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Surveillance of *Aeromonas sobria* and *Aeromonas hydrophila* from commercial food stuffs and environmental sources

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Abstract

In tropical countries like India, commercial food items are often contaminated by various food-borne pathogens. Present research work reports the surveillance of *A. sobria* and *A. hydrophila* from commercial food stuffs and environmental sources across Tamil Nadu and Kerala, India. Samples were aseptically collected throughout the year and processed for isolation and identification of *A. sobria* and *A. hydrophila*. Isolates of *Aeromonas* were characterized for arrays of biochemical and phenotypic traits and finally assayed for antibiotic susceptibility test. A total of 71 suspected *Aeromonas* strains were isolated from 154 commercial food and environmental samples. Upon biochemical characterization of these isolates, 56(79%) were identified as *A. sobria* and remaining 15(21%) isolates were *A. hydrophila*. Upon detailed biochemical and phenotypical investigation, distinguishable results were obtained on esculin hydrolysis, acid production from L- arabinose, amylolytic, lipolytic and nuclease activities. All the isolates were 100% resistance to ampicillin, carbenicillin, cephalothin and clindamycin; 100% sensitive to colistin and moderate to cefuroxime, chloramphenicol, nalidixic acid, neomycin and nitrofurantoin. The present research suggested that colistin could be useful for motile Aeromonas infection but there has also been prevalence of multi drug resistant strains of Aeromonads in the Sothern states of India. 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.

Keywords: Aeromonas hydrophila, Aeromonas sobria, Antibiotic susceptibility

INTRODUCTION

Motile mesophilic *Aeromonas sobria* and *A. hydrophila* are ubiquitous, aerobic, Gram negative bacteria. These bacteria are frequently isolated from various food products such as fish and shellfish, raw meat, vegetables and raw milk and have a broad host spectrum, with both cold-and warm-blooded animals, including humans. However, seafood products are among the ideal substrates for proliferation of *Aeromonas* [1]. Among all motile *Aeromonas*, *A. hydrophila* is considered to be very common in fresh water and is associated with various diseases and syndrome in fishes particularly catfishes [2,3]. Variations in the incidence level of *A. hydrophila* and *A. sobria*, the most common *Aeromonads* in food is attributed by secondary contaminations such as handling, usage of polluted/non-hygenic water, storage of sea foods in inadequate facilities [4].

In general, a large numbers of unidentified food poisoning cases are caused by species of motile *Aeromonads* mostly ignored during investigations of outbreaks. But, in the last few years, motile *Aeromonads* family are considered as re-immerging enteric pathogens by FDA [5]. Two factors appear to be affecting the significance of *Aeromonas* species as a re-emerging pathogen, one factor is to consumer driven demand for less processed and more natural foods containing fewer additives, that has in turn grown emphasis on refrigeration, as the primary means for controlling microbial growth and other factor is to increase in water lever during the warm season [6]. Very limited reports on prevalence of motile *Aeromonas* from food and environmental samples are available and therefore there is a need to characterize these re-immerging enteric pathogens. Present study reports the surveillance of motile *A. sobria* and *A. hydrophila* isolated from commercial food and environment samples intended for human consumption and to determine their antimicrobial susceptibility patterns.

MATERIALS AND METHODS

Sample collection

A total of 154 samples consisting vegetables, commercial food items, raw fish, milk and water were acquired for the study. Vegetables and commercial food items were collected from randomly selected retail shops and supermarkets of Coimbatore, Tamil Nadu and Ernakulam, Kerala in a regular consumer packages and immediately transferred to the laboratory conditions for microbiological analysis. Fishes and water samples were collected from Bhavani Sagar Dam, Sathyamangalam, Tamil Nadu in presterilized polyethylene bags and transported to the laboratory in an ice chest.

