Mangrove Ecosystems: An Adopted Habitat for Pathogenic *Salmonella* spp.

Krupali V. Poharkar[1], Savita Kerkar[1*], Dilecta D'Costa[2], Swapnil Doijad[2] and S.B. Barbuddhe[2]

ABSTRACT: Mangroves are affected by industrial and anthropogenic factors. Although mangroves have been widely studied, investigations of pathogens that may affect public health significance are largely lacking even while incidences of diseases linked with the consumption of mangrove-associated food have increased. A total of 150 samples of water, sediment, and biota were collected from ten mangrove ecosystems in Goa, India. Total viable counts of pathogens such as *E. coli, Listeria, Salmonella*, and *Vibrio* spp. ranged from 1.25 to 3.9 $\times$ 10^3 cfu/ mL, which were above the relevant standards. *Salmonella* counts were the highest at 3.1 to 3.9 $\times$ 10^3cfu/mL, with a prevalence of 40%. Considering its high prevalence, the virulence of *Salmonella* spp. was studied. The *invA* gene was detected in 35% of the *Salmonella* isolates by polymerase chain reaction (PCR). The findings suggested that pathogens adapt to this habitat, resulting in contamination of the indigenous fauna. *Water Environ. Res.*, **88,** 264 (2016).

KEYWORDS: bacteria, bacterial diseases, mangrove ecosystems, pathogens, waste disposal, water analysis.

doi:10.2175/106143016X14504669767733

Introduction

Mangroves are complex, unique, and highly productive ecosystems typically found in tropical and subtropical intertidal regions of the world. Mangroves ecosystems are biologically important (Singh, 2012) and provide food, shelter, and nursery areas for fish, birds, crustaceans, and other marine life. Worldwide data show that the fish catch from mangrove ecosystems contributed 10 to 67% of total fish catch and up to 100% of shrimp catch (Walter et al., 2014). The mangrove ecosystem forms a bridge between terrestrial and marine ecosystems and harbors unique microbial diversity (Brown et al., 1997; Sahoo and Dhal, 2008). These ecosystems are highly affected by anthropogenic stressors such as unsustainable fishing practices, habitat loss, and eutrophication (Duke et al., 2007). Because of rapid industrialization and wastewater discharge, mangroves are receiving an ever-increasing load of various biological and chemical contaminants (Bordalo et al., 2002; Hood et al., 1982). Many pathogens, such as *Escherichia coli, Listeria monocytogenes, Salmonella* spp., and *Vibrio* spp., have been reported to cause contamination in mangrove-originated food because of wastewater and other human activities (Hatha et al., 2004; Ukwade, 1990). Humans are consuming the affected biota, thus increasing the spread of these pathogens.

Because *Salmonella* contamination of seafood is common, it is a public health issue of concern (Grisi and Lira, 2010; Upadhyay et al., 2010). In most humans, salmonellosis typically occurs from consumption of contaminated food or water (EUFIC, 2006). *Salmonella* causes a range of serious human diseases such as enteric fever, headache, gastroenteritis, diarrhea (sometimes bloody), and bacteremia (Foley et al., 2007; Lotfy et al., 2011). The source of *Salmonella* in the environment increasingly is being investigated as a potentially significant reservoir of *Salmonella* transmission (Schutze et al., 1999)**.** Several domestic and wild animals can serve as potential carriers of *Salmonella*. With dispersal of such animal excreta in environment, this bacterium is spread (Stevens et al., 2009). Such fecal wastes eventually get mixed with the water bodies, resulting in contamination of the ecosystem and the food chain (Thong et al., 2002). Handling these carrier animals also has been responsible for the transmission of *Salmonella* (Lal et al., 2012). Exposure to wastewater-related pathogens in marine and estuarine environments typically occurs through incidental ingestion of contaminated water during recreational or commercial activities, such as fishing, swimming, and boating and through consumption of raw or partially cooked food caught or harvested in contaminated water (Brands et al., 2005; Martinez-Urtaza et al., 2004; Schutze et al., 1999). It has been suggested that contaminated soils, sediments, water, and wildlife may play a significant role in the transmission of *Salmonella* spp. to humans (Haddock et al., 1993; Schutze et al., 1998). Cruise ships often directly discharge untreated wastewater in the mangrove environment, thereby introducing pathogens such as *Salmonella* directly into these ecosystems. *Salmonella* is a facultative intracellular pathogen, and the *invA* gene is conserved among *S. enterica* isolates (Murugkar et al., 2003). It has been suggested that the *invA* gene is the predominant virulence gene necessary for the expression of virulence in the host.

In Goa, tourism boat cruises, casinos, trawlers, and iron-ore barges travel through these estuarine waters, affecting the mangroves. Once *Salmonella* gains entry, it can survive over long periods of months or even years after it reaches the soil and aquatic environment (Winfield and Groisman, 2003). Comparative studies have shown that persistence and dissemination of *Salmonella* are analogous in saltwater and freshwater fish (FAO, 2010).

Recently, several incidences have been reported in relation to *Salmonella* and seafood. In Mangalore, India, an outbreak of food poisoning affected 34 people after eating fish contaminated by *S. wertevreden* (Antony et al., 2009). The tuna exported from

[1] Department of Biotechnology, Goa University, Taleigao Plateau, Goa 403 206 India

[2] ICAR Research Complex for Goa, Old Goa 403 402 India

[*] Department of Biotechnology, Goa University, Taleigao Plateau-Goa, 403 206 India; e-mail: savitakerkar@gmail.com.

