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Studies on *Salmonella* Isolates from Fishery Products

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Salmonella isolates obtained from fishery products were studied with respect to their sensitivity to antibiotics, ionizing radiation and chlorine water treatment. The optimum pH range for the growth of the isolates was determined. The isolates were also characterized for pathogenicity related genes based on PCR assay. None of the 34 isolates under study were resistant to 16 antibiotics tested. All isolates were sensitive to chlorine water treatment (25 ppm) when treated in saline, but they were resistant when treatment was in organic medium. Optimum growth of all isolates was observed in the pH range of 5 to 7; however, *S. typhimurium* and *S. worthington* could grow even at pH 4. The PCR based identification of pathogenicity related genes *invA* and *spvC* showed that both genes were present in *S. gallinarum* and *S. enteritidis* and only *invA* was present in *S. typhimurium* and *S. worthington*. D_{10} value for these serovars irradiated in brain heart infusion broth was in the range of 270 to 289 Gy.

Key words: Antibiotic resistance, chorine water, ionizing radiation, *Salmonella*, PCR.

Salmonella is an important bacterial pathogen associated with food borne illness in most of the countries of the world. They are widely reported to be present in a variety of foods including seafood. Nambiar and Iyer (1991) have reported prevalence of 16 different serotypes of *Salmonella* like *S. adelaide, S. barendrup, S. chingola, S. cerro, S. nchanga, S. oslo and S. mbandaka* in frozen shrimp and frozen fish samples from Kochi. In another study, 40% of squid samples and 28.5% of shrimp samples from Mumbai were found to be contaminated with *Salmonella* (Kamat *et al.*, 2003). *Salmonella* contamination was also reported from 42% samples of freshwater prawns and serotypes detected were *S. typhimurium* and *S. worthington* (Bandekar *et al.*, 2004). As in the case of marine fishery, aquaculture industry has also been facing several problems that include diseases in the farms due to bacterial as well as viral infections of fish and shellfish. In intensive aquaculture there is a problem of spread of infection due to overcrowding. To check infection, antibiotics are generally used. Indiscriminate use of antibiotics can have adverse consequences by promoting the selection and prevalence of antibiotic resistant strains (Threlfall, 2002). Among various *Salmonella* serovars, *S. typhimurium* is of particular concern because of the recent emergence of strain resistant to ampicillin, chloramphenicol, streptomycin and tetracycline designated as definitive type MR-DT104 (Vahaboglu, 2001). Angulo and Griffin (2000) have suggested that the resistance determinants of MR-DT104 might have emerged among bacteria in aquaculture and have been horizontally transferred to *S. typhimurium* DT104. The aquacultured products can also be source of various bacterial, viral and protozoan pathogens. The contamination can

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occur from various sources such as water, feed, pond, soil, bird droppings and other live forms of surrounding ecosystems (Hus *et al.*, 2000).

Chlorine treatment can not guarantee elimination of pathogens which may be present deep in the tissues. Majority of the *Salmonella* spp. are neutrophilic; however, a number of strains have been found which can not only survive but also grow in acidic conditions due to adoptive response that is inducible by incubation in acidic condition (Gorden & Small, 1993).

Reflecting a complex set of interaction with its host, *Salmonella* species require multiple genes for full virulence (Marcus, 2000). *InvA*, one of the genes present on the chromosome, is responsible for invasion of epithelial cells of intestine (Millis *et al.*, 1995). The *spv* operon present on plasmid appears to promote survival and rapid growth of *Salmonella* in host thereby increasing virulence (Guling, 1993A). All *Salmonella* serovars seem to contain *invA* gene (Chiu & Ou, 1996) and some contain virulence plasmid. Attempts were made to identify the presence of specific pathogen related genes by PCR (Cerro *et al.*, 2003).

