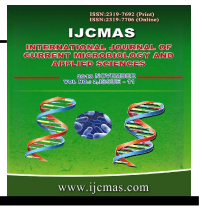
Int.J.Curr.Microbiol.App.Sci (2013) 2(11): 300-306

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 11 (2013) pp. 300-306**
http://www.ijcmas.com



Original Research Article

Diagnosis of virulent strains of motile *Aeromonas* from commercial food

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A B S T R A C T

This research investigation was carried to isolate and identify the virulent strains of motile *Aeromonas* from commercial food products across Tamil Nadu, India. A total of 389 food samples were aseptically collected throughout the year and processed for the isolation and identification of motile *Aeromonas* by performing arrays of morphological, biochemical and phenotypical tests, along with their virulence determination by polymerase chain reaction. A total of 72 (18.50%) tentative isolates of *Aeromonas* were identified as *A. sobria* 34 (47.22%) and *A. hydrophila* 38 (52.77%). In duplex PCR, extracellular haemolysin gene (*ahh*1) was detected from 14 (41.17%) isolates of *A. sobria* and 21 (55.26%) isolates of *A. hydrophila*, whereas, *A. hydrophila* aerolysin gene (*aer*A) gene was detected along with the *ahh*1 gene in 26 (68.42%) isolates of only *A. hydrophila*. In single step PCR, *A. sobria* haemolysin gene (*asa*1) was detected from 31 (91.17%) isolates of *A. sobria*. None of the isolates were positive for the *A. caviae* haemolysin gene (*cav*1) of *A. caviae*. The present investigation suggested the occurrences of virulent strains of motile *A. sobria* and *A. hydrophila* in human consumable food and food products impending a high risk of food borne illness.

Keywords

Aeromonas hydrophila; Aeromonas sobria; Food; PCR; Virulence.

Introduction

Aeromonas are ubiquitous, aerobic, gram negative, rod shaped bacteria which are able to survive and multiply even at very low temperatures. Fourteen species of *Aeromonas* have been described till date, of which motile species like *A. hydrophila, A. caviae, and A. veronii* biovar *sobria* have been associated with numerous human diseases (Walker, 2003), also linked with many diseases in both cold-and other warm-blooded animals. Among the diseases that are associated with the *Aeromonas*, gastroenteritis and wound infections are significant. Gastroenteritis typically occurs after the ingestion of contaminated water or food, whereas wound infections result from exposure to contaminated water. Foods of animal origin, seafood, fish and vegetables have also been considered as major carrier of

Aeromonas infections (Mattick and Donovan, 1998). There are several report that suggested that motile *Aeromonas* isolates can carry either one or multiple virulence factors (e.g. endotoxins, aerolysin/hemolysin, proteases, lipases, DNases, adherence factors etc.) that may play an important role in the development of disease, either in humans or in fishes (Castro-Escarpulli *et al*., 2003).

In this perspective, the detection of the *Aeromonas* requires rapid and specific methods, which assist in the control of potentially pathogenic microorganisms to enter from various food sources to human populations. Even though PCR is very effective with pure culture, its applications to food samples are limited by the complex composition of food matrices that can inhibit the assay and it cannot differentiate the DNA from live cells or dead cells. To defeat these problems, enrichment of sample and a sample preparation step earlier to the PCR analysis is necessary. The present research has been undertaken with the aim to isolate and identify the motile *Aeromonas* species and to standardize a rapid, sensitive and specific PCR based assay to detect virulence strains of motile *Aeromonas* from various food and food products.

Materials and Methods

Sample collection

A total of 389 samples comprise of meat, fish products, milk and dairy products, raw vegetables and products, bakery products, beverage and fermented rice products were investigated. All the samples were procured from various randomly selected local retail shops, markets and departmental store of Salem, Erode, Tiruppur, Namakkal and Coimbatore Districts of Tamil Nadu, India and immediately transferred to the laboratory for microbiological analysis.

Isolation of Bacteria

Commercial food samples (5 grams each) were weighed and homogenised for 2 min in 50 ml of alkaline peptone water. Samples were inoculated aseptically into sterile Brain heart infusion broth (Himedia, Mumbai) and incubated aerobically at 28°C for 24h. Bacterial colonies were purified based on the size, shape, colour and patterns of haemolysis and non haemolysis on 5% sheep blood agar by incubating at same temperature for 24h. For selective isolation, purified colonies were also streaked into Aeromonas selective agar with supplements (Himedia, Mumbai) and Ampicillin dextrin agar with selective supplements (Himedia, Mumbai) and incubated at above mentioned temperature for 24h. Finally, isolates were maintained on nutrient agar slants for various microbiological studies.