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Isolation process and media details

Vegetables and commercial food samples (five grams each) were weighed aseptically and homogenized for 2 min in 50 ml of alkaline peptone water and inoculated into sterile brain heart infusion (BHI) broth (HiMedia, Mumbai). Five grams of fresh water fish samples (skin, gills, and air sack) weighed aseptically were homogenized for 2 min in 50 ml alkaline peptone water and inoculated in BHI broth. Water samples (five millilitre each) were directly inoculated in 50 ml BHI. After aerobic incubation at 28°C for 24hr, inoculums from BHI broth media were streaked onto 5% sheep blood agar (SBA) (HiMedia, Mumbai) and incubated aerobically at 28°C for 24hr. Both β haemolytic and non-hemolytic colonies showing similar morphologies of *Aeromonas* were selected and purified by repeated streaking and re-streaking on SBA plate. Suspected colonies were also streaked onto ampicillin dextrin agar with selective supplement (HiMedia, Mumbai) and *Aeromonas* selective agar with supplements (HiMedia, Mumbai) for specific and selective isolation of *Aeromonas* strains. All the pure isolates were maintained onto nutrient agar and were subjected to various microbiological, biochemical and phenotypical tests.

Biochemical identification

Suspected colonies of *Aeromonas* were identified by performing the biochemical tests like motility, Gram's staining, indole, oxidase, catalase, Voges–Proskauer, triple sugar iron agar, acid production from L-arabinose, m-inositol, d-manitol, salicin, esculin hydrolysis, lysine decarboxylase, and arginine dihydrolase [7]. All tests were read after incubation at 28°C for 24 hr.

Phenotypic study of virulence factors

Single colony of bacterial isolates was freshly grown in 3ml of nutrient broth and was incubated at 28°C for 24hr. The cell density was adjusted to 1.5×10^8 cells per ml. Ten micro litres of this suspension were added to several media for the phenotypic characterization of virulence factors. All isolates were tested in duplicate, and when results were different, a third experiment was carried out to resolve the discrepancies.

Hemolytic activity

Isolates were tested for β-haemolytic activity on 5% sheep blood agar plates (HiMedia, Mumbai). A loop full of freshly grown culture was streaked onto the plates and incubated at 28°C for 24hr. The presence of a clear colourless zone surrounding the colonies indicated β-hemolytic activity [8].

Amylolytic activity

Amylase enzyme was detected in 1% (w/v) agarose (Genei, Bangalore) gel media containing 0.4% (w/v) starch in phosphate buffer saline (PBS). Ten microliters of each suspension was placed in 4-mm-diameter wells cut into an agarose gel and incubated at 28°C for 24hr. The presence of a transparent zone around the colonies indicated amylase activity.

Lipolytic activity

Lipase activity was determined by placing ten micro litres of each grown culture suspension into wells cut into 1% (w/v) agarose in phosphate buffer saline containing 1% L-α-lecithin (HiMedia, Mumbai) and incubated at 37°C for 48 hr [9]. The presence of a transparent zone around the colonies indicated lipase activity.

Casein hydrolysis

Proteolytic activity was tested on Mueller–Hinton agar (Oxoid, UK) containing 10% (w/v) skimmed milk (HiMedia, Mumbai) by streaking a loop full of freshly grown culture onto the plates and incubating at 28°C for 24hr [10]. The presence of a transparent zone around the colonies indicated caseinase activity.

Gelatinase activity

Gelatinase activity was assayed by a radial diffusion method [10], using 3% gelatine (HiMedia, Mumbai) in 1% agarose gel. Ten microliters of each suspension was placed in 4-mm-diameter wells cut into an agarose gel and incubated at 28°C for 24hr. Then, the plates were immersed in a saturated ammonium sulphate solution at 70°C to precipitate unhydrolysed gelatine. The presence of a transparent zone around the colonies indicated gelatinase activity.

Nuclease activity

Extracellular nucleases (DNases) were determined on DNase agar plates with 0.005% methyl green. Drop of five microliters of each broth culture was placed onto the plates and incubated at 28°C for 24hr. A pink colour halo around the colonies indicated positive nuclease activity.

Congo red dye uptake

The ability of bacteria to take up Congo red dye was determined on agar plates supplemented with 50 µg/ml of Congo red dye. Drop of five microliters of each suspension was placed onto the plates and incubated at 28°C for 24hr. Orange colonies were considered positive [11].