Radiation processing can play a major role in ensuring security and safety of fish and fishery products by eliminating pathogenic microorganisms. Radiation processing of frozen seafood with 4 kGy dose can ensure elimination of various pathogens including *V. parahaemolyticus* and *Salmonella* (Nerkar & Bandekar, 1990). Therefore, radiation processing of seafood will help in ready acceptance of fishery products abroad, which otherwise are rejected due to the presence of food borne pathogens.

The present work was conducted to study *Salmonella* serovars obtained from fishery products. Effect of chlorine water, antibiotic susceptibility, radiation sensitivity and optimum pH for growth were studied. All strains were tested for the presence of pathogenicity related genes by PCR using primers for *inv*A and *spv*C.

Materials and methods

The isolation of *Salmonella* was carried out as described previously (Bandekar *et al*, 2004). In brief, samples were homogenized and pre-enriched in Lactose Broth (LB). Selective enrichment for *Salmonella* was carried out in Rappaport Vassiliadis (RV) and Tetrathionate Broth (TB). The enriched cultures were streaked on Xylose Lysine Deoxycholate Agar (XLDA), Hektoen Enteric Agar (HEA), and Bismuth Sulphite Agar (BSA). The presumptive positive colonies were tested further by a series of biochemical tests to confirm presence of *Salmonella*. The serology of these cultures to identify species was carried out at Salmonella & Escherichia Centre, Central Research Institute, Kasauli, HP 173205. Total 31 *Salmonella* isolates were obtained from 6 out of 18 samples (12 fresh water prawns and 6 cuttle fish) of fishery products screened. From 5 fresh water prawn samples, *S. typhimurium, S. worthington,* and from 1 sample of cuttle fish *S. enteritidis* and *S. gallinarum* were isolated. At least 3 isolates from each of the four serovars (*S. typhimurium, S. worthington, S. enteritidis* and *S. gallinarum*) representing all *Salmonella* positive samples were selected and 13 isolates were tested for antibiotic sensitivity. For chlorine sensitivity, effect of pH, radiation sensitivity and for PCR detection one representative of each serovars of *Salmonella* was used. *E. coli* ATCC 35218 and *S. typhimurium* MTCC 98 were used as standard cultures. Brain Heart Infusion Broth (BHI), Peptone Water, antibiotic discs and Plate Count Agar (PCA) were from Hi Media, India; Tryptic Soya Agar (TSA), Tryptic Soya Broth (TSB), Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) were from Difco, Becton Dickinson, USA; Taq polymerase Enzyme, dNTP and primers were from Bangalore Genei, India.

Chlorine water (25 ppm available chorine) was prepared in phosphate buffer (pH 7) by using commercially available sodium hypochlorite containing 32% chlorine. Test organisms were grown in BHI for 20 h at 37°C. Culture was diluted in saline as well as in BHI to give approximately 10^7cfu /ml and 1 ml of this was added to 9 ml of phosphate buffer with and without final concentration of 25 ppm chlorine and kept at melting ice temperature for 10 min. After the incubation, appropriate dilutions were plated on PCA and the colonies were counted after 48 h at 37°C.

S. typhimurium, S. worthington, S. gallinarum and S. enteritidis isolates were screened for antibiotic sensitivity using a panel of 16 antibiotics by Agar Disk Diffusion Method as described by Bauer *et al.*, (1996). This technique involved inoculating a single colony from TSA Plates into 10 ml of MHB, incubating the culture overnight at 37°C and preparing a 1:10 dilution of overnight culture in 0.1% peptone-water. Five colonies from each isolates were taken. A sterile swab was immersed in the diluted suspension and swabbed over the entire surface of a number of MHA plates. The plates were held at room temperature for 10 min, and antibiotic disc containing 16 different antibiotics were dispensed. The zone of inhibition was measured after 24 h at 37°C.The diameter of these zones were recorded to the nearest mm and classified as resistant (little or no inhibition of the growth) or susceptible (wide clear zone, complete inhibition of the growth). The type and concentrations of antibiotics in the concentration discs were as follows: Ampicillin, 10 µg; Ciprofloxacin, 5µg; Chloramphenicol, 10 and 30µg; Ceftriaxone, 30µg; Chlorotetracycline, 30µg Cephalothin, 30µg; Ticarcillin, 75µg; Enrofloxacin, 30µg; Kanamycin, 30µg; Nalidixic acid, 30µg; Ofloxacin, 2µg; Streptomycin, 10 and 30µg; Sulphamethizol, 300µg; Tetracycline, 10 and 30µg; Trimethoprim, 5, 10, 25 and 30µg and Oxytetracycline, 30µg.

