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HEMOLYTIC ACTIVITY AND ANTIBIOTIC RESISTANCE OF *AEROMONAS* SP. ISOLATED FROM MARKETED FISH.

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ABSTRACT

The present work highlights the important incidence of *Aeromonas* sp., with hemolytic activity, antimicrobial resistance and multiple antibiotic resistance **(**MAR) isolated from freshwater marketed fish (*Labeo rohita*) intended for human consumption in Silchar town. Hence, a total of 114 samples were collected of which 45 isolates were isolated. Strain differentiation of *A. hydrophila* was done on the basis of different sugar fermentation tests used in this study. The haemolytic activity tests were performed on blood agar containing 5% sheep red blood cells and of the 45 *Aeromonas* sp. strains that were isolated, 35 (77.7%) isolates exhibited β-haemolysis and the number of isolates showing α-haemolysis (8; 17.7%) and γ-haemolysis (2; 4.4%) was relatively small. Antibiotic resistance results revealed that all the *A. hydrophila* strains were 100% resistant to ampicillin. Besides this, more than 80% of the strains were found to be resistant to penicillin, ofloxacine and gentamycin. Kanamycin, nalidixic acid, chloramphenicol and tetracycline were recorded with a resistance percentage between 28-32% and resistance towards erythromycin (52%) and neomycin (8%) was also observed. Finally, the percentage occurrence of antibiotic resistance of MAR index was calculated and a MAR index value of more than 0.2 was observed, thus suggesting that all the isolates originated from high-risk source of contamination.

KEYWORDS: Fish, haemolytic activity, antibiotic resistance.

INTRODUCTION

Foods of animal origin commonly like seafoods and vegetables have always been accompanied by *Aeromonas* sp. infections (Altwegg *et al*., 1991; Kirov, 1986; Mattick and Donovan, 1998). It has been observed that some strains of *Aeromonas* are enteropathogenic

and possess virulence factors as enterotoxins, cytotoxins, haemolysins and invasins and this association between haemolysin production and enterotoxicity is none other than the *Aeromonas* sp. which can grow and release enterotoxin and haemolysin even at fridge temperature conditions (Sharma & Kumar, 2012). Today many, researchers have confirmed the presence of β-haemolytic aeromonads in fish products (Tsai & Chen, 1996; Radu *et al.*, 2003; Thayumanavan *et al.*, 2003; Sharma & Kumar, 2012) and a special attention is being paid on *A. hydrophila* as a causative agent of foodborne gastroenteritis and fish products as this is the commonest vector of this microbial pathogen (Thayumanavan *et al.*, 2003; Hatha *et al.*, 2005). Also, the wide spread of these bacteria has been considered as a public health threat due to the fact that *Aeromonas* infections are provoked by consumption of contaminated water and food (Kirov, 1986). Gastroenteritis being the commonest infections provoked by *Aeromonas* sp., which may appear as diarrhoea, antibiotics has been applied widely not only in the human and veterinary medicine, but as growth promoters in animal breeding as well. A number of investigations have indicated that the wide and irrelevant antibiotics usage leads to genes selection encoding the resistance and thus, this resistance maybe the result of a bacterial modification and enzymatic inactivation. Multiple antibiotic resistance (MAR) too, among *Aeromonas hydrophila* strains has been reported from many parts of the world (Vila, *et al*., 2002) and under these circumstances, it will be worthwhile to find out the prevalence of antibiotic resistance of the *Aeromonas* strains that may be considered as an emerging pathogen and to identify the high-risk source.

MATERIALS AND METHODS

Bacterial stains and growth conditions

The bacterial strains were isolated from samples of freshwater fish (*Labeo rohita*) and identified to the species level by biochemical methods (Carnahan *et al*., 1991) by using routine methods including motility test (+), Gram coloring (-), Voges-Proskauer (VP) test (+), Urease (-), D-glucose fermentation (37^{o}C/24 h) (+), catalase activity (+), SIM (+), Starch hydrolysis (+), LAO (+), etc. The isolates were further identified by (Cruickshank et al., 1985) performing esculine hydrolysis, L-arabinose utilization, glucose gas production, indol, gelatinase activity, ornithine-decarboxylase activity, H_2S separation from cystein, ability for aerobic and anaerobic growth, etc. Working cultures of the microorganisms were kept on nutrient agar (Hi-Media) slants at refrigerated temperature and routinely cultivated in Brain Heart Infusion broth (BHI) (Hi-Media) at 28°C and isolated on ADA agar (Ampicillin Dextrin Agar, Hi-Media).

Determination of hemolytic activity

The haemolytic activity tests were performed on blood agar containing 5% sheep red blood cells. Typical colonies of each sample were grown for 4–5 h in brain heart infusion broth (Merck, Germany). The presence of haemolysis in agar plates was detected after 24-hour incubation at 37^0C according to Singh & Sanyal (1992).

