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Prevalence of Multiple Drug Resistant Pathogenic Bacteria in Cultured Black Tiger Shrimp (*Penaeus monodon* Fabricius)

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Abstract: The shrimp sector is the second largest export earner for Bangladesh, which is a major contributor in the national economy of Bangladesh. It also provides millions of employment. Every year a huge amount of shrimps are rejected to export due to its pathogenic bacterial contamination. A comparative study regarding prevalence and antimicrobial susceptibility of pathogenic bacteria in the black tiger shrimp (*Penaeus monodon* Fabricius) were analyzed in terms of coliform, fecal coliform, Salmonella-Shigella and *Vibrio spp*. Total coliform, total faecal coliform, total Salmonella-Shigella and total vibrio counts were ranged from 6×10^2 to 8.6×10^5 cfu/gm, 0.0 to 3.8×10^5 cfu/gm, 0.0 to 3.7×10^5 cfu/gm and 0.0 to 2.5×10^3 cfu/gm, respectively. Among 11 tested isolates selected from shrimp, 7 isolates displayed multidrug resistance to more than three antibiotics. Inadequate fish storage facilities, limited use of ice to preserve the shrimp, improper and unhygienic handling of shrimp could be considered as some of the factors contributing to the occurrence of these pathogens. The main reason of antibiotic resistance could be the contamination of shrimps with the antibiotic resistant bacteria through the environment and human handling.

Key words: Food safety · Shrimp · Salmonella-Shigella · *Vibrio* · Faecal colifrm · Antimicrobial resistance · Multiple drug resistance

INTRODUCTION

Shrimps are one of the most important commodities of the global fishery trade [1] and this sector is the second largest export earner (next to readymade garment and knitwear) for Bangladesh. It provides millions of employment and earns more than USD 428 million annually [2]. However, a lot of fishery resources are simply wasted due to lack of appropriate post harvest technology [3]. Food security is a complex issue, where various factors pose a condition of risk to fish food safety and they range from contamination from the environment where it is caught up to contamination by the consumer before eating [4]. Black tiger shrimp, which occupies about76% of the total export of shrimp from Bangladesh, may contain many bacteria of public health importance like *Escherichia coli, Salmonella, Shigella* and *Vibrio spp*. Presence of such pathogenic bacteria in shrimp renders it unfit and dangerous for human consumption. Consumption of raw or undercooked seafood is the factor most commonly associated with infection [5]. So, fresh shrimp should have as low as possible amount of bacteria that is not harmful to human.

The development of antimicrobial resistance among pathogenic bacteria has emerged as a major public health concern, which has led to an intensification of discussion about the prudent use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture [6]. Antimicrobial agents have been applied to the shrimp feed and water in large quantities primarily to treat and prevent diseases in farmed shrimps. Consequently, antimicrobial agents persist in sediment and aquatic environments, leading to deteriorated environmental conditions and conferring antimicrobial resistance to the sediment

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bacteria. Of particular concern is the indiscriminate use of antibiotics leading to the development of multiple-antibiotic-resistant pathogenic bacteria in shrimps and humans [7]. In this case, shrimps could serve as delivery vehicles of antimicrobial resistance to pathogenic bacteria from aquatic environments to humans and from one country to another.

The present study was an attempt to help the surveillance of the antimicrobial resistance status of farm raised black tiger shrimp and therefore, the purpose of this investigation was also to determine the status of multiple drug resistant pathogenic bacteria in cultured black tiger shrimp.

MATERIALS AND METHODS

Sampling Area and Period of Study: The black tiger shrimp samples were collected from Kaliganj Upazilla in Satkhira district located at the south western part of Bangladesh where most of the shrimp farming is carried out. The study area lies between the latitudes 22°27'34N and longitudes 89°01´37E. The samples were collected in July, 2011. Besides these, shrimp samples were collected from the different markets of Savar and Dhaka in order to compare them with the shrimp gher (farm) samples.

Sample Collection: The shrimp samples were collected individually in pre-sterilized polyethylene bags and brought to the laboratory as soon as possible for the analysis. To avoid further contamination, during transportation from the source to the laboratory, samples were carried by special sterile bags packed in insulated box with ice to maintain the temperature around 4 to 6 °C.