0.1ml of twenty hour old cultures of different serovars (~10^9 cfu/ml) was inoculated into BHI tubes of pH range 3, 3.5, 4, 5, 7, 8.5 and 9. Tubes were incubated for 20 hr at 37°C and growth was measured in terms of optical density at 600 nm.

Salmonella serovars were inoculated in BHI, grown for twenty h at 37°C and the culture was diluted in BHI to obtain 10^7 cfu of *Salmonella* / ml. 1.5 ml of diluted culture was dispensed in microfuge tubes and irradiated in melting ice at 100, 200, 400 and 600 Gy doses in Gamma cell 220 (AECL, Canada, Dose Rate 11.5 Gy/min). Appropriate dilutions were made and plated on PCA to obtain surviving population. The colony forming units were counted after 24-48 h at 37°C. D_{10} value was calculated from the graph, as the dose required to reduce initial population of cells by one log cycle.

The culture was streaked on PCA plates and well isolated colony was selected and suspended in 100 µl of distilled water and boiled for 10 min. After a brief spin of 30 s, supernatant was taken as target. The primers for *Salmonella* specific genes, *invA* and *spvC*, were synthesized and PCR was performed as described by Cheng *et al.* (1996). The PCR mixture consisted of 2 µl of 10 x PCR amplification buffer containing 1.5mM $MgCl_2$: 200µM (each) of the four deoxyribonucleoside triphospates, 1 µM each of primer pairs, 1.25 units of Taq polymerase; total volume was made up to 25 µl with double –distilled water. The mixture was subjected to 30 PCR cycles in Eppendorf Master Cycler Gradient. The parameters for amplification were as follows: 1 min at 94° C to denature target DNA prior to the first cycle of PCR, denaturation for 30 s at 94° C, annealing of primers for 30 s at 56° C, and primer extension for 2 min at 72° C. The PCR products were electrophoresed in 2% agarose gel.

Results and Discussion

Treatment with 25 ppm chlorine resulted in elimination of all *Salmonella* cells

when treatment was carried out in saline. The controls were kept without the addition of chlorine wherein there was no change in cell number after 10 min of incubation (Data not shown). Therefore, effect observed was due to chorine treatment. However, only one log cycle kill was observed when chlorine treatment was given in BHI medium (Table 1). This could be due to the reaction of chlorine with organic matter present in the medium. Fish is known to be rich in organic matter that can neutralize chlorine and hence chlorine wash may not be an effective disinfectant treatment. In fish processing industry, all processing is carried out at melting ice temperature (0-4^{0}C); therefore, the cultures were treated with chlorine water at melting ice temperature. In control cells, maintained under same conditions at 0-4^{0}C without chlorine, no change in cell number was observed These results are consistent with previous report by Andrews *et al.* (2002) who demonstrated that treatment with chlorine wash reduced aerobic bacterial numbers by only 1-2 log cycle in shrimp when chlorine level were >20 ppm. General practice in fish processing industry is to use 25 ppm chlorine water for washing of all the equipments and surfaces; whereas a concentration of 5 ppm chlorine water is used for washing fish.