Antibiotic susceptibility test

The bacterial strains were screened against various commercially available antibiotics for susceptibility test by disc diffusion method [12]. The antibiotics and their concentrations used for this study were as follows, Ampicillin (10µg), Carbenicillin (100µg), Cefuroxime (30µg), Cephalothin (30µg), Chloramphenicol (30µg), Clindamycin (2µg), Colistin (10µg), Nalidixic acid (30µg), Neomycin (30µg), Nitrofurantoin (100µg) (HiMedia, Mumbai). The zone of inhibition was recorded after 24–48h of incubation at 28^0C. *Escherichia coli* (MTCC 723), *A. hydrophila* (MTCC 646) and *A. sobria* (MTCC 1608) were used as controls. Each isolate was tested thrice against the antibiotics and the positive results were noted down.

RESULTS AND DISCUSSION

A total of 71(46%) isolates of *A. sobria* and *A. hydrophila* were tentatively identified out of 154 commercial food stuffs and environment sources obtained from Kerala and Tamil Nadu. Biochemical tests revealed the isolation of 56(79%) *A. sobria* and 15(21%) *A. hydrophila* (Fig. 1). Out of 56 *A. sobria*, 43(77%) were isolated from commercial food stuffs distributed in vegetables (5%), bakery products (12%), fresh chicken (14%), frozen chicken (16%), fish pickle (25%) and frozen fish (28%) samples, while, remaining 13(23%) were distributed in fresh water fish (2%), raw milk (28%) and lake water (70%). Out of 15 *A. hydrophila,* 10(67%) were isolated from commercial food stuffs distributed in bakery products (11%), frozen chicken (11%), frozen fish (22%), fish pickle (22%) and fresh chicken (14%) samples and rest 5(33%) were distributed in raw milk (17%), lake water (33%) and fresh water fish (2%). The percentage population of *A. sobria* was higher in compare to *A.*

hydrophila and were obtained largely from commercial food samples. The origin and subsistence of various *Aeromonas* species in aquatic environments, including ground water, surface waters, drinking water and wastewater [13] and in foods, like fresh grocery products, seafood, raw meats, packaged ready-to-eat meats, and even in raw milk [14] have been reported.