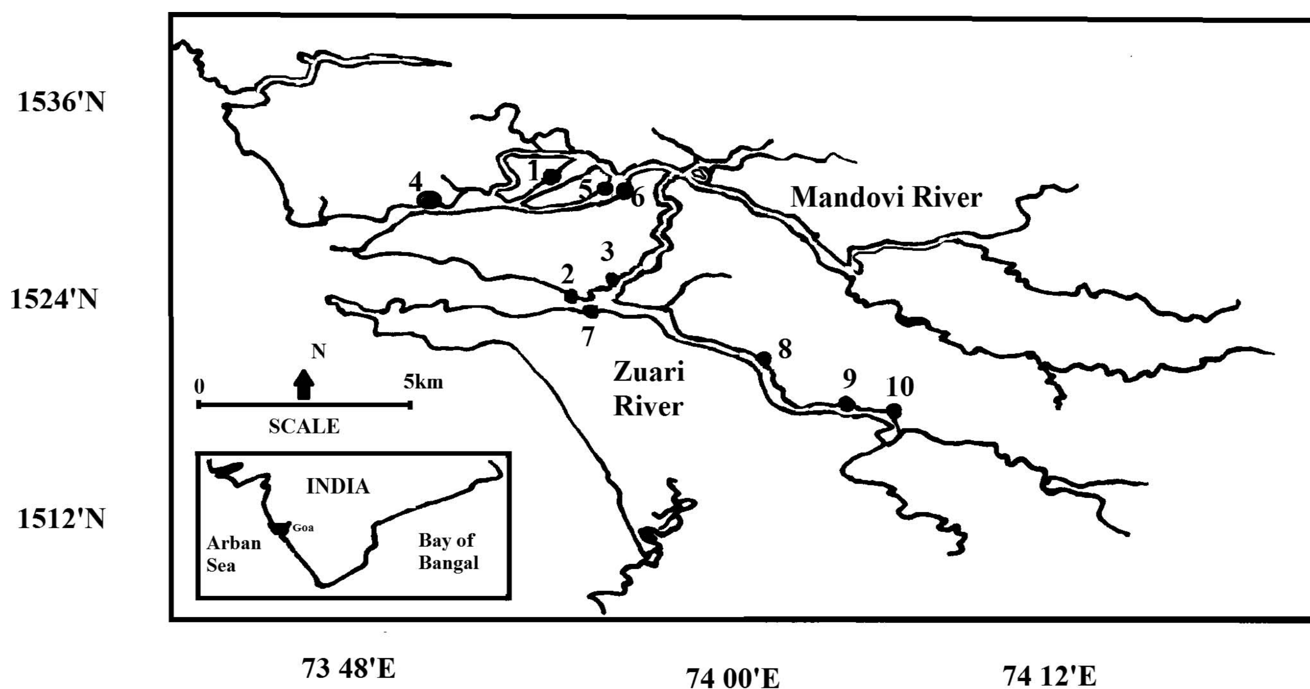

Figure 1—Map of the study area showing 10 sampling sites of mangrove areas.

India were attributed to more than 250 illnesses in 24 states and the District of Columbia (FDA, 2012). The U.S. waterborne salmonellosis case load has been estimated at 1.2 billion cases per year (CDC, 2012). In the last three years, more than 10 *Salmonella* outbreaks have been reported annually from seafood consumption (CDC, 2012).

Many people depend on food from mangrove ecosystems; however, this ecosystem has not been investigated for the occurrence of foodborne pathogens. The state of Goa has 2000 ha of mangroves. Of these, 700 ha of mangroves are found along the Mandovi estuary, 900 ha along Zuari estuary, and 200 ha along the Cumbarjua canal (ENVIS, 2012). The Goa marine fishery yields 85 000 to 90 000 tons per year, of which approximately 37 000 tons is exported. Approximately 200 species of marine and estuarine fish, 60 species of crabs, and a dozen species of oysters, clams, bivalves, and mussels are found at this location (Kamat, 2011). In India, widespread occurrence of *Salmonella* contamination in seafood through diverse routes has been reported (Kumar et al., 2009). In this study, researchers investigated the occurrence of human pathogens, especially *Salmonella* spp., in the mangroves of Goa. Researchers aimed to quantify their abundance in mangrove ecosystems of Goa, to determine the degree of pathogenicity of the isolates in-vitro, and to differentiate their expression of virulence.

Materials and Methods

Sampling Area. Sampling was carried out at ten different sites as seen in Figure 1 along the estuaries of Goa (India) in the mangroves ecosystems, concentrating specifically on sites from where most of the seafood gets collected by the local fisher folk. Sampling sites were across Mandovi (15°21′ to 15°31′ N and 73°45′ to 73°49′ E) and Zuari estuary (15°25′ N and 15°25′ E). The area is comprised of subtropical mangroves, intertidal mudflats, commercial fishponds, and industrial drainage channels. In addition, these mangrove areas are also highly intervened by tourism boats and mining barges.

Sampling. A total of 150 mangrove samples (sediments, $n = 60$; water, $n = 60$) and mangrove biota including oyster ($n = 7$), prawns ($n = 3$), crabs ($n = 5$) and fish ($n = 15$) were collected in two seasons (pre-monsoon and post-monsoon). Water and sediment samples were collected at 10-cm depth in sterile tubes. Mangrove biota samples were collected from the catches of local fishermen in sterile polythene bags and transported chilled in an ice-box to the laboratory, where they were further processed for total viable count and isolation of *Salmonella* spp. as applicable.

Physicochemical Characterization. Physiochemical parameters such as temperature and pH were determined onsite using a field multimeter (Cole-Parmer); total dissolved solids (TDS) and dissolved oxygen (DO) was determined as described by Trivedi et al. (1986). Salinity was measured using a handheld refractometer (ATAGO). The data was analyzed using a paired t-test.

Total Viable Count. Bacterial abundance was determined by selective media counts. Water samples (1 mL) and mangrove sediment samples (1 g) were 10-fold serially diluted in sterile sea water. Two consecutive dilutions (from 10^{-2} and 10^{-4}) of 100 uL were transferred to respective selective agar in triplicates. *E. coli, Salmonella* spp., *Listeria* spp., and *Vibrio* spp. were plated on EMB agar, Hektoen Enteric agar, PALCAM (Polymixin Acriflavin Lithium chloride Ceftazidime Asculin Mannitol) agar, and

Thiosulphate citrate bile salts sucrose (TCBS), respectively. Plates were incubated for 24 hours at 37°C.

Enrichment and Isolation of *Salmonella* spp. The ISO 6579 method was used for the isolation of *Salmonella* spp. Approximately 1 mL of water and 1 g of sediment were inoculated in tetrathionate brilliant green bile broth. Mangrove biota (i.e., prawns, fishes, crabs, oyster) were macerated in sterile phosphate buffer saline (PBS), then the extract was inoculated in tetrathionate brilliant green bile broth and incubated overnight at 37°C for enrichment. A loopful of enriched broth was streaked on Hektoen Enteric agar and incubated at 37°C for 24 hours. Green colonies with dark centers were suspected as *Salmonella* spp. Presumptive isolates were stored at 4°C in nutrient broth. These presumptive *Salmonella* isolates were further confirmed by Gram staining, sugar fermentation tests and growth on triple sugar iron agar (TSI).

