Prevalence of Multidrug Resistant *Salmonella* in Shrimp of Dhaka City

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ABSTRACT

*Salmonella* contamination in shrimp is a matter of concern nowadays in Bangladesh. The present study was aimed at investigating the occurrence of *Salmonella* and their antibiotic resistance pattern in shrimp from different markets in Dhaka city. A total of 105 shrimp samples were collected from randomly selected markets. *Salmonella* was detected by conventional methods. All *Salmonella* isolates were subjected to antimicrobial susceptibility testing and plasmid profiling. The prevalence of *Salmonella* was found very low (n = 6, 5.71%). After detailed biochemical and serological characterization, the isolates were identified as *Salmonella enterica* subsp. *Salamae* (A4), *Salmonella bongori* (G3), *Salmonella enterica* subsp *typhi* (J3), *Salmonella enterica* subsp. *enterica* (K4), *Salmonella enterica* subsp. *houtenae* (O3) and *Salmonella enterica* subsp. *arizonae* (T5). All isolates were susceptible to chloramphenicol, ciprofloxacin and doxycycline. The highest resistance was found to ampicillin (100%) followed by erythromycin (83.33%), gentamicin 33.33%), nitrofurantoin (16.67%) and nalidixic acid (16.67%). Plasmid profiles showed the presence of small plasmids of 3.4 to 1 Kbp in all isolates. The results suggest that shrimps consumed in Dhaka city may be contaminated with multi-drug resistant plasmid-harboring *Salmonella*.

Key words: Shrimp, *Salmonella*, antibiotic resistance, plasmid profiling

INTRODUCTION

*Salmonella* are considered to be major food borne pathogens and are responsible for food poisoning to animals and humans. Salmonellosis is the consequence of increased consumption of raw or slightly cooked as well as contaminated food, but human to-human transmission and direct animal-to-human transmission can also occur (Prost and Riemann, 1967). Although the majority of infections result in self-limited disease; however, in immune-compromised patients, neonates and elderly-infection requires antibiotic treatment (Mead et al., 1999; Van et al., 2007). Rapid use of antibiotics is one of the recent causes of emergence of drug resistant *Salmonella* strains. In veterinary practice, antibiotics are used in livestock production, disease prevention and as growth promoting feed additives (Swartz, 2002; Sun, 1984). The antibiotics, their metabolites and bacteria may get into water bodies from poultry farms (Singer and Hofacre, 2006). Aquatic environment becomes contaminated with antibiotic resistant bacteria from Integrated broiler chicken-fish farms (Petersen et al., 2002). The fatality rate due to antibiotic resistant *Salmonella* is twenty-one times greater than that of infection with non-antibiotic resistant *Salmonella* strains.
Aquatic environments act as the major sources of Salmonella transmission to hosts (Cherry et al., 1972). In the shrimp processing industry, the processed shrimps are usually contaminated by coastal water used for handling and processing of sea food (Iyer and Joseph, 1980).

Shrimp is becoming an important commodity in the global fishery trade because of its increasing demand and competitive price (Bhaskar et al., 1995). Antimicrobial drugs are frequently used to inhibit disease outbreaks in shrimp farming (Holmstrom et al., 2003). Rapid use of antibiotics in shrimp hatcheries may cause development of antibiotic resistant bugs that can infect both animal and human (Khachatourians, 1998; Willis, 2000). Plasmids are extra-chromosomal DNA found in bacteria and sometimes there is a correlation between plasmid and antibiotic resistance (Radu et al., 1998; Kagiko et al., 2001). Salmonella most often carry the resistance gene in their plasmid and spread drug resistance to another bacterial population through conjugation (Halawani and Shohayeb, 2008).

The present study aimed at isolation and characterization of different species of Salmonella from shrimp of Dhaka city and investigation of their resistance patterns against different antimicrobial drugs and study of their plasmid biology.

