Molecular detection of *Salmonella* Typhimurium and its antibiotic gram from marketed raw fish of inland fisheries sold in Aizawl, Mizoram, India

Devajani Deka, M Das and H Bayan

Abstract

The study aimed to detect *Salmonella* spp in 100 numbers of raw fish samples from inland fisheries sold in local markets of Aizawl. *Salmonella* was isolated and presumptively identified by using conventional bacteriological method and the *Salmonella* serovars were further confirmed by detection of species specific 16S-rRNA gene and serotyping. The PCR positive *Salmonella* serovars were screened for the presence of virulence associated genes (*inv*A and *sta*) and antimicrobial susceptibility. From the 10 phenotypically positive *Salmonella* strains, 4 strains were confirmed as *Salmonella* by PCR (16S-rRNA gene) and serotyped as *Salmonella* Typhimurium. All the 4 (100%) strains were subsequently positive for *inv*A and *sta* genes. The antimicrobial sensitivity profile revealed that the *Salmonella* Typhimurium strains were sensitive to Amikacin, Gentamicin, Ofloxacin, Cefazidime, Chloramphenicol and Imipenem and resistant to Tetracycline and Ciprofloxacin.

Keywords: Antibiogram, Mizoram, raw fish, *Salmonella* typhimurium, virulence genes

Introduction

Fish is loaded with many essential nutrients mainly good quality protein, iron, vitamin D and calcium along with unsaturated fat called omega-3 fatty acid with huge availability in most of the countries worldwide. The role of fisheries is increasingly recognized by national and global development policy makers for alleviation of hunger and malnutrition. In 2015, fish contributed for about 17 per cent of animal protein consumed by the global population and fish provided about 3.2 billion people with almost 20 percent of their average per capita intake of animal protein [8]. Globally, India holds 3rd position in fisheries and second in aquaculture and the per capita annual consumption of fish in India is 9kg in 2015 as per National Fisheries Development Board. Besides the capture fisheries, recent developments in inland fisheries make different kinds of fishes more available and favourite in most parts of world. In many developing countries with water and fishery resources, fish serves as an important source of nutrition, livelihoods and income for the rural poor who suffer from malnutrition including micronutrient deficiencies [31].

Like the raw meat, egg and other food products of animal origin, fish may also transmit many bacterial, viral and other microorganisms to human if appropriate food safety measures are not adapted. The hazardous factors may enter fish production chain during handling of fishes like catching, transportation, and marketing, slaughtering and processing for consumption [12]. Therefore, fish and other aquatic life forms are vulnerable to different kinds of environmental hazards [25]. Fish and fishery products are frequently contaminated with bacterial pathogens and have been recognized as an important carrier of food-borne pathogens [34]. Fishes are known to transmit *Salmonella* spp., *Staphylococcus* spp. and *Aeromonas* spp. which are the causal agents of human food borne infection and intoxication [11]. Common pathogens that are found in Indian seafood are *Salmonella*, *Vibrio*, *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* [26]. *Salmonella* infection in human and other animals occur due to the ingestion of under cooked fish contaminated during production and processing. *Salmonella* in fresh water fishes has been related to the faecal contamination of water from where fishes were harvested. The microbial contamination of fishes and detection of *Salmonella* in pond fishes has been recorded in some studies [5, 21]. Thus, fish is very much prone to be contaminated with different microorganisms which might threat consumer’s health.
Mizoram has witnessed a good growth in fish production in recent years. Aquaculture has been contributing 90% of total fish production of 7630 MT during 2016-17. The pond productivity of fish is 1280 kg/ha/yr in 2016-17 which is still far below than the national average [13]. Detection of the bacterial pathogen can be done by isolation and identification using traditional cultural method in combination with molecular tool such as polymerase chain reaction (PCR) [10]. Therefore, the present piece of work was carried out to detect *Salmonella* in raw fish from inland fisheries sold in local markets of Aizawl, Mizoram, its virulence associated genes and antibiogram profile.

