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The Occurrence of Emerging Human Pathogen *Shewanella algae* in Shrimp Seafood

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ABSTRACT

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Shewanella algae is an emerging pathogen with an increasing rate of association in clinical samples worldwide. Exposure to marine and brackish waters has frequently been linked to human infection. The present study has examined the prevalence of the bacterium in shrimp which is a brackish water crop. The bacterium was isolated from about 8% of the shrimps' samples cultured and sold in different parts of West Bengal, India. The isolates produced hemolysin and were thus considered virulent. An increase in the occurrence of human infections caused by *S. algae* and a significant presence of the bacterium in shrimp warrants need for higher surveillance of the pathogen in clinical samples.

Introduction

Shewanella algae are one of the emerging bacterial pathogens (Dey *et al.*, 2015). The bacterium causes a wide range of human infections from acute gastroenteritis to osteomyelitis, skin and soft tissue ulcer, ear and eye infection, inflammation conditions of bones and joints with pre-existing wounds which may lead to bacteraemia and sepsis,

especially among immuno-compromised patients from different parts of the world (Nozue *et al.*, 1992; Domínguez *et al.*, 1996; Holt *et al.*, 1997; Botelho-Nevers *et al.*, 2005; Jampala *et al.*, 2015; Shu-Ying-Tseng *et al.*, 2018).

In association with other pathogens, it has been implicated in neonatal sepsis, meningitis, pneumonia, infective

endocarditic, post-operative peritonitis and chronic obstructive pulmonary disease (COPD) with acute exacerbation (Dhawan *et al.*, 1998; Mukhopadhyay *et al.*, 2007; Charles *et al.*, 2015; Torri *et al.*, 2018). Although the majority of the *S. algae* isolates show susceptibility to aminoglycosides, quinolones, third-generation cephalosporins, and β -lactamase inhibitors, resistance to multiple antibiotics or antimicrobials are increasingly being reported raising serious concerns (Holt *et al.*, 2005; Botelho-Nevers *et al.*, 2005; Jampala *et al.*, 2015). The bacterium produces a battery of virulence factors such as adhesion, lipopolysaccharide, siderophores, production of exoenzyme, hemolysins and tetrodotoxins (Martino *et al.*, 2003; Paździor, 2016).

Several *Shewanella* species are a natural inhabitant of marine and brackish water but can adapt to freshwater (Winn *et al.*, 2006) and fish, shellfish and other seafood have been reported to harbor the bacterium (S.Y. Tseng *et al.*, 2018). *S. algae* have been detected from human borne illness while other species, especially, *S. putrefaciens* is more associated with fish disease. In Japan, the bacterium was originally isolated from red alga which produces tetrodotoxin (Simidu *et al.*, 1990) and the incidences of *Shewanella* infection were increased drastically in the following years due to consumption of raw fish (Otsuka *et al.*, 2007). Furthermore, individuals with hepatobiliary diseases have been more infected with *S. algae* infection from ingestion of raw seafood in Asian countries like Japan, China, Vietnam, Thailand, etc. (Liu *et al.*, 2013; Tseng *et al.*, 2018). As such, water, fish and shellfish might be the major sources of the bacterium to human.

Human cases of *S. algae* infection are increasingly being reported from India and other countries in recent years. Increasing

water temperature due to global warming has been linked to it (Bauer *et al.*, 2019); making people of tropical countries more prone to the infection than their temperate counterparts (Tseng *et al.*, 2018; Bauer *et al.*, 2019).

Exposure to the marine environment and consumption of seafood has frequently been associated with human infections. Shrimp is one of the most traded items globally with an annual turnover of almost 4 million tonnes in 2018 (FAO, 2018). West Bengal is maritime and one of the major shrimp producing states of India with an annual production of 76543 tonnes during FY 2017-18 and whereas in India the annual production of shrimp and prawn was 682142 metric tonnes in quantity during 2018-19 (MPEDA, Govt. of India). The per capita consumption of fish and shellfish was 9 kg during 2017-18 (DOF, Govt. of India). *S. algae* infection in humans has also frequently been reported from this country, suggesting a probable link between human infection and shrimp (Jampala *et al.*, 2015). The objective of this study was to examine the prevalence of *S. algae* in shrimps to find a probable source of human infection and towards the safety of the shrimp growers, sellers and consumers.