In-Vitro Determination of Virulence. All the *Salmonella* isolates (60) were screened for the presence of the invasive gene (*invA*) as virulence-associated genes. The PCR was performed as described by Amini et al. (2011). The DNA extraction was carried out by the snap chill method. Overnight grown (1 mL) bacterial culture was centrifuged at 10 000 rpm for 10 minutes. The pellet was then suspended in 100 μL of sterile distilled water and kept in boiling water for 10 minutes. The suspension was later transferred to –20°C for 10 minutes. The treated cell suspension was centrifuged at 10 000 × g for 10 minutes. The supernatant was used as a DNA template and tested for presence of the *invA* gene by PCR analysis. Primers used for the *invA* gene were as per Amini et al. (2010), forward primer 5-ACAGTGCTCGTTTACGACCTGAAT-3 and reverse primer 5-AGACGACTGGTACTGATCAT-3. The reaction mixture was prepared for total volume of 25 μL containing 2.5 μL of 10X PCR buffer, 1.5 mM of $MgCl_2$, 2 mM of dNTP mixture, 0.5 μM of each primer, and 50 ng of DNA template. The reaction conditions were set as initial denaturation at 94°C for 2 minutes followed by denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and final extension at 72°C for 2 minutes. Positive control was maintained by *S. typhi* MTCC 733, and negative control was maintained without any DNA. The amplified DNA product was analysed by electrophoresis on 1.5% agarose gels stained with ethidium bromide and visualized under Alpha-Imager Gel Doc system.

Virulence Associated Gene Expression Study. Virulence-associated gene *invA* was investigated for the level of expression by determining the amount of respective mRNA present. Four randomly selected mangrove isolates bearing the *invA* gene were studied.

RNA Extraction. The RNA extraction was performed using the Purelink RNA extraction kit (Invitrogen). Bacterial cultures were grown in Brain Heart Infusion (BHI) broth at 37°C overnight, and RNA was extracted as per manufacturer's instructions. The quality and quantity of RNA was assessed by use of the A_{260}/A_{280} ratio by Nanodrop (Thermo-fisher) and further confirmed on 2% agarose gel. To ensure removal of contaminated DNA, 200 ng of RNA was treated with DNAse I (Bangalore GeNei) at a concentration of 1 U/μg of RNA for 15 minutes at 42°C temperature. Aliquots were made from extracted RNA and stored at –20°C.

Real Time PCR. Real-time quantitative RT-PCR (qRT-PCR) was performed with the one-step SYBR Green Quantitative RT-PCR Kit (Sigma Aldrich), according to the manufacturer's instructions. Primer used for the *invA* gene of *Salmonella* spp. is as mentioned above for PCR and primers used for 23s rRNA as a housekeeping gene were F: GTGTCAGGTGGGCAGTTTG, R: CATTCTGAGGGAACCTTTGG (Romanova et al., 2006). For each sample, 50 ng of total RNA was used in the assay and both genes were tested with the same panel of RNA samples. Negative control reactions were performed by adding deionized water. All standards and samples were run on 96-well reaction plates with the MyiQ2™ Two-Color Real-Time PCR Detection System (Bio-Rad). Reactions were prepared in a total volume of 25 μL containing 50 ng of RNA, 1.25 μL of each primer of concentration 0.5 uM, 12.5 μL of SYBR Green master mix, 0.25 μL of RT mix, and the final volume was adjusted to 25 μl by adding RNase/DNase-free sterile water. The cycle conditions were as follows: reverse transcription at 48°C for 30 minutes for synthesis of cDNA, DNA polymerase activation, and RT enzyme inactivation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, primer annealing at 53°C for 30 seconds, elongation at 72°C for 30 seconds. This cycle was followed by a melting curve analysis, ranging from 55°C to 95°C, with temperature increasing steps of 1°C every 10 seconds. Baseline and threshold values were automatically determined for all plates using the iQ5 optical detection software version 2.1. The obtained data were analyzed using iQ5 optical detection software version 2.1.

Results

Physicochemical Analysis of Water Samples. During the pre-monsoon season, (March to May) water temperature ranged from 29°C to 30.6°C; in the post- monsoon season (October to December), the range was 27 to 28.5°C. Salinity variations ranged from 28 to 35 psu in the pre-monsoon. The highest values being 35 in May. During October to December, salinity was lower than during the pre-monsoon season. In the pre-monsoon and post-monsoon seasons, the dissolved oxygen (DO) level varied from 5.4 to 6.8 mg/L, and 6.1 to 7.4 mg/L, respectively. Total dissolved solids were highest in the month of May at 50.8 and were the lowest in October at 27.2 g/L. The minimum pH value was 5.5 during pre-monsoon season, and the maximum pH was 7.2 during the post-monsoon season (Table 1). The pre-monsoon values for water temperature, salinity, dissolved oxygen, total dissolved solids, and pH were significantly different from post-monsoon values ($p < 0.05$).

Total Viable Count. The abundance of pathogenic micro-organisms were identified based on the typical colony morphology on selective media used as follows: on EMB agar, purple colored colonies with green metallic sheen were noted as *E. coli;* on Hektoen Enteric agar, green colonies with black center as *Salmonella* spp.; on PALCAM, grey greenish colonies as *Listeria* spp.; on TCBS yellow and green colonies were reported as *Vibrio* spp. Their mean abundance and total counts per milliliter are tabulated in Table 2 .The trend observed was: *Salmonella* > *Vibrio* > *E. coli* > *Listeria*.

Isolation and Characterization. Out of 150 samples (water, sediments, and biota) collected, the bacteria were further screened for the occurrence of *Salmonella* spp. as described by ISO 6579 method. It was found that 60 out of 150 samples (40%) were positive for *Salmonella* spp., which was confirmed biochemically by sucrose, glucose, lactose fermentation test and triple sugar iron agar test (Cheesbrough, 1984). Out of the

Table 1—Physiochemical parameters during pre-monsoon and post-monsoon seasons.