**Materials and methods**

A total of 100 numbers of raw fish sample of different species from inland fisheries sold in local markets Aizawl, Mizoram were collected during October, 2015 to March, 2016. About 100 grams of fish flesh was aseptically collected from each fish and processed for isolation and identification of *Salmonella* as per the standard guidelines from ISO 6579:2002 with slight modification. Different stages for isolation and identification of *Salmonella* involved pre-enrichment, selective enrichment, selective plating, Gram’s staining and a set of biochemical tests. Twenty-five gram of fish flesh was aseptically ground and added with 225 ml of buffered peptone water (BPW) and incubated at 37°C for 18 hours for pre-enrichment. One ml of pre-enriched broth was transferred into tubes containing 10 ml selenite cysteine broth and incubated at 37°C for 24 hours. A loop-full of enriched culture was streaked onto selective agar plates of Xylose lysine deoxycholate agar (XLD) and Brilliant green agar (BGA) and incubated at 37°C for 24 hours. Gram negative bacterial colonies with specific morphological characteristics were biochemically analysed for Indole, Methyl red, Voges-Proskauer, Citrate utilisation and TSI as per the method described by Quinn et al. (1994) [22].

All the phenotypically positive strains were further screened for *16S*-rRNA genus specific PCR based detection of *Salmonella*. The template DNA was prepared from the pure cultured bacterial strains by using boiling and snap chill method. The bacterial isolates were grown in five ml single strength Luria Bertani (LB) broth and incubated at 37°C for 24 hours under constant shaking. After incubation, one ml of the bacterial broth culture was taken in a sterile micro-centrifuge tube and centrifuged at 8000 rpm at 4°C for 8-10 minutes. The bacterial pellet thus obtained was washed thrice with sterile normal saline solution (NSS, 0.85% w/v) by centrifuging at 8000 rpm at 4°C for five minutes and finally pellet was re-suspended in 100μl of nuclease free sterile distilled water. The bacterial suspension was boiled for 15-20 minutes in a boiling water bath followed by immediate chilling for 15 minutes at -20°C. The lysate was centrifuged again at 5000 rpm for five minutes to sediment the cell debris and the supernatant was used as template DNA for PCR assay as per standard method.

The *16S*-rRNA gene was amplified (Master Cycler Gradient, Bio Rad) by using published oligonucleotide primers (Eurofins Genomics India Pvt. Ltd., Bangalore, India) which flanked a 480bp segment in reserved species specific gene sequence. The *16S*-rRNA gene positive *Salmonella* strains were processed for detection of virulence associated genes namely invA and smn. Oligonucleotide primers used for detection of the targeted genes are given in Table-1. All the PCR mixtures consisted a final volume of 25μl containing 12.5 μL 2X Dream taq PCR Master Mix Mgc2 (20 mM), 1 μl (10 pmol) each of forward and reverse primer, 5 μl of template DNA (culture lysate) and nuclease free water to make up the volume 25 μl. The cycling condition of *16S*-rRNA gene and virulence genes are presented in Table-2. The final amplified products were analyzed by horizontal submarine electrophoresis with one per cent(w/v) agarose gel in 1X TAE buffer (Tris acetate 0.04 M, EDTA 0.001 M and pH adjusted to 8.0) [28].

### Table 1: Oligonucleotide primers used in PCR for detection of different genes of *Salmonella*.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Base pair</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>16S</em>-rRNA</td>
<td>F: TAT CTG GCT ATC GCT GGC AGT G \ R: TCC GCT AAT CTT TTG GCA ACC</td>
<td>480</td>
<td>Whyte et al. (2002) [15]</td>
</tr>
<tr>
<td>Sm</td>
<td>F: TGGTGTCGTATCCTGCGGAACC \ R: ATTCGTGAAACCCTGCTTGTC</td>
<td>617</td>
<td>Murugkar et al. (2003) [19]</td>
</tr>
<tr>
<td>invA</td>
<td>F: TGAAATTATGCGCCAGTTCGGGCAAC \ R: TCATCGCACCCTGCAAAGGAAACC</td>
<td>284</td>
<td>Rahn et al. (1992) [24]</td>
</tr>
</tbody>
</table>