Sample Processing: 250 gm of shrimp sample were blended aseptically. From the blending portion, 10 gm sample and 90 ml sterile normal saline (0.9% w/v NaCl) solution were taken in a conical flask to make a homogenous suspension. Samples were serially diluted and thoroughly mixed by using Vortex-mixture and analyzed for microbial contamination.

Bacteriological Analysis: Four different types of media, namely; MacConkey agar medium (Difco, USA) for total coliform count, mFC agar medium for total faecal coliform count, S-S agar medium for total Salmonella-Shigella count and Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) for total vibrio count were used. Standard plate count (SPC) method was used for inoculation technique, described by APHA [8]. Except mFC agar all of the inoculated plates were incubated at 35 °C for 24 to 48 hours. The mFC agar plates were incubated at 44.5 °C for 24 to 48 hours.

Antimicrobial Susceptibility Testing: Bacterial susceptibility to antimicrobial agents was performed by the disk diffusion method using guidelines established by Bauer *et al.* [9] (1966). A total of 12 antibiotic discs (Oxoid Ltd., Basingstoke, Hampshire, UK) with Streptomycin 10 µg, Erythromycin 15 µg, Chloramphenicol 30µg, Ciprofloxacin 5µg, Penicillin 10 µg, Norfloxacin 10µg, Ampicillin 10 µg, Kanamycin 30 µg, amoxicillin 10µg, Azithromycin 15 µg, cephalexim 30 µg and sulphamethoxazole 25 µg, were used. Within 15 min of the application of the discs, the plates were inverted and incubated at 37 °C. After 16-18 h of incubation, the plates were examined and the diameters of the zones of complete inhibition to the nearest whole millimeter were measured. The zone diameter for individual antimicrobial agents was then translated into susceptible, intermediate and resistant categories according to the interpretation table of the Oxoid Ltd., Basingstoke, UK.

Grouping of the Multiple Drug Resistant (MDR) Isolates: Based on the occurrence of resistance to more than three drugs the isolates of each sampling sites were grouped as multiple drug resistant isolates [10].

RESULTS AND DISCUSSION

A total of seven mixed samples of the black tiger shrimps have been analyzed. Table 1 illustrates the total coliform count (TCC), total faecal coliform count (TFCC), total Salmonella-Shigella count (TS-SC) and total vibrio count (TVC) of different shrimp samples. All of the tested samples were contaminated with coliform and the total coliform count ranged from 6×10^2 to 8.6×10^5 cfu/gm. The total fecal coliform count (TFCC) was ranged from 0.0 to 3.8×10^5 cfu/gm and the fecal coliforms were absent in gher shrimp samples. The total Salmonella-Shigella count (TSSC) was ranged from 0.0 to 3.7×10^5 cfu/gm.

Presence of coliform in shrimp samples indicates sewage contamination. Yousuf *et al.,* [11] reported that, the coliform counts ranged between 5.4×10^2 and 8.5×10^5 cfu/gm for the shrimp collected from the markers in Dhaka city. According to International Commission on the Microbiological Specification of Foods [12], maximum acceptable limit of total coliform and faecal coliform for shrimp 10^2 and 10 cfu per gram, respectively and

Table 1: Enumeration of Total coliform count (TCC), total faecal coliform count (TFCC), total Salmonella-Shigella count (TS-SC) and total vibrio count (TVC) count in shrimp samples collected from shrimp Ghers of Satkhira and different markets of Dhaka city

Sample No.	Sample Source	(TCC)Cfu/gm	(TFCC)Cfu/gm	(TSS)Cfu/gm	(TVC)Cfu/gm
Sample-01	Gher (live)	6×10^2	0.0	0.0	0.0
Sample-02	Gher (Dead)	1.4×10^3	0.0	0.0	2×10^2
Sample-03	Savar Market	3.6×10^5	4.9×10^4	3.3×10^4	0.0
Sample-04	Mohammudpur Town Hall market	8.6×10^5	2.4×10^5	3.7×10^5	1.9×10^3
Sample-05	Mohammudpur Town Hall market	6.7×10^5	3.8×10^5	3.5×10^4	3×10^2
Sample-06	Mohammudpur Krishi Market	2.4×10^5	2.3×10^5	2.7×10^4	0.0
Sample-07	Mohammudpur Krishi Market	3.9×10^5	2.5×10^5	2.2×10^5	2.5×10^3

