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Article

Occurrence of *Salmonella* and *Vibrio* species in fresh fishes collected from different markets of Mymensingh, Gazipur and Sherpur districts of Bangladesh and their characterization

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Abstract: This study was undertaken to determine total viable count (TVC), total salmonella count (TSC) and total vibrio count (TVibC) in fresh fishes collected from markets of Mymensingh, Gazipur and Sherpur districts. Isolation and identification of *Salmonella* spp. and *Vibrio* spp. was also done from pangas (n=20), tilapia (n=20) and koi (n=20) collected from different markets of Mymensingh, Gazipur and Sherpur districts. Samples were cultured on plate count agar to determine TVC, xylose-lysine deoxycholate agar (XLD) to determine TSC and thiosulfate citrate bile salt sucrose agar (TCBS) to determine TVibC. The mean value of logarithm colony forming units (CFU±SD/g) of TVC, TSC and TVibC in pangas were log 9.09±0.616, log 5.32±0.391 & log 3.14±0.557CFU/g; log 8.46±0.441, log 5.26±0.589 & log 3.59±0.823 CFU/g and log 7.58±0.466, log 3.28±0.493 & log 2.88±0.386 CFU/g of Mymensingh, Gazipur and Sherpur, respectively. Similarly, the mean values of TVC, TSC and TVibC in tilapia were log 6.60±0.790, log 3.59±0.388 & log 3.75±0.176 CFU/g; log 6.55±0.553, log 3.26±0.502 & log 3.67±0.021 CFU/g and log 6.74±0.372, log 3.44±0.411 & log 3.05±0.609 CFU/g of Mymensingh, Gazipur and Sherpur, respectively and in koi were log 7.51±0.537, log 3.49±0.459 & log 3.35±0.390 CFU/g; log 7.66±0.752, log 3.25±0.465 & log 3.59±0.581 CFU/g and log 7.13±0.393, log 3.27±0.384 & log 3.43±0.297 CFU/g of Mymensingh, Gazipur and Sherpur respectively. The targeted *Vibrio* spp. and *Salmonella* spp. were isolated and identified from collected fishes. All the isolates of *Salmonella* were confirmed by targeting genus specific histidine transport operon gene. Antimicrobial sensitivity test was done for all the isolates of *Salmonella* and *Vibrio* species by disc diffusion method. Out of forty five isolates of *Salmonella*, seven were found multidrug resistant. The bacteria isolated from fish were of public health significance as well as responsible for spoilage of fish.

Keywords: fresh fishes; *Salmonella* species; *Vibrio* species; characterization

1. Introduction

Bacteria present in fish are responsible for economic loss due to mortality. Some pathogens are also known to cause zoonotic disease to the handlers and final consumers. Human infection results from contamination of hands and utensils during processing operations especially at evisceration. Fishes are also known to transmit *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp. and *Aeromonas* spp. which are the causal agent of human food-borne infection and intoxication (Gold and Salit, 1993).

Bacteria found in fish are classified into non-indigenous and indigenous bacteria. The non- indigenous bacteria include *Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus, Salmonella* spp., *Shigella* spp. and *Escherichia coli.* On the other hand, indigenous bacteria include *Vibrio* spp., *Staphylococcus aureus,*

Salmonella, Vibrio parahaemolyticus, Shigella, Aeromonas, Yersinia and *Pseudomonas* (Clucas and Ward, 1996).

Pangas (*Pangasius pangasius*), Tilapia (*Oreochromis mossambicus)* and Koi (*Anabas testudineus*) are generally fresh water fishes. These fishes are found in small rivers, canal and swamp. Koi fish is the most popular and tasty fish among all fishes of Bangladesh. Now a days commercial fish farming in pond is very popular. *Aeromonas* spp. and *Pseudomonas* spp. are deadly bacteria that live in every pond causing life threatening bacterial infection such as ulcer, fin rot and tail rot of fishes. Poor water quality cause burn off the slime coat or stress the fish making it more susceptible to bacterial infection.