Physiochemical parameters											
Pre-monsoon						Post-monsoon					
Sample Site	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Total dissolved solids (gm/L)	Salinity (o/oo)	Sample Site	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Total dissolved solids (gm/L)	Salinity (o/oo)
1	30	6.4	5.65	42.9	28.90	1	27.	6.5	6.35	27.0	17.00
2	29	5.5	5.43	52.2	31.20	2	28	6.4	6.13	31.2	24.49
3	30	6.1	5.45	47.9	34.49	3	27	7.0	5.92	35.1	26.42
4	30	6.0	5.46	45.8	33.40	4	26.5	6.0	7.29	29.2	20.22
5	30.6	6.5	5.33	38.8	34.98	5	27	7.2	6.46	30.4	25.24
6	30	6.2	6.44	39.2	30.15	6	28.5	7.1	5.84	34.0	26.10
7	30	5.8	6.86	50.8	34.40	7	27	6.0	7.05	32.6	18.00
8	31	6.0	5.52	31.0	32.90	8	29	6.0	7.10	31.1	20.00
9	31	5.9	6.48	39.2	31.29	9	27	7.2	7.25	30.4	26.00
10	30	6.0	5.55	50.0	30.58	10	27	6.1	6.55	24.6	19.45

60 isolates, 32 were from mangrove water, 21 from the sediments, and 7 from the biota.

In-Vitro Determination of Virulence. The *Salmonella* isolates (60) were screened for the presence of the invasive gene by PCR method as described by Amini et al. (2010). Out of 60 isolates, 21 isolates (35%) were found to be positive for the invasive gene (Figure 2).

Gene Expression Studies. The expression of the *invA* gene was studied to determine if there was any change in the expression. Randomly selected four isolates were tested along with *S. typhi* MTCC 733 as a standard strain of *Salmonella* spp. The expression of the *inv* gene was normalized by using 23S rRNA gene. The data obtained was then analyzed by iQ5 software version 2.1. The gene expression study showed that expression of the *invA* gene in wild strains of *Salmonella* spp. was comparatively lower than *S. typhi* MTCC733 strain as seen in Figure 3.

Discussion

Mangroves provide an excellent breeding, feeding, and growth habitat and have long served as important nurseries for many fish species (Brown, 1997; Dekate et al., 2011; Kimirei et al., 2013). Mangrove leaf litter provides an important nutrient base for the food web. In addition, mangroves provide a unique ecological environment for diverse bacterial communities. Sustained human activity and pollution has affected the microbes living in these ecosystems, adding or depleting the type of microbial flora present (Ristori et al., 2007). Disposal of human waste may introduce new pathogens to mangroves that may tolerate saline and harsh environments and tend to persist (Tam, 1998; Chandran et al., 2005). Attri et al. (2011) have reported mangrove ecosystems to be reservoirs for heavy metals, which also gain entry via various anthropogenic activities.

The mangroves span the Goan coastline and are significantly affected by human activities and industrialization. Local residents explore the area for edible mollusks and crustaceans, which they sometimes consume raw. Mangrove biota includes *Meretrix* spp., *Crassostrea* spp., *Penaeus* spp., *Scylla serrata*, and *Mugil cephalis*, which are in great demand and are harvested by locals and exported or sold locally, without further processing. Researchers have investigated different aspects of mangroves; the effects of relevant pathogens on public health, however, remain unclear. In Goa, there have been reports of an increase of waterborne diseases such as diarrhea, hepatitis, and typhoid because of inadequacies in the drinking water system in the state (Planning Commission Government of India, 2011). Discharge of urban and industrial waste affects the number of fecal indicator bacteria and enteric pathogens in estuaries (Touron et al., 2007). Fecal pollution in water is monitored by enumerating the coliforms to predict the presence of pathogens (Efstratiou et al., 2009); significant wastewater contamination would lead to higher numbers of indicator bacteria in waterbodies. In this study, we have enumerated common pathogenic bacteria such as *E. coli*, *Listeria*, *Salmonella*, and *Vibrio* spp. The counts of fecal indicators and human pathogenic bacteria were found to 1.25 to

Table 2—Mean abundance and total viable counts (TVC) per milliliter (mL) of pathogenic bacteria form mangrove environment (EMB = eosin methylene blue agar; HE = hektoen enteric agar; PALCAM = polymixin acriflavin lithium chloride ceftazidime asculin mannitol agar; TCBS = thiosulphate citrate bile salt sucrose agar).

		TVC (CFU/mL) $\times 10^3$		TVC (CFU/mL) $\times 10^3$	
		Pre-monsoon		Post-monsoon	
Pathogens	Selective agar	Sediment	Water	Sediment	Water
E. coli	EMB	1.8(±0.20)	2.5(±0.09)	2.1(±0.56)	2.1(±0.67)
Listeria spp.	PALCAM	1.25(±0.12)	0.9(±0.02)	1.3(±0.27)	1.25(±0.12)
Salmonella spp.	HE	2.2(±0.34)	3.1(±1.1)	3.9(±0.34)	3.2(±0.15)
Vibrio spp.	TCBS	2.2(±0.19)	2.6(±0.34)	3.0(±0.1.5)	3.8(±0.1.2)

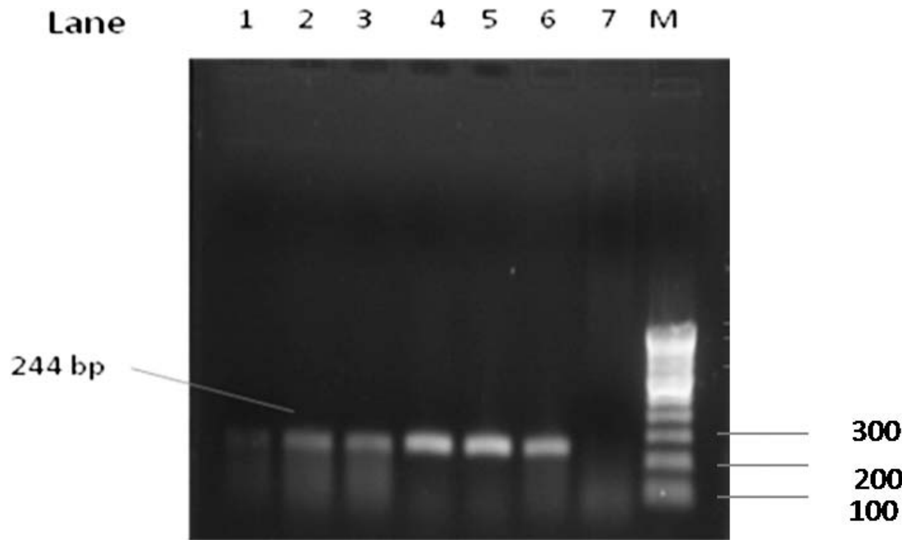

Figure 2—*Salmonella* spp. isolates showing amplification of *inv* gene. Lanes 1 to 5 showing amplification of *inv* gene; lane 6 = positive control *S. typhi* MTCC 733; lane 7 = negative control; lane M = 100bp DNA ladder.

