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Occurrence of *Cronobacter sakazakii* in seafood and its environment, Kerala, India

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ABSTRACT

The presence of these invasive pathogens in the seafood is a safety risk to handlers in the seafood production chain. However, the risk can be mitigated through hygienic handling of the fish and fishery products from pond to plate. In this study of *Cronobacter sakazakii* (*C. sakazakii*) was assessed from different seafood in different sampling sites *viz*., retail fish markets, supermarkets, fish landing centers, aquaculture farms of Ernakulum district, Kerala, India. One hundred and four seafood samples were collected and screened for the *C. sakazakii* both by microbiological and molecular methods. Overall, 8.7 % of samples harbored *C. sakazakii*. *C. sakazakii* was detected in fish products. A total of nine presumptive colonies of *C. sakazakii* were confirmed by PCR targeting *saka*, *crono*, and *ess* specific genes. The study also emphasizes that more fish products must be screened for understanding the prevalence of *C. sakazakii* and the survival kinetics of the pathogens in fish products.

Key words: *Cronobacter sakazakii, PCR, Prevalence, Fish and fisheries products*

Introduction

C. sakazakii is a recognized food-borne human pathogen emerging at the global level. Formerly, this bacterium was known as "yellow-pigmented *E. cloacae*" (Farmer *et al.,* 1980) and later as *C. Sakazakii* honouring Riichi Sakazakii, a Japanese bacteriologist (Iversen and Forsythe, 2003). *C. Sakazakii* belongs to the family *Enterobacteriaceae* (Drudy *et al.,* 2006) and this newly formed genus *Cronobacter* has six species of which *C. sakazakii* is a potent opportunistic pathogen. *C. sakazakii* causes a wide range of disease conditions *viz*., septicemia, meningitis, and necrotizing enterocolitis in infants and mortality range between 20 and 80% (Zhu *et al.,* 2011; Norberg *et al.,* 2012). The organism is widely distributed in the plant sources, and aquatic environment and has been isolated mainly from infant formulation and milk products, cereals, vegetables, and beverages (Kornacki *et al*., 1998; Muytjens *et al*., 1980 ; Cottyn *et al.,* 2001; Kuzina *et al.,* 2001; Kandhai *et al.,* 2004). However, the outbreaks of the diseases are more linked to the powdered infant formulations (Iversen and Forsythe, 2004; Ray *et al.,* 2007; Norberg *et al.,* 2012). Although the incidence of C. *sakazakii* contamination in meat products, raw milk, and ready-to-eat food is widely reported (Watanabe *et al., 1994*; Montgomery *et al.,* 2002), scant information is available in the seafood (fish and fishery products) (Miranda *et al.,* 2003).

Several methods were adopted, revised and followed for the isolation and identification/ detection of *C. sakazakii* viz., pre-enrichment (buffered peptone water), enrichment (Enterobacteriaceae Enrichment, EE broth; modified Lauryl sulfatetryptose broth, (mLSTB), selective plating (VRBA, EMB), chromogenic medium (Druggan-Forsythe-Iversen agar; *E. sakazakii* chromogenic plating medium) and followed by biochemical and molecular confirmation (Iversen *et al.,* 2007; Iversen *et al.,* 2008 and Restaino *et al.,* 2006). The proposals of combining culture-dependent and independent methods were also followed after several revisions by involving PCR in the enrichment step (Chen *et al.,* 2009). For the PCR confirmation, the genes viz., 16S *rRNA*, *rpoB*, *gyrB*, *cgcA*, and *ompA* were commonly used for *C. sakazakii* detection (Li *et al.,* 2016). This method of combined culture dependent and independent is currently recommended by FDA for the detection of *C. sakazakii* in powdered infant formulations. Therefore, the present work aimed to investigate the occurrence of *C. sakazakii* in fish fishery products using conventional microbiological and molecular methods and to also assess the antimicrobial resistance.

Materials and Methods

A total 104 numbers of seafood fresh/dry samples which included, shellfish, finfish, water and ice from local fish markets, fish landing centers, supermarkets and aquaculture farms were screened for the occurrence of *C. sakazakii*. Details of samples and the location are shown in (Table 1). The collected samples were brought to the laboratory in sterile polythene bags. Briefly 10 g sample was enriched in 90 mL(1:10) Buffer peptone water (BPW, pH 7.4) overnight at 37 °C and enriched samples were streaked on to Brilliance *Enterobacter sakazakii* agar (DFI formulation) DFI and *Enterobacter sakazakii* chromogenic plating agar (R&F) for selective isolation of *C. sakazakii* as per a modification of USFDA, BAM protocol by excluding PCR confirmation of direct BPW enrichment broth (Restaino *et al*., 2006; Kilonzo-Nthenge *et al*., 2012). After 24 h of incubation at 37°C blue/green colonies (Fig 1a & 1b) were picked for biochemical testing *viz*., Gram's reaction, ability to acidify (methyl-á-D-glucopyranoside), Indole test, Catalase test, and further for malonate, maltitol, melezitose, inositol, dulcitol, and lactose utilization test done as per (Chen *et al.,* 2009). MGP positive and lactose, malonate and maltilol utilizing isolates with oxidase negative, indole, dulcitol, melizitose non-utilization and inositol variable reactions isolates cultures were further confirmed by PCR.