Fish is involved in active and passive transfer of a wide range of bacterial infection and intoxication to humans, underscoring the need to investigate public health risk associated with fish. Prophylactic use of antibiotics and growth promoters in intensive fish feed is responsible for development of antibiotic resistance in consumers, which is an important public health issue in recent time. Understanding the main risk factors and how to reduce them is therefore essential for developing best management practice to safeguard public health. Therefore, this study was designed with a view to assess the occurrence of *Salmonella* and *Vibrio* species in fresh fishes collected from different markets of Mymensingh, Gazipur and Sherpur districts.

2. Materials and Methods

2.1. Collection and transportation of samples

A total of 60 samples were collected from 20 different markets of Mymensingh, Gazipur and Sherpur districts. During the collection of samples precautionary measures were maintained to avoid touch and ice box were used to maintain cool chain. The samples were then brought to the laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh.

2.2. Processing and enrichment of samples

Aseptic measures were undertaken during the sampling procedure to prevent contamination of the samples. Skin, gill, intestine of fish, mud and water samples were collected. These samples were minced and grinded together. Ten gm of samples were homogenized with 90 ml of 0.1% peptone water and 0.1 ml of homogenized sample was streaked on to selective agar media and incubated at 37ºC for 24 hours.

2.3. Determination of total viable count (TVC), total salmonella count (TSC) and total vibrio count (TVibC)

Hundred microliter (0.1 ml) of each ten fold diluted sample was transferred and spreaded to plate count agar (PCA), Xylose lysine deoxycholate agar (XLD agar) and Thiosulfate citrate bile salt sucrose agar (TCBS) using a sterile pipette and a sterile glass spreader. The incubated plates were then kept in an incubator at 37°C for 24-48 hours. The number of colonies in a particular dilution was multiplied by the dilution factor to determine TVC, TSC and TVibC, which were expressed as mean logarithm colony forming units (CFU±SD/g).

2.4. Isolation of target bacteria

Initially samples were enriched in nutrient broth at 37º C for 24 hours. The overnight cultures were streaked on XLD agar for Salmonella and TCBS agar for *Vibrio*. Inoculated plates were incubated at 37º C for 24 hours. Single well defined colony was further sub-cultured until pure culture was obtained.

2.5. Identification of target bacteria

Cultural, morphological, and biochemical characteristics were studied to identify the bacterial flora. The cultural characteristics or colonial morphology of the bacteria grown on the SS, XLD and TCBS agar were recorded. Gram's staining was performed to study the morphology and staining characteristics of the bacteria according to the method described by Cheesbrough (1985). Biochemical tests, such as sugar fermentation, motility, catalase, methyl red (MR), voges-proskauer (VP) and indole tests were performed to identify the bacteria (Cheesbrough, 1985).

2.6. Molecular identification by polymerase chain reaction (PCR)

Bacterial DNA template was prepared by using boiling method (Englen and Kelley, 2000).

All the samples were examined by two pairs of primers (Table 1) to detect Histidine transport operon gene of *Salmonella* spp. PCR reactions were carried out using a thermocycler (ASTEC, Fukuoka, Japan) with the following programme: initial denaturation with 1 cycle of 5 min at 94°C, 30 cycles each consisting of denaturation with 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 45 seconds and

a final extension step of 5 min at 72 °C. PCR products were separated on 2% agarose gel, stained with ethidium bromide and photographed using a gel documentation system (BioRad, USA).

Table 1. Primer used in this study.

Primer	Sequence (5'-3')	Target of gene	Amplicon size (bp)	Reference
Upper strand	ACTGGCGTTATCCCTTTCTCTGGTG	Histidine transport operon gene of *Salmonella*	496	Cohen *et al.* 1993
Lower strand	ATGTTGTCCTGCCCCTGGTAAGAGA			

2.7. Antibiotic sensitivity test

All isolates were tested for antimicrobial drug susceptibility against eight commonly used antimicrobials by disc diffusion method according to the guidelines of Clinical and Laboratory Standard Institute (CLSI), 2012. The zones of growth inhibition was compared with the zone size interpretative tables provided by Clinical and laboratory Standards Institute (CLSI, 2012). Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretative standards provided by CLSI (2012).

2.8. Statistical analysis of experimental data

The data on total viable count (TVC), total vibrio count (TVibC) and total salmonella count (TSC) obtained from the bacteriological examination were analysed in completely randomised design (CRD) using SPSS Software (Version 16, 2007). The differences between means were evaluated by Duncan's Multiple Range Test. Correlation among TVC, TVibC and TSC were also evaluated.