### Table 2: Thermal cycling conditions used for detection of different genes of *Salmonella*.

<table>
<thead>
<tr>
<th>Stages of PCR</th>
<th>Different genes of <em>Salmonella</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>16S</em>-rRNA</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C for 45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>59°C for 45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 45 sec</td>
</tr>
<tr>
<td>Final extension for 1 cycle</td>
<td>72°C for 6 min</td>
</tr>
<tr>
<td>No of cycle</td>
<td>30</td>
</tr>
</tbody>
</table>

The *16S*-rRNA gene positive *Salmonella* strains were serotyped on the basis of their somatic antigen at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India. All the PCR positive *Salmonella* strains were tested for phenotypic antimicrobial sensitivity pattern by *in vitro* antimicrobial sensitivity test through disc diffusion method [1]. A panel of 12 commonly used antimicrobial agents were tested namely; Ampicillin (AMP, 10), Gentamicin (GEN, 10), Amikacin (AK, 30), Ciprofloxacin (CIP, 5), Norfloxacin (NX, 10), Ofloxacin (OF, 5), Ceftriaxone (CTR, 30), Cefazidime (CAZ, 30), Cephalexine (CTX, 30), Chloramphenicol (C, 30), Tetracycline (TE, 30) and Imipenem (IPM, 10) (Hi-Media, Mumbai, India). Briefly, isolates were grown in a shaking water bath at 37°C Overnight and then the
bacterial suspension was spread over the entire surface of Mueller-Hinton agar plates and antibiotic disks were applied on the surface of the medium and incubated at 37°C for 18-24 hours. *Salmonella* strains were evaluated as susceptible and resistant according to the diameter of the zone of inhibition.

**Results and discussion**

From the 100 numbers of raw fish samples collected from different unorganized fish markets of Aizawl, 10 *Salmonella* strains were detected presumptively by conventional bacteriological method. From the 10 phenotypically positive *Salmonella* strains, four strains were found to be positive for genus specific gene (16S-rRNA) of *Salmonella* (Figure 1). All the four strains were serotyped as *S. Typhimurium* indicating the 4 per cent prevalence rate of the organism in raw fish sold in local markets.

According to Center for Disease Control and Prevention, *Salmonella* is the leading cause of bacterial foodborne illness causing approximately 1.4 million nontyphoidal illnesses, 15,000 hospitalizations and 400 deaths in USA annually. Thus, *Salmonella* is a pathogen responsible for severe foodborne infections which can be introduced into the fish production chain through inadequate handling/ hygiene or contact with contaminated water although it is not a biological contaminant originally reported in fish [9]. Its waterborne transmission has been well documented [8]. *Salmonella* has been isolated from fish and fishery products from India, though it is not psychotrophic or indigenous to the aquatic environment [18]. The occurrence of *Salmonella* in fish from other studies was found to be much higher than the findings of present study. However, the incidence of *Salmonella* infection due to aquatic food consumption is still low compared with salmonellosis associated with other foods while detection of *Salmonella* spp. in aquatic food cannot be skipped as it is responsible for much food borne gastroenteritis. Beshisu et al. (2019) [2] indentified *Salmonella Enteridis* (24.40%) and *S. Typhimurium* (31.40%) from shrimps sold in open market of Delta and Edo state, Nigeria. The occurrence of *Salmonella* serovars has also been reported in fresh water fish and sea foods from different parts of India. Kumar et al. (2008) [15] detected 23.20 per cent prevalence of *Salmonella* with 27 different serovars predominantly including *Salmonella Typhimurium* among all other serovars from seafood. *Salmonella* was also reported from the intestines of Silver Carp fish due to the microbial contamination of fishes grown in ponds in and around Calcutta [21].

