SUMMARY & CONCLUSION
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_Salmonella_ serovars are important food borne pathogens and often isolated from seafood worldwide. The incidences of _Salmonella_ in seafood have been reported in India and abroad. Present study was mainly focused on _Salmonella_ serovars in seafood of Cochin. This investigation consisted of three main parts. The first part deals with the prevalence and distribution of _Salmonella_ serovars in seafood. The second part deals with biochemical and molecular characterization of _Salmonella_ serovars isolated from seafood and the final part covered the development of rapid and sensitive molecular methods for detection of _Salmonella_ from seafood. The important findings of this study are summarized as follows.

5.1 Prevalence of _Salmonella_ in seafood

A total of 443 seafood samples consisting of pelagic fish (n=79), demersal fish (n=52), shrimp (n=86), lobster (n=25), crab (n=38), clam (n=41), mussel (n=31), oyster (n=27), squid (n=23), cuttlefish (n=21), and octopus (n=20) samples from the fish markets and landing centres of Cochin over a period of 4 years, from 2003 to 2007 were analyzed for presence of _Salmonella_. Isolation and identification of _Salmonella_ from seafoods was carried out by BAM, USFDA and ISO methods. The prevalence of _Salmonella_ was maximum in clams (34.1%) followed by mussel (31%), fish (30.2%) and shrimps (29.0%) samples. These values were higher, compared to prevalence of _Salmonella_ in crab (10.5%), oyster (14.8%), squid (17.3%), and octopus (15.0 %) samples and the lowest incidence of _Salmonella_ was
noted in lobster samples (8.0%). The results further demonstrated that of a total of 443 seafood samples analyzed, an overall of 24.3% seafood were contaminated with *Salmonella*. The study also showed that lactose broth was comparatively superior for the isolation of *Salmonella* from seafood, compared to BPW. A comparison of different selective media indicated the Rappaport Vassiliadis (RV) and xylose lysine desoxycholate (XLD) agars were the most efficient media in the recovery of *Salmonella* in seafood.

### 5.2 Identification of *Salmonella* serovars from seafood

serovars prevalent in seafood and also pointing out a need for more robust serotyping facility in the country as some of the isolates could not be serotyped in India.

5.3 Biotyping of *Salmonella* based on utilization of sugars and amino acids

Bio-typing of *Salmonella* isolates was done based on Bergey’s manual of systematic Bacteriology. Ten most predominant *Salmonella* serovars such as *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Bareilly, *Salmonella* Braenderup, *Salmonella* Lindenburg, *Salmonella* Mbandaka, *Salmonella* Ohio, *Salmonella* Irumu isolated from seafood were biotyped based on utilization of different sugars. A total of 12 sugars viz., dulcitol, glucose, lactose, maltose, mannose, mannitol, sucrose, cellobiose, arabinose, raffinose, trehalose, and xylose were used in this study to determine the sugar utilization pattern. All *Salmonella* serovars formed biotype S1 pattern, based on utilization of arabinose, dulcitol, glucose, maltose, mannose, raffinose, trehalose, and xylose sugars. The results further showed that none of the serovars utilized cellobiose, lactose, and sucrose. Utilization of other carbon sources such as inositol, salicin, sorbitol, citrate, and tartrate were found to be variable for different serovars.

The ten most prevalent *Salmonella* serovars isolated from seafood were characterized based on utilization of different amino acids viz., arginine, lysine, ornithine, valine, and phenylalanine. Results revealed the presence of four amino acid biotypes (A1, A2, A3, and A4) for *Salmonella* Weltevreden, *Salmonella* Rissen, *Salmonella* Typhimurium, *Salmonella* Bareilly, *Salmonella* Lindenburg, *Salmonella*
Mbandaka *Salmonella* Irumu, and 2 biotypes (A1 and A3) were obtained in *Salmonella* Derby and *Salmonella* Braenderup strains.

### 5.4 Antibiotics resistance in *Salmonella* serovars

All *Salmonella* serovars were assayed for antibiotic susceptibility by disc diffusion assay on Muller Hinton agar. The isolates were tested against all major commercial antibiotics viz., sulphonamides, quinolones, beta-lactams, cephalosporins, tetracyclines, aminoglycosides, macrolides, and chloramphenicol. Results showed that all *Salmonella* serovars were 100% resistant to erythromycin. But, antibiotic resistance was not observed against ampicillin, ciprofloxacin, chloramphenicol, gentamicin, and kanamycin. *Salmonella* Lindenburg, *Salmonella* Rissen, *Salmonella* Takoradi and *Salmonella* Typhi isolates were resistant towards nalidixic acid. Sixteen out of 29 *Salmonella* serovars were resistant against sulphamethizol. Present study also determined the multi-drug resistance (MDR) in *Salmonella* serovars of seafood origin and results highlighted MDR in 49.3%, 31.8%, 10%, 0.4% of *Salmonella* isolates towards erythromycin and sulphamethizol (2 drug), erythromycin, sulphamethizol and carbenicillin (3 drug), erythromycin, sulphamethizol, carbenicillin and oxytetracycline (4 drug), erythromycin, sulphamethizol, carbenicillin, oxytetracycline and nalidixic acid (5 drug), respectively.