Table 1. Effect of chlorine water treatment on *Salmonella* isolates

Medium	Organism	Control Log cfu/ml	Chlorine water treatment (25ppm)
Culture in saline	*S. typhimurium*	7.9	ND
	S. worthington	7.1	ND
	S. enteritidis	7.2	ND
	S. gallinarum	7.1	ND
Culture in BHI	*S. typhimurium*	7.8	6.4
	S. worthington	7.1	6.1
	S. enteritidis	7.2	6.2
	S. gallinarum	7.0	6.4

Log values are mean of three sets of independent experiments. (n=6, p <0.001)
ND- Not detected

However, in spite of chlorine water treatment, bacterial pathogens were found to be present in 42 % of the processed fishery products. (Bandekar et al., 2004)

Our studies showed that none of the isolates obtained from aquacultured fishery products were resistant to tested antibiotics. Organisms were sensitive even to lower concentrations of chloramphenicol (l0µg), tetracycline (10µg), trimethoprim (5µg) and streptomycin (10µg) (Table 2). The survival of these strains in freshwater fish and shellfish could be due to cutting down of antibiotic use in aquaculture due to threat of ban on seafood with antibiotic trace from importing countries (Anon, 2003). Also, it is possible that the contamination might have occurred during processing. Ministry of Commerce and Industry (MOCI), Government of India has issued a notification on maximum residue limits of antibiotics and heavy metals in marine products for export. (Notification SO 792 (E) dated August 17, 2001). According to the notification five antibiotics viz., chloramphenicol, furazolidone, neomycin, nalidixic acid and sulphamethoxazole are banned and no residue should be present in animal body. From India, *S. paratyphi A* resistant to chloramphenicol and cotrimoxazole (Chandal *et al.*, 2000) and *S. typhi* resistant to chloramphenicol, ampicillin, sulphamethaxazole and trimithoprim (Sudarsana *et al.*, 1992) have been reported from clinical isolates. There are many reports about emergence of antibiotic resistant strains due to indiscriminate use of antibiotics (Duffy *et al.*, 1999; Randall *et al.*, 2001). In intensive aquaculture large amount of antibiotics are generally used. As per the news report issued by Institute Of Agriculture And Trade Policy an estimated 204,000 to 433,000 pounds of antibiotics are used annually in the production of seafood sold in US, this includes antibiotics from the same classes that doctors depend on treating sick humans (Bendrook, 2002).

Table 2. Antibiotic sensitivity of *Salmonella* serovars.

Antibiotic	Symbol	Concentration µg /disc	Zone of inhibition (mm) Mean+S.D *S. typhimurium*	*S. worthington*	*S. gallinarium*	*S. entritidis*
Cephalothin	Ch	30	25.6+1.5	22.4+1.5	21.4+1.1	20.2+0.4
Nalidixic acid	Na	30	25.0+2.2	23.8+1.3	22.4+1.8	22.0+1.0
Tetracycline	T	30	23.3+1.7	20.6+0.8	22.0+2.0	20.6+1.6
Ampicilllin	A	10	21.8+1.4	20.0+1.5	19.4+1.3	18.2+0.8
Ciprofloxacin	Cf	5	38.8+0.8	36.0+0.7	33.2+1.9	31.2+1.4
Chloramphenicol	C	30	31.0+0.7	29.2+1.1	29.2+2.5	28.0+1.0
Chloramphenicol	C	10	25.4+0.5	23.6+1.1	24.4+2.4	23.8+1.1
Ceftriaxone	Ci	30	30.4+0.5	30.2+0.4	30.2+2.4	29.6+0.8
Sulphamethizole	Sm	300	21.0+3.5	25.4+0.7	26.0+2.3	24.8+1.9
Streptomycin	S	25	23.4+1.1	21.8+0.8	20.4+0.5	25.4+0.8
Streptomycin	S	10	18.4+2.0	20.0+0.7	18.0+1.0	18+0.7.0
Enrofloxacin	Ex	10	34.6+0.5	32.8+1.3	31.2+1.4	30.4+0.5
Chlorotetracycline	Ct	30	22.8+1.6	20.6+0.5	21.6+1.9	21.2+0.8
Ticarcilin	Ti	75	24.0+2.7	23.8+1.1	23.6+1.8	21.4+1.1
Kanamycin	K	30	25.2+0.8	21.6+0.5	23.6+1.8	21.4+2.6
Oxytetracycline	O	30	18.2+1.7	17.6+0.5	19.0+1.4	18.0+1.2
Ofloxacin	Of	2	31.2+1.8	32.2+0.8	31.6+2.0	29.0+1.7
Trimethoprim	Tr	30	32.4+0.5	33.0+0.5	31.8+2.0	32.2+1.3
Trimethoprim	Tr	25	31.2+0.4	30.4+1.7	30.2+1.7	30.4+0.5
Trimethoprim	Tr	10	27.0 +1.2	29.8 +1.6	25.0 +2.7	25.6 +1.1
Trimethoprim	Tr	5	25.0+ 0.5	22.4+ 0.5	24.2+ 1.3	22.4+ 1.1