Antibiotic sensitivity test

Pure cultures were grown in brain heart infusion broth (BHIB) (Hi-Media, Mumbai, India) for sensitivity testing. Mueller Hinton agar (Hi-Media) was used for all solid media. Disc diffusion method for antibiotic susceptibility was conducted as described by Bauer *et al*., (1966). The *A. hydrophila* strains were tested against the following antibiotic discs (HiMedia): Ampicillin 10 mcg; Penicillin 10 mcg, Neomycin 30 mcg, Chloramphenicol 30 mcg; Gentamycin 10 mcg; Kanamycin 30 mcg, Nalidixic acid 30 mcg; Erythromycin 15 mcg; Ofloxacin 30 mcg and Tetracycline 30 mcg. After enrichment in BHIB for 6–8 h at 37^0C, 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 prediffusion time, the plates were incubated at 37^0C for 18–24 h. After the incubation period, the diameter of the inhibition zones was measured and compared with the interpretive chart of Performance Standards for Antimicrobial Disk Susceptibility Tests, Dec. 1993 (Hi-Media) and classified as resistant, intermediate and sensitive.

Multiple Antibiotic Resistance (MAR)

The MAR index when applied to a single isolate is defined as a/b, where ‘a’ represents the number of antibiotics to which the isolate was resistant and ‘b’ represents the number of antibiotics to which the isolate was exposed. MAR index value higher than 0.2 is considered to have originated from high-risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used. MAR index value of less than or equal to 0.2 considered as the origination of strain from animals in which antibiotics are seldom or never used (Krumperman, 1985).

RESULTS

A total of 114 samples were collected from fresh market fish intended for human consumption of which 45 isolates isolated grown in Ampicillin Dextrine agar (ADA) medium produced honey-yellow coloured, round, small to medium, convex, elevated and transparent

colonies. After Gram staining, a series of biochemical tests and sugar fermentation tests confirmed as *Aeromonas hydrophila*, *A. sobria* and *A. caviae*.

Microscopically, *Aeromonas* sp. was observed as Gram negative short rod, motile by polar flagella exhibiting positive reaction towards oxidase, catalase, produced gas and acids from glucose, utilize citrate for growth and produced acetoin and indole; reduced nitrate, showed positive reaction towards gelatinase test, DNase test, lysine and arginine decarboxylase test; esculin hydrolysis test, methyl red (MR) test; showed negative reaction towards ornithine decarboxylase test, Voges proskauer (VP) test, urease test and DL-lactate utilization test. The isolated isolates utilized glucose, dextrose, lactose, D-mannitol, sucrose, Dmaltose, trehalose, starch, D-galactose, D-ribose, salicin, sorbitol, rhamnose, D-mannose, L-arginine and arabinose; did not ferment D-sorbose, L-rhamnose, D-melibiose, m-inositol, raffinose, adonitol, xylose and dulcitol; did not reduce ferrous sulfate to ferric sulfate and did not produce H2S gas.

The presence of hemolysin was determined by the formation of zones of alpha or beta or gamma around their colonies. Table 1 represents the results from the haemolytic activity of *Aeromonas* sp. isolates. Of the 45 *Aeromonas* sp. strains that were isolated 35 (77.7%) isolates exhibited β-haemolysis. The number of isolates showing α-haemolysis (8; 17.7%) and γ-haemolysis (2; 4.4%) was relatively small (Table 1).

Table1: **Hemolytic activity of *Aeromonas* sp.**

Origin	**Number of isolates**	**Hemolytic activity**		
Fresh Market fish	45	α	β	γ
		(8) 17.7%	(35) 77.7%	(2) 4.4%

Antibiotic susceptibility test

All the 45 isolates from marketed fish samples were tested against 10 antibiotics and the results exhibited the existence of multiple antibiotic resistances amongst the *A. hydrophila* strains.

In the present study, it was observed that all the *A. hydrophila* strains were 100% resistant to ampicillin. Besides ampicillin, more than 80% of the strains were found to be resistant to penicillin, ofloxacine and gentamycin. Kanamycin, nalidixic acid, chloramphenicol and

tetracycline were recorded with a resistance percentage between 28-32% and resistance towards erythromycin (52%) and neomycin (8%) was also observed.

Multiple Antibiotic Resistance (MAR)

The Multiple Antibiotic Resistance (MAR) was calculated and ranged from 0.1-0.3. Since the MAR index value of more than 0.2 is considered to be high risk source of contamination, therefore in the present study, the percentage occurrence of antibiotic resistance of MAR index were compared and it was found that all the strains isolated exhibited a MAR index value of more than 0.2 thus suggesting that all the isolates originated from high-risk source of contamination.

