibrio*, but the causative agent is different, the treatment may not be effective, and the disease may be left uncontrolled. Inappropriate and inefficient use of antibiotics including improper antibiotic selection, dose, application methods, or mislabeled commercial feed could be the causes of antimicrobial drug resistance. All the above can be prevented with the use of prescribed antibiotics at the right dosage, training of farmers about antibiotic application, diagnostic support to farmers, and use of probiotics or other alternatives such as bacteriophages to combat bacterial disease [1]. Multi-stocking and high stocking density is another potential contributor to the problem. Farmers frequently added post larvae during the stocking season at regular intervals, and this increases the risk for transmitting disease by contaminated post larvae [3]. In Bangladesh, surface water run-off is a concern and pond water is regularly exchanged with the environment. Best practices are needed to ensure AMR is not spread to the coastal environment as well as continued surveys to detect new AMR bacteria. Future work should test the surrounding local waters for these bacteria and additional AMR.

5. Conclusions

Five multidrug-resistant Enterobacterales: *Proteus penneri*, *Proteus alimentorum*, *Morganella morganii*, *Enterobacter hormaechei* subsp. *xiangfangensis* and *Plesiomonas shigelloides*, were present in the two Bagherhat shrimp farms studied. This is the first documented case of *Enterobacter hormaechei* subsp. *xiangfangensis* in shrimps collected in shrimp farms of Bangladesh. Moreover, from the nine antimicrobials tested, in 88.9% of the cases at least one isolate was resistant. Despite the low number of samples analyzed, gathered results emphasize that shrimp farms in Bagerhat may have a problem with antimicrobial resistant bacteria, which could have negative impacts on shrimp quality and consumers health.

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