Materials and Methods

Sample collection

A total of 112 fresh shrimp samples which included *Penaeus monodon*, *P. indicus*, *L. vannamei* were collected from different domestic retail markets in and around Kolkata, West Bengal.

These shrimps are produced locally in coastal districts of the states and a part of the product is marketed fresh locally. The samples were kept in sterile plastic bags, brought to the laboratory under ice cover and processed without delay.

Bacterial isolation

The whole shrimp samples were cut into small pieces by sterile scissors. The muscles, gut, hepato-pancreatic tissues were homogenized to 10% w/v in sterile PBS and 5 ml of the homogenate was enriched in 100 ml marine broth (MB) 2216 (BD Difco™, USA). After 48 h incubation at 30°C a loop full of enrichment culture was streaked on to marine agar (MA) 2216 (BD Difco™, USA) and incubated at 30°C for 2 days. Orange-yellow or pink colonies with smooth, circular and convex surface and entire margin were selected and grown to pure culture (Tseng *et al.*, 2018).

Identification of the bacteria

The bacterial isolates were identified by Gram staining, biochemical reactions such as oxidase, catalase reaction, hydrogen sulfide (H₂S) and indole production, utilization of carbohydrate (arabinose, glucose, and sucrose) and fatty acids, growth in 6.5% NaCl following standard methodologies (Khashe and Janda 1998; Vogel *et al.*, 2005).

Molecular identification of bacteria

The bacterial gene encoding 16S rRNA was PCR amplified using universal bacterial 27f (5'-AGAGTTTGATCCTGGCTCA G-3') and 1492r (5'-GGTACCTTGTTACGACTT-3') primers. The template DNA was obtained by extracting the genomic DNA using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) from a fresh colony grown on marine agar 2216. The PCR reaction was performed in 50 µl volume containing 25 µl of Red Taq Ready Mix (Sigma), 0.2 µ each of forward & reverse primers and template DNA. The following cycle was used for PCR reaction: initial denaturation at 95°C for 1 minute, followed by 30 cycles at 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 2

minutes and a final extension at 72°C for 5 minutes. The PCR products were visualized on a 1.5% agarose gel; a 100 bp DNA ladder (Sigma) was included on each gel for product size estimation. PCR products were gel purified using a QIAquick gel extraction kit (Qiagen) and sequenced by the Sanger sequencing method. Sequenced data were edited and aligned using CodonCode Aligner software. The identification of the isolates was determined following the BLAST search of sequence homology in NCBI GenBank and RDP databases. The phylogenetic tree of the identified bacteria from the nearly complete 16S rDNA gene sequences was prepared with gene sequences of reference/type strains using MEGA6 software.

Haemolysin production

Haemolysin production by the bacterial isolates was examined by streaking of bacteria on 5% sheep blood agar (HiMedia, India). The plates were incubated at 37°C and examined for hemolytic activity.

Results and Discussion

A total of 14 presumptive *Shewanella* bacterial strains were initially isolated from 112 shrimp samples. Based on cell morphology and biochemical reactions 11 strains, isolated from a total of 9 samples, were identified as *S. algae*. The prevalence of *Shewanella* sp. in different shrimp species collected from different markets of West Bengal is given in Table 1. The bacteria were detected in all the shrimp species examined: 8.11% of *Penaeus monodon*, 11.76% of *P. indicus*, and 4.76% of *L. vannamei* shrimp samples were contamination with *S. algae*.