DISCUSSION

Aquatic environments are used world widely for water supply, energy production, irrigation, navigation, aquaculture, and primary and secondary contact activities. Nevertheless, in the last decades these environments have been threatened by human misuse with detrimental consequences for mankind as a whole (Bruke *et al*., 1982). Though Fish and fish products are of primary significance for humans due to their nutritional and health value (Stratev *et al*., 2013) but they are very highly prone to contamination and transmit microbial pathogens, which normally inhabit the aquatic environment (Seethalakshmi *et al.*, 2010). The production of hemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads (Santos *et al*., 1999). In the present study, haemolytic proteins were isolated from *Aeromonas* sp. and β- haemolysins which are among the essential virulence factors (Pandey *et al.*, 2010) besides playing an important role in the pathogenesis of human and fish diseases were studied (Castro-Escarpuli *et al.*, 2003). Thus, 45 *Aeromonas* sp. strains were isolated, of which 77.7% exhibited β-haemolytic activity. The tested 35 β-haemolytic isolates were identified as *A. hydrophila*. These observations where high percentage of haemolytic *A. hydrophila* strains in fish products have been observed are similar to those also reported by other researchers (Thayumanavan *et al.*, 2003; Radu *et al*., 2003; Tsai & Chen, 1996). Furthermore, β -haemolysin in *Aeromonas* sp. has been suggested to be a cytotoxin; (Barer *et al*., 1986, Husslein *et al*., 1988, Ljungh *et al*., 1981) a recent report on the cytotoxicity of *A. caviae* strains indicates that most of them possess this property and the failure by earlier workers to detect this was probably due to the use of medium containing higher levels of glucose, which has deleterious effects on the organism (Namdari and Bottone, 1989).

Identification of *Aeromonas* sp. requires the use of non-conventional biochemical assays which are often time consuming and require long incubation, thus in the present study, attempts were made to identify the minimal characteristics for the use in clinical laboratories (Abbott *et al*., 1992). Hence, based on observations recorded in the present investigation, the use of three tests for the identification of *Aeromonas* sp. with about 90% accuracy that has been recommended were followed which includes the tests of esculin hydrolysis, Voges-Proskauer and gas from glucose. Furthermore, the *Aeromonas* isolates were also identified up to species level by using Aerokey II (Carnahan *et al*., 1991) which included tests of acid from arabinose and sucrose and indole production besides the above three tests. *A. hydrophila* (33; 73.33%) and *A. sobria* (11; 24.44%) were the most frequently isolated species from apparently healthy marketed fishes meant for human consumption (Santos *et al*., 1988).

Though fish is nutritive, they act as a vehicle for pathogenic bacteria naturally occurring in the aquatic environment. *Aeromonas* sp. has been recognized as potential or emerging foodborne pathogens for more than 20 yrs. They are most commonly isolated from aquatic animals and associated with economic loss in fish culture worldwide (Seethalakshmi *et al.*, 2010). *Aeromonas* was isolated using the selective medium supplemented with ampicillin where only the resistant colonies were observed. In the present study, it was observed that all the *A. hydrophila* strains were resistant to ampicillin which was similar with the findings of Vila *et al.*, 2002; Castro-Escarpuli *et al.*, 2003; Radu *et al.*, 2003; Hatha *et al.*, 2005. Besides ampicillin, more than 80% of the strains were found to be resistant to penicillin, ofloxacine and gentamycin (Orozova *et al*., 2008). Many reports have stated that *A. hydrophila* was susceptible to gentamycin from different food sources (Radu *et al.*, 2003) but presently this variation in this study maybe because of different environmental and geographical conditions prevailing in the N.E region of India. Also, chloramphenicol which is widely used for controlling clinical strains of *A. hydrophila* in aqua culture, but in contrast, a 24% of chloramphenicol resistant *A. hydrophila* in marketed fish foods was observed in the present study whereas Vivekanandhan *et al*., 2002 found 3.7% resistant. Kanamycin, Nalidixic Acid and tetracycline were recorded with a resistance percentage between 28-32%. In earlier reports of Chang and Bolton (1987) it has been found that more percentage of Asian isolates of *A. hydrophila* and *A. sobria* were resistant to tetracycline than Australian isolates. Besides this, also many authors have reported tetracycline resistant strains of *A. hydrophila* isolated from different sources (Tsai & Chen, 1996) along with a contrast findings recording that

tetracycline suspectible isolates were noted from environmental and clinical sources. Resistance towards erythromycin (52%) and neomycin (8%) was also recorded.

The emergence of bacterial isolates that are resistant to an antimicrobial agent represents a continuing ecological battle to achieve a natural host-parasite balance. As new antibiotics are developed and used, resistant strains often develop due to its indiscriminate use. Thus, multidrug resistant bacterial pathogens are the serious problem now–a-days faced by the clinicians. When such multiple antibiotic resistant strains enter the community, and hybridize with non-MAR index value of 0.2 or above is said to originate from high-risk source of contamination (Krumperman, 1985). Hence, in the present study, all the strains isolated showed a MAR index value of more than 0.2, which implies that all the isolates originated from high-risk source of contamination. Finally, the wide usage of antibiotics and chemotherapeutics in the fish-breeding farms for prevention or treatment of diseased fish, as well as their usage as food additives applied through the fodder or their solution in water directly has increased the antibiotic resistance among the pathogenic bacteria (Vila *et al*., 2002).

CONCLUSION

The present work highlights an important incidence of *Aeromonas* sp. in fishes intended for human consumption in Silchar city and the high presence of virulence factors and antimicrobial resistance detected in these strains should not be underestimated.

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