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Haemolytic and Multidrug Resistant *Aeromonas hydrophila* Cross Contamination in Retail Seafood Outlets of Coimbatore, South India

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Abstract: The incidence of *Aeromonas hydrophila* in retail seafood outlets as a source of cross-contamination in Coimbatore, South India, was studied for a period of one year from February 2000 to January 2001. A total of 179 strains of *A. hydrophila* were isolated. The maximum incidence (89.8%) of *A. hydrophila* was recorded in wash-water samples followed by platform, balance, butcher's hand, cutting board and knife. It was recorded that 84.9% of the strains were haemolysin producers. They were resistant to bacitracin and all were sensitive to both chloramphenicol and ciprofloxacin. As the number of recognised disease causing organisms originating from the aquatic environment has been increasing in recent years, aeromonads should be carefully monitored in foodstuffs as a possible source of food borne infections.

Key words: *Aeromonas hydrophila*, cross contamination, haemolysin

Introduction

Aeromonas species are widely distributed in the aquatic environment, including raw and processed drinking water (Holmes *et al*., 1996) and have been isolated frequently from various food products such as fish, shellfish, raw meat, vegetables and raw milk (Palumbo, 1996). Motile aeromonads are considered to be emerging food-borne pathogens and it has been shown that some *Aeromonas* food isolates can produce different virulence factors, not only at optimal growth temperature, but also at refrigeration temperatures (Merino *et al*., 1995). Although the exact role of these virulence factors in the pathogenesis of motile *Aeromonas* is still not fully elucidated, representatives of these taxa have been incriminated in cases of human gastroenteritis, particularly in children younger than two years, the elderly and immunocompromised patients (ICMSF, 1988; Joseph, 1996). Further, *Aeromonas* species have been frequently associated with travellers diarrhoea (Hanninen, 1993; Yamada *et al*., 1997). Consequently, aeromonads should be carefully monitored in foodstuffs as a possible source of food borne infections.

Seafood products are considered as the prime source for the motile aeromonads (Abeyta *et al*., 1990; Araujo *et al*., 1990; Neves *et al*., 1990). Among the species belonging to the genus *Aeromonas*, one of the most important and widely studied species is *Aeromonas hydrophila*, being responsible for a variety of fish pathological conditions termed collectively as *Aeromonasis* (Ghittino, 1985), occur in both wild and farmed fish (fish breeding ponds, tropical aquaria, etc.).

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Food borne illness has generally a major public health impact. Retail seafood outlets are considered as the prime source for food borne illness. Food handlers and poor hand washing practices have been implicated as the source of food borne diseases out break. Generally contaminated water and hands are the major sources of microbial contamination in the food services. One of the main ways of bacterial pathogens spread is cross-contamination, which frequently occurs in retail outlets, through improper handling of foodstuffs, cutting boards, utensils and even by hands. The term cross-contamination means spreading of microorganisms, particularly pathogens from raw seafood to uncooked/fresh seafood. Sources of contamination include the immediate surroundings, other foods, utensils and/or individuals handling the foods. Cross-contamination, a leading cause of food borne illness is the transfer of harmful bacteria to food from improperly handled food, During food handling and preparation, microorganisms on raw foods can be transferred to various surfaces such as cutting boards and water faucet spigots (Ak *et al*., 1994; Miller *et al*., 1996; Zhao *et al*., 1998). Pether and Gilbert (1971) and Scott and Bloomfield (1990) reported that various bacteria survive on hands, cloths and utensils for hours or days after initial contact with the microorganisms. This is notably true when handling of raw meat, poultry and seafood. These foods and their juices should be kept away from other seafood, especially raw, ready-to-eat foods. The numbers of recognized organisms, originating from the aquatic environment and causing disease in humans has been increasing in recent years. The epidemiology of the food borne illness is rapidly changing as newly recognized emerging pathogens or re-emerging pathogens increase in prevalence or become associated with new food vehicles. Poor hygienic practices after harvesting may also lead to contamination and they will lead to the cross-contamination of the seafood products. The incidence of multiple antibiotic resistance bacteria in the retail seafood outlets is a serious problem in the view of public health.

The present investigation has aimed to assess the possible sources of *A. hydrophila* cross-contamination in few selected retail seafood outlets of Coimbatore city, South India. Attention has been given to haemolytic and multidrug resistant motile *Aeromonas* species because of the indication of pathogenic potential, though non-haemolytic aeromonads have also been implicated as human pathogen (Namdari and Battone, 1990).