The identified *S. algae* strains were oxidase-positive, indole negative, citrate-negative, urease positive, produced black coloration in butt portion of Triple Sugar Iron agar slant

due to hydrogen sulfide (H₂S) production. The isolates had grown on Salmonella-Shigella agar (SS agar) at 42 °C, and in presence of 6.5% NaCl and were beta-hemolytic on 5% sheep blood agar, but failed to produce acid from sucrose, maltose, and arabinose. Detailed biochemical reactions of three strains are given in Table 2.

Identification of the isolates was further confirmed from 16S rDNA sequences and three sequences have been submitted to the GenBank database (Accession nos. JQ265998, JQ26605, and JQ266007). The 16S rDNA sequences of the isolates showed

the highest % identity with *S. algae* and moderate genetic distance from some of the other *Shewanella* species reported from different parts of the world (Fig. 1).

This is possibly the first report showing the presence of *S. algae* in shrimp. Shrimp is one of the commonly available, affordable and most traded seafood globally as well as in this part of India and the presence of this emerging human pathogen raises a serious public health concern. The bacterium was detected in as many as 8% of the shrimp samples tested, which matches the findings of Tseng *et al.*, (2018).

Table.1 Prevalence of *S. algae* in different shrimp samples examined

	Scientific name	Habitat ^a	Place of sample collection	Number of samples examined	Number of samples contaminated	Percentage of Samples contaminated
Tiger shrimp	<i>Penaeus monodon</i>	Marine & estuarine water	Garia (Kolkata)	20	0	0.00
White shrimp	<i>P. indicus</i>	Marine & estuarine water	do	6	1	6.25
Vannamei shrimp	<i>L. vannamei</i>	Marine & coastal water	do	12	0	0.00
Tiger shrimp	<i>P. monodon</i>	Marine & coastal water	Sealdah (Kolkata)	20	2	10.00
Tiger shrimp	<i>P. monodon</i>	Marine & estuarine water	Diamond Harbour	18	3	16.66
Tiger Shrimp	<i>P. monodon</i>	Marine & coastal water	Kakdwip	16	1	6.25
White Shrimp	<i>P. indicus</i>	Marine & coastal water	Sealdah (Kolkata)	11	1	9.09
Vannamei Shrimp	<i>L. vannamei</i>	Marine & estuarine water	Namkhana	9	1	11.11
Total				112	9	8.04

^aHabitat of the shrimp species as per record

Table.2 Biochemical characteristics of *S. algae* strains

Test	Bacterial strain ID		
	KUGWB02	KUHWB220	KUKWB16
Oxidase	+	+	+
Catalase	+	+	+
Ornithine decarboxylase	+	+	+
OF (Glucose)	Oxidation	Oxidation	Oxidation
Acid production from glucose	-	-	-
Acid from arabinose	-	-	-
Acid from sucrose	-	-	-
H ₂ S production	+	+	+
Growth in 6.5% NaCl	+	+	+
Indole production	-	-	-
Urease	+	+	+
Beta Hemolysis	+	+	+

^a (+) indicates positive reaction, (-) indicates negative reaction

Table.3 Summary of human case reports of *S. algae* infection in India

City	Year	Number of affected persons	Comorbidities	Sign/Symptom	Exposure to seawater/ seafood	Reference
Manipal, Karnataka	2007	02	-	Vomiting, abdominal pain, Pneumonia	No	Mukhopadhyay <i>et al.</i> , 2007
Tirupati Andhra Pradesh	2010	05	Diabetes, cellulites, ulcers, breast carcinoma, diarrhoea	Ulcers, Gastroenteritis	unknown	Sharma <i>et al.</i> , 2010
Delhi	2011	01	Non healing ulcers	Ulcers	unknown	Goyal <i>et al.</i> , 2011
Dibrugarh , Assam	2010-2011	02	none	Bloody Diarrhoea	Fish	Nath <i>et al.</i> , 2011
Bengaluru Karnataka	2014	01	Chronic draining osteomyelitis	Squamous cell carcinoma	water	Sumathi <i>et al.</i> , 2014
Hubli Karnataka	2012		none	Acute gastroenteritis, bloody diarrhoea	unknown	Dey <i>et al.</i> , 2015
Kochi, Kerala	2010-2014		Hypertension, peripheral vascular occlusive disease, Hypertension Diabetes	Chronic ulcer, Gangrene and cellulitis of left toe	no	Jampala <i>et al.</i> , 2015
Pondichery	2015		Newborn	Sepsis	No	Charles <i>et al.</i> , 2015