MATERIALS AND METHODS

Sample collection: A total of 105 samples were collected from seven local markets (15 samples from each) of Dhaka city namely Anandabazar Kacha Bazar, Farmgate Kacha Bazar, Hatirpur Kacha Bazar, Kazipara Kacha Bazar, New Market Kacha Bazar, Sheikhpara Kacha Bazar and Taltola Kacha Bazar. Samples were collected in sterile poly-bags, labeled and transferred to the laboratory for examination within 1-2 h. Salmonella detection was done by culture technique (Rall et al., 2005).

Enrichment of sample: Shrimp samples (25 g) were homogenized with 225 mL of Buffered Peptone Water (HIMEDIA, India) in a stomacher for one minute. The samples were pre-enriched in the same media for 18-20 h at 35°C. For selective enrichment, 1 mL portions from pre-enriched broth was transferred into two tubes containing 10 mL of Selenite-Cystine Broth (HIMEDIA, India) and Tetrathionate Broth (TT) (HIMEDIA, India) respectively, contents were mixed well and incubated at 35°C for 24 h.

Isolation: Selectively enriched samples from both the Selenite-Cystine Broth and Tetrathionate Broth were streaked onto Xylose Lysine Desoxycholate (XLD) Agar (HIMEDIA, India), Salmonella Shigella (SS) Agar (HIMEDIA, India) and Bismuth Sulfite Agar (BSA) (HIMEDIA, India). Inoculated dishes were incubated at 35°C for 24 h. After incubation, suspicious colonies were confirmed with biochemical tests including Triple Sugar Iron test, Lysine Iron Agar test and Urease test. The urease negative cultures were then serologically confirmed by slide agglutination test using polyvalent somatic (O) antisera kit (Remel Europe Ltd, UK) as described by Mandal et al. (2004). These urease negative and serologically confirmed isolates were transferred to nutrient agar slants for further analysis.

Biochemical characterization: Urease negative cultures were subjected to biochemical tests according to the procedures recommended in Bergey's Manual of Determinative Bacteriology, 8th edition. The shape and Gram reaction were microscopically observed using 18 hours old cultures
from agar slant. The biochemical tests used were IMViC (Indole, Methyl red, VP and Citrate) test, motility test, catalase test, oxidase test, fermentation of lactose, sucrose, arabinose, trehalose, xylose and mannose.

**Antimicrobial susceptibility test:** Antimicrobial susceptibility of *Salmonella* isolates were tested using agar disc diffusion assay as described by Clinical and Laboratory Standards Institute (NCCLS, 2000). Pure colonies of isolated Salmonellae were emulsified in normal saline and turbidity was matched with 0.5 McFarland turbidity standards. Antibiotics used were Ampicillin 25 μg, Amikacin 30 μg, Chloramphenicol 30 μg, Ciprofloxacin 5 μg, Doxycycline 30 μg, Erythromycin 15 μg, Gentamicin 30 μg, Nalidixic acid 30 μg, Nitrofurantoin 300 μg and Tetracycline 30 μg. Antibiotic discs were placed on Muller Hinton Agar (HiMEDIA, India) plates seeded with *Salmonella*. After incubation at 35°C for 24 h, zone size of inhibition on petri dishes was measured. Susceptible and resistant isolates were defined according to the criteria suggested by NCCLS (2000).

**Plasmid extraction and agarose gel electrophoresis of extracted plasmid:** Plasmids were extracted by using Modified alkaline lysis technique developed by Kado and Liu (1981). Nutrient broth (2 mL) was inoculated with one loop full culture and incubated at 37°C for overnight. From the inoculated broth, 1.5 mL of the culture was transferred in to a micro centrifuge tube and centrifuged at 12000 rpm for 10 min at room temperature. The supernatant was discarded and the pellet was re-suspended in 20 μL Kado-Buffer by vigorous shaking. Freshly prepared 100 μL lysis mixture solution was added to the suspension and mixed very gently by inverting the tubes until a homogenous mixture was obtained. The tubes were placed in to a water bath and incubated 30 min at 56°C. Just after incubation 100 μL phenol:chloroform:iso-amyl alcohol (25:24:1) solution was added and again mixed gently until a uniform white color was formed. Then the tubes were centrifuged at 13000 rpm for 15 min. The supernatant was then extracted very carefully. When the plasmid DNA extraction was completed, 15 μL plasmid DNAs and 2 μL blue juice (Invitrogen) were mixed and loaded on 0.8% agarose gel together with the Molecular weight size marker: Plasmid of *E. coli* V517 was used as ladder for the determination of plasmid size. The plasmid DNAs were run on 2 mm-thick vertical 0.8% agarose gel in 1X Tris Borate EDTA-buffer solution under 50 V/15 min followed by 100 V/2 h electrophoresis conditions. To view the plasmid pattern, agarose gel was stained with ethidium bromide solution (0.5 mg mL⁻¹) for 20 min and after washing with sterile water for 15 min, the stained DNA was visualized in a Gel Documentation System (Alpha Innotech).