Table 2: Diameter of zone of inhibition (in mm) of selected isolates with different antibiotics discs

	Mean Diameter of the Zone of Inhibition (mm) of selected isolates										
	Coliform			Fecal coliform		Salmonella-Shigella sp.			Vibrio spp.		
Antibiotic discs	C_1	C_2	C_3	F_1	F_2	SS_1	SS_2	SS_3	V_1	V_2	V_3
Group I-Inhibitors of cell wall synthesis											
Amoxycillin(10µg)	21	0	0	0	0	0	0	0	16	23	21
Penicillin (10µg)	13	9	0	0	0	0	0	0	11	21	19
Ampicillin (10µg)	0	13	0	0	0	0	0	0	17	22	24
Cephalexin (30µg)	0	14	0	18	15	0	0	16	18	15	15
Group II-Inhibitors of protein synthesis											
Kanamycin (30µg)	27	18	21	18	16	18	22	26	21	19	24
Erythromycin (15µg)	0	19	15	0	0	12	0	19	15	29	29
Azithromycin (15µg)	29	25	23	11	0	19	19	21	28	33	27
Chloramphenicol (30µg)	9	27	23	26	22	21	0	17	25	29	29
Streptomycin (10µg)	21	23	22	16	16	18	13	16	19	18	21
Group III-Inhibitors of nucleic acid synthesis											
Norfloxacin (10µg)	27	26	29	0	0	21	24	27	32	32	31
Sulphamethoxazole (25µg)	25	21	19	0	0	13	15	18	29	35	24
Ciprofloxacin(5µg)	29	14	26	0	0	18	28	31	35	39	35

E. coli should not be present. Therefore, the bacterial load found in this study for shrimp is beyond the reference value, which indicates their unacceptability as food from public health point of view. Kumar *et al.* [13] reported that the contamination by *E. coli* was higher in the seafood samples collected from the landing centers and from fresh fish markets than from frozen shrimp samples collected from processing plants. The presence of coliform suggests the practices of inadequate hygienic measures, mishandling, improper storage and above all unhygienic condition of the markets [14]. Khan [15] also found contamination carried over from the source of water, poor hygiene and sanitation condition of the processing premises. The use of poor quality water and improper storage conditions may contribute to the high occurrence of *E. coli* [16-18]. Water used for washing and ice for chilling seafood may also be contaminated with *E. coli* [19].

Salmonella-Shigella was observed in market shrimp samples but was absent in shrimp samples collected from shrimp Gher. Yousuf *et al.,* [11] reported that the Salmonella-Shigella (SS) counts ranged between $0.15x10^2$ to $1.1x10^4$ cfu/gm in shrimp collected from gher and shops. Contamination can occur at multiple steps along the food chain including production, processing, distribution, retail marketing and handling/preparation [20]. *Vibrio spp.* was detected in the shrimp samples at a relatively low level as compared to other organisms. Areerat *et al*, [21] reported that *Vibrio l*evels from harvested shrimps ranged between 1.30×10^3 and 1.44×10^5 cfu/g which was higher than the present study. These results reveal that the black tiger shrimp had an exposure to very unhygienic condition of different stages. It may start from hatcheries and other subsequent processes like farming area (gher/pond), processing station, transportation and market place. Health status of working personnel's and their poor sanitation practices are also responsible.

Table 3: Antimicrobial resistance to selected isolates from shrimp.

