Fishery Technology 56 (2019) : 164 - 167

Research Note

Need for an Optimized Protocol for Screening Seafood and Aquatic Environment for *Shigella* sp

N. Ranjit Kumar*, V. Murugadas, Toms C. Joseph, K. V. Lalitha, A. K. Basha, T. Muthulakshmi and M. M. Prasad

ICAR-Central Institute of Fisheries Technology, P.O. Matsyapuri, Cochin - 682 029, India

Shigella sp are known to cause shigellosis, an acute enteric infection affecting over 164.7 million people accounting for 69% mortality in children below 5 years of age globally (Kotloff et al., 1999; WHO, 2005) and 80% of these cases are registered in Asia (Bardhan et al., 2010). India is endemic to Shigellosis (Taneja & Mewara, 2016) and the most common species reported is *Shigella flexneri*. Increase in seafood trade and consumption may potentiate the spread of this bacterium. *Shigella* is not a native flora of fish and Shigellosis outbreaks may occur by consumption of uncooked shrimp and tuna salad contaminated with *Shigella* sp (Novotny et al., 2004). The main mode of transmission in humans is through oral-faecal route through contamination of food and water by the infected people. Further, the infective dose of this bacterium is as low as ten cells and this makes *Shigella* a highly contagious pathogen (Du Pont et al., 1989). Few reports are available on the isolation, identification and molecular confirmation of *Shigella* sp from seafood (Reeve et al., 1989; David et al., 2009; Iwamoto et al., 2010; Sujatha et al., 2011; Sichewo et al., 2013; Obaidat & Salman, 2017) and there is difficulty in differentiating *Shigella* from *E. coli*. Hence, the present study was undertaken to assess the occurrence of *Shigella* sp in various seafood, ice and fishery environments and to determine the suitability of the standard method for identifying *Shigella* in seafood.

A total of 223 samples comprising of fresh seafood, dried fishery products, ice from local markets, water and soil from aquaculture ponds were collected and screened for *Shigella* sp in Ernakulam district, Kerala, India, (Table 1). Isolation was carried out as described in Bacteriological Analytical Manual (FDA, 2001). Multiple plating media *viz*. MacConkey, Xylose Lysine Desoxycholate (XLD) and Hektoen Enteric (HE) agar were used for selective isolation. Plates were incubated at 37°C for 18-24 h. On the basis of morphology, presumptive colonies were picked from agar plates *i.e.*, colourless colonies (2-3 mm) from MacConkey, green colonies (2-3 mm) from HE agar and red colonies (1-2 mm) from XLD agar respectively. After checking the morphology by Gram staining, 1377 isolates were subjected to different biochemical tests (Table 2). Isolates showing characteristic biochemical reactions of *Shigella* were confirmed by PCR targeting invasion plasmid antigen (*ipaH*) gene specific for *Shigella* sp. The primers used for detecting *ipaH* were: forward-5'CGGTCAGCCACCCTCTGAG3' and reverse-5'CTTGACCGCCTTTCCGATACC3' yielding an amplicon of size 613 bp (Binet et al., 2014). Ten μl of PCR products were loaded on a 2% agarose gel and run at 100 V for 1 h. Three μl Generuler 100 bp DNA ladder was used as marker along with positive and negative controls. *ipaH* gene was not detected in any of the isolates.

Shigella sp was isolated in fish harvested from Winam Gulf of Lake Victoria, Kenya (David et al., 2009). Similarly, Sujatha et al. (2011) and Sichewo et al. (2013) isolated *Shigella dysenteriae* from edible fishes from India and Zimbabwe respectively. These reports were based on morphological identification of *Shigella* sp on specific agar plates as followed in this study. Morphological identification is merely not sufficient for confirmation of *Shigella* sp and Bacteriological Analytical Manual recommends molecular as well as serological identification (FDA,

Received 21 October 2017; Revised 22 March 2019; Accepted 28 March 2019

*E-mail: nranjeetkumar@gmail.com

© 2019 Society of Fisheries Technologists (India)

Table 1. Details of samples studied for isolation of *Shigella*

Sample	No	Presumptive Isolates picked from plates	Biochemically confirmed isolates (FDA, 2001)
Fish*	95	512	45
Shell fish**	52	276	21
Dry fish***	18	61	4
Soil from Aquaculture pond	20	128	0
Water from Aquaculture pond	20	156	0
Ice	18	244	15
Total	223	1377	85

*Fish- Indian mackerel (*Rastrelliger kanagurta)*, Indian oil sardine (*Sardinella longiceps*), ribbion fish (*Trachipterus trachypterus*), Bluefin trevally (*Caranx melampygus)*, Indian Anchovy (*Stolephorus indicus)*, grey mullets (*Mugil cephalus)*, flatfish (*Cynoglossus cynoglossus*), *Megalaspiscordyla*, Tilapia (*Oreochromis mossambicuss*), thryssa (*Thryssa malabarica)*, *Nemipterus* sp, Pearl Spot (*Etroplus suratensis*), Skipjack tuna (*Katsuwonus pelamis*), snapper (*Lutjanus lutjanus*); barracuda (*Sphyraena jello*)

**Shellfish- *Metapenaeus* sp, *Penaeus indicus*, sea crab (*Portunus pelagicus*), Tiger prawn (*Penaeus monodon*), King prawn (*Metapenaeus affinis*); molluscs- clam (*Meretrix meretrix*) meat, Squid (*Loligo duvaucelii)*

***Dry fish products- prawn (*Acetes indicus*), anchovy (*Stolephorus indicus)*, flatfish (*Cynoglossus cynoglossus*), ribbon fish (*Trachipterus trachypterus*).