**RESULTS AND DISCUSSION**

Some preventive measures against *Salmonella* spp have been implemented in Bangladesh. However, Salmonellosis is still a global challenge to public health. Results of this study showed that, a total of six different *Salmonella* strains (from 105 samples) isolated and identified from the seven (7) local markets of Dhaka city are a serious cause for concern since they are of public health significance. On the basis of their biochemical characteristics, these isolates were identified as *Salmonella enterica* subsp. *salaeman* (A4), *Salmonella bongori* (G3), *Salmonella enterica* subsp *typhi* (J3), *Salmonella enterica* subsp. *enterica* (K4), *Salmonella enterica* subsp. *houtenae* (O3) and *Salmonella enterica* subsp. *Arizonae* (T5). Unfortunately, these data concern only the capital of Bangladesh and reinforce the need for similar studies elsewhere in Dhaka. Several studies have
been done on prevalence of *Salmonella* in the tropics (Bhaskar *et al.*, 1998; Wan Norhana *et al.*, 2001; Jeyasekaran and Ayyappan, 2002) and the isolates prevalent in this study are similar to the studies where *Salmonella* have been reported from shrimp (Bhaskar *et al.*, 1998; Wan Norhana *et al.*, 2001; Murachman and Darius, 1991). Studies by Iyer and Varma (1990), Bhaskar *et al.* (1998) and Wan Norhana *et al.* (2001) emphasized that *Salmonella* is a natural micro flora of the shrimp culture practice.

In this study the size of zone of inhibition of every antibiotic disc was measured in millimeters. The zones of inhibition were compared with zone diameter interpretive standards from NCCLS, 2000 (Table 1). Among all the antibiotics tested, all the isolates were found resistant to ampicillin (100%) followed by erythromycin (83.33%), gentamycin (33.33%) and 16.67% isolates were resistant to amikacin, nitrofurantoin and nalidixic acid. All the isolates were sensitive to chloramphenicol, ciprofloxacin and doxycycline, while 66.67% of isolates were intermediate resistant to amikacin, followed by gentamycin (50%), nitrofurantoin (16.67%) and tetracycline (16.67%). Nalidixic acid and Tetracycline sensitivity were found in 83.33% of the isolates; Nitrofurantoin sensitivity in 66.67% and amikacin, erythromycin and gentamycin sensitivity in 16.67% of the isolates (Fig. 1). Several investigators have used antimicrobial susceptibility typing of *Salmonella* strains for epidemiological purposes. The isolates which were resistant to four or more separate classes of antimicrobial drugs were defined as multidrug resistant (Butaye *et al.*, 2006). The incidence of resistance (i.e., resistant to two drugs) and multidrug resistance (i.e., resistance to four or more drugs) of all *Salmonella* strains isolated is presented in Table 2.