In recent years, PCR has been used to study the distribution of genes present in *Salmonella* serovars associated with seafood in India [15, 4]. In the present study, all the *S. Typhimurium* strains were found to be positive (100%) for the virulence genes, *invA* and *stn* (Figures 2-3). These two genes are invariably present in all *Salmonella* serovars which can be used as PCR based detection tool for quick and less laborious identification of the organism. The *invA* and *stn* genes are responsible for invasiveness and enterotoxin production which make *Salmonella* an obligatory pathogen. Similar to the present findings, 100 per cent positivity to *invA* and *stn* gene in *Salmonella* isolates from fish was reported in an earlier study [32]. However, 100 per cent positivity of *invA* gene in *Salmonella* isolates from fishes was also reported [3, 29]. Kshirsagar et al. (2014) [14] reported that *Salmonella* strains originating from raw beef and offal were positive for *invA* and *stn* genes.

The antimicrobial resistance pattern among *Salmonella* strains isolated from environmental sources and food shows a variable incidence rate of resistant strains obtained from developed and developing countries. These observational studies varied in methodology and spectrum of antibiotics used by different investigators which might contribute to the high degree of variation in resistance pattern. Resistance to antimicrobials by *Salmonella* spp. can be transferred mainly due to consumption of food contaminated with antibiotics or eating food contaminated with faeces of animal or human carriers, who continue to suffer from the disease after various
incomplete or failed treatments [27].

The antibiogram study on Salmonella Typhimurium strains showed 100 per cent sensitivity to Gentamicin, Amikacin, Ofloxacin, Ceftazidine, Chloramphenicol and Imipenem where as all the strains were found to be completely resistant against Ciprofloxacin and Tetracycline. The Salmonella Typhimurium strains were 75 per cent resistant to Cephalaxin, 50 per cent to Ampicillin and 25 per cent to Norfloxacin and Ceftriaxone (Figure 4).

Fig 4: Antibiogram of Salmonella Typhimurium strains (n=4) isolated from fish

Martinez-Urtaza et al. (2004) [16], Kumar et al. (2008) [15] and Setti et al. (2009) [21] reported that 9, 82 and 49.10 per cent Salmonella strains isolated from marine environment showed antimicrobial resistance from Spain, India and Morocco, respectively. Seel et al. (2016) [30] reported that 86.95 per cent strains of S. Typhimurium were found to be resistant to Azithromycin and 91.30 per cent to Erythromycin while strains were 100 per cent sensitive to Ciprofloxacin and Gentamicin, 82.62 per cent to Norfloxacin and 86.95 per cent to Streptomycin. Elhadi (2014) [7] reported that Salmonella were highest resistant against Tetracycline (90%) followed by Ampicillin (70%) and Amoxycillin-clavulanic acid (45%). Beshiru et al. (2019) [2] reported that all Salmonella species recovered were resistant to Penicillin and Erythromycin with 100 per cent sensitivity to Cefotaxime, Cephalothin, Colistin and Polymyxin B. In a study by Rahimi et al. (2011) [23], Salmonella showed the highest resistance against Nalidixic acid (47.40%) followed by Tetracycline (36.80%), Streptomycin (15.80%), Trimethoprim (15.80%) and Ciprofloxacin (5.30%).

The occurrence of antimicrobial resistance in Salmonella was probably an indication of their frequent usage both in livestock, fish and human. Studies conducted in different parts of India and other countries have also indicated that the increase in the proportion of drug-resistant Salmonella isolates could be due to the irrational use of antimicrobials and inappropriateness of the prescription and dispensing methods in both the veterinary and public health setups [32, 33]. The contamination of fishes with antimicrobial resistant Salmonella in the present study might have resulted from the runoff water contaminating the ponds, use of animal and poultry offal as feed, indiscriminate use of antibiotics, improper handling of fishes during catching, storage, transportation and retailing.

Conclusion
The detection of antimicrobial resistant S. Typhimurium in fresh water fishes from Mizoram indicated the probable public health hazard contributed by irrational use of antibiotics in animal, fish and human, improper fish production management and handling.

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