The crude DNA extracted from TSB broth culture (overnight) 1500 µl of isolated cultures was taken

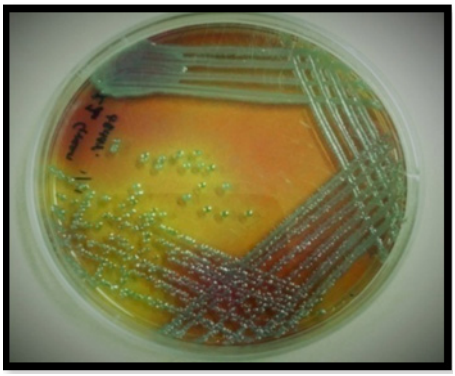

Fig. 1a. *Cronobacter* colonies on R&F agar blue-black to blue-grey, 1 to 2 mm in diameter, and raised (convex) with or without clear halos

Table 1. Details of sampling location and their GPS coordinates

S.No	Sampling Location	GPS coordinates
1	Cherai Roadside Market	10°08′02.3"N, 76°11′47.1"E
2	Munambam Landing Centre	10°10′57.4"N, 76°10′05.9"E
3	Thevara Fish Market	9°56′39.4"N, 76°17′38.8"E
4	Kalamassery fish market	10°03′10.3"N, 76°19′19.1"E
5	Kadavanthra Retail Market	9°58′11.8"N, 76°17′42.2"E
6	Ettumanoor, Vaikkom	9°40′08.6"N, 76°33′22.6"E
7	Murinjupuzha, Bismi	9°49′20.2"N, 76°23′23.6"E
8	Broadway Market	9°58′52.4"N, 76°16′40.4"E
9	Kumbalangii Farm	9°51′04.2"N, 76°17′07.8"E
10	Munambam	10°10′58.4"N, 76°10′13.6"E
11	Vaikkom	9°45′17.3"N, 76°23′18.1"E
12	Vypin	9°58′56.0"N, 76°14′34.1"E

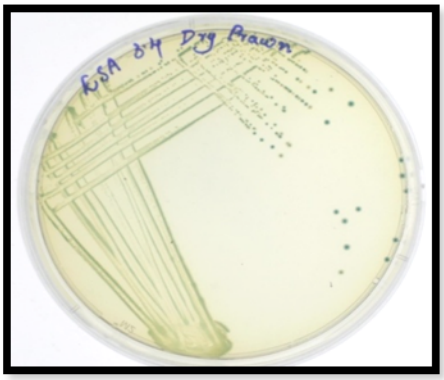

Fig. 1b. Cronobacter colonies on DFI agar.

directly in 2ml microfuges and centrifuged at 8000rpm for 10 min and the aqueous wash was discarded. To the cell pellet 500 µl of 1X TE buffer was added and washed and the same step was repeated. To the washed cell pellet, 500 µl of 1X TE buffer was added and kept in a dry bath for 10min at 95 °C for cell lysis and the extracted crude DNA was shifted to -20°C for future use. Three sets of specific primers *saka*, *crono* and *ess* gene (Active, ILS, India) of *Cronobacter sakazakii* was used for DNA amplification by PCR was carried out in a 25 µl final volume in 0.2 ml PCR tubes (Veriti Thermal cycler). Briefly, reaction mix was prepared by adding 50 ng DNA, 10 pmol of each forward and reverse primer, 200 µM dNTPs mix, 0.75U Taq polymerase, 1.5 mM $MgCl_2$, 1x buffer and volume was made up to 25 µl with sterile nuclease-free water. Primers and amplicon sizes of the three genes were CronoF-GGGATATTGTCCCCTGAAACAG, CronoR–CGAGAATAAGCCGCGCATT (amplicon-199bp; Chen *et al*., 2009); SakaF-ACAGGGAGCAGC TTGCTGC and Saka RTCCCGCATCTCTGCAGGA (amplicons-952bp); ESSRF–GGATTTAACCGTGA ACTTTTCC & ESSRR -CGCCAGCGATGTTAGA AGA (amplicon - 469bp). PCR cycling conditions followed for the three types of PCR were 95 °C for 3 min; 35 cycles of 95 °C for 20s - 60s each; Variable 52 °C for 20s for*crono* gene, 50 ºC for *saka* gene, 60ºC for *ess* gene; 72 °C for 60s based on length; with a final extension of 5 minutes at 72°C. The PCR products were run on 2% Agarose gels and electrophoresis in (Fig. 2).