In the present study, about all (83.33%) *Salmonella* strains isolated from shrimp showed resistance to erythromycin. This is in accord with the study done by Wan Norhana *et al.* (2001) where *S. enterica* serovar Weltevreden, *S. enterica* serovar Hvitvingfoss, *S. enterica* serovar Litchfield, *S. enterica* serovar Agona, *S. enterica* serovar Paratyphi, *S. enterica* serovar Benin and *S. enterica* serovar Java isolated from shrimp were resistant to erythromycin. However, *Salmonella enterica* subspp. *enterica* (K4) isolated from Kazipara kacha bazar showed multiple antibiotic

### Table 1: Antibiotic resistance patterns of *Salmonella* spp isolated from shrimp

<table>
<thead>
<tr>
<th><em>Salmonella</em> spp.</th>
<th>Amikacin</th>
<th>Ampicillin</th>
<th>Chloramphenicol</th>
<th>Ciprofloxacin</th>
<th>Doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em> subsp. <em>salamae</em> (A4)</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella bongori</em> (G3)</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>typhi</em> (G3)</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> (K4)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>houtenae</em> (G3)</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>arizonae</em> (T5)</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

### Table 2: Antibiotic resistance patterns of *Salmonella* spp isolated from shrimp

<table>
<thead>
<tr>
<th><em>Salmonella</em> spp.</th>
<th>Erythromycin</th>
<th>Gentamycin</th>
<th>Nalidixic acid</th>
<th>Nitrofurantoin</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em> subsp. <em>salamae</em> (A4)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella bongori</em> (G3)</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>typhi</em> (G3)</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> (K4)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>houtenae</em> (G3)</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. <em>arizonae</em> (T5)</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>I</td>
</tr>
</tbody>
</table>

S: Sensitive; I: Intermediate (%); R: Resistance (%)
resistances (four antibiotics) compared to other _Salmonella_ strains which were resistant to two antibiotics only. The occurrence of multiple antibiotic resistances could be due to the presence of chicken farm nearby that maybe using different types of antibiotics. Antibiotics are used in poultry as therapeutics as well as growth promoters. According to Singer and Hofacre (2003), antibiotics and their metabolites as well as bacteria can spread from poultry farms into waterways. In addition, poultry litter can also help in their dissemination onto open field. Petersen _et al_. (2002) have reported that integrated broiler chicken-fish farms contributed to antimicrobial-resistant bacteria in a pond environment. The antibiotic residues from the nearby chicken farm could have led to multiple antibiotic resistances observed in the present survey. Ampicillin is not commonly used in shrimp culture. In the present study, all the isolates were found resistant to Ampicillin. So, there is a possibility that these _Salmonella_ could have acquired resistance from other places. The other possibility of the presence of antibiotic resistant bacteria could be the use of probiotics. According to Mathur and Singh (2005), commensal bacteria may act as reservoirs of antibiotic resistant genes that can be transferred to pathogenic bacteria. Therefore, the use of probiotics in the shrimp hatchery from where shrimps were supplied to the studied markets may have led to the incidence of multidrug resistant bacteria. Several Investigators reported that resistance to different antimicrobial agents was mediated by a large plasmid. This plasmid was not found in the strains; however, this observation may not exclude an epidemiological relationship between all isolates because plasmids can be readily lost or acquired (Morosini _et al_.., 1995).
The isolated *Salmonella* showed different plasmid profile; the sizes of plasmid obtained ranged from 1.0 to 3.7 Kbp (Fig. 2). Table 2 shows the distribution of plasmids, nearly all the isolates harbored a plasmid of approximately 1.0 Kbp. From the plasmid profile of the identified isolates it is showed that large plasmid was not found which could mediate antibiotic resistances. All isolates were commonly resistant to Ampicillin. Among the antibiotics used, the isolates were resistant to 20-40% drugs.

CONCLUSION

The development of antimicrobial resistance in Gram negative bacteria (e.g., *Salmonella*) constitute a public health risk, as it may potentially affect the efficacy of drug treatment in humans (Angkititrakul *et al.*, 2005; Glynn *et al.*, 1998; Carraminana *et al.*, 2004; Nygard *et al.*, 2008; Wegener *et al.*, 1999; Abdellah *et al.*, 2009). Therefore, it is necessary to avoid abundant use of antimicrobial drugs in shrimp hatcheries as the resultant resistant strains can be passed to human through food products. These results indicate that presence of *Salmonella* resistances to antimicrobial drugs is common in shrimp. Further studies are needed in a greater population to identify the sources and causes of this drug resistance.

REFERENCES