Morphological, biochemical and phenotypical characterization

Suspected colonies of *Aeromonas* were identified based on the colony morphology, pattern of haemolysis/ non haemolysis, motility test, Gram's and flagellar staining followed by arrays of biochemical tests like indole production, Voges–Proskauer test, esculin hydrolysis, cytochrome oxidase, catalase test, triple sugar iron agar slant test for gas and acid production from glucose, sucrose and lactose and hydrogen sulphide production (Altwegg, 1989) and phenotypic characterization like haemolytic, amylolytic, lipolytic, casein hydrolysis,

gelatinase activity, nuclease activity, congo red dye uptake were studied (Das *et al*., 2012).

Virulence determination of motile *Aeromonas* by polymerase chain reaction

To determine the virulence factors present in the isolates of motile *Aeromonas*, all the tentative isolates were screened with the *Aeromonas hydrophila* aerolysin (*aer*A; 309bp) and extracellular hemolysin genes (*ahh*1; 130bp) by duplex PCR and *Aeromonas sobria* hemolysin gene (*asa*1; 249bp) and *Aeromonas caviae* hemolysin gene (*cav*1; 381bp) by single step PCR according to Das *et al*. (2013). All the oligonucleotide primers for the conserved regions of *aer*A, *ahh*1, *asa*1 and *cav*1 genes were commercially synthesised (Eurofins Genomics Pvt. Ltd, Bangalore). *A. hydrophila* (MTCC 646) and *A. sobria* (MTCC 3613) were used as positive controls and *Escherichia coli* (MTCC 723) were used as negative controls. There were three sets of PCR.

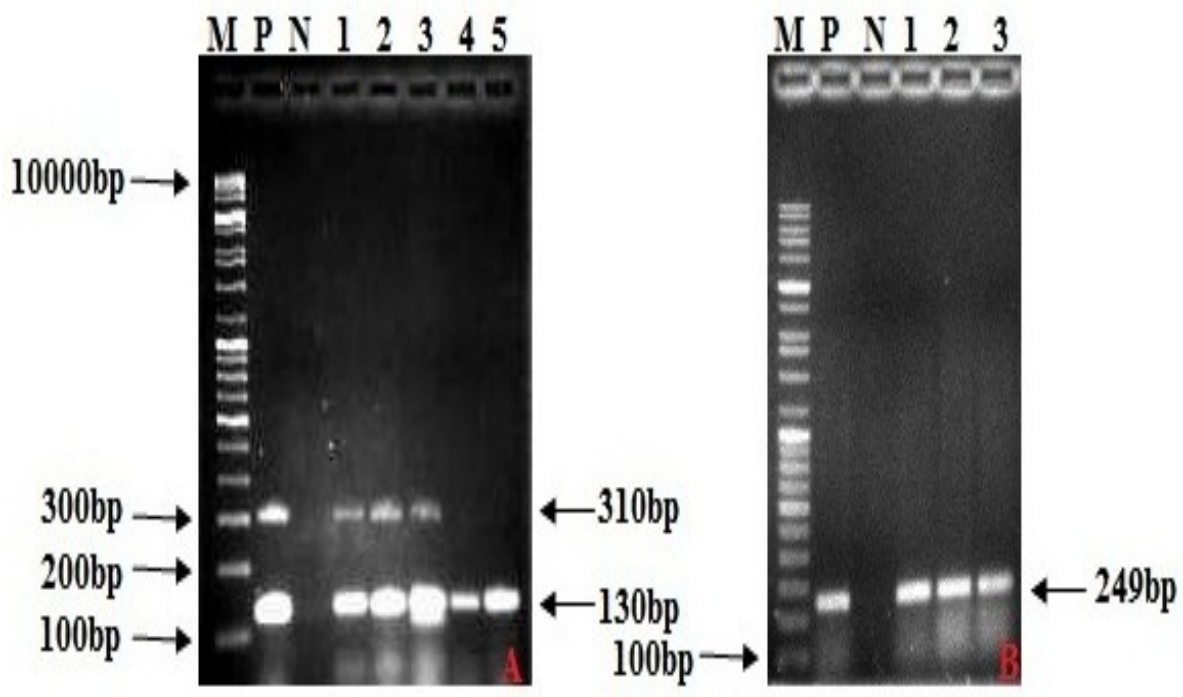
PCR assay and agarose gel electrophoresis