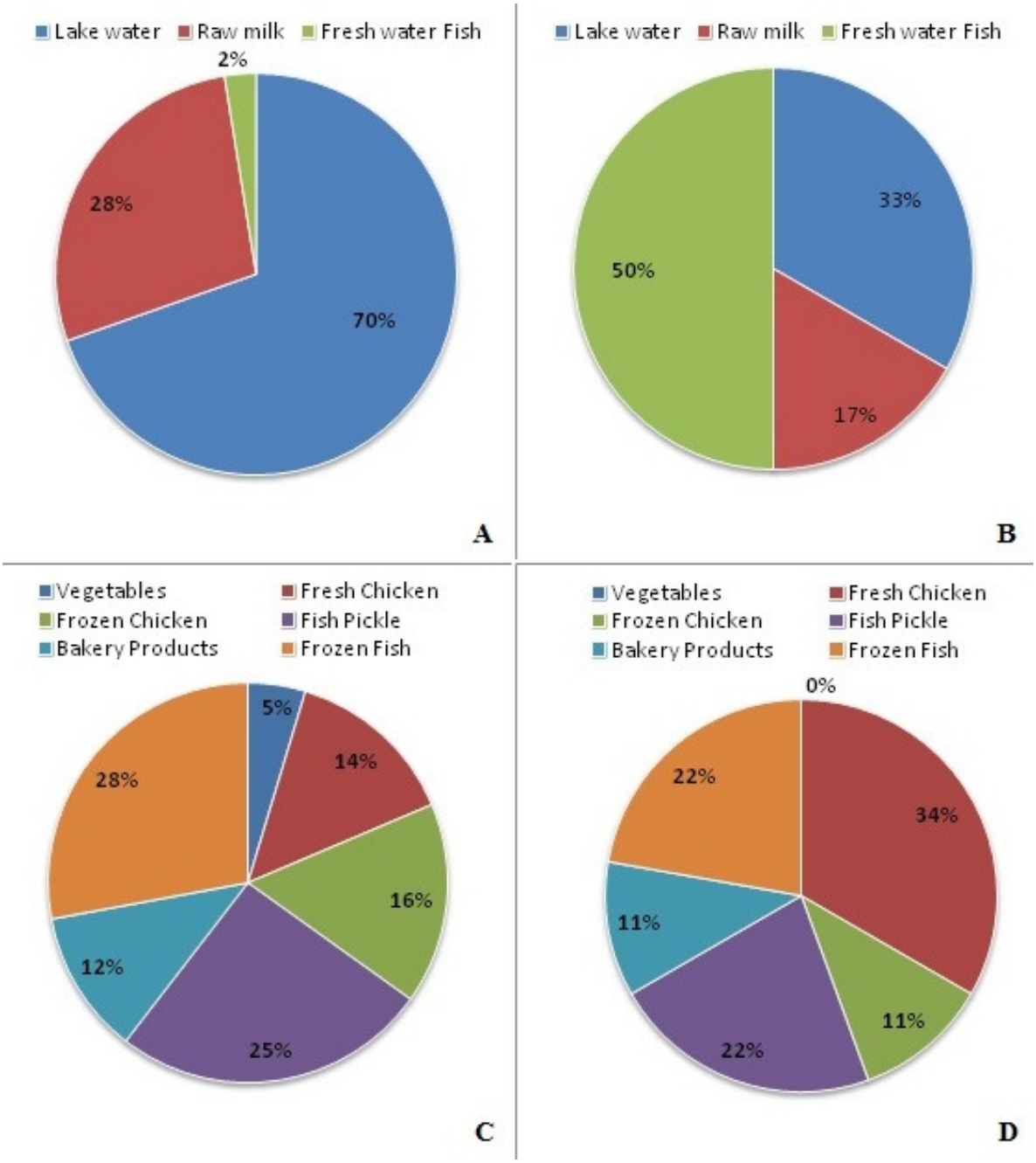

Fig. 1. Isolation frequencies of *A. sobria* and *A. hydrophila* from environmental sources and commercial food stuffs
A: *A. sobria* from environmental sources; B: *A. hydrophila* from environmental sources; C: *A. sobria* from commercial food stuffs; D: *A. hydrophila* from commercial food stuffs

All the *Aeromonas* isolates showed positive for indole production, oxidase, catalase, and VP test (Table 1, Fig. 2). None of the *Aeromonas* isolates from environmental sources fermented lactose in TSI. About 95% *A. sobria* and 75% of *A. hydrophila* isolates from food samples fermented lactose. Fermentation of arabinose was observed in 57% isolates, whereas, fermentation of sucrose, m-inositol, d-mannitol was observed in 96% irrespective of their source (Table 1). Esculin hydrolysis was observed in 25% of the total *A. sobria* isolates and 95% *A. hydrophila* of total isolates from environmental samples. But, none of the *A. sobria* isolated from food samples hydrolysed esculin (Table 1). A report faintly similar to the biochemical test results obtained through our study was also observed by [15].

Table 1. Salient biochemical characters of *A. sobria* and *A. hydrophila*

Biochemical Tests	Percentage of isolates positive			
	A. sobria		*A. hydrophila*	
	Environmental sources	Commercial food stuffs	Environmental sources	Commercial food stuffs
Motility	90	90	85	90
Gram staining				
Gram negative rod	100	100	100	100
Indole	94	95	80	96
Oxidase	87	89	91	90
Catalase	94	99	94	96
Voges- Proskauer's	20	15	75	86
Triple sugar iron agar				
Fermentation of lactose / sucrose	0	95	0	75
Production of H_2S	100	100	100	100
Acid production from				
L-arabinose	20	25	90	92
m-inositol	88	99	99	99
D-manitol	87	99	99	99
salicin	75	75	70	70
Esculin hydrolysis	25	0	95	0
Lysine decarboxylase	99	99	99	99
Arginine dihydrolase	85	99	99	99

