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MOLECULAR DETECTION AND CHARACTERIZATION OF *SALMONELLA* SPP. ISOLATED FROM FRESH FISHES SOLD IN SELECTED UPAZILA MARKETS OF BANGLADESH

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ABSTRACT

Aquatic environments are the major reservoirs of *Salmonella*. Therefore, fishery products have been recognized as a major carrier of food-borne organism. Fish is known to harbor bacteria of public health significance. Aquatic environments are known to influence the bacterial loads in the harvested fish. The present work was undertaken for molecular detection and characterization of *Salmonella* species isolated from fresh fishes sold in different markets of Jamalpur,Tangail, Kishoreganj and Netrokona districts of Bangladesh. The isolates were identified by their morphological, cultural and biochemical characteristics with standard reference organisms, and molecular methods. Out of 20 pangas fish (*Pangasius* spp.) samples the number of samples found to be positive for *Salmonella* spp. was 14 (70%); of 20 koi fish (*Anabas* spp.) samples this number was 17 (85%); and of 20 tilapia fish (*Oreochromis* spp.) samples it was 15 (75%). All the isolates of *Salmonella* were confirmed by targeting genus specific histidine transport operon gene. Antimicrobial susceptibility test was performed to know the susceptibility and resistance patterns of the isolates to different antimicrobial agents. Results of antimicrobial susceptibility test shows that 40 (86.95%) isolates were found to be resistant to azithromycin, 42 (91.30%) were resistant to erythromycin. On the other hand all isolates were 100% susceptible to ciprofloxacin and gentamicin, 38 (82.62%) were susceptible to norfloxacin, 40 (86.95%) were susceptible to streptomycin.

Key words: *Salmonella* spp., Histidine transport operon gene

INTRODUCTION

With a population of more than 30,000 known species, fish forms the biggest group in the animal kingdom that is used for the production of animal-based foods. About 700 of these species are commercially fished and used for food production. Furthermore, some 100 crustacean and 100 molluscan species (for example mussels, snails and cephalopods) are processed as food for humans in fish industry. However, some fishery product processed in a modern fish industry which is a technologically advanced and complicated industry in line with any other food industry, and with the same risks of product being contaminated with pathogenic organisms. Fish is a popular food item in Bangladeshi food menu. It provides good source of animal protein. This sector contributes 60% of the daily per capita animal protein intake, 22.60% of agricultural GDP, and 3.69% of total GDP. Total fish production in Bangladesh in 2013-14 was 3548 thousand metric tons, of which 83.20 % was from inland fisheries and 16.80% was from marine fisheries (Bangladesh Economic Review, 2015). 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. 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 *E. coli*. On the other hand, indigenous bacteria include *Vibrio* spp, *Staphylococcus aureus, Salmonella, Shigella, Aeromonas, Yersenia* and *Pseudomonas* (Clucas and Ward, 1996). *Bacillus*, *Proteus*, *Pseudomonas*, *Klebsiella*, *Streptococcus*, *Salmonella*, *Staphylococcus*, *Micrococcus*, *Serratia* and *Escherichia* are found in the skin and intestine of fish (Tiamiyu *et al*., 2011*)*. The bacteria from the surface of fresh water fish known to carry *Acineto bactercalcoaceticusae, A hydrophila, A. bestiarum, A. caviae, A. jandaei, A. schubertii, A. veronii*, *Entero bacteraerogenes, E. coli* and *Flavobacterium (*Zmyslowska *et al.*, 2001). The bacteria present in gill include *E. coli, Citrobacter* spp., *Enteriobacter* spp. and *Klebsiella* spp.

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The Microbiology of skin and gastro intestinal tract of fish has been studied by many researchers. Fish can be spoiled from both outer surface and inner surfaces. After fish is being caught the immune system collapses with its eventual death, bacteria can proliferate freely on the skin surface and the stomach. The walls of intestine do break down sufficiently for bacteria to move into the flesh through the muscle fiber (Kaneko, 1971). The main objectives of the present research work were isolation and identification of bacteria from fish samples and antimicrobial susceptibility patterns of isolated bacteria with the detection of *Salmonella* spp. based on histidine transport operon gene PCR.