3. Results and Discussion

In this study TVC of three types of fish from three districts were determined to know the bacterial load present in these samples. The mean and standard deviation of the total viable count (TVC), total salmonella count (TSC) and total vibrio count (TVbiC) in pangas, tilapia and koi of Mymensingh, Gazipur and Sherpur districts are summarized in Tables 2, 3 and 4. The result of total viable count in three districts retail markets were differed significantly ($P<0.01$). Tiamiyu *et al*. (2011) reported 6.09±0.65 Log CFU microbial load in skin sample of fish. Higher TVC of fish was recorded in all occasions of this present study. The permissible count of heterotrophic bacteria in the 1 cm^2 of skin ranges from 10^2 –10^7 or bacteria of log 10 CFU/cm^2 $\leq$ 5.70 according to International Commission on the Microbiology Specification of Foods (Zmyslowka *et al*., 2000). The higher TVC of fish in this study might be resulted from consumption of bacteria by the fish for long time through food and water. The survival of these bacteria is dependent on the conditions prevailing in the aquatic environment and fish are often simply their hosts (Zmyslowka *et al*., 2000).

The interpretation of total salmonella count in three districts were differed significantly ($P>0.01$) as shown in Tables 2, 3 and 4. Similarly, the interpretation of total vibrio count in three districts were also differed significantly ($P>0.01$) as shown in Tables 2, 3 and 4.

Table 2. Determination of mean and standard deviation for microbiological quality of pangas at different markets of three districts.

Place of collection	TVC (Log CFU/g)	TSC (Log CFU/ g)	TVbiC (Log CFU/ g)
Mymensingh	9.09±0.616[a]	5.32±0.391[a]	3.14±0.557[b]
Gazipur	8.46±0.441[a]	5.26±0.589[a]	3.59±0.823[a]
Sherpur	7.58±0.466[b]	3.28±0.493[b]	2.88±0.386[c]
LSD	0.708	0.311	0.180
Level of sig.	**	**	**

** = Double asterisk (**) means significant at 1% level of probability

In a column figures with same letter do not differ significantly ($p>0.05$) whereas figures with dissimilar letters differ significantly (as per DMRT).

LSD= Least Significant Difference

All counts are expressed in logarithms and CFU/g.

Table 3. Determination of mean and standard deviation for microbiological quality of tilapia at different markets of three districts.

Place of collection	TVC (Log CFU/g)	TSC (Log CFU/ g)	TVbiC (Log CFU/ g)
Mymensingh	6.60±0.790[a]	3.59±0.388[a]	3.75±0.176[a]
Gazipur	6.55±0.553[a]	3.26±0.502[b]	3.67±0.021[a]
Sherpur	6.74±0.372[a]	3.44±0.411[ab]	3.05±0.609[b]
LSD	0.184	0.185	0.425
Level of sig.	NS	**	**

** = Double asterisk (**) means significant at 1% level of probability; NS = Not significant
In a column figures with same letter do not differ significantly (p>0.05) whereas figures with dissimilar letters differ significantly (as per DMRT).
LSD= Least Significant Difference
All counts are expressed in logarithms and CFU/g.

Table 4. Determination of mean and standard deviation for microbiological quality of koi at different markets of three districts.

Place of collection	TVC (Log CFU/g)	TSC (Log CFU/ g)	TVbiC (Log CFU/ g)
Mymensingh	7.74±0.610[a]	3.71±0.405[a]	3.25±.328[b]
Gazipur	7.66±0.752[a]	3.25±0.465[b]	3.59±0.581[a]
Sherpur	7.13±0.393[b]	3.27±0.384[b]	3.43±0.297[a]
LSD	0.189	0.151	0.168
Level of sig.	**	**	**

** = Double asterisk (**) means significant at 1% level of probability
In a column figures with same letter do not differ significantly (p>0.05) whereas figures with dissimilar letter differ significantly (as per DMRT).
LSD= Least Significant Difference
All counts are expressed in logarithms and CFU/g.

