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DETECTION OF MULTIDRUG RESISTANCE *AEROMONAS HYDROPHILA* IN MARINE RUPCHANDA FISH SOLD AT LOCAL FISH MARKETS AT MYMENSINGH CITY IN BANGLADESH

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Abstract

This study was conducted for detection of multidrug resistance *Aeromonas hydrophila* in marine Rupchanda (Pampus chinensis) sold at local fish markets at Mymensingh city, Bangladesh. Rupchanda (n=30) were collected from three local fish markets. Intestine (n=30) and gill (n=30) samples were aseptically collected and inoculated into alkaline peptone water for enrichment at 37°C for 8 hrs. Enriched culture was streaked onto thiosalfate bile salt sucrose (TCBS) agar to isolate bacteria. Identification of bacteria was performed by routine bacteriological tests and polymerase chain reaction assay. The antibiogram profile of bacteria was investigated against nine commonly used antibiotics such as: gentammicin, chloramphenicol, ampicillin, erythromycin, streptomycin, tetracycline, cefalexin, azithromycin and ciprofloxacin by disc diffusion method. A total of 10 *Aeromonas hydrophila* were identified and all isolates were found resistant to ampicillin and cefalexin. Data of this study suggest that Rupchanda harbour multidrug resistance *Aeromonas hydrophila* which may cause public health problem if enter into human food chain.

Keywords*:* Multidrug resistance, *Aeromonas hydrophila*, Rupchanda fish, Bangladesh.

INTRODUCTION

Fish carries various types of bacterial flora in gills, gut and skin. *Aeromons hydrophila* is a ubiquitous and opportunistic bacterium that constitutes part of the normal flora of fish. It has been reported both from freshwater and marine environments. It is a Gram negative facultative anaerobic bacterium. It causes diseases in fish at the time of stress (Peters et al., 1988). It has been associated with several disease conditions in fish such as fin rot, tail rot and *haemorrhagic septicaemia*. It causes gastroenteritis and localized wound infection in humans (Nemetz and Shotts, 1993). Fish spoilage is caused by *Aeromonas* spp. due to the action of its extracellular microbial enzymes such as haemolysin, enterotoxins, cytotoxins, lipases and proteases (Farag, 2006). During the last years, the interest to *Aeromonas* spp. extended beyond the boundaries of fish pathology due to the increased incidence of human disease caused by *Aeromonas* after consumption of contaminated foods. Motile aeromonads are emerging food pathogens as some isolates could produce virulence factors not only at optimum temperatures, but also under cold storage conditions (Neyts et al., 2000). Waters receive antimicrobial agents from human and animal waters which results in the emergence of multidrug resistance (MDR) bacterial flora in the aquatic environment (Morita et al., 1994). The multidrug resistance was reported in the genus of *Aeromonas* (Albert et al., 2000; Palu et al., 2006).

A. hydrophila were detected in fresh water fishes and prawns sold at fish markets in Bangladesh (Rahim et al., 1984; Rahim and Aziz, 1994). *Rupchanda* (Pampus chinensis) is a popular and costly sea fish in Bangladesh. It is harvested from the Bay of Bengal of Bangladesh. This fish is regularly sold in the domestic fish markets throughout Bangladesh. Both dried and fresh forms of this fish have a huge demand among the consumer levels including the tourists. Rupchanda is also exported from Bangladesh to the foreign countries. In order to predict the hazard for consumers' health no study has been conducted so far in Bangladesh on the status of *A. hydrophila* in Rupchanda fish sold at the local fish markets. The objectives of this study were (i) Isolation and identification of *A. hydrophila* from Rupchanda sold in the local fish markets at Mymensingh city and (ii) determination of antibiogram profile of *A. hydrophila* against nine commonly used antibiotics.

Materials and Methods

Collection of samples: Rupchanda (n=30) were collected from three local fish markets such as Kewatkhali fish market (n=10), Kamal Ranjit (KR) market (n=10) and Mesuabazar markets (n=10) located at Mymensingh city. The samples were packed into sterile polyethene bags in an ice box and transported to the Department of Microbiology and Hygiene at the Bangladesh Agricultural University (BAU), Mymensingh for bacteriological study.

Processing of samples: Gill (n=30) and intestine (n=30) of Rupchanda were aseptically collected. Intestinal samples were cut into small pieces using sterile scissors and mixed with 4.5 ml APW and grinded by pestle and mortar to prepare homogenous

suspension. Gill swab samples were collected using sterile cotton swabs.