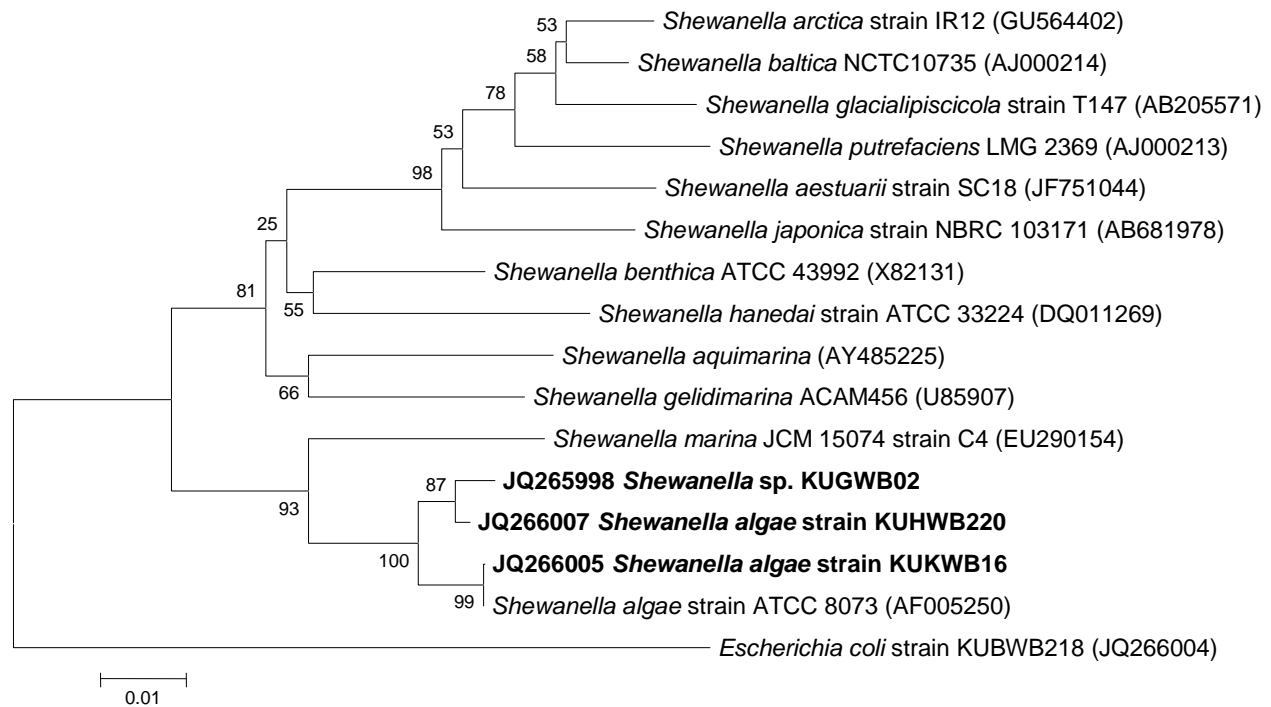


Fig.1 Phylogenetic tree of *S. algae* strains constructed by the neighbor-joining method (Saitou N. and Nei, 1987) and 1000 bootstrap replicates. Taxonomic positions of the present isolates (shown in bold) are compared with reference strains of few other *Shewanella* species. The evolutionary distances were computed by MEGA 6 (Tamura *et al.*, 2013) program using the Kimura 2-parameter method (Kimura, 1980)

Members of the genus *Shewanella* are widely found in marine and brackish water environments which include benthic and intertidal zones, sediments, terrestrial environments, oilfield wastes (Melvold, 2017). These shrimps are cultured in brackish water and it is assumed that the bacterium has come to shrimp from its culture environment. However, *Shewanella* can well adapt to freshwater environments and has been detected from the freshwater tilapia fish farm (Lu and Levin, 2010). In the present study, the bacterium was detected in all the shrimp species examined, with a higher presence in *P. indicus* and lower presence in *L. vennamei*. However, due to an insufficient number of total and contaminated samples, the difference in the level of contamination in different shrimp species was insignificant and we consider all the three species more or less equally contaminated.