Two targeted bacteria *Vibrio* spp. and *Salmonella* spp. were isolated from three types of fish from three districts. The results of isolation are in agreement with the findings of Adebayo-Tayo *et al.* (2012).
Salmonella spp. was found in 75% of the tested samples and the highest count recorded was log 5.89 CFU/g in pangas collected from Mymensingh. Out of the 60 samples, 42 were contaminated with *Vibrio* spp. and highest count was log 4.37 CFU/g in pangas collected from Gazipur. This value is higher than the findings of Mamun (2014). This increase might be the result of exposure of fishes in unhygienic condition during different stages of cultivation and collection. It may start from hatcheries and other subsequent processes like farming area, processing station, transportation and market place. Health status of working personnels and their poor sanitation practices are also responsible. The presence of *Salmonella* and *Shigella* in the fish samples may be due to exposure to flies (Mensah *et al.*, 2002) and prevailing poor sanitary conditions in the markets. *Salmonella* and *Shigella* are well-known important human pathogens. Their presence is unacceptable because of their attendant health risks (Mensah *et al.*, 2002). Fishes are prone to bacterial contamination and could cause health risk to consumers (Wafaa *et al.*, 2011). The food poisoning associated with consumption of fish and shellfish either raw or slightly cooked, contaminated with *Vibrio* spp. causes intestinal infection characterized by diarrhea, abdominal cramps, sickness, vomiting, fever and severe headache (Merwad *et al.*, 2011, Espineira *et al.*, 2010*).* The samples of fish and shellfish analyzed bacteriologically showed varying degree of *Vibrio* contamination.
Morphology of *Vibrio* spp. on TCBS agar plate recorded in this study was similar to the findings of Khan *et al.* (2007). In Gram's staining, bacteria exhibited curved rod shaped appearance, which was similar to the findings of Faruque *et al.* (2008). In this study, the colonies of *Salmonella* spp. on SS agar plate were opaque, translucent with black centers, which were similar to the findings of Hossain (2002). In Gram's staining bacteria exhibited short rods, Gram negative, single or paired arrangement. Similar findings were also reported by Samad (2005). *Salmonella* spp. produced acid by fermenting dextrose, maltose and mannitol and did not ferment lactose and sucrose. *Vibrio* spp. produced acid by fermenting dextrose, maltose, sucrose and mannitol except lactose. *Salmonella* spp. gave positive reaction to MR and negative reaction to VP and indole production tests and

Vibrio spp. gave positive reaction to MR and indole production tests and negative reaction to VP test that were similar to the findings described by Zaman *et al.* (2013).

Molecular detection of *Salmonella* spp. was performed by histidine transport operon gene based PCR method. All isolates gave specific amplification (496 bp). The result of PCR is shown in Figure 1. These findings are in agreement with the result of Nadia *et al.* (2011). Noah *et al.* (1993) also detected *Salmonella* at genus level.

Results of antimicrobial susceptibility test for *Vibrio* spp. are presented in Table 4. All *Vibrio* isolates were susceptible to azithromycin, gentamicin, norfloxacin, and tetracycline and resistant to amoxycillin. Most of the isolates susceptible to streptomycin (95.24%) and erythromycin (97.61%) and intermediate to ciprofloxacin (88.10%). Almost similar antimicrobial profiles were also reported by Hossain *et al*. (2012). Results of antimicrobial susceptibility test for *Salmonella* spp. are summarized in Table 5. As regards to *Salmonella* spp., all isolates were susceptible to azithromycin, ciprofloxacin, gentamicin and norfloxacin. Most of the isolates were intermediate to erythromycin (91.11%) and tetracycline (86.67%) and resistant to amoxycillin (93.33%). These findings of the present study are in agreement with the result of Frech and Schwarz (1998); and Chugh and Suheir (1983).

In case of *Vibrio* spp. all isolates were resistant to one antimicrobial agent (AMX). In case of *Salmonella* spp., out of 45 isolates, 38 (84.44%) isolates were resistant to one antimicrobial agent (AMX). Moreover 1 (2.22%), 3 (6.67%) and 1 (2.22%) isolates were resistant to two antimicrobial agents (AMX-E), (AMX-TE) and (E-TE) respectively. Furthermore 2 (4.44%) isolates were resistant to three antimicrobial agents (AMX-E-TE). These results are shown in Tables 6 and 7.