MATERIALS AND METHODS

Sample collection

A total of 60 fresh fish samples (20 pangas fish, *Pangasius* spp.; 20 koi fish, *Anabas* spp. and 20 tilapia fish, *Oreochromis* spp.) were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh. The samples were collected from July 2015 to December 2015 and investigation was carried out following collection. The collected milk samples were immediately transported on ice to the Microbiology Laboratory at the Department of Microbiology and Hygiene of BAU and District Veterinary Hospital, Sirajgonj for bacteriological analysis.

Isolation and identification of *Salmonella* spp.

10 gm of sample were taken from skin, gills and intestine by cutting with a sterile scalpel. Then the samples were grinded by using mortar and pestle. Then grinded fish samples were performed 10 fold dilution using 0.1% peptone water and diluted samples were spread onto XLD agar (HiMedia®, India) incubated at 37°C for overnight. Then the presumptive colonies of *Salmonella* spp. were sub-cultured onto SS agar and XLD agar to get pure culture. These isolates were preserved for further bacterial identification. The isolates were identified as *Salmonella* spp. on the basis of Gram staining, colony morphology on XLD agar (HiMedia®, India), SS agar, biochemical characterization of the isolates (using sugar fermentation test, indole and MR-VP test). Further the isolates were detected by histidine transport operon gene based PCR.

Bacterial Genomic DNA extraction

DNA template was prepared by boiling method briefly 250 µl distilled water was taken into eppendorf tube and a pure bacterial colony was picked up and mixed with the distilled water. The tubes then transferred to boiling water and boiled for 10 minutes then immediately transferred to ice for cold shock about 10 minutes and then centrifuged at 10000 rpm for 10 minutes. Supernatant were collected and used as DNA template during PCR.

Identification of *Salmonella* spp. by PCR

PCR reaction was performed to detect *Salmonella* spp. by histidine transport operon gene based PCR. Two different primers pairs were used for this purpose, histidine transport operon gene (F 5'-ACTGGCGTTATCCCTTTCTCTGGT-3' and R 5' ATGTTGTCCTGCCCCTGGTAAGAG-3') according to the methods described by Cohen *et al. (*1993). Each 20 µl reaction mixture consists of 3 µl genomic DNA, 10 µl PCR master mixtures (Promega, USA), and 1 µl of each of the two primers with the final volume adjusted to 20 µl with 5µl of nuclease free water. Amplification was done by initial denaturation at 95°C for 5 minutes, followed by denaturation at 94^0C for 30 sec, annealing temperature of primers was 56°C for 30 sec and extension at 72°C for 45 sec. The final extension was conducted at 72°C for 5 minutes. The total reaction was performed at 30 cycles. The amplified PCR products were resolved by electrophoresis in 2% agarose gel at 100v for 30 minutes, stained with ethidium bromide and finally visualized under UV trans-illuminator.

Antibiotic sensitivity test

All isolates separated from pangus fish (*Pangasius* spp.), koi fish (*Anabas* spp.) and tilapia fish (*Oreochromis* spp.) were tested for antimicrobial drug susceptibility against antimicrobial agents by disc diffusion method according to the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2012). Sensitivity pattern of the isolates were determined against ciprofloxacin, azithromycin, gentamicin, amoxycillin, streptomycin, erythromycin, tetracycline and norfloxacin. Antimicrobial testing results were recorded as sensitive, intermediate sensitive and resistant according to zone diameter interpretative standards provided by CLSI (2012).

RESULTS AND DISCUSSION

A total of 60 samples were collected for the isolation of bacteria from fresh fishes (pangas fish, *Pangasius* spp.; koi fish, *Anabas* spp. and tilapia fish, *Oreochromis* spp.) sold from different upazilla markets of Bangladesh. Out of 20 koi fish (*Anabas* spp.) samples 85% (n=17) were contaminated with *Salmonella* spp., out of 20 tilapia fish (*Oreochromis* spp.) samples 75% (n=15) were contaminated with *Salmonella* spp and out of 20 pangas fish (*Pangasius* spp.) samples 70% (n=14) were contaminated with *Salmonella* spp. In xylose-lysine deoxycholate (XLD) agar the *Salmonella* spp. produced blackish centered colony (Figure 1). In salmonella-shigella (SS) agar the *Salmonella* spp. produced small, round smooth colony with black centre (Figure 2). In Gram's staining, organism revealed as pink colored short rod shaped bacteria arranged in single or paired (Figure 3). These isolates were positive for catalase and coagulase test. In catalase test; Hydrogen peroxide was broken-down into water and oxygen. Production of oxygen was indicated by bubble formation, whereas the negative control did not produce any bubble.

