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Research Article

Characterization and serosurveillance of *Aeromonas hydrophila* infection in disease affected freshwater fishes

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ABSTRACT

Fish, the fastest food producing sector in the world is one of the richest sources of animal protein. However, diseases are the major bottleneck which causes severe economic loss. Therefore routine surveillance of diseases is extremely important to predict and minimize the adverse effect caused by outbreak, epidemic, and pandemic situations, by adopting suitable and accurate preventive measures. In recent times, serosurveillance the gold standard programme is routinely used to measure the population immunity which in turn complements the traditional disease surveillance methods. Serological studies used to investigate the acquisition of various infections in a population, measure the induction of an immune response in the host, evaluate the persistence of antibody etc. Herein, for the first time possibly an attempt has been made to study the seroprevalence of *Aeromonas hydrophila* which is widely associated with a wide range of diseases like epizootic ulcerative syndrome, dropsy, fin rot, infection dropsy and hemorrhagic septicemia in several freshwater species. Further, the serological findings were corroborated with bacteriological studies by isolating the *A. hydrophila* from the disease affected fish exhibiting typical sign and symptoms of aeromoniasis. In this study, 90 sera samples from 10 different disease affected and/or survived freshwater fish species were processed to find out the presence of *A. hydrophila* antibodies. The findings showed that fish like *Labeo rohita, Catla catla, Cyprinus carpio, Hypopthalminthys molitrix etc.*, were highly seropositive and hence indicated their susceptibility towards this pathogen. Therefore the study provides the evidence that serosurveillance studies could be useful in identifies and they adopting suitable control measures against major diseases of fishes.

INTRODUCTION

Diseases are inherent, albeit integral component of any living system and are mostly reported after their outbreaks. Therefore surveillance of diseases which is involved systematic collection, analysis and interpretation of data is extremely important for adopting appropriate prophylaxis measures. There are several methods for surveillance of diseases. Amongst, "serosurveillance" is routinely used method to monitor disease in wide range of animals. Serosurveillance is an important component of any comprehensive surveillance system not only for accessing natural infection in animals but also for evaluating post vaccination immunity. It is the gold standard for accessing immunity in a population, induced both by post immunisation/vaccination and natural infection. Serosurveillance programmes are successfully used to detect natural infection in many animal systems (Albina *et al.*, 2000; Hsueh *et al.*, 2004; Jungersen *et al.*, 2005; Koppel *et al.*, 2007; Tum *et al.*, 2015).

Like any other living` system, diseases are major bottleneck in the progress and sustainability of aquaculture industry (Van Hai, 2015). Over the years intensive and stressful rearing conditions make the farmed aquatic animals especially fish highly susceptible to diseases leading to high mortality and ultimately severe economic losses (Dias *et al.*, 2016; Plant, & La-Patra, 2011) . Among several pathogens, *Aeromonas hydrophila* is a great concern since it is responsible for variety of diseases like hemorrhagic septicemia, abdominal edema, exopthalmia, ulcers, dropsy, motile aeromonas septicemia (MAS), red sore diseases and respiratory infections etc in several fish species like carp, tilapia, perch, salmon, catfish, and other fish species (Citarasu *et al.*, 2011; Esteve *et al.*, 1995; Huang *et al.*, 2015; Janda, & Abott, 2010; Joseph, &

Carnahan, 1994; Praveen *et al.,* 2016; Sarkar, & Rashid, 2012).

The pathogen is very fatal and is associated not only with a number of complications in fresh water fish species but also many times responsible for 100 % morbidity and mortality leading to severe economic loss. Herein, this study provides microbial and serological evidences of a prospective association of *A. hydrophila* in the outbreak of disease/infection in various freshwater fish species.

MATERIALS AND METHODS

Sample collection

Aeromoniasis is a major and frequently occurring disease of freshwater fishes. Therefore, samples from fishes suffering with this disease and/or survived were collected from different fish farms situated at various parts of Odisha, India for microbiological and serological studies.