Enrichment of samples: Swab of gill was inoculated into a test tube containing 4.5 ml of Alkaline Peptone Water (APW) and incubated at 37°C for 8 hrs. Intestinal samples (0.5g) were separately inoculated into test tubes containing 4.5 ml APW and incubated at 37°C for 8 hrs.

Isolation of bacteria: One loopful of enrichment culture of gill and intestine was separately streaked duplicate onto thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia, India) and incubated aerobically at 37°C for 24 hrs. Single colony grown onto the TCBS agar was further sub cultured onto TCBS agar until pure cultures were obtained.

Identification of bacteria: Identification of bacteria was conducted by observing cultural characteristics and colony morphology on the TCBS agar and growth of bacteria into nutrient broth containing 0% and 6% sodium chloride (NaCl). Gram's staining method, motility test, sugar fermentation and biochemical tests (oxidase test, catalase test, citrate test, indole test and MR-VP test) were performed to identify bacteria.

Molecular detection of bacteria by PCR: A genus specific PCR assay was performed to identify *Aeromonas* spp. by amplifying 276-bp fragment of lipase gene (Delamare et al., 2012).

Antibiotic sensitivity test: Antibiogram profile of 10 *A. hydrophila* isolates was done against five different antibiotics such as: Gentamicin, Azithromycin, Ciprofloxacin, Ampicillin, Cefalexin, Erythromycin and Chloramphenicol (Himedia, India). The antibiotics sensitivity testing was carried out according to instructions of the Clinical and Laboratory Standard Institute (CLSI, 2011).

RESULTS

Isolation and identification of *A. hydrophila*

Gill (n=30) and intestine (n=30) samples of Rupchanda were streak onto Thiosulfate Citrate Bile salt (TCBS) agar to isolate *Aeromonas* spp. Yellow shin colony was seen on the TCBS agar after 24 hrs incubation at 37°C which are characteristics of the genus *Aeromonas*. All isolates produced β- hemolysis on blood agar media. *Aeromonas* isolates grew in nutrient broth with (6%) or without (0%) addition of NaCl. In Gram's staining *Aeromonas* isolates were found Gram negative, rod-shaped and arranged in single and pair. Sugar fermentation and biochemical tests confirmed *Aeromonas* isolates as *A. hydrophila* (Table 1).

Table 1: Summary of sugar fermentation, motility and biochemical test results for *Aeromonas hydrophila*

Name of tests	Results of this study	Results of Bergey's Manual*	Results of other investigators		Interpretation
			Results	References	
1. Sugar fermentation					
Dextrose	A	A	A	Kannan *et al. (*2011)	
Sucrose	A	A	A	Popovic *et al.* (2000)	
Lactose	A	NS	A	Chandrakanthi *et al.* (2000)	
Maltose	A	A	A	Popovic *et al.* (2000)	
Mannitol	A	A	A	Chandrakanthi *et al.* (2000)	
2. Motility test using hanging drop method	Motile	Motile	Motile	Chandrakanthi *et al.* (2000)	
3. Biochemical tests					
Oxidase	+	+	+	Kaysner and Depaola (2004)	
Catalase	+	+	+	Kannan *et al.* (2011)	
Citrate	+	NS	+	Kannan *et al.* (2011)	
Indole	+	NS	+	Al- Fatlawy *et al.* (2013)	
MR	+	NS	+	Chandrakanthi *et al.* (2000)	
VP	+	+	+	Kaysner and Depaola (2004)	

Aeromonas hydrophila

A=Acid, + = positive, NS= Not stated, MR= Methyl Red, VP= Voges Proskauer, *= Bergey's Manual of Systematic Bacteriology (Krieg, 1984)

Molecular detection of ***A****eromonas* spp. was performed by PCR a**s**say. *Aeromonas* spp. was confirmed by ampl**i**fication of the expected 276 bp amplic**o**n of lipase genes in all isolates (Fig. 1).