2001). Therefore, PCR was carried out for 85 isolates after biochemical identification targeting *ipaH* gene. *ipaH* gene was not detected in any of the isolates. Similar findings was also reported by Mokhtari et al. (2012), where *Shigella* was absent in seafood samples collected from Nabeul, Tunisia screened by both conventional and molecular methods. Even though the isolates showed morphological characteristic of *Shigella*, *ipaH* gene was absent when PCR amplication for the gene was performed.

This result further heightened the need to study the recovery of *Shigella* from spiked seafood with published methods. This validation study was carried out with *S. flexneri* (ATCC 12022) separately for raw and sterile fish in triplicate to elucidate the effect of background microflora on isolation. Freshly grown culture of *S. flexneri* was harvested and the density ($O.D_{600}$) was adjusted to 0.5 using spectro-

Table 2. Biochemical tests carried out to identify suspected *Shigella* isolates

Biochemical Test	Result
Gram Staining	Gram negative rod shaped bacteria
Motility	Non-motile
Oxidase	Negative
Catalase	Positive
Urea hydrolysis	Negative
Methyl Red (MR)	Positive
(Voges–Proskauer) VP	Negative
H_2S production on triple sugar iron (TSIA) slants	Negative
Sugar fermentation	
Glucose	Acid-Postive Gas-Negative
Sucrose	Negative
Lactose	Negative
Citrate utilization	Negative
Mucate utilization	Negative
Sodium acetate utilization	Negative
Amino acid (lysine) utilization	Negative

photometer (corresponding to 2.6 X 10^6 cfu ml^{-1}). Then serially diluted bacterial inoculum was spiked at level of 10^1, 10^2, 10^3 and 10^4 cfu g^{-1} of fish samples. Isolation, identification from the spiked samples was performed as described earlier. Presumptive colonies from each dilution (10^1, 10^2, 10^3 and 10^4 cfu g^{-1}) from selective plates were picked and identified with biochemical tests and molecularly confirmed by PCR assay (Fig. 1). The same method was performed for raw fish under similar conditions. The spiked enriched broth of raw fish (Fig. 2) produced amplicon of 613 bp which is specific for *ipaH* gene albeit, individual colonies were not detected on agar plates.

PCR based molecular method was found significant in detecting the presence of *Shigella* sp from the raw spiked fish, since the conventional culture based methods are inefficient in isolation of *Shigella* sp from food owing to presence in low numbers and in stressed condition (Lindqvist, 1999). Similar spiking studies for isolation of *Shigella* from enriched broth were also reported (Senachai et al.,

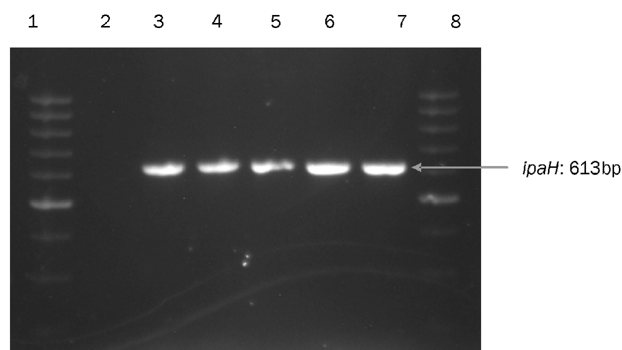

Fig. 1. PCR for *ipaH* gene of suspected *Shigella* sp isolated from sterile spiked sardine.

Lanes 1 and 8: DNA ladder (100 bp); Lane 2: Negative control (Nuclease free distilled water); Lanes 3-6: Suspected Shigella isolates picked out from sterile spiked sardine; Lane 7: Positive control (*Shigella flexineri*-ATCC 12022).

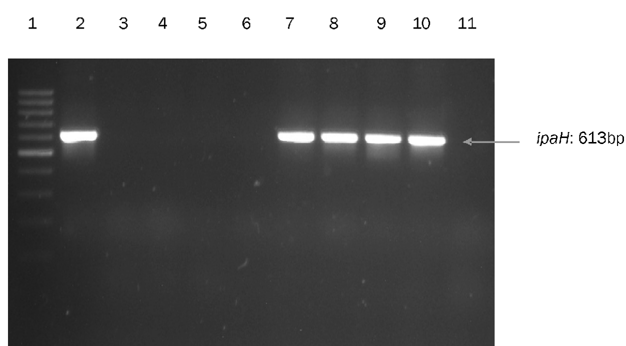

Fig. 2. PCR for *ipaH* gene of suspected *Shigella* sp and enriched broth of raw spiked sardine

Lane 1: DNA ladder (100 bp); Lane 2: Positive control (*Shigella flexineri*, ATCC-12022); Lanes 3-6: Suspected Shigella isolates picked from raw spiked sardine; Lane 7-10: Enriched broth of raw spiked sardine at 10^1, 10^2, 10^3 and 10^4cfug^{-1}; Lane 11- Negative control (nuclease free distilled water).