The genus *Shewanella* is a gram-negative, oxidase-positive, catalase-positive, H_2S producing bacillus. *S. algae* and *S. putrefaciens* are two prominent species which have been studied in environmental samples, fish, and human, etc. The main distinguishing features between two species are *S. algae* can grow at $42^\circ C$ and in 6% NaCl and reduces nitrate, and does not produce acid from maltose (Holt *et al.*, 2005).

The present isolates showed typical biochemical reactions which were one of the bases of their identification as *S. algae*. Although *S. algae* produce several virulence factors, hemolysin production is easy to test and has often been found in clinical isolates (Holt *et al.*, 2005). A good number of *S. algae* isolates show β -hemolysis on 5% blood agar after 72 incubation at $37^\circ C$; production of hemolysin is a virulence mechanism and has

been correlated with clinical occurrences and severity of infection (Charles *et al.*, 2015).

Different *Shewanella* species have been identified from the gut of several wild caught Mediterranean fish species and were able to colonize the gut of zebrafish in an exposure study indicating the presence of the bacteria as part of fish gut microflora (Jammal *et al.*, 2017). *S. putrefaciens*, a common halophilic species associated with fish spoilage, cause skin disorders and haemorrhages in internal organs of marine and freshwater fishes (Kozinińska and Pekala 2004; Paździor, 2016). Unlike *S. putrefaciens*, *S. algae* have often been associated with human infections.

The present isolates produced hemolysin suggesting their virulent nature and there is a strong possibility that they might be associated with human infections. Table 3 shows that *S. algae* have been associated with several human ailments in India in last one decade: although a source of infection was not traced in many cases, the predominance of such cases in coastal and fish-eating states suggest a possible association of water and/or fish/shellfish with human infections. Seafood has been identified as the source of infection in a few cases (Nath *et al.*, 2011).

During the period from 1999 to 2017, a large number of *S. algae* infection cases have been reported from Asian countries like China, Japan, Malaysia and Iran, and water was an important source of contamination in 64% of cases (Tseng *et al.*, 2018). In India also *Shewanella algae* have been implicated in serious health hazards in humans like bacteraemia, otitis media, cellulitis, gastroenteritis, abscesses, soft tissue infections and wound infections directly through the exposure of seawater or consumption of raw seafood (Dey *et al.*, 2015).

Although the bacterium often causes disease with other pathogens, *S. algae* were the only pathogen in about 60% of human cases (Melvold, 2017). *S. algae* infections are more common in immuno-compromised patients, however, severe necrotizing fasciitis and bacteraemia have been recorded in healthy but stressed individuals exposed to sea with warmer water temperatures making it a potentially virulent organism (Bauer *et al.*, 2019).

There is a possibility that *Shewanella* infection is under-reported in developing countries like India due to lack of adequate infrastructure facilities for proper identification of this rare bacteria and lack of awareness which creates misidentification of this bacteria as *Pseudomonas* sp. (Shamanna *et al.*, 2018). The prevalence of the bacterium in about 8% of shrimp, which is very popular and affordable seafood, is quite high and alarming. This raises a significant risk of infection not only of the consumers but also for fish farmers and those involved in shrimp processing and marketing.

Thus, there is a need for increased surveillance for this pathogen in human clinical samples. Proper cooking, cleaning of processing and kitchen environments, and high level of personnel hygiene are recommended to reduce the chance of *S. algae* infection.

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