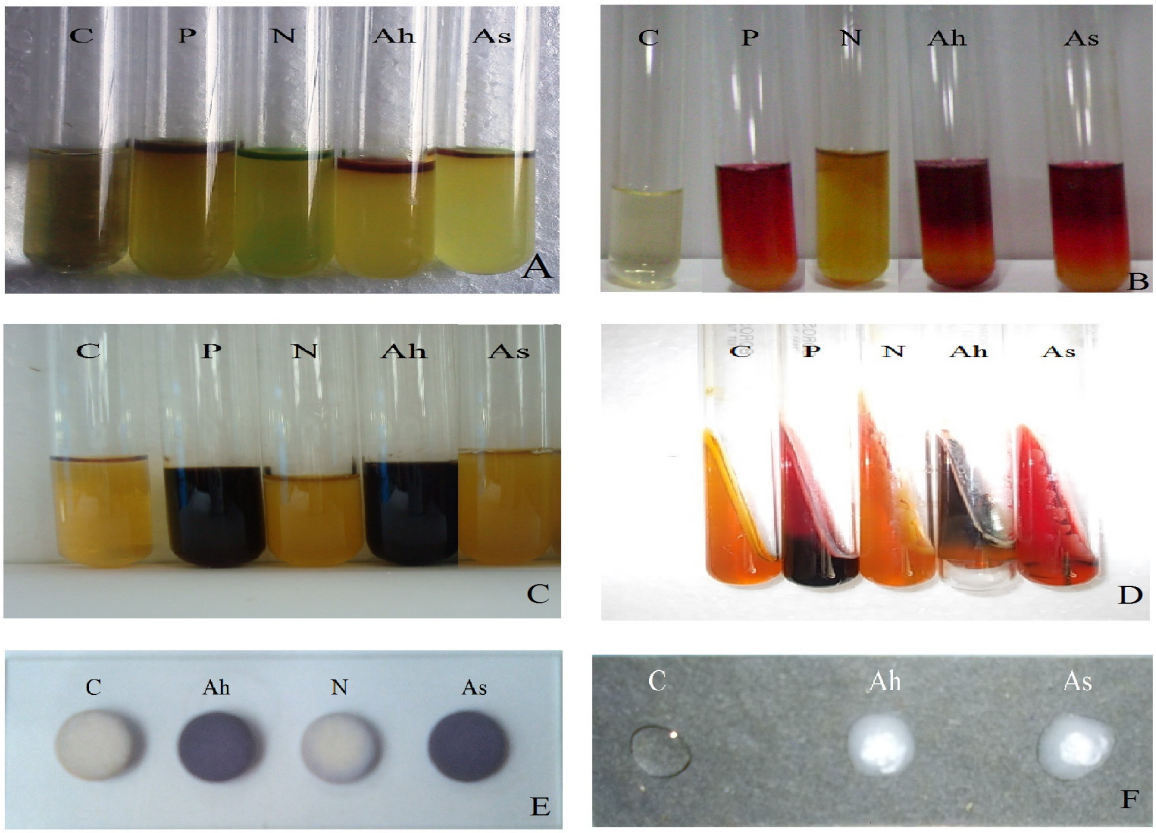

Fig. 2. Biochemical characteristics of *A. sobria* and *A. hydrophila*
A: Indole test; B: Voges Proskauer's test: C: Esculin hydrolysis test; D: TSI test; E: Oxidase test; F: Catalase test; c: Media control; P: Positive control; N: Negative control; As: *A. sobria*; Ah: *A. hydrophila*

Hemolysis on sheep blood agar revealed, both hemolytic and non hemolytic colonies of *Aeromonas*. *A. sobria* isolates were found producing more β-hemolytic colonies than *A. hydrophila*. It has been observed that about 78% isolates of *A. sobria* were β-hemolytic, whereas, 55% isolates of *A. hydrophila* were β-hemolytic. More than 87% of *A. sobria* and 40% *A. hydrophila* isolates were found positive for amylase, caseinase, gelatinase, lipase and nuclease activities irrespective of sources. Similar phenotypic detection of virulence factors from *Aeromonas* isolates was reported [16,17].