Materials and Methods

The investigation was conducted for a period of one year from February 2000 to January 2001. A total of 59 retail seafood outlets from different locations in Coimbatore city, South India were selected for the study and samples were collected between 08.00 and 09.00 in the morning, with out prior intimation to the retail outlets. Swabbing of surfaces (knife, cutting board, balance, platform and butchers hand) was done with sterile cotton swabs and the swabs were introduced to alkaline peptone water in screw cap containers. Wash-water was mixed thoroughly; approximately 10 mL of the water was collected aseptically and transferred to a screw cap bottle containing 30 mL of alkaline peptone water. The samples were transferred to the laboratory within 1 h of collection and incubated at 37°C for 16 to 24 h. After incubation, 5 μL of the enriched culture was streaked on Starch Ampicillin Agar (SAA) plates and incubated at 37°C for 18-24 h. Yellow to honey coloured, oxidase and catalase positive colonies were taken and tested in Kaper's multi test medium (Kaper *et al*., 1979). Appearance of an alkaline surface and acid butt on the bottom of the tube after 24 h at 37°C was considered positive for the presence of *A. hydrophila*.

Haemolysin Assay

A. hydrophila strains were cultured in 5 mL of brain heart infusion broth (BHIB, HiMedia, India) and incubated at 37°C for 16 to 18 h. Supernatant fluids were carefully removed after centrifugation at 10,000 X g for 30 min at 4°C. One hundred micro liters of supernatant fluid was mixed with an equal volume of 2% (v/v) suspension of rabbit erythrocytes in a 96 well V-bottom micro titre tray. The mixture was incubated for 1 h at 37°C and then for 1 h at 4°C. An erythrocyte suspension in BHIB was included in each assay as a negative control. Haemolysin production was recorded by visual inspection. After incubation, complete/partial lysis of blood cells was considered as haemolysin positive and button formation of blood cells at the bottom of the micro titre tray was considered as haemolysin negative.

Determination of Antibiotic Resistance

The disc diffusion method of antibiotic susceptibility testing was conducted as described by Bauer *et al.* (1966). *A. hydrophila* strains were tested against the following antibiotic discs (HiMedia, India): bacitracin, 10 units; chloramphenicol, 30 µg; ciprofloxacin 5 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; methicillin, 5 µg; nalidixic acid, 30 µg; neomycin, 30 µg; novobiocin, 30 µg; polymyxin-B, 300 µg; rifampicin, 5 µg; streptomycin, 10 µg; tetracycline, 30 µg; trimethoprim, 5 µg; vancomycin, 30 µg. Following enrichment in BHIB for 6-8 h at 37°C, the cultures were streaked on Mueller Hinton agar plates using a cotton swab. With an antibiotic disc dispenser, the discs were placed on the agar surface sufficiently separated so as to avoid overlapping of the inhibition zones. After 30 min of pre-diffusion time, the plates were incubated at 37°C for 18-24 h. After incubation, the diameter of the inhibition zone was measured and compared with the interpretive chart of Performance Standards for Antimicrobial Disk Susceptibility Tests, Dec. 1993 (HiMedia, India) and classified as resistant, intermediate or sensitive.

Results and Discussion

In recent years, the number of reports on the prevalence of *Aeromonas* species in various food products, especially from retail seafood outlets and from various geographical regions have increased significantly. The incidence of *A. hydrophila* in retail seafood outlets from various parts of the world has been reported by several authors (Altwegg *et al.*, 1990; Carnahan and Joseph, 1993; Sugita *et al.*, 1994; Huys *et al.*, 1995; Netys *et al.*, 2000; Vivekanandhan *et al.*, 2002). The incidence of *A. hydrophila* in freshly caught seafood has also been reported (Thayumanavan *et al.*, 2003). However, the incidence of this organism as a source of cross-contamination in retail seafood outlets in India has not been reported. In this study, samples were taken from 59 retail outlets and 179 strains of *A. hydrophila* were isolated (Table 1 and 2). Bacteriological analysis showed that 89.8% of the wash-water samples, collected from the retail seafood outlets were positive for the presence of *A. hydrophila* (Table 1), followed by platform (61%), balance (44.1%), butcher's hand (40.7%), cutting board (35.6%) and knife (32.2%). The incidence of *A. hydrophila* in this study was not a seasonal dependent one (Table 2). The reasons (according to our observations in the retail outlets) for the maximum incidence of *A. hydrophila* in wash-water samples are likely to be: (i) the practice of spraying water (from the wash-water container) on fish and knife before cutting the fish, (ii) after cutting the fish, washing of the knife and hands in same water and (iii) poor cleaning of containers leading to biofilm formation on the inner surfaces of the wash-water containers.