Isolation of Aeromonas hydrophila

The involvement of *A. hydrophila* in the disease outbreak was studied by isolating the bacteria from blood samples of septicemic fish, abdominal fluid from dropsy and from fishes with typical aeromoniasis sign and symptoms by standard microbiological procedure using specific Aeromonas isolation agar (AIA) and also in Tryptone soya agar (TSA) medium (Hi-media, India). After isolation, bacteria were identified biochemically and also by using anti-*A. hydrophila* serum raised in rabbit.

Serum samples

A total of 90 sera samples were collected from 10 different fish species showing typical signs of *A. hydrophila* infection as well as fish survived after disease outbreak in the pond. These sera samples were treated as test sera. Since it was difficult to get sera from 100 % disease free fish for use as control, 3 healthy fish from each fish species, reared in controlled conditions with added antibiotics were used as control sera.

Pathogen used

Three *A. hydrophila* strains were used in the present study. Two virulent strains of *A. hydrophila* (Ahv & Ahm) isolated from diseased fish and maintained at Department of Biotechnology, NOU, Baripada, Odisha was used in this study. Besides another non-pathogenic strain (Ahr) isolated from the water sample of fish cultured pond was also used in this study.

Preparation of antigen

The whole cell antigens of all the three strains of *A. hydrophila* were prepared for serological studies. Briefly, mass culture of individual strains of *A. hydrophila* was made by separately inoculating the culture onto 100 ml of nutrient broth (Hi-media, India). After incubation, the cultures at 30 ^{0}C for overnight, individual bacterial culture broth was inactivated by 1 % (v/v) formalin solution at 4 ^{0}C for 18 h and then viability was checked by streaking on nutrient agar plates. After inactivation cultured broth was centrifuged at 5000 × rpm for 10 minutes at 4 ^{0}C. Finally whole cell antigen suspension of individual *A. hydrophila* strains was prepared after three times washing the pellet with phosphate buffer saline (PBS, pH 7.2).

Raising of anti-A. hydrophila hyperimmune sera

Hyperimmune sera against whole cells antigen of *A. hdrophila* strain (Ahm) was raised in New Zealand White rabbit as per the method of (Dukpa et al. 2011). Briefly, rabbit was intramuscularly injected with 0.5 ml of bacterial whole cell (10^9 CFU ml^{-1}) antigen emulsified with equal volume of Freund's complete adjuvant followed by two booster doses with Freund's incomplete adjuvant at fortnight intervals. The rabbit was bled for serum through ear veins, 15 days after second booster.

Detection of A. hydrophila antibody

Spot agglutination test

Agglutination reaction was done by mixing 10µl of individual *A. hydrophila* antigen (Ahm, Ahv, Ahr) separately with equal volume (10 µl) of serum sample obtained from immunized rabbit as well as infected/disease survived test fish sera. The percentage of seropositivity against each strain of *A. hydrophila* was calculated (Swain *et al.,* 2003).

Agglutination titre

The agglutination titre of immunized sera raised in rabbit with homologous Ahm antigen as well as heterologous antigens *i.e.,* Ahv & Ahr was determined. Similarly the agglutination titre of test sera tested obtained from infected/disease survived fishes against all the three strains of *A. hydrophila* was also conducted. The agglutination titre was determined in 'U'-shaped microtitre plates by two-fold serial dilution of 50 µl serum (immunized /test) was made with equal volume of PBS (pH 7.2) in each well, to which 50 µl of formalin-killed antigen of individual *A. hydrophila* (Ahv/Ahm/Ahr @10^7 CFU ml^{-1}) suspension was added. The plates were incubated for 2 h or kept for overnight at room temperature, if result is not observed after 2 h.

Determination of antibody titre

The antibody titre was calculated as the reciprocal of the highest dilution of serum (immunized/test sera) showing complete agglutination of the bacterial cells.