	Antimicrobial resistance to selected isolates from shrimp											
	Coliform			Fecal coliform			Salmonella-Shigella sp.			Vibrio spp.		
Antibiotic discs	R(%)	M(%)	S(%)	R(%)	M(%)	S(%)	R(%)	M(%)	S(%)	R(%)	M(%)	S(%)
Group I-Inhibitors of cell wall synthesis												
Amoxycillin(10µg)	66.66	0	33.33	100	0	0	100	0	0	0	33.33	66.66
Penicillin (10µg)	100	0	0	100	0	0	100	0	0	33.33	0	66.66
Ampicillin (10µg)	100	0	0	100	0	0	100	0	0	0	0	100
Cephalexin (30µg)	100	0	0	0	50	50	66.66	33.33	0	0	66.66	33.33
Group II-Inhibitors of protein synthesis												
Kanamycin (30µg)	0	0	100	0	50	50	0	0	100	0	0	100
Erythromycin (15µg)	33.33	0	66.66	100	0	0	66.66	33.33	0	0	33.33	66.66
Azithromycin (15µg)	0	0	100	100	0	0	0	0	100	0	0	100
Chloramphenicol(30µg)	33.33	0	66.66	0	0	100	33.33	33.33	33.33	0	0	100
Streptomycin (10µg)	0	0	100	0	0	100	0	33.33	66.66	0	0	100
Group III-Inhibitors of nucleic acid synthesis												
Norfloxacin (10µg)	0	0	100	100	0	0	0	0	100	0	0	100
Sulphamethoxazole (25µg)	0	0	100	100	0	0	0	66.66	33.33	0	0	100
Ciprofloxacin(5µg)	33.33	0	66.66	100	0	0	0	33.33	66.66	0	0	100

Table 4: Antibiotic resistance patterns of selected isolates from shrimp

No. of resistant antibiotics	No. of isolates	Antimicrobial Resistance patterns	Origin of isolates	Resistance Classification
5	2	P-AMP-CL-E-C		
		AML-P-AMP-CL-CIP	Coliform	MDR
4	1	AML-P-AMP-CL	Coliform	MDR
8	2	AML-P-AMP-E-AZM-NOR-SXT-CIP		
		AML-P-AMP-E-AZM-NOR-SXT-CIP	Fecal coliform	MDR
6	1	AML-P-AMP-CL-E-C	Salmonella-Shigella spp.	MDR
5	1	AML-P-AMP-CL-E	Salmonella-Shigella spp.	MDR
3	1	AML-P-AMP	Salmonella-Shigella spp.	NMDR
1	1	P	Vibrio spp.	NMDR
0	2	_	Vibrio spp.	NMDR
Total	11			

symbols: MDR-multiple drug resistance, NMDR-non multiple drug resistance, AML-amoxycillin, P-penicillin, AMP-ampicillin, CL-cephalexin, K-kanamycin, E-erythromycin, AZM-azithromycin, C-chloramphenicol, S-streptomycin, NOR-norfloxacin, SXT-sulfamethoxazole, CIP-ciprofloxacin.

A total of 11 isolates were selected for antimicrobial resistance test. Table 2 shows the mean diameter of the zone of inhibition (mm) of selected isolates. Typically, the raw data were interpreted based on the available CLSI (Clinical and Laboratory Standards Institute) data and zone diameter interpretive standards. Though antibiotics use has its advantages, the intensive and extensive use of antibiotics has lead to the emergence of antimicrobial resistance.

Table 3 shows the percentage of isolates in resistant, medium and sensitive to different tested antibiotics. All the coliform isolates were resistant (R: 100%) to penicillin, ampicillin, cephalexin and no coliform isolates were resistant (R: 0%) to kanamycin, azithromycin, streptomycin, norfloxacin and sulphamethoxazole. All the fecal coliform isolates were resistant (R: 100%) to amoxycillin, penicillin, ampicillin, erythromycin, azithromycin, norfloxacin, sulfamethoxazole, ciprofloxacin and no fecal coliform isolates were resistant (R: 0%) to cephalexin, kanamycin, chloramphenicol, streptomycin. Tricia *et al.,* [22] reported 43% isolates of *E. coli* were resistant to ampicillin but no isolate was found resistant to gentamicin. Daini and Adesemowo [23] found the resistance of *E. coli* from Nigeria in 54% and 88% strains against gentamicin and tetracycline, respectively. In the present study higher percent of coliform isolates (100%) were resistant to penicillin, ampicillin, cephalexins, are higher than the previous records [24-27].