The incidence of *A. hydrophila* on the fish platform (where the fish were usually kept displayed in the retail outlets) was 61%. As soon as the fish was removed from the basket, they were kept on the platform of the shop for display. Little attention was given to cleaning of either the platform or the surface of the fish or on the flies.

Before and after cutting the fish, the fish was weighed. During that time also, there were many chances for the transfer of many food borne pathogens, including *A. hydrophila* from the fish to the trays of the balance. The incidence was recorded as 44.1% from the balance samples. About 40.7% of Butcher's hand samples were found with *A. hydrophila*. Chen *et al.* (2001) reported that washing

Table 1: Incidence of *A. hydrophila* in retail seafood outlets as a source of cross-contamination

Source	No. of samples	Positive	%
Balance	59	26	44.1
Butcher's hand	59	24	40.7
Cutting board	59	21	35.6
Knife	59	19	32.2
Platform	59	36	61.0
Wash water	59	53	89.8

Table 2: Incidence of *A. hydrophila* in various sources of retail seafood outlets as a source of retail seafood cross contamination from February 2000 to January 2001.

	Sources																	
	Knife			Cutting board			Balance			Platform			Butcher's hands			Wash-water		
Months	A	P	%	A	P	%	A	P	%	A	P	%	A	P	%	A	P	%
February, 2000	5	1	20.0	5	1	20.0	5	2	40.0	5	3	60.0	5	2	40.0	5	5	100.0
March	6	2	33.3	6	3	50.0	6	3	50.0	6	4	66.7	6	3	50.0	6	5	83.3
April	4	2	50.0	4	1	25.0	4	1	25.0	4	2	50.0	4	1	25.0	4	4	100.0
May	5	2	40.0	5	2	40.0	5	2	40.0	5	3	60.0	5	2	40.0	5	4	80.0
June	5	1	20.0	5	2	40.0	5	2	40.0	5	3	60.0	5	2	40.0	5	5	100.0
July	5	1	20.0	5	2	40.0	5	2	40.0	5	3	60.0	5	2	40.0	5	3	60.0
August	4	2	50.0	4	1	25.0	4	3	75.0	4	2	50.0	4	2	50.0	4	4	100.0
September	5	2	40.0	5	2	40.0	5	2	40.0	5	3	60.0	5	2	40.0	5	5	100.0
October	6	2	33.3	6	2	33.3	6	3	50.0	6	4	66.7	6	2	33.3	6	5	83.3
November	5	1	20.0	5	2	40.0	5	3	60.0	5	3	60.0	5	2	40.0	5	5	100.0
December	4	1	25.0	4	1	25.0	4	1	25.0	4	3	75.0	4	2	50.0	4	3	75.0
January, 2001	5	2	40.0	5	2	40.0	5	2	40.0	5	3	60.0	5	2	40.0	5	5	100.0
Total	59	19	32.2	59	21	35.6	59	26	44.1	59	36	61.0	59	24	40.7	59	53	89.8

Table 3: Production of haemolysin by *A. hydrophila* isolated from retail seafood outlets

Source	No. of isolates	Haemolysin producers (%)
Knife	19	78.9 (n = 15)
Cutting board	21	80.6 (n = 17)
Balance	26	80.8 (n = 21)
Platform	36	86.1 (n = 31)
Butcher's hand	24	83.3 (n = 20)
Wash water	53	88.7 (n = 48)
Total	179	84.9 (n = 152)

Table 4: Antibiotic resistance of *A. hydrophila* isolated from retail seafood outlets

Antibiotics	Resistance (%) (n = 179)
Bacitracin	100.0
Chloramphenicol	0.0
Ciprofloxacin	0.0
Erythromycin	90.8
Gentamicin	5.0
Kanamycin	93.1
Methicillin	92.4
Nalidixic acid	12.8
Neomycin	90.6
Novobiocin	90.4
Polymyxin-B	84.9
Rifampicin	93.6
Streptomycin	6.4
Tetracycline	46.9
Trimethroprim	59.7
Vancomycin	78.0

hands according to the food code recommendations (FDA, 1999) reduces the level of the *E. aerogenes*, it did not eliminate this organism, from hands initially contaminated with ~10^7CFU. Under these conditions, the washed hands remained a potential source of cross-contamination. The incidence of *A. hydrophila* from the cutting boards (in all the retail outlets, they were using the wooden cutting boards only) and knifes were recorded as 35.6 and 32.2%, respectively. We observed in all the retail outlets that the butchers were not taking much care on the cleaning of boards and their hands after each and every use. They were not also wearing the gloves while processing the fish. The knives were always kept sharp and often create some marks on the cutting boards while cutting the fish. It would lead to the adherence not only the small muscle particles of the fish, but also the organisms on to the board. Here the organism might come either from the fish or from the wash-water and if they are able to adhere to the hands and knives, they did serve as one of the potential sources for the cross-contamination.