3.9×10^3 cfu/mL. There are no regulations on the presence of such pathogens in environmental bodies such as mangroves. The total counts observed in this study were far greater than acceptable limits when compared with the relevant standards defined by environmental agencies such as United States Environmental Protection Agency (U.S. EPA, 2010, 2014). The bacterial abundance was higher during post-monsoon season compared to the pre-monsoon with an observed trend of *Salmonella*>*Vibrio*>*E. coli*>*Listeria. Vibrio* spp. Researchers have found this trend to be typical in such ecosystems (Thompson et al., 2003). In the mangroves in this study, there was a high prevalence of *Salmonella* spp., which has been associated with diseases in Goa (Planning Commission Government of India, 2011). This study focused on *Salmonella,* one of the deadliest pathogens, and its virulence to determine its prevalence in the mangroves of Goa (India) and its potential to contaminate seafood through water and sediments. Sampling was carried out for two seasons (pre-monsoon and post-monsoon) in 2011 and 2012. A total of 60 (40%) out of 150 samples were suspected positive for *Salmonella* spp. A similar study is reported by Grisi et al. (2010) in an industrially affected mangrove habitat from Paraiaba do Norte rover (Brazil), which had a 25% occurrence of *Salmonella* spp. Therefore, contamination of mangrove-derived food with high numbers of *Salmonella* at potential food harvesting zones does occur. Contamination of crab meat and other associated fish has been linked with the presence of *Salmonella* spp. in the mangrove area (Grisi et al., 2010; Lotfy et al., 2011). Disposal of human fecal waste has been shown to be related to the occurrence of fecal coliforms in mangroves (Abbu et al., 2007). In this study, the mangroves are highly influenced by human activities such as the disposal of humans waste from boats of tourist cruises and casinos, iron ore transporting barges, industrial waste, and others. Therefore, these sources may contribute to the influx of pathogens in the estuarine waters and into the mangroves. The ability of *Salmonella* spp. to tolerate salt and low pH helps them

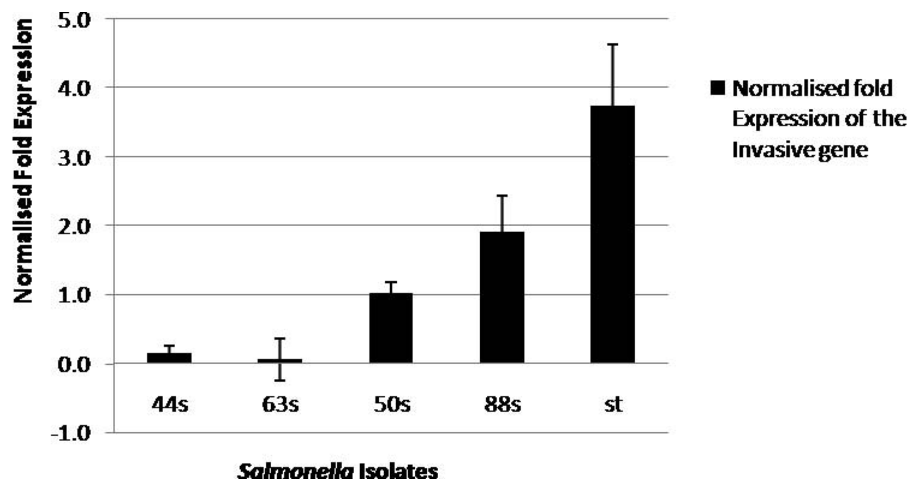

Figure 3—Normalized expression of invasive gene of the randomly selected Salmonella isolates obtained from mangrove environment; 44s, 63s, 50s, 88s = wild strains from mangrove, standard *S. typhi* MTCC 733.

to sustain the harsh environment and thus to persist (Hermans, 2007).

Out of the 60 isolates, 22 (36.6%) were from the pre-monsoon season and 38 (63.3%) were from the post-monsoon sampling, which implies their prevalence throughout the year. Occurrence of *Salmonella* is influenced by environmental parameters such as temperature, rainfall, and salinity (Lemarchand and Lebaron, 2003; Martinez-Urtaza, 2004). In this study, *Salmonella* counts were higher in the post-monsoon season, while temperature and salinity values were higher in the pre-monsoon season. The upwelling and turbulence of water and seepage and runoff from the land and estuaries during the monsoon season may contribute to an increase in *Salmonella* spp., which corresponds with the high numbers obtained in the present study. This seasonal pattern of prevalence of *Salmonella* has been reported by Martinez-Urtaza (2004), where the greatest incidence was observed during the months of October to December, which is comparable to our findings. An increase in the frequency of *Salmonella* spp. is noted with an increase in wastewater pollution in water (Polo et al., 1998; Efstratiou et al., 2009). The study revealed high pathogenic bacterial contamination in the mangrove ecosystem. Detection of the *invA* gene among the *Salmonella* isolates is the most sensitive and accurate method for detection of pathogenic *Salmonella* spp. (Malorney et al., 2003). *Salmonella* is a facultative intracellular pathogen, and the *invA* gene is highly essential for virulence of *Salmonella* (Khan et al., 1999). To confirm the pathogenicity of the isolates, the virulence-associated gene, invasive gene (*invA*), was screened. Presence of the *invA* gene among 21 isolates out of 60 confirmed the prevalence of pathogenic *Salmonella* spp. in the mangrove ecosystem of Goa.