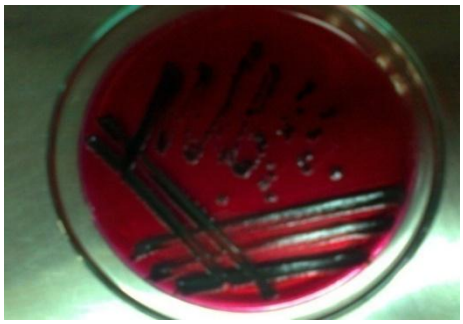

Figure 1. *Salmonella* spp. produces round black center colony in XLD agar media.

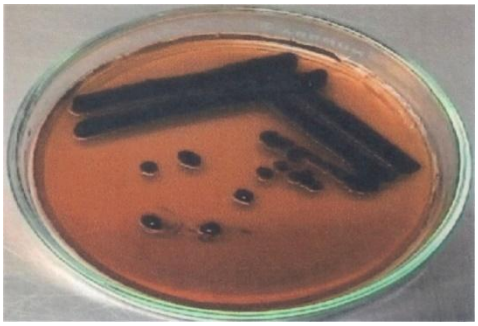

Figure 2. Translucent black smooth, small round colonies of *Salmonella* spp. on SS agar media.

Molecular detection of *Salmonella* spp. was performed by histidine transport operon gene based PCR method. Specific 496 bp fragment of targeted histidine transport operon gene was amplified successfully (Figure 4). Each genus was subjected to antimicrobial susceptibility test by disc diffusion method against 8 most commonly used antimicrobial agents. 40 (86.95%) isolates were found to be resistant to azithromycin, 40 (91.30%) were resistant to erythromycin. Furthermore, all isolates were susceptible to ciprofloxacin and gentamicin, 38 (82.62%) were susceptible to norfloxacin, 40 (86.95%) were susceptible to streptomycin (Figure 5)

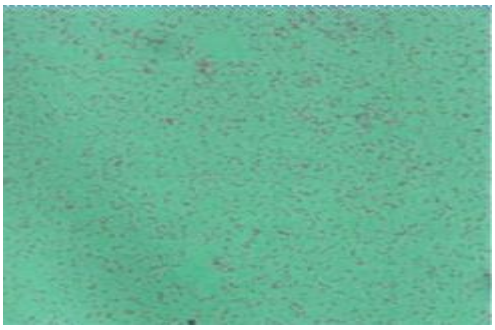

Figure 3. Gram-negative very short plump, rods of *Salmonella* spp. (100X).

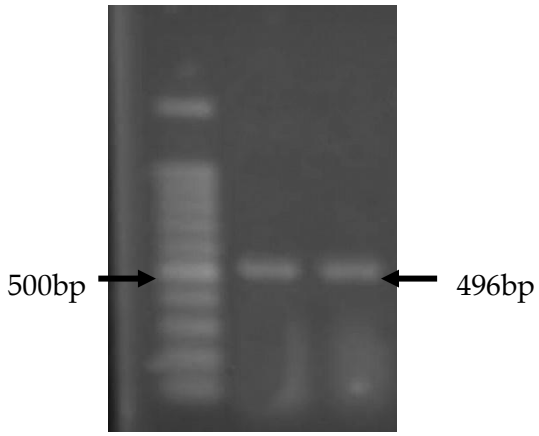

Figure 4: Detection of *Salmonella* spp. by histidine transport operon gene based PCR. Lane 1:100bp DNA marker, lane 2, 3: DNA of *Salmonella* spp.