All serovars grew in pH range of 5 to 8.5. However, the optimum pH for these organisms is in the range of pH 7-8.5. But *S. typhimurium* and *S. worthington* also grew at acidic pH (Fig. 1). Several large outbreaks of food borne illness have been traced back to bacterial contamination of animal feed (Crump *et al.*, 2002). Fish processing wastes as well as products prepared from fish processing wastes such as fish silage and fish meals are very often used as ingredients of aqua-feed and animal feed. Fish silage is prepared by ensilation process by lowering the pH and fermenting fish processing waste. Low pH can prevent the bacterial growth or eliminate certain bacteria but at times there is a possibility of survival of acid adapted bacterial pathogens (Haapapuro *et al.*, 1997). Acid adapted cells were found to have increased tolerance to various stresses including heat, salt and organic acid (Tosun and Gonul, 2003). In this context, presence of *S. typhimurium* and *S. worthington* able to grow at low pH is significant indicating that some further processing is required to

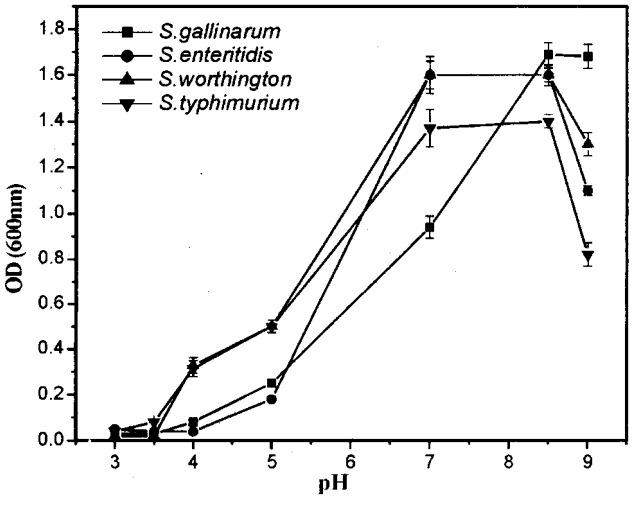

Fig. 1. pH optima for *Salmonella* isolates. (Values are average of three independent experiments: n=6.)

eliminate the pathogens, especially when fish silage is used as one of the ingredients in the feed.

The PCR analysis showed that *invA* gene, a gene shown to be involved in invasion of host cells during *Salmonella* infection, was present in all four serovars of *Salmonella*. However, *spvC* gene was found to be present only in *S. gallinarum* and *S. enteritidis* (Fig. 2). Both these serovars were isolated from cuttle fish. *Salmonella* plasmid virulence (*spv*) genes are shown to be important for enhancement of the virulence of *Salmonella* strains leading to systemic infection in animal models (Libby *et al.*,2002). Presence or absence of virulence plasmid does not influence the pathogenicity of the bacteria but presence of plasmid increases growth rate of the *Salmonella* within host cells (Guling, 1993A). However, Saxena *et al* (2004) have shown a positive correlation between virulence of the organism and the presence of plasmid and amplification of *Vir* gene. Five *Salmonella* serovars are known to carry virulence plasmid *viz S. typhimurium, S. cholerae-suis, S. dublin, S. enteritidis and S. gallinarum-pullorum* (Guling, 1993B).