Less than, 15% of *A. sobria* and *A. hydrophila* respectively were positive for congo red dye uptake activity (Table 2). The involvement and phenotypic expression of virulence factors contributing to pathogenicity of *A. sobria* and *A. hydrophila* isolates were shown in Fig. 3. In the present study, the proteolytic activity of the isolates was evaluated by determining caseinase and gelatinase production, which was almost similar in both *A. sobria* and *A. hydrophila*. It has been observed that proteolytic activities of *Aeromonas* isolated from environmental samples were lesser than the isolates from commercial food samples. The proteolytic activity of *A. hydrophila* and *A. sobria* has been correlated with its ability to induce pathology in food products [18]. A mild difference of result was seen in amylolytic, lipolytic and nuclease activity. The phenotypic attributes of isolates thus confirmed in a way the mode of bacterial attack on host cell leading to infections.

Table 1. Phenotypic characteristics of *A. sobria* and *A. hydrophila*

Test	*A.sobria* % positive		*A.hydrophila* % positive	
	Environmental sources	Commercial food stuffs	Environmental sources	Commercial food stuffs
Hemolysis				
β-hemolytic	58	98	24	85
Amylolytic activity	90	96	10	40
Casein activity	44	97	36	99
Gelatinase activity	54	99	58	99
Lipolytic activity	95	97	24	8
Nuclease activity	97	99	15	11
Congo red dye uptake activity	5	20	3	26