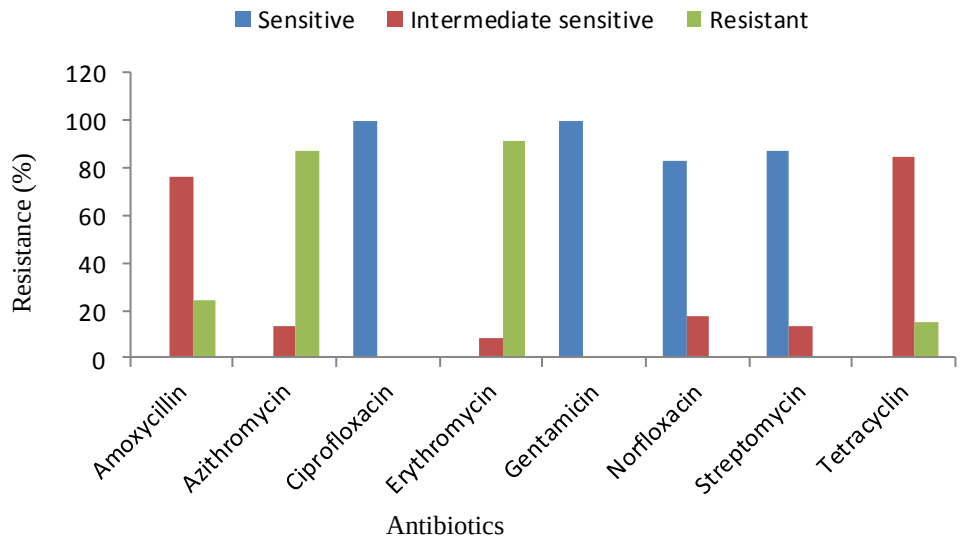

Figure 5. Antibiotic resistance patterns of *Salmonella* spp. against commonly used antibiotics in Bangladesh

The present study was designed for identification and characterization of *Salmonella* spp. from fresh fish sample. Total 60 samples (20 pangas fish, *Pangasius* spp.; 20 koi fish, *Anabas* spp. and 20 tilapia fish, *Oreochromis* spp.) were collected and analyzed. Out of 20 pangasius fish samples 14 samples were revealed the positive result for *Salmonella* spp.; of 20 koi fish samples 17 samples were revealed the positive result for *Salmonella* spp.; of 20 tilapia fish samples, 15 samples were revealed the positive result for *Salmonella* spp. *Salmonella* spp. were identified and were confirmed by cultural examination, morphological studies, staining characters and biochemical tests and finally PCR were performed for the amplification of histidine transport operon gene of isolated bacteria.

Salmonellosis is still a global challenge to public health. Salmonellosis is an important food borne infective disease worldwide, occurring mostly as sporadic cases in families or as outbreaks. Now a days Fish and fish products are the important source of *Salmonella* spp. *Salmonella* spp. is a recognized human pathogen and its waterborne transmission has been well documented (Cabral, 2010). Poultry and poultry products have been the most commonly implicated foods to cause infection in human. Although meat and meat products, milk and milk products, and water have also been associated with large outbreaks of salmonellosis (Inatsu *et al*., 2013).

Results of antimicrobial susceptibility test showed that most of the isolates of *Salmonella* were susceptible to ciprofloxacin (100%), gentamicin (100%), streptomycin (86.95%) and norfloxacin (82.62%). Most of the isolates were intermediate susceptible to tetracycline (84.78%) and amoxycillin (76.08%) and resistant to erythromycin (91.30%) and azithromycin (86.95%). These findings are slightly correlated to Swati *et al*. (2015) and Mahmuda *et al*. (2010).

CONCLUSION

Fish is an important source of animal protein for human diet. Fish are susceptible to a wide variety of bacterial pathogen. Many of these bacteria are capable of causing human infection and intoxication. The microbiological hazard of *Salmonella* contamination of fish and fish products during storage and improper handling or cooking of fish can lead to human food-borne illness. Hence, there is need to monitor the contamination levels of *Salmonella* as well as other zoonotic pathogens throughout the year to safeguard public health. The present work was undertaken for molecular detection and characterization of *Salmonella* species isolated from fresh fishes sold in different markets of Bangladesh. Out of 60 samples of pangus fish (*Pangasius* spp.), koi fish (*Anabas* spp.) and tilapia fish (*Oreochromis* spp.) of 20 each 14 (70%), 17 (85%) and 15 (75%) samples were positive for *Salmonella* respectively. The isolates were identified by their morphological, cultural properties, biochemical

characteristics with standard reference organisms and molecular methods. All the isolates of *Salmonella* were confirmed by targeting genus specific histidine transport operon gene. Results of antimicrobial susceptibility test showed that most of the isolates of *Salmonella* were susceptible to ciprofloxacin (100%), gentamicin (100%), streptomycin (86.95%) and norfloxacin (82.62%). Most of the isolates were intermediate susceptible to tetracycline (84.78%) and amoxycillin (76.08%) and resistant to erythromycin (91.30%) and azithromycin (86.95%). Resistant pattern against broad spectrum antibiotic (e.g., erythromycin and azithromycin) depicts an alarming situation which needs special consideration.

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