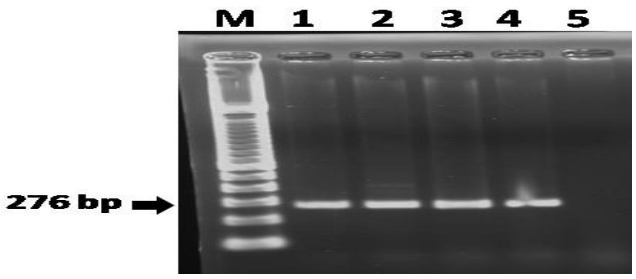

Fig. 1: PCR assay to am**p**lify *lipase* gene of *Aeromonas* spp.of marine Rupchada. Lane M: 1 kb DNA marker (Prom**e**ga, USA); lanes 1, 2, 3 and 4: DNA of *Aer**o**monas* spp. Isolated from marine Rupchada, lane 5: Negative control without DNA.

Prevalence of *Aeromonas hydrophila* in Rupchanda

Aeromonas hydrophila were isolated from gill and intestine samples of marine Rupchada. A **t**otal of 10 *Aeromonas hydrophila* were **i**solated from gill and intestine of marine Rupchada. The prevalence of ***A****. hydrophila* in gill and int**e**stine was 13.33% and 20%, respe**c**tively. The overall prevalence of *A. hydroph**i**la* in marine Rupchada was 33.33% (Ta**b**le 2). Prevalence of *A. hydrophila* was 40% in Kewatkhali fish market, 60% in KR market and 20% in Mesuabazar markets.

Table 2: Prevalence of *Aeromonas hydrophila* in gill and intestine samples of marine Rupchada

Name of samples	No. of Samples tested	No. of culture positive samples	Prevalence (%)	Overall Prevalence (%)
Gill	30	4	13.33	33.33
Intestine	30	6	20	

Antibiotic susceptibility profiles

All 10 isolates of *A. hydrophila* were found sensitive to seven antibiotics such as Gentamicin, Azithromycin, Ciprofloxacin, Tetracycline, Chloramphenicol, Erythromycin & Streptomycin and resistant against two antibiotics such as Ampicilin and Cephalexin (Table 3).

Table 3: Results of antibiotic sensitivity test of *Aeromonas hydrophila* isolated from Rupchada

Name of fish markets	No of isolates tested	No of isolates sensitive to antibiotics							No of isolates resistant to antibiotics	
		E	AZM	GEN	C	CIP	TE	ST	AMP	CN
Kewatkhali	4	4	4	4	4	4	4	4	4	4
KR market	4	4	4	4	4	4	4	4	4	4
Meshua bazar	2	2	2	2	2	2	2	2	2	2

GEN= Gentamicin, AZM= Azithromycin, CIP= Ciprofloxacin, AMP= Ampicillin and CN= Cefalexin, ST= Streptomycin, TE= Tetracycline C= Chloramphenicol, E= Erythromycin.

Discussion

The genus *Aeromonas* is widely distributed in aquatic environment and increasingly reported as a primary pathogen of human and lower vertebrates. Many of these bacteria are capable of causing human infection and intoxication. *Aeromonas* has been reported as an etiological agent in variety of human infections including gastroenteritis and extra intestinal infections. *Aeromonas hydrophila* causes disease in marine Rupchada under stress conditions or in association with infection by other pathogens. Bacterial causal agents of human disease transmitted from fish when used as a food or by handling them. This causes risks for public health, especially for immuno compromised individual children and aged person. Hence, there is need for public enlightment, campaign and general education to assist in curtailing the outbreak of diseases in human through ingestion of the bacteria along with fish. The purpose of the present work was to isolate, identify and determine the antibiotic profile of *A. hydrophila* of marine Rupchanda sold at three different fish markets of Mymensingh.

A total of 30 Rupchanda fishes were collected three local fish markets. Gill (n=30) and intestine (n= 30) were collected aseptically and inoculated into alkaline peptone (APW) water for enrichment at 37℃ for 8 hrs. Enriched cultured was streaked into TCBS agar to isolate bacteria. Identification of bacteria was performed by cultural characteristics, Gram's staining, sugar fermentation and biochemical tests and polymerase chain reaction (PCR) assay. The antibiogram profiles of bacteria were investigated against nine commonly used antibiotics (Gentamicin, Cefalexin, Ampicillin, Azithromycin, Erythromycin, Streptomycin, Tetracycline, Chloramphenicol and Ciprofloxacin) by disc diffusion method (CLSI, 2011).