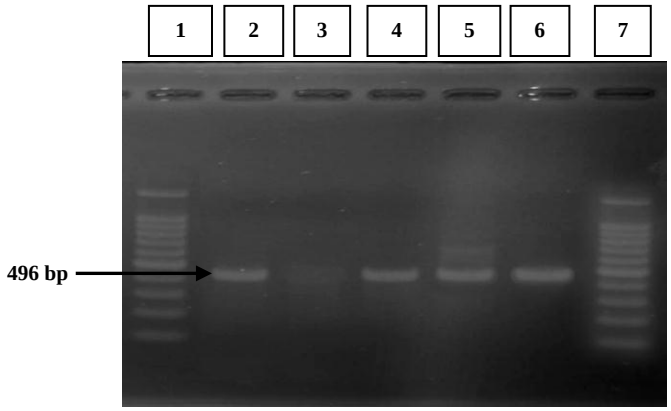

Figure 1. PCR assay to amplify histidine transport operon gene of *Salmonella* spp. isolates from fish samples. Lane 1 and 7. 100 bp size DNA marker; lane 2. Positive control; lane 3. Negative control without DNA; lane 4. *Salmonella* spp. isolate from pangas; lane 5. *Salmonella* spp. isolate from tilapia and lane 6. *Salmonella* spp. isolate from koi.

Table 5. Results of antimicrobial susceptibility test for *Vibrio* spp.

Name of isolates	**No. (%)**							
***Vibrio* spp.(n=42)**	AMX	AZM	CIP	E	GEN	NOR	S	TE
Susceptible	0 (0)	42 (100)	5 (11.90)	41 (97.61)	42 (100)	42 (100)	40 (95.24)	42 (100)
Intermediate	0 (0)	0 (0)	37 (88.10)	1 (2.39)	0 (0)	0 (0)	2 (4.76)	0 (0)
Resistant	42 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

AMX= Amoxycillin, AZM= Azithromycin, CIP= Ciprofloxacin, E= Erythromycin, GEN= Gentamycin, NOR= Norfloxacin, S= Streptomycin, TE= Tetracycline

Table 5. Results of antimicrobial susceptibility test for *Salmonella* spp.

Name of isolates	No. (%)							
***Salmonella* spp.(n=45)**	AMX	AZM	CIP	E	GEN	NOR	S	TE
Susceptible	0 (0)	45 (100)	45 (100)	0 (0)	45 (100)	45 (100)	38 (84.44)	0 (0)
Intermediate	3 (6.67)	0 (0)	0 (0)	41 (91.11)	0 (0)	0 (0)	7 (15.54)	39 (86.67)
Resistant	42 (93.33)	0 (0)	0 (0)	4 (8.89)	0 (0)	0 (0)	0 (0)	6 (13.33)

AMX= Amoxycillin, AZM= Azithromycin, CIP= Ciprofloxacin, E= Erythromycin, GEN= Gentamycin, NOR= Norfloxacin, S= Streptomycin, TE= Tetracycline

Table 6. Results of antimicrobial resistance patterns of *Vibrio* spp.

Isolates	Resistance profiles	No. of isolates (%)
Vibrio spp.(n=42)	No resistance demonstrated	-
	Resistant to 1 agent (AMX)	42(100)
	Resistant isolates	n=42(100)

Table 7. Results of antimicrobial resistance patterns of *Salmonella* spp.

Isolates	Resistance profiles	No. of isolates (%)
	No resistance demonstrated	-
	Resistant to 1 agent (AMX)	38(84.44)
Salmonella spp.(n=45)	a. Resistant to 2 agents (AMX-E)	1(2.22)
	b. Resistant to 2 agents (AMX-TE)	3(6.67)
	c. Resistant to 2 agents (E-TE)	1(2.22)
	Resistant to 3 agents (AMX-E-TE)	2(4.44)
	Resistant isolates	n=45(100)

4. Conclusions

This study confirmed the occurrence of *Salmonella* and *Vibrio* species in fresh fishes collected from different markets of Mymensingh, Gazipur and Sherpur districts of Bangladesh. This study also recorded the presence of multidrug resistant *Salmonella* spp. in fish that is alarming with regards to food safety and public health point of view.

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Conflict of interest

None to declare.

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