It was set up in 25µl reaction volume containing 12.5 µl of 2× PCR master mix (Promega, USA) containing 4mM magnesium chloride, 0.4 mM of deoxynucleotide triphosphates (dNTPs), 0.5U of *Taq* DNA polymerase, 150mM trishydrochlroric acid, pH 8.5 (Promega, USA), 2µM of primers (*ahh*1-F and ahh1-R), 1.5µM of primers (*aer*A-F and *aer*A-R; *asa*1-F and *asa*1-R) and 1µM of primers (*cav*1-F and *cav*1-R) and 2.5µl of template DNA. The PCR reactions were performed in thermal Cycler (Eppendorf, USA). After initial denaturation at 94°C for 5 min, the amplification cycle (35×) for *ahh*1, *aer*A and *asa*1 genes had denaturation, annealing and extension at 94°C, 59°C and 72°C for 30s, 30s and 30s respectively, while amplification cycle (30×) for *cav*1 gene had denaturation, annealing and extension at 94°C, 65°C and 72°C for 2 min, 1 min and 1 min respectively. In each PCR, final extension was performed at 72°C for 10 min. The PCR amplicons (5µl) were electrophoresed in 1.5% agarose gel in TAE (tris-acetic acid-EDTA, pH 8) buffer, stained with ethidium bromide and observed in gel doc system (Universal Hood, BIORAD, Italy).

Results and Discussion

A total of 72 (18.50%) isolates of *Aeromonas* consisting of meat (28), fish products (15), milk and dairy products (10), raw vegetables and products (7), bakery products (6), beverages (5) and fermented rice products (1) were tentatively identified out of 389 food samples obtained from the Salem, Erode, Tiruppur, Namakkal and Coimbatore Districts of Tamil Nadu, India (Data table not shown). The origin and subsistence of various *Aeromonas* species in aquatic environments, including ground water, surface waters, drinking water and wastewater (Holmes *et al*., 1993) and in commercial foods (Das *et al*., 2012), like fresh grocery products, seafood, raw meats, packaged ready-to-eat meats, and even in raw milk (Palumbo, 1996) have been reported. After detailed morphological, biochemical and phenotypical analysis, 34 (47.22%) and 38 (52.77%) isolates of 72 were identified as *A. sobria* and *A. hydrophila* respectively (Table 1). Similar isolation and identification of *A. sobria* and *A. hydrophila* from the commercial food samples and cat fish infections have been

Figure.1 Virulence determination of motile *Aeromonas* by PCR. A: *ahh*1 gene (130bp) and *aer*A *gene* (310bp); B: *asa1* gene (249bp)