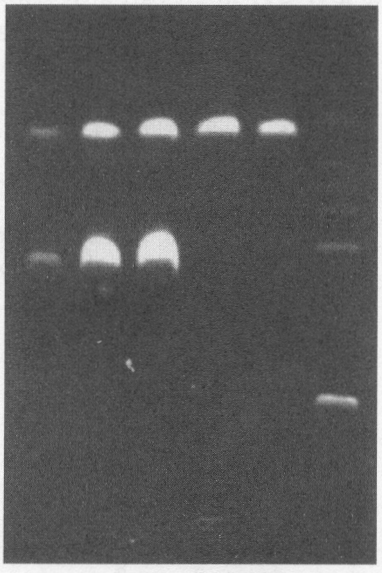

Fig. 2. Identification of pathogenicity related genes, *invA, spvC*. Lane 1-6. Marker, *S. typhimurium, S. worthington, S. gallinarum, S. enteritidis, S. typhimurium MTCC98*

In the present study, *S. typhimurium* isolate did not show presence of *spvC* gene which is in agreement with the findings of Guiney (1995) who observed that all *S. typhimurium* strains do not show presence of virulence plasmid.

D_{10} of four isolates, *S. typhimurium, S. worthington, S. enteritidis and S. gallinarum,* irradiated in BHI were found to be 276, 289, 284 and 270 Gy, respectively (Fig. 3). Among the isolates, *S. gallinarum* was the most sensitive to radiation. None of the isolates showed high radiation resistance. *S. typhimurium* and *S. enteritidis* were reported to have D_{10} values of 225 to 250 Gy when the cells were irradiated in phosphate buffer and 300 and 450 Gy in shrimp homogenate. Dose of 4 kGy completely eliminated *Salmonella* from frozen pre-packed shrimp (Nerkar and Bandekar, 1990). Even low dose of 1 to 2 kGy can assure microbial safety for food (Venugopal *et al.*, 1999). Present studies confirm that treatment with low dose of gamma radiation can eliminate *Salmonella* from fishery products. Due to diverse nature of fishery production, processing, and trade, there is always a possibility of contamination. *Salmonella* is considered as a zero tolerance pathogen since every known strain of this bacterium is shown to be pathogenic

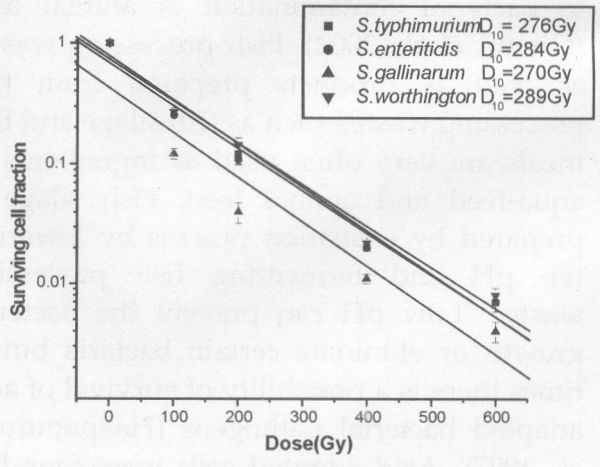

Fig. 3. Radiation sensitivity of *Salmonella* isolates. (Values are average of three independent experiments; n=6)

to humans. Therefore, any food lot found to be positive for Salmonella is rejected. Radiation processing can effectively eliminate this pathogen.

The present results indicate that processing methods like radiation treatment may be adopted instead of chlorine water treatment for complete elimination of pathogenic organisms from fishery products. Even though *Salmonella* isolates were sensitive to antibiotics more isolates need to be tested for any emergence of antibiotic resistant strains. Organisms that can grow in acidic range need to be taken into account for fish waste utilization. It is the first report of the surveillance of pathogenicity related genes both on chromosome and plasmid in *Salmonella* population from Indian subcontinent. The surveillance of incidence of pathogenicity related genes is of great importance because it will help in understanding of the new virulence genes and their distribution in the population.

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