2013; Binet et al., 2014). Mokhtari et al. (2012) isolated and identified *Shigella* sp from foods of different origin based on both culture dependent and culture independent (PCR) methods and concluded that employing PCR based method improved the detection rates of *Shigella*. Although reports are available on the presence of *Shigella* in seafood, no study has confirmed detection of Shigella by using PCR based methods which are similar to this study. In conclusion, this study indicates the need for development of harmonized protocol for isolation of *Shigella* from seafood and aquatic environment and also need to study the effect of background flora on isolation. This study also indicates that enrichment PCR may be performed along with conventional isolation procedure.

Acknowledgments

Authors are grateful to the Indian Council of Agricultural Research, New Delhi, India, for the financial support and to the Director, ICAR-Central Institute of Fisheries Technology, Cochin, India, for providing all the facilities required for the present study and for permission to publish the research note.

References

Binet, R., Deer, M. D. and Uhlfelder, S. J. (2014) Rapid detection of *Shigella* and enteroinvasive *Escherichia coli* in produce enrichments by a conventional multiplex PCR assay. Food Microbiol. 40: 48-54

Bardhan, P., Faruque, A. S. G., Naheed, A. and David, A. (2010) Decrease in Shigellosis-related deaths without Shigella sp. Specific interventions, Asia. Emerg. Infect. Dis. 16(11): 1718-1723

David, O. M., Wandili, S., Kakai, R. and Waindi E. N. (2009) Isolation of *Salmonella* and *Shigella* from fish harvested from the Winam Gulf of Lake Victoria, Kenya. J. Infect. Dev. Countries. 3(2): 99-104

Du Pont, H. L., Levine, M. M., Hornick, R. B. and Formal S. B. (1989) Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. 159: 112-1128

FDA (2001) *Shigella*. In: Bacteriological Analytical Manual Online. Authors: Wallace H. Andrews and Andrew Jacobson. Chapter 6

Iwamoto, M., Ayers, T., Mahon, B. E. and Swerdlow, D. L. (2010) Epidemiology of seafood-associated infections in the United States. Cli. Microbiol. Rev. 23(2): 399-411

Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L. and Sansonetti, P. J. (1999) Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull. World Health Organ. 77: 651-66

Lindqvist, R. (1999) Detection of *Shigella* sp in food with a nested PCR method-sensitivity and performance compared with a conventionalculture method. J. Appl. Microbiol. 86: 971-978

Mokhtari, W., Nsaibia, S., Majouri, D., Hassen, A. B., Gharbi, A. and Aouni, M. (2012) Detection and characterization of *Shigella* species isolated from food and human stool samples in Nabeul, Tunisia, by molecular methods and culture techniques. J. Appl. Microbiol. 113: 209-222

Novotny, L., Dvorska, L., Lorencova, A., Beran, V. and Pavlik, I. (2004) Fish: a potential source of bacterial pathogens for human beings. Vet. Med. Czech. 49(9): 343-358

Obaidat, M. M. and Bani Salman, A. E. (2017) Antimicrobial Resistance Percentages of Salmonella and Shigella in Seafood Imported to Jordan: Higher Percentages and More Diverse Profiles in Shigella. J Food. Prot. 80(3): 414-419

Reeve, G., Martin, D. L., Pappas, J., Thompson, R. E. and Greene, K. D. (1989) An outbreak of shigellosis associated with the consumption of raw oysters. New Engl. J. Med. 1990; 321(4): 224-7

Senachai, P., Chomvarin, C., Wongboot, W., Boonyanugomol, W. and Tangkanakul, W. (2013) Duplex PCR for detection of *Salmonella* and *Shigella* spp in cockle samples. Southeast Asian J. Trop. Med. Public Health. 44(5): 866-874

Sichewo, P. R., Robert, K. Gono, R. K., Muzvondiwa, J. V. and Sizanobuhle, N. (2013) Isolation and identification of pathogenic bacteria in edible fish: A case study of Fletcher dam in Gweru, Zimbabwe. Int. J. Sci. Res. 2(9): 269-272

Sujatha, K., Senthilkumaar, P., Sangeetha, S. and Gopalakrishnan, M. D. (2011) Isolation of human pathogenic bacteria in two edible fishes, *Priacanthus hamrur* and *Megalaspis cordyla* at Royapuram waters of Chennai, India. Indian J. Sci. Technol. 4(5): 539-541

WHO (2005) Guidelines for the control of shigellosis, including epidemics due to *Shigella dysenteriae* type 1. Geneva

Taneja, N. and Mewara, A. (2016) Shigellosis: Epidemiology in India. Indian J. Med. Res. 143(5): 565-576