Activation and repression of the invasion gene in *Salmonella* is a complex phenomenon involving many steps of regulation (Jones et al., 2005). Regulations must predict the influence of environmental parameters on the invasive gene expression. Environmental conditions such as oxygen stress, high osmolarity, and alkaline pH can modulate the expression of the *invA* gene (Bajaj et al., 1995). These environmental factors may mediate a small DNA-binding protein, altering the DNA supercoiling and, therefore, transcription (Jones et al., 2005). The exact mechanism of regulation of the *invA* gene is still unclear. Considering these aspects, an attempt was made to find the difference in expression of the *invA* gene among the *Salmonella* spp. from the mangroves. To determine the change in expression of the *invA* gene from mangrove strains, a comparison was made with a standard strain (*S. typhi* MTCC 733 strain). The experiment was repeated three times, and each strain was checked in triplicate to confirm the results. Although the standard and the mangrove *Salmonella* strains were grown in similar conditions, the mangrove strains showed a lower expression of the *invA* gene. This could be attributed to the mobilization of *Salmonella* spp. from a natural mangrove ecosystem into an in-vitro experimental condition.

Thus, it is inferred that environmental conditions dictate the expression of certain genes, and such expression may be inherited in subsequent generations of *Salmonella* spp. This may result in an alien species adapting and adopting the mangrove ecosystem as its natural habitat and, in turn, becoming an indigenous organism. Thus, the presence of the *invA* gene in the *Salmonella* spp. associated with the infected mangrove ecosystem is a threat to the humans consuming food products from this ecosystem.

Conclusions

This study indicates the high prevalence of pathogenic *Salmonella* spp. in the mangrove ecosystem of Goa in both pre- and post-monsoon periods. Human interference may add pathogens directly or indirectly to the mangroves. The ability of *Salmonella* spp. to tolerate salinity could cause its continued prevalence in the mangrove ecosystems. Continued acclimatization of *Salmonella* to this environment may result in creation of an indigenous niche, supporting permanent persistence of pathogenic strains in these otherwise pristine ecosystems. Mangrove ecosystems may become significant reservoirs for pathogenic strains. Persistence of virulent species of *Salmonella* in these environments may contaminate associated food, which could create a potential threat to human health. Seasonal monitoring in mangrove ecosystems could improve understanding of these productive marine coastal areas that are vulnerable to human effects.

Acknowledgments

The authors are thankful to the Director, ICAR Research Complex for Goa and Head of the Department of Biotechnology for providing the necessary facilities. The research work is supported by grants from Department of Biotechnology, Government of India to SBB (BT/01/CEIB/11/VI/13).

Submitted for publication March 28, 2014; revised manuscript submitted November 25, 2014; accepted for publication March 2, 2015.

References

Abbu, A. A.; Lyimo, T. J. (2007) Assessment of Fecal Bacteria Contamination in Sewage and Non-sewage Impacted Mangrove Ecosystems along the Coast of Daresalaam. *Tanz. J. Sci.*, **33,** 1-16.

Amini, K.; Salehi, T. Z.; Nikbakht, G.; Ranjbar, R.; Amini J.; Ashrafganjooei, S. B. (2011) Molecular Detection of *invA* and *spv* Virulence Genes in *Salmonella* Enteritidis Isolated from Human and Animals in Iran. *Afr. J. Microbiol. Res.*, **4,** 2202-2210.

Antony, B.; Dias, M.; Shetty, A. K.; Rekha, B. (2009) Food Poisoning due to *Salmonella* Enterica Serotype Weltevreden in Mangalore. *Ind. J. Med. Microbiol.*, **27,** 257-258.

Attri K.; Kerkar S. (2011) Seasonal Assessment of Heavy Metal Pollution in Tropical Mangrove Sediments (Goa, India). J. Ecobiotechnol., **3,** 09-15.

Bajaj, V. C.; Hwang, C.; Lee, C. A. (1995) *hilA* is a Novel *ompR/toxR* Family Member that Activates the Expression of *Salmonella typhimurium* Invasion Genes. *Mol. Microbiol.*, **18,** 715-727.

Bordalo, A. A.; Onrassami, D. (2002) Survival of Faecal Indicator Bacteria in Tropical Estuarine Waters (Bangpakong River, Thailand). *J. Appl. Microbiol.*, **93,** 864-871.

Brands, D. A. (2005) Prevalence of *Salmonella* spp. in Oysters in the United States. *Appl. Environ. Microbiol.*, **71,** 893-897.

Brown, B. E. (1997) *Integrated Coastal Management: South Asia. Department of Marine Sciences and Coastal Management*. University of Newcastle: Newcastle upon Tyne, United Kingdom.

Center of Disease Control and Prevention (CDC) (2004) Annual Summer Report for *Salmonella* http://www.cdc.gov/ncidod/dbmd/phlisdata/salmtab/2004/*Salmonella*AnnualSummary%2 (accessed Oct. 10, 2014).

Center of Disease Control and Prevention (CDC) (2012). http://www.cdc.gov/salmonella/general (accessed June 22, 2013).

Chandran, A.; Hatha, M. A. A. (2005) Relative Survival of *Escherichia coli* and *Salmonella* Typhimurium in a Tropical Estuary. *Water Res.*, **39,** 1397-1403.

Cheesbrough, M. (1984) Biochemical Testing of Organisms In: Medical Laboratory Manual for Tropical Countries. Ed. *ELBS Edition*, **2,** 58-69.

Dekate, H. M.; Yeragi, S. S.; Yeragi, S. G. (2011) Biodiversity and Distribution of Prawn Juveniles in Mangroves Habitat of Mumbri Creek of South Konkan, Maharashtra. *Life Sci. Leaflets,* **21,** 992-995.

Duke, N. C.; Meynecke, J. O.; Dittmann, S.; Ellison, A. M.; Anger K. (2007) A World Without Mangroves? *Science*, **317,** 41-42.

ENVIS (2012) Mangroves in Goa. http://www.goaenvis.nic.in/mangrove.htm (accessed June 1, 2013).

European Food Information Council (EUFIC) (2006) Some Animal Diseases and their Possible Impact on Food Safety. http://www.eufic.org/article/en/food-safety-quality/animal-health/expid/review-animal-diseases/ (accessed June 23, 2013).

Efstratiou, A. M.; Mavridou, A.; Richardson, C. (2009) Prediction of *Salmonella* in Seawater by Total and Faecal Coliforms and Enterococci. *Mar. Pollut. Bull.*, **58,** 201-205.