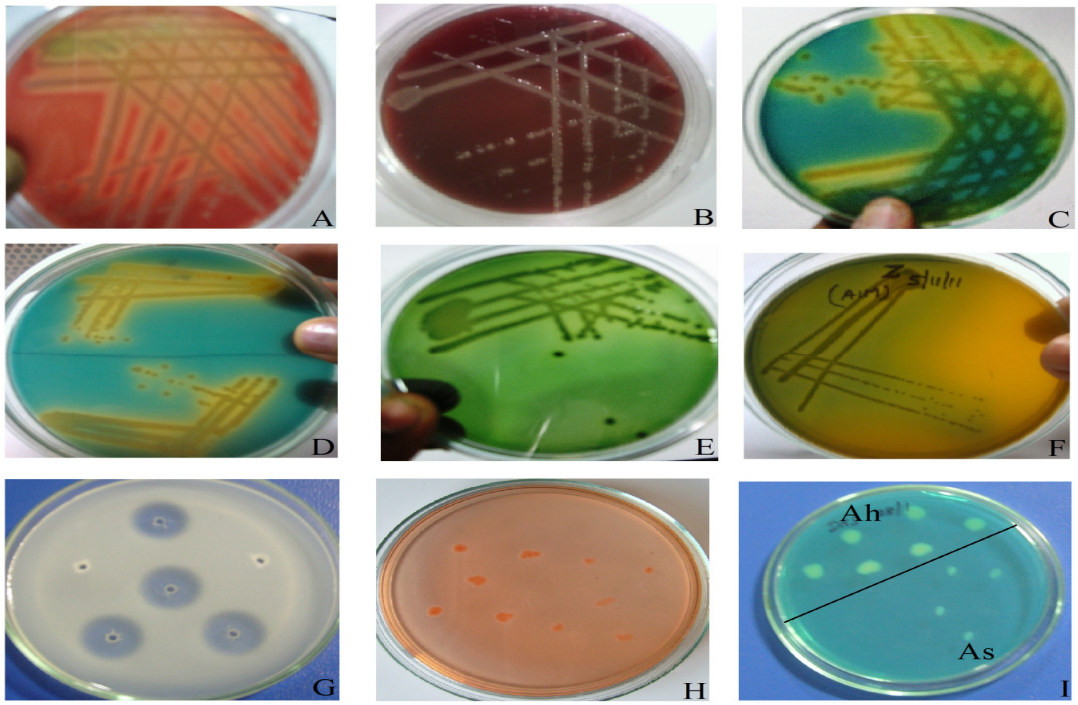

Fig. 3. Phenotypic expression of virulence factors of *A. sobria* and *A. hydrophila*
A: Hemolytic activity; B: Non hemolytic activity; C : *Aeromonas hydrophila* in Ampicillin dextrin agar; D : *Aeromonas sobria* in Ampicillin dextrin agar; E : *Aeromonas hydrophila* in Aeromonas Selective isolation agar; F : *Aeromonas sobria* in Aeromonas selective isolation agar; G : Gelatinase test; H : Congo red dye uptake test; I : DNase test; As: *A. sobria*; Ah: *A. hydrophila*

Antibiotic susceptibility of *Aeromonas* isolates against various antibiotics was performed through disc diffusion method. All the *A. sobria* and *A. hydrophila* isolates were 100% resistant to ampicillin, carbenicillin, cephalothin and clindamycin [16] and 100% sensitive to colistin [17] (Fig. 4). Moderate result was observed with cefuroxime, chloramphenicol, nalidixic acid, neomycin and nitrofurantoin. The percentage of resistance of *A. sobria* against cefuroxime, chloramphenicol, nalidixic acid, neomycin and nitrofurantoin were found to be 70%, 84%, 12%, 80% and 60% respectively whereas, *A. hydrophila* were resistant to 27%, 55%, 10%, 75% and 43% respectively against the same antibiotics.

Thus, colistin was found most potent antibiotic among all antibiotic used in this study also leaves a door open for the application as an active drug against the *Aeromonas* infections [19]. In this study, cefuroxime, nalidixic acid and nitrofurantoin were found 100% resistant to *A. hydrophila* which is completely deviating the earlier findings where all these drugs were highly sensitive [17]. This result clearly indicated the prevalence of multi drug resistant strains of both *A. sobria* and *A. hydrophila* [20] in commercial food stuffs and environmental sources across the Tamil Nadu and Kerala, India.

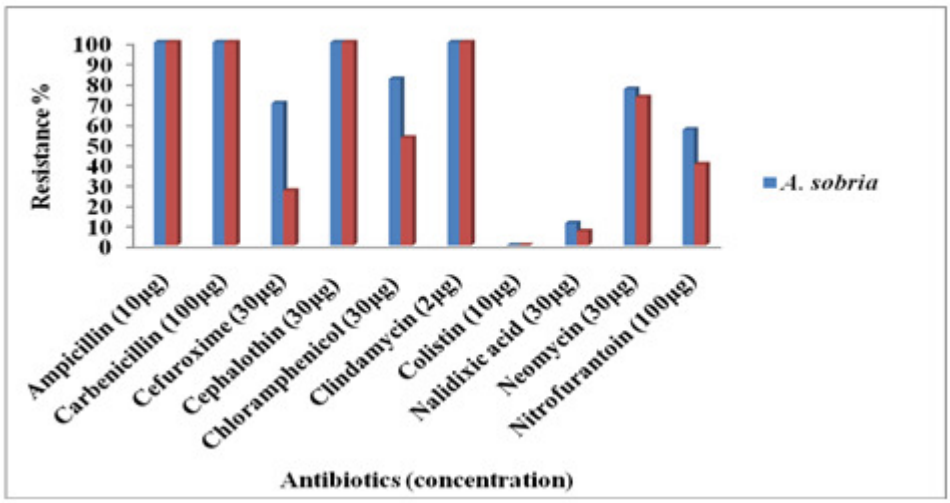

Fig. 4. Antibiotic susceptibility test of *A. sobria* and *A. hydrophila*

CONCLUSION

Present investigations confirmed the surveillance of *Aeromonas* species in consumable food items and environmental samples collected. The bacteria are able to grow and develop in refrigerated conditions and also in baked foods. A matter of concern for food preservation is in need as food infections can lead to health related issues in both humans and animals. The highlights on raise in *Aeromonas* infections provided by our study could be helpful in large awareness about these facts. A more emphasis on the development of control measures and medications as in the form of vaccines could be formulated.

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