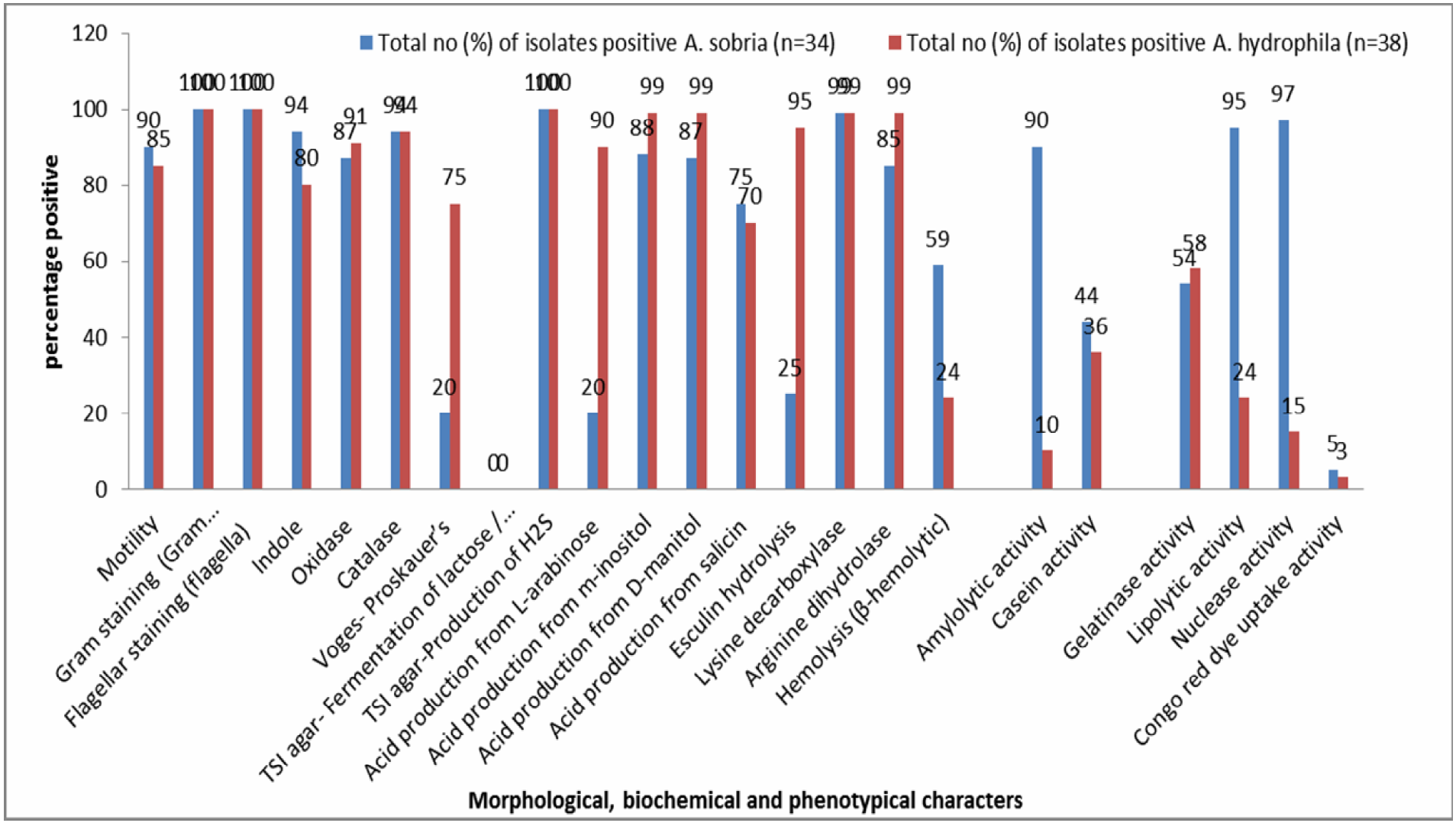


In figure A, P: positive control (MTCC 646), N: negative control (MTCC 723), Lane 1-5: field isolates; In figure B, P: positive control (MTCC 3613), N: negative control (MTCC 723), Lane 1-3: field isolates; Lane M: High range DNA marker

reported from India (Shome *et al*., 2006; Lakshmanaswamy *et al*., 2010; Das *et al*., 2012) and in other countries.

In duplex PCR, *ahh*1 gene was detected from 14 (41.17%) isolates of *A. sobria* and 21 (55.26%) isolates of *A. hydrophila*, but *aer*A gene was detected along with the *ahh*1 gene in 26 (68.42%) isolates of only *A. hydrophila* (Figure 1). In single step PCR, *asa*1 genes were detected from 31 (91.17%) isolates of *A. sobria* (Figure 1). None of the isolates were positive for the *cav*1 gene of *A. caviae*. Detection dual genes like *ahh*1 and *aer*A from the isolates of *A. hydrophila* (Das *et al.*, 2013) and the detection of *asa*1 from the isolates of *A. sobria* have been reported (Das *et al.*, 2013) from various clinical cases of animal and human origin (Wang *et al*., 2003). Similar to the present study researchers have not detected none isolates and *cav*1 genes from the food sample (Das *et al*., 2013). In this research, out of 72 isolates of Aeromonas, 29 (40.27%) were found haemolytic in 5% sheep blood agar and rest 43(59.72%) were non haemolytic. However, same isolates when tested for the presence of various haemolysin and aerolysin (like haemolysin) genes viz. *ahh*1, *aer*A and *asa*1 by PCR showed 100% positive result, suggested that the isolates those were phenotypically negative for haemolysin production, can be positive for that gene by PCR,

Figure.2 Morphological, Biochemical and phenotypical characterization of motile *A. sobria* and *A. hydrophila*



indicating that other factors affect gene expression (Wang *et al*., 2003) has been reported. The analysis of virulence factors produced by the two species of motile Aeromonas by PCR suggested that single isolates those carried the genes encoding for multiple virulence factors can be dangerous and more prone to cause food borne illness in human. PCR based identification of virulent strains of motile *Aeromonas* appeared to be very useful, sensitive and less time consuming method than traditional microbiological identification system (Das *et al*., 2013). The results aided our efforts to prove the strong occurrences of *A. sobria* and *A. hydrophila* as food borne pathogens in human consumable foods than in the environmental samples.

The research investigations confirmed the significant occurrences of motile and virulent *Aeromonas* like *A. sobria* and *A. hydrophila* in consumable food and food products. It is a high matter of concerned now for the food industries to use natural food preservatives to control the growth of these emerging food borne pathogens before entering into the food chain. The research data provided by our study showed that *Aeromonas* infections in food and food products has to be treated seriously as food infections can lead to various health hazards both in human and animal populations.

Acknowledgement

The authors wish to acknowledge the financial grant vide reference no. 20/AICTE/RIFD/RPS (POLICY-1)28/2012-13 under Research Promotion Scheme of All India Council of Technical Education (AICTE), New Delhi to execute this research work as a part of the funded project. Authors would also like to convey thanks to Bannari Amman Educational Trust for the kind support.

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