Of 179 strains, 152 (i.e., 84.9%) were found to be haemolysin producers (Table 3). It was recorded that 88.7% of the isolates, taken from wash-water were found to be haemolysin producers, followed by isolates taken from platform, butchers' hand, balance, cutting board and knife with 86.1, 83.3, 80.8, 80.6 and 78.9%, respectively. Oliver *et al.* (1981) isolated 40 strains of *A. hydrophila* from fish and most of them were positive for haemolysin. Callister and Agger (1987) also studied that all of the strains of *A. hydrophila* isolated from retail grocery store products were highly cytotoxic at 35°C. Tsai and Chen (1996) reported haemolytic activity in 87.5% and 100% of the strains of *A. hydrophila* isolated, respectively from fish and prawns of Taiwan. Thayumanavan *et al.* (2003) reported that 78.4% of *A. hydrophila*, isolated from freshly caught fish and prawn were haemolysin producers. Almost all enteropathogenic strains of *A. hydrophila* produce haemolysin and enterotoxins (Burke *et al.* 1981). *A. hydrophila* isolated from infected as well as healthy fish in Malaysia also produced haemolysin (Yadav *et al.*, 1992). These reports strongly support our findings.

The release of multiple antibiotic resistant organisms through faeces may ultimately pave the way for the contamination of fish and shellfish in the aquatic environment (Grabow *et al.*, 1973, 1976) and through food chain they may be transferred to any level. The emergence of resistance will be accelerated by the clinical use of antibiotics (Chaudhary *et al.*, 1996) and plasmids confer resistance to more than one drug (Annear and Grubb, 1972; Lacey *et al.*, 1974). A higher level of resistance to various antibiotics with reference to *A. hydrophila* was also reported (Pettibone *et al.*, 1996). Antibiotic susceptibility tests showed that all the strains were resistance to bacitracin, followed by rifampicin (93.6%), gentamicin (93.1%), while all strains were sensitive to chloramphenicol and ciprofloxacin (Table 4). These results were similar to the findings of Thayumanavan *et al.* (2003). Vivekanandhan *et al.* (2002) also reported that 99 and 100% of the strains, respectively isolated from fish and prawn were resistant towards bacitracin and 4.4% and all the strains from fish and prawn, respectively were sensitive to chloramphenicol. Ansary *et al.* (1992) and Pettibone *et al.* (1996) also reported chloramphenicol resistant strains of *A. hydrophila*, while in this study, no such resistance was recorded. It was found that 93.1% of the isolates were resistant to kanamycin, while Ramteke *et al.* (1993) and Pettibone *et al.* (1996) have not reported the kanamycin resistant strains. Gentamicin resistant strains were recorded as 5%, while Vivekanandhan *et al.* (2002) reported 8.2 and 3.9% of *A. hydrophila* strains, respectively from fish and prawns resistant to gentamicin. The findings of Vivekanandhan *et al.* (2002) and Thayumanavan *et al.* (2003) supports our study in this regard, These reports indicate that geographical location and local selective pressure influences levels of antibiotic resistance.

The role of *Aeromonas* species in food poisoning is still in controversial and the number of potential enterotoxins that have been suggested over the years may indicate that the pathogenesis of *Aeromonas* infections is complex and multifactorial (Kirov, 1997; Granum *et al.*, 1998; Chopra and Houston, 1999). The present data clearly confirm the widespread sources of haemolysin positive and multi-drug resistant *A. hydrophila* in retail seafood outlets. In India, food additives like garlic, onion, turmeric etc are added while cooking to both vegetarian and non-vegetarian foods and they have been identified as antimicrobial agents and foods are also properly boiled. There have been no reports of food related *A. hydrophila* out breaks in India. However, due to westernization in food trends, there are few shops selling half cooked, half boiled and ready to eat foods. These foods, particularly seafood, there are many chances for out breaks. Quantifying the probability of bacterial transfer associated with various steps in the food preparation process may provide the scientific basis for risk management strategies to reduce, prevent, or eliminate cross-contamination in the retail seafood outlets. Much care should be taken while buying, cooking and also while consuming fresh or half boiled seafood.

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