### 5.5 Plasmid profiling of *Salmonella* isolates

and large plasmids by Alkaline lysis (Mini preparation) and Kado and Liu (1981) methods. Plasmids were isolated from *Salmonella* serovars such as *Salmonella Typhimurium*, *Salmonella Derby*, *Salmonella Braenderup*, *Salmonella Lindenburg*, and *Salmonella Mbandaka* isolates. Large Megadalton plasmids were isolated in *Salmonella Typhimurium* and *Salmonella Derby* isolates. A total of nine plasmid profiles were obtained from different *Salmonella* serovars associated with seafood. *Salmonella* serovars such as *Salmonella Weltevreden*, *Salmonella Rissen*, *Salmonella Barielly*, *Salmonella Irumu*, *Salmonella Ohio*, *Salmonella Oslo*, and *Salmonella Typhi* did not harbour plasmids. Serovars without plasmid were placed under profile I. *Salmonella Typhimurium* showed 3 plasmid profiles (I, Ila, and IIIa) and harboured both small and large plasmids. *Salmonella Derby* and *Salmonella Braenderup* exhibited 3 plasmids profiles, however, each serovar harboured different plasmids of different sizes. *Salmonella Braenderup* harboured five plasmids of 1.5, 2.1, 3.5, 3.8, 4.1, and 9 kb in sizes. Plasmid profile of *Salmonella Lindenburg* isolates was observed to be most diverse in nature as five different plasmid profiles (I, II b, III b, III c, and IV d) were detected and plasmid profile IV d was detected in *Salmonella Mbandaka* isolates.

**5.6 PCR-ribotyping of *Salmonella* serovars**

Four most predominant *Salmonella* serovars viz., *Salmonella Weltevreden* (n = 22), *Salmonella Rissen* (n = 21), *Salmonella Typhimurium* (n = 18) and *Salmonella Derby* (n = 17) isolated from seafood were fingerprinted based on PCR-ribotyping pattern. *Salmonella Weltevreden* and *Salmonella Rissen* isolates exhibited three to four band patterns ranging from 700 to 1000 bp in both serovars, whereas, 700 to 900 bp ribotype patterns were observed in *Salmonella*
Typhimurium and *Salmonella* Derby. There were three ribotypes profile in *Salmonella* Rissen, and four major ribotype patterns were observed in *Salmonella* Derby and *Salmonella* Weltevreden strains.

### 5.7 ERIC-PCR assay for *Salmonella* serovars

*Salmonella* Weltevreden (n = 22), *Salmonella* Rissen (n = 21, *Salmonella* Typhimurium (n = 18) and *Salmonella* Derby (n = 17) isolated from seafood were molecular typed based on ERIC-PCR assay. DNA fingerprinting pattern of ERIC-PCR was analyzed with the Gel Compar II, Applied Maths BVBA, Belgium. UPGMA cluster analysis of ERIC-PCR profile in *Salmonella* Rissen and *Salmonella* Weltevreden showed the clonal variation with in the serovars. The level of similarity used for defining a type was set a 95%. The minimum Dice coefficient value for ERIC-PCR was observed at 32.87 % and 36.21 % for *Salmonella* Weltevreden and *Salmonella* Rissen, respectively, whereas, 44.5 and 47.5 % Dice coefficient values were obtained for *Salmonella* Typhimurium and *Salmonella* Derby, respectively. Sixteen different banding profile was observed for *Salmonella* Rissen, and six isolates (SR361, SR362), (SR429, SR520), and (SR415, SR428) showed similar homology (100%) with in the pair.

### 5.8 Discrimination indices of different typing methods

The discriminatory power of the fingerprinting methods was calculated using Simpson’s index of diversity and expressed as the index of discrimination (Hunter and Gaston, 1988). Based on three different PCR-ribotype patterns the discrimination index of PCR-ribotypes for *Salmonella* Rissen was observed at 0.668, whereas, ERIC-PCR discrimination was attained at 0.969. The combined (PCR-ribotype & ERIC-PCR) index was reached at 0.974. Similarly, lower discrimination
index (0.680) was observed for *Salmonella* Weltevreden by PCR-ribotyping and combined index was recorded at 0.988. The combined discrimination indices obtained for *Salmonella* Typhimurium and *Salmonella* Derby by different typing methods was at 0.974 and 0.905, respectively.