Table 4 summarizes antibiotic resistance patterns and multiple drug resistance (more than three antibiotics) results in selected pathogenic isolates from shrimp. In the present study, among 3 isolates of coliform, 100% (3/3) displayed multidrug resistance (MDR) (more than three antibiotics) which is higher than the findings on multiple

drug resistance of *E. coli* strains as reported from Bangladesh and other parts of the world [28-31]. Highest number of resistant antibiotics (5 antibiotics) was observed in 2 selected isolates of coliform. Among 2 isolates of fecal coliform, 100% (2/2) displayed multidrug resistance (MDR) and highest number of resistant antibiotics (8 antibiotics) was observed in 2 isolates.

All of the tested isolates among total salmonella-shigella spp. were resistant (R: 100%) to amoxycillin, penicillin and ampicillin and no Salmonella-Shigella isolates were resistant (R: 0%) to kanamycin, azithromycin, streptomycin, norfloxacin, sulfamethoxazole and ciprofloxacin. Among 3 isolates of total salmonella-shigella spp. 66.66% (2/3) displayed multidrug resistance (MDR) and highest number of resistant antibiotics (6 antibiotics) was observed in 1 isolate. A higher percentage of antibiotic resistance of Salmonella-Shigella isolates in aquaculture shrimps has been evidenced by Le *et al*. [32] from their study carried out in Vietnam. They reported a wide use of antibiotics in aquaculture, which may result in residues of antibiotics in water and mud and subsequently, the development of antibiotic resistance in bacteria in the environment. Fonseka and Ranjini [33] observed *Salmonella* isolated from farm shrimps in SriLanka were not resistant to any kind of antibiotic tested such as ampicillin 10 (µg), cephaloridine 5 (µg), colistin sulphate 25 (µg), gentamicin 10 (µg), streptomycin 10 (µg), sulphatriad 200 (µg), tetracycline 25 (µg) and cotrimoxazole 10 (µg).

In this study, highest resistance (R: 33.33%) among total *Vibrio* spp isolates was observed to penicillin and all the Vibrio spp. isolates were sensitive to maximum tested antibiotics. Among 3 isolates of total vibrio spp. no isolates displayed multidrug resistance (MDR) and highest number of resistant antibiotics (1 antibiotic) was observed in 1 selected isolate. But Adeleye *et al.* [34] reported that resistance to 10, 9, 8 drugs occurred in the majority of the *Vibrio* isolates from seafood. This may be due to structural variation in the organisms. Ferrini *et al.,* [35] reported that 82% of *Vibrio* isolated from fish settings as well as both national and imported seafood showed resistance to ampicillin, against which only a low percentage of the isolates 28.57% were resistant. In a previous study on potentially pathogenic vibrios isolated from seafood, Ottaviani *et al.,* [36] also found that those bacteria were sensitive to chloramphenicol (90% of the total), sulfamethoxazole and ciprofloxacin; conversely, they were resistant to ampicillin, penicillin, amoxicillin and cephalexin. Bacterial resistance to ampicillin was the most frequently detected among *Vibrio* species isolated from fishery products [37] as well as from a shrimp hatchery [38] and shrimp farms [39]. Khan *et al*. [28] reported that 100% isolate of enteropathogenic *Vibrio spp* from shrimp in Bangladesh were found to show resistance to erythromycin, penicillin, ampicillin and kanamycin.

Among all 11 (100%) isolates, 7 (63.64%) displayed multidrug resistance (MDR). The main reason of antibiotic resistance of pathogenic bacteria may be the application of antibiotics in shrimp farming and release of shrimp pond effluent to estuarine ecosystems or post harvest contamination of shrimps with the antibiotic resistant bacteria through the environment and human handling. In general bacteria can develop resistance for antibiotics which share similar structures [40]. Van de Boogard and Stobberingh [41], reported that due to indiscriminate exploitation of antimicrobial agents, such high incidence of multi drug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antibiotic saturated environment.

CONCLUSION

Shrimp samples were contaminated with coliform, faecal coliform, salmonella-shigella and vibrio and the ranges of pathogenic bacteria exceeded the limit of standard food value. Among all 11 (100%) isolates, 7 (63.64%) displayed multidrug resistance (MDR) to more than three antibiotics.

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