Results and Discussion

This is study of prevalence of *C. sakazakii* in fishery products of retails markets and fish landing centers in Ernakulum, Kerala. The possible source of con-

Table 2. Occurrence of the fish and fishery products and different landing Centres of Ernakulum District, Kerala

Types of sample	No. of Samples	Percent Occurrence
Raw Finfish	33	9.09
Raw Shellfish	19	5.26
Dry Finfish	15	26.66
Dry Shellfish	14	7.14
Ice	13	Nil
Water	10	Nil
Total	104	8.65

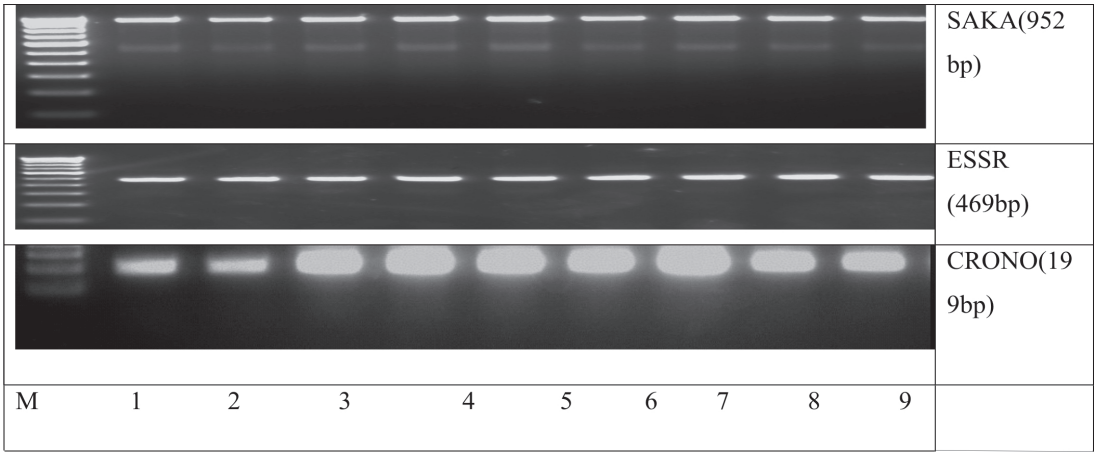

Fig. 2. PCR result of three genes of *C.sakazakii* in nine samples of dry and fresh fish. Lane M: Gene Ruler 100bp Ladder (Thermo Scientific, USA); lane 1: Shrimp; lane 2: Cobia 1; lane 3: Hilsa; lane 4: Dry anchovy1; lane 5: Dry anchovy2; lane 6: Dry shrimp; lane 7: Mackerel; lane 8: Dry prawn; lane 9: Crab

tamination to seafood is post-harvest handling in unhygienic conditions. In the present study, *C. sakazakii* was identified in 8.6% (9/104) of the samples, among them 4 were dried fishes/ shrimp This pathogen is associated with a variety of domestic food and food processing factories. Total 104 numbers of Seafood fresh/dry samples which include shellfish, finfish, water and ice from local markets, fish landing centers, supermarkets were screenedemploying biochemical and molecular methods. With 9 positive among 104 samples screened which amounts to 8.7% prevalence of *C. sakazakii* **(Table 2)**. This investigation addresses the isolation of *C. sakazakii* in nine samples (8.6%) in fish and fishery products namely Shrimp, Cobia, Hilsa, dry anchovies, mackerel, dry prawn. The presumptive Cronobacter isolates of 28 from 104 samples on *Cronobacter sakazakii* agar (Oxoid, Fig. 1a) appeared either dark green or weak green having a green center with a white/yellow border on Cronobacter sakazakii agar (Himedia, Fig 1b) appear with the red background were observed to be Gram-negative, non-spore forming rods, with catalase production, oxidase test negative and utilization of MGP. The cultures characterized for the biochemical characters revealed that all the isolates were biochemically similar viz., positive reactions for MGP Test, lactose fermentation, mannitol utilization, and other tests such as Oxidase, indole, dulcitol, malonate, melibiose utilization reactions. Variable inositol utilization was observed, 5 out of 9 and none of the isolates of *C. sakazakii* utilized inositol.

Cronobacter has also been recovered from a range of foods including meats, poultry, rice and other grains, vegetables, herbs and spices, dry food ingredients, breads, milk, cheese, sausage, tofu and sour tea (7; 8). This study asks for a nationwide surveillance of this emerging pathogen in fishery products and compares the diversity and elucidates the possible entry of this pathogen into the seafood. More surveillance programs need to be designed for dried fishery products and also survival kinetics of this pathogen in the dried fishery products for the better implementation of the control measures. This may be attributed to the more recovery in desiccated milk powders and also desiccation and heat tolerance of the pathogen compared to others. The key areas prioritized for future research are establishing critical control points for the management of *C. sakazakii*, and knowledge on possible virulence factors of *C. sakazakii* and improved detection methods to identify the reservoirs and contribute to eliminate this pathogen from the final fish product. (Kilonzo-Nthenge *et al*., 2012) detected *C. sakazakii* in 26.9% of domestic kitchens samples in Tennessee, USA.

Conflicts of Interest: The authors declare no conflict of interests.

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