**5.9 PFGE analysis of *Salmonella* Weltevreden and *Salmonella* Typhi isolates**

Different strains of *Salmonella* Weltevreden and *Salmonella* Typhi isolated from seafood were analyzed based on the pulsed field gel electrophoresis (PFGE) profile to ascertain the genetic relatedness among different isolates of *Salmonella* Weltevreden and *Salmonella* Typhi. PFGE analysis of *Salmonella* Weltevreden strains exhibited four main restriction patterns (X1, X2, X3 and X4) for *Xba*I restriction enzyme. *Salmonella* Weltevreden showed 14 to 18 restriction fragments whereas, *Salmonella* Typhi showed 12 to 14 DNA fragments. PFGE pulsotype of *Salmonella* Typhi revealed the two restriction patterns with *Xba*I and profile X2 was identified in 5/7 isolates. PFGE analysis demonstrated the intra serovar strain variation, hence, highlighted the multiple clones of the test isolates present in seafood.

**5.10 Characterization of virulence genes in *Salmonella* serovars**

Takoradi, *Salmonella* Virchow, *Salmonella* Washington, *Salmonella* Weltevreden, *Salmonella* Worthington, *Salmonella* II (2 serovars), *Salmonella* IIIa, *Salmonella* IIIb, and *Salmonella* VI were *Salmonella* VI from seafood harboured three targeted virulence genes (*invA*, *stn* and *fimA* gene) and produced desirable 284 bp, 260 bp and 85 bp gene amplicons, respectively. Exceptions were also observed, *Salmonella* arizonae (IIIa) strains did not exhibit the presence of *fimA* gene and weak *invA* gene was observed in *Salmonella* Emek and *Salmonella* Lindenburg isolates.

### 5.11 Development of an eight hour PCR method for detection of *Salmonella* in seafood

Detection of *Salmonella* serovars from seafood was carried out with a different enrichment period, viz. 0, 2, 4, 6, and 8 h prior to PCR assay. All seafood samples were negative for *Salmonella* by PCR at zero hour. After 2 h enrichment, shrimp, mussel and edible oyster samples were positive for *Salmonella* and, an overall 5 % of seafood were found to be positive for *Salmonella*. With increase in enrichment periods to seawoods to 4, 6, and 8 h, showed improvement in detection rate as 14, 28, and 34 % respectively. The eight hour PCR exhibited 37/110 seafood samples positive for *Salmonella*, while, 27/110 seafood samples were positive for *Salmonella* by USFDA culture method. The results revealed that the newly developed 8 h PCR method was more sensitive than the culture method.

### 5.12 Comparison of culture, ELISA and PCR methods

A total of 215 seafood samples from different fish market and landing centers of Cochin were analyzed for the presence of *Salmonella* by culture (USFDA), ELISA and PCR methods. Results from the three assays were statistically analysed using software package SPSS 12.0 for Windows (SPSS Inc., Chicago,
USA). These three methods were considered as raters and the kappa coefficient was calculated to test the agreement. Based on kappa coefficient, the results were interpreted, as having fair agreement (0.21 to 0.40), moderate agreement (0.41-0.60), substantial agreement (0.61-0.80) and perfect agreement (0.81 to 1.0) between the raters. The comparison of different detection methods such as culture, ELISA and PCR showed that PCR was most sensitive for detection of *Salmonella* in seafood.

### 5.13 Development of the real-time assay for quantitative detection of *Salmonella* in seafood

Real-time PCR assay for was developed for the quantitative detection of *Salmonella* in seafood. Assay was first developed for DNA from pure *Salmonella* culture and seeded fish and shrimp samples. The quantitative detection of *Salmonella* in naturally contaminated shrimp and fish samples was carried out. The minimum detection sensitivity was 0.005 pg of the pure DNA in a PCR reaction, which corresponds to the genome of one *Salmonella* cell. The linear range of detection spanned from 7 log cycles of pure DNA ranging from 0.5 μg to 0.005 pg and further dilution did not provide any specific product. The minimum detection level in spiked seafood was 20 cfu/g. This method could quantify the level of *Salmonella* load in the naturally contaminated samples and observed that the natural contamination level of 0.25 to 9000 pg of *Salmonella* genome/g in seafood samples. The results obtained from the real-time PCR highlighted the presence of *Salmonella* cells <100 cfu/g of seafood samples. This assay would be ideal for rapid enumeration of *Salmonella* in seafood.
Suggestions for further research

1. Molecular source tracking of *Salmonella* serovars in seafood to identify the origin of *Salmonella* contamination in seafood.

2. Determine the prevalence of inherent *Salmonella* serovars in marine animals.

3. Expression studies of the *Salmonella* virulence genes to determine the pathogenicity levels in different *Salmonella* serovars.