In the present study, TCBS agar was used for isolation of *Aeromonas* spp. TCBS agar supports the growth of both *Vibrio* spp. and *Aeromonas* spp. (Kaysner and Depaola, 2004). The most frequently isolated species of *Vibrio* in fresh water are *V. cholera* and *V. mimicus* (Fouz *et al.*, 2002). In TCBS agar *V. cholera* produce yellow colour colony and *V. mimicus* produce green colour colony (Kaysner and Depaola, 2004). *Aeromonas* spp. on the other hand also produce yellow colour colony on TCBS agar. In this study, DNA extracted from yellow colour colony grown on TCBS agar successfully amplified 276 bp fragment of lipase gene confirmed bacterial isolates belonged to *Aeromonas* spp. (Delamare *et al.*, 2012). Additionally, differential diagnosis between *Vibrio* spp. and *Aeromonas* spp. were also performed by Gram's staining technique, lactose fermentation test and VP test. *Vibrio* spp. are Gram negative, comma shaped or curved rod, non- lactose fermenter and VP negative (Brooks *et al.*, 2007; Islam *et al.*, 2013; Kaysner and Depaola, 2004; Jayashinghe *et al.*, 2008). In this study, bacteria isolated from Rupchada were Gram negative, rod shaped, lactose fermenter and VP test positive which are characteristics for *A. hydrophila*. Another differential diagnosis carried out to differentiate *Vibrio* spp. from *Aeromonas* spp. by their growth characteristics in nutrient broth containing 0% and 6% NaCl (Kaysner and Depaola, 2004; Jayashinghe *et al.*, 2008). *V. cholera* and *V. mimicus* cannot grow in nutrient broth containing 6% NaCl.

Ashiru *et al.*, (2011) isolated *A. hydrophila*, *A. caviae* and *A. sobria* from Tilapia fish and Catfish. These three species of *Aeromonas* can be differentiated by lactose and sucrose fermentation tests. *A. hydrophila* can ferment lactose and sucrose. On

the contrary, *A. caviae* and *A. sobria* cannot ferment lactose and sucrose (Ashiru *et al.,* 2011). In this study, bacterial isolates of Rupchada fermented lactose and sucrose confirming their identity as *A. hydrophila.* In present study, 10 *A. hydrophila* were isolated and identified from Rupchada. The prevalence of *A. hydrophila* in marine Rupchada in this study was 33.33%. In Iran, 13.89% prevalence of *A. hydrophila* in Rupchanda was reported by Khamesipour *et al.,* (2014). Vivekanandhan, (2005) reported 17.62% prevalence of *A. hydrophila* in Rupchanda in India. In the current study, gill and intestine samples were screened for *Aeromonas* spp. since these samples were also analyzed by other investigators (Lijon *et al.*,2015; Ashiru *et al.,* 2011; Jayasinghe *et al.,* 2008). This study detected the presence of *A. hydrophila* in gill and intestine samples of Rupchada. The prevalence of *A. hydrophila* was the highest in intestine (20%) followed by gill (13.33%). Vivekanandhan (2005) also recorded the highest prevalence of *A. hydrophila* in the intestine (38.43%) as compared to and gill (29.10%).

A. hydrophila are sensitive to ciprofloxacin and gentamicin (Ko *et al.,* 2003; Truong *et al.,* 2008; Overman, 1980) and resistant to ampicilin (Geiss and Freij, 1989 and Overman, 1980) Cefalexin. In this study, all *A. hydrophila* were found to be sensitive to ciprofloxacin, gentamicin and azithromycin and resistant to ampicillin and cefalexin. Similar antibiotic resistance patterns of *A. hydrophila* isolated from fresh water prawn were reported by Lijon *et al.* (2014). Antibiotic resistance frequencies and profile varied according to the source of the strains (Ko *et al.,* 1996). The widespread use of antibiotics in the aquaculture systems and agricultural sectors in Bangladesh may act as the source of antibiotics diffusion into the sediment (Sorum, 2006). Sometimes fishes are treated with some antibiotic solutions to extend their shelf life. The broad spectrum antibiotics, tetracyclines, chloramphenicol etc. have been used to extend the shelf life of fish (Balachandran, 2001).

The results of this study indicate that *A. hydrophila* are prevalent in marine Rupchada. Data of antibiogram profiles suggest that marine Rupchada harbors multidrug resistant *A. hydrophila.*

CONCLUSIONS

The results of the current study indicated that the marine Rupchada sold at the local fish markets of Mymensingh city carry multidrug resistant *A. hydrophila* which may cause health hazard in consumers if enter into the human food chain.

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