RESULTS AND DISCUSSION

Aeromonas hydrophila one of the most virulent pathogen, is responsible for a variety of infections leading to colossal economic losses to the aquaculture (Janda, & Abott, 2010; Kumar *et al.,* 2016; Laith, & Najiah, 2013; Lallier, & Higgins, 1988; Monette *et al.,* 2006; Peatman *et al.,* 2018; Plant, & La-Patra, 2011). Nonetheless, the bacterium could be a pathogen to other animals like amphibians, reptiles, mammals, including humans (Janda, & Abott, 2010; Lallier, & Higgins, 1988; Plumb, & Hanson, 2011; Stratev, & Odeyemi, 2016; Trott, 2013). The

detection of microbial pathogens or their specific antibody in the host is the pre-requisite for the diagnosis of any infectious disease, and the detection of antibodies through serodiagnosis provides a unique means to ascertain pre-exposure to pathogens (Valassina *et al.*, 2003). Therefore serosurveillance of Aeromonas infection could be very critical in adopting appropriate remedial measures. During the study, different fish species were found to suffer/infected with typical *A. hydrophila* infection like tail, rot/fin rot, ulcerative condition, dropsy, septicemic condition. Samples from these aeromoniasis suspected fish species were processed for isolation of *A. hydrophila*. A total of 17 bacteria were isolated and identified biochemically as well as serologically (Table 1). Out of 17 bacterial isolates, 15 were identified to be *A. hydrophila* both by biochemical and serological test. One bacteria each isolated using TSA medium from diseased *Cirrhinus mrigala* and *Anabas testudineus* samples on TSA medium was found to be different biochemically and also failed to agglutinate anti-*A. hydrophila* sera.

In this study, *A. hydrophila* was invariably isolated from found all the aeromoniasis suspected disease conditions. Out of 17 isolates from disease affected fishes, 15 were confirmed biochemically as well as serologically using anti- *A. hydrophila* immunized rabbit sera to be *A. hydrophila*. Further bacteriological findings collaborated with the serosurveillance study. Herein, bacteriological findings were corroborated with serological findings. Fish species belonging to *Labeo rohita, Catla catla, Cyprinus carpio, Hypopthalminthys molitrix* were highly seropositive towords *A. hydrophila.* The high agglutination titre of *L. rohita, C. catla* and *A. testudineus* sera against Ahv and Ahm and sera of *Piaractus brachypomus, Channa striata* sera against Ahv, indicated the possible pre-exposure of these pathogens either single or mixed form in the culture environment. However, a higher percentage of test sera from *C. carpio, C. striata, A. testudineus C. mrigala* and *P. brachypomus* didn't agglutinate any of the *A. hydrophila*. However, this percentage could be narrowed down and more accurate if we use more sensitive assays like ELISA as viewed by Swain *et al.* (2003). While fish sera of naturally infected fish and disease survived fish exhibited strong agglutination reaction with high antibody titre against particular *A. hydrophila* strain(s), no agglutination reaction and/or titre could be detected against several test sera against other strain(s) of *A. hydrophila.*

During the study, a total of 90 sera samples from 10 different fish species were collected and processed serologically to detect the presence of anti-*A. hydrophila* antibodies. Out of 10 fish species, fish species *Labeo calbasu, Ctenopharyngodon idella* were found to be 100 % seropositive towards Ahv and Ahm respectively (Table 2). Similarly sera samples from *L. rohita, C. catla, H. molitrix, C. carpio* and *P. brachypomus* showed highly seropositive towards various strains of *A. hydrophila*. Among the 3 tested *A. hydrophila* strains, the highest percentage of seroprevalence was recorded against Ahm irrespective of fish species. Similarly among different fish species, test sera obtained from *C. idella* and *C. carpio* didn't agglutinate Ahv and Ahr while the test sera obtained from *C. striata* and *A. testudineus* failed to agglutinate Ahr. On the contrary, 50.0