FAO (1988) *Worldwide Compendium of Mangrove Associated Aquatic Species of Economic Importance*; FAO Fisheries Circular No.814 (FIRI – C814). FAO Publishing Management Service: Rome, Italy.

FAO (2010) *Report of the FAO Expert Workshop on the Application of Biosecurity Measures to Control* Salmonella *Contamination in Sustainable Aquaculture Mangalore, India, 19–21 January 2010*; FAO Fisheries Circular, No. 937. FAO Publishing Management Service: Rome, Italy.

Foley, S. L.; Lynne, A. M. (2007) Food Animal-Associated Salmonella Challenges: Pathogenicity and Antimicrobial Resistance. *J. Animal Sci.*, **86,** 173-187.

Galán, J. E.; Ginocchio, C.; Costeas, P. (1992) Molecular and Functional Characterization of the *Salmonella* Invasion Gene *invA*: Homology of *InvA* to Members of a New Protein Family. *J. Bacteriol.*, **174,** 4338-4349.

Grisi Lima de, T. C. S.; Lira K. G. (2010) The Abundance of Some Pathogenic Bacteria in Mangrove Habitats of Paraiba do Norte Estuary and Crabmeat Contamination of Mangrove Crab *Ucides cordatus*. *Braz. Arch. Biol. Technol.*, **53,** 227-234.

Haddock, R. (1993) The Origins of Infant Salmonellosis. *Am. J. Public Health,* **83,** 772.

Hatha, A. A. M.; Chandran, A.; Rahiman Mujeeb, K. M. (2004) Prevalence of Diarrheginic Serotypes of *Escherichia Coli* the Cochin Estuary, along West Coast of India. *Indian J. Mar. Sci.*, **33,** 238-242.

Heinitz, M. L.; Ruble, R. D.; Wagner, D. E.; Tatini, S. R. (2000) Incidence of Salmonella in Fish and Seafood. *J. Food Protect.*, **63,** 579-592.

Hermans Armand P. H. M. (2007) Stress Response and Virulence in *Salmonella Typhimurium* a Genomics Approach. Thesis Wageningen University, Wageningen, **90** (8), 504-572

Hood, M. A.; Ness, G. E. (1982) Survival of *Vibrio Cholerae* and *Escherichia coli* in Estuarine Waters and Sediments. *Appl. Environ. Microbiol.*, **43,** 578-584.

International Standard Organisation ISO 6579. (1993). *Microbiology: General Guidance on Methods for The Detection of* Salmonella, 3rd Ed. International Organization for Standardization: Geneva, Switzerland.

Jones, B. D. (2005) *Salmonella* Invasion Gene Regulation: A Story of Environmental Awareness. *J. Microbiol.*, **43,** 110-117.

Kamat, N. (2011) The Neglected Fish of Goa. *The Navhind Times*, Panorama. Panjim, Goa. www.navhindtimes.in/panorama/neglected-fish-goa (accessed March 20, 2014).

Khan, A. A.; Nawaz, M. S.; Khan, S.; Sernigelia, C. E. (1999) Detection of Multidrug Resistant *Salmonella* Typhimurium DT104 by Multiplex Polymerase Chain Reaction; *FEEMS Microbiol. Lett.*, **182,** 355-360.

Kimirei, I. A.; Nagelkerken, I.; Mgaya, Y. D.; Huijbers, C. M. (2013) The Mangrove Nursery Paradigm Revisited: Otolith Stable Isotopes Support Nursery-to-Reef Movements by Indo-Pacific Fishes. *PLoS One.*, **12,** 8.

Kramer, M.; Herwaldt, B.; Craun, G.; Calderon, R.; Juranek, D. (1996) Surveillance for Waterborne-Disease Outbreaks—United States, 1993–1994. *Centers for Disease Control and Prevention Surveillance Summaries*, **12,** 1-33.

Kumar, R.; Surendran, P. K.; Thampuran, N. (2009) Distribution and Genotypic Characterization of Salmonella Serovars Isolated from Tropical Seafood of Cochin, India. *J. Appl. Microbiol.*, **106,** 515-524.

Lal, A.; Hales, S.; French, N.; Baker, M. (2012) Seasonality in Human Zoonotic Enteric Diseases: a Systematic Review. *PloS One,* **7,** e31883.

Lemarchand, K.; Lebaron, P. (2003) Occurrence of *Salmonella* spp. and *Cryptosporidium* spp. in a French Coastal Watershed: Relationship with Faecal Indicators. *FEMS Microbiol. Lett.*, **218,** 203-209.

Lotfy, N. M.; Hassanein, M. A.; Gawad, G. E.; Taweel, E.; Bassem, S. M. (2011) Detection of *Salmonella* spp. in Aquatic Insects, Fish and Water by MPN-PCR. *World J. Fish Mar. Sci.,* **1233,** 58-66.

Malorny, B.; Hoorfar, J.; Bunge, C.; Helmuth, R. (2003) Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard. *Appl. Environ. Microbiol.,* **69,** 290-296.

Martinez-Urtaza, J. M.; Liebana, E.; Garcia-Migura, L.; Perez-Pineiro, P.; Saco, M. (2004) Characterization of *Salmonella enterica* Serovar Typhimurium from Marine Environments in Coastal Waters of Galicia (Spain). *Appl. Environ. Microbiol.,* **70,** 4030-4034.

Martinez-Urtaza, J.; Saco, M.; Novoa de, J.; Perez-Piñeiro, P.; Peiteado, J.; Lozano-Leon, A.; Garcia-Martin O. (2004) Influence of Environmental Factors and Human Activity on the Presence of *Salmonella* Serovars in a Marine Environment. *Appl. Environ. Microbiol.,* **70,** 2089-2097.

Murugkar, H. V.; Rahman, H.; Dutta, P. K. (2003) Distribution of Virulence Genes in *Salmonella* Serovars Isolated from Man & Animals. *Ind. J. Med. Res.*, **117,** 66-70.

Planning Commission Government of India (2011) Goa Development Report. Academic Foundation: New Delhi, India. http://www.academicfoundation.com (accessed June 13, 2014).

Polo, F.; Figueras, M.; Inza, I.; Sala, J.; Fleisher, J.; Guarro J. (1988) Relationship between Presence of *Salmonella* and Indicators of Faecal Pollution in Aquatic Habitats. *FEMS Microbiol. Lett.*, **160,** 253-256.

Ristori, C. A.; Iaria, S. T.; Gelli, D. S.; Rivera, I. N. (2007) Pathogenic Bacteria Associated with Oysters(*Crassostrea brasiliana*) and Estuarine Water along the South Coast of *Braz. Int. J. Environ. Health Res.*, **17,** 259-269.

Rodriguez-Lazaro, D.; Hernandez, M.; Esteve, T.; Hoorfar, J.; Pla, M. (2003) A Rapid and Direct Real-Time PCR-Based Method for Identification of *Salmonella* spp. *J. Microbiol. Meth.*, **54,** 381-390.

Romanova, N. A.; Wolffs, P. F. G.; Brovko, L. Y.; Griffiths, M. W. (2006) Role of Efflux Pumps in Adaptation and Resistance of Listeria monocytogenes to Benzalkonium Chloride. *Appl. Environ. Microbiol.*, **72,** 3498-3503.

Ruimy, R.; Breittmayer, V.; Boivin, V.; Christen, R. (1994) Assessment of the State of Activity of Individual Bacterial Cells by Hybridization with a Ribosomal RNA Targeted Fluorescently Labelled Oligonucleotidic Probe. *FEMS Microbiol. Ecol.,***15,** 207-213.

Sahoo, K.; Dhal, N. K. (2009) Potential Microbial Diversity in Mangrove Ecosystem: A Review. *Ind. J. Mar. Sci.*, **38,** 249-256.

Salehi, T. Z.; Mahzounieh, M.; Saeedzadeh, A. (2005) Detection of *invA* gene in Isolated *Salmonella* from Broilers by PCR Method. *Int. J. Poultry Sci.,* **8,** 557-559.

Schutze, G.; Flick, E.; Pope, S.; Lofgren, J.; Kirby, R. (1995) Epidemiology of Salmonellosis in Arkansas. *South. Med. J.,* **88,** 195-199.

Schutze, G.; Kirby, R.; Flick, E.; Stefanova R.; Eisenach, K.; Cave, M. (1998) Epidemiology and Molecular Identification of *Salmonella* Infections in Children. *Arch. Pediat. Adol. Med.,***152,** 659-664.

Schutze, G. E.; Sikes, J. D.; Stefanova, R.; Cave, M. D. (1999) The Home Environment and Salmonellosis in Children. *Pediatrician,* **103**.

Singh, L. J. (2012) Mangrove Plant Diversity in Bay Islands, India and its Significance. In *National Conference On Biodiversity: One Ocean-Many Worlds of Life*, Kumar, P.; Singh, P.; Srivastava R. J.; Dubey R. K., eds.; Uttar Pradesh State Biodiversity Board: Lucknow, India.

Stevens, M.; Humphrey, T.; Maskell, D. (2009) Molecular Insights into Farm Animal and Zoonotic *Salmonella* Infections. *Royal Society of London. Series B, Biol. Sci.,* **364,** (1530), 2709–2723.

Tam, N. F. Y. (1998) Effects of Wastewater Discharge on Microbial Populations and Enzyme Activities in Mangrove Soils. *Environ. Pollut*., **102,** 233-242.

Thompson, F. L.; Iida, T.; Swings, J. (2003) Biodiversity of Vibrios. *Microbiol. Mol. Biol. Rev*., **68,** 403-431.

Thong, K. L.; Goh, Y. L.; Radu, S.; Noorzaleha, S.; Yasin, R.; Koh, Y. T.; Lim, V. K. E.; Rusul, G.; Puthucheary, S. D. (2002) Genetic Diversity of Clinical and Environmental Strains of *Salmonella* Enteric Serotype. Weltevreden Isolated in Malaysia. *J. Clinic. Microbiol*., **40,** 2498-2503.

Touron, A.; Berthe, T.; Gargala, G.; Fournier, M.; Ratajczak, M.; Servais, P.; Petit, F. (2007) Assessment of Faecal Contamination and the Relationship Between Pathogens and Faecal Bacterial Indicators in an Estuarine Environment (Seine, France). *Mar. Pollut. Bull*., **54,** 1441-1450.

Trivedi, R. K.; Goel, P. K. (1986) The Chemical and Biological Method for Water Pollution Studies. *Environ. Pub. Karad,* 37–78.

Ukwade I. M. (1990) Microbiological Studies of the Man Mangrove Oyster *Crossostrea gasar* from an Experimental Fish Form of the Lagas Lagoon, Nigeria. Nigerian Institute for Oceanography and Marine Research Lagos; Niomr Technical Paper No. **55,** 1-10.

Upadhyay, B. P.; Utrarachkij, F.; Thongshoob, J.; Mahakunkijcharoen, Y.; Wongchinda, N.; Suthienkul, O.; Khusmith, S. (2010) Detection of *Salmonella invA* gene in shrimp enrichment culture by polymerase chain reaction. *SE Asian J. Trop. Med.,* **41,** 426-435.

U.S. Environmental Protection Agency (U.S. EPA) (2010) Standard Analytical Protocol for Salmonella Typhi in Drinking Water 600/R-10/133. http://oaspub.epa.gov/eims/eimscomm.getfile?p_download_id=499264 (accessed November 2014).

U.S. Environmental Protection Agency (U.S. EPA) (2014) Control of Pathogens and Vector Attraction in Sewage Sludge http://www2.epa.gov/sites/production/files/documents/625R92013ALL.pdf (accessed November 2014).

U.S. Food and Drug Administration (FDA) (2012) Investigation of Multistate Outbreak of *Salmonella* Bareilly and *Salmonella* Nchanga Infections Associated with Nakaochi Scrape AA or AAA Frozen Raw Tuna from India http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm298741.htm#update (accessed November 2014).

Walters, B.; Rönnbäck, P.; Kovacs, J.; Crona, B.; Hussain, S.; Badola, R.; Primavera, J.; Barbier, E.; Dahdouh-Guebas, F. (2009) Erratum to Ethnobiology, Socio-economics and Management of Mangrove Forests: A Review, *Aquat. Bot.,* **89,** 220-236.

Winfield, M. D.; Groisman, E. A. (2003). Role of Nonhost Environments in the Lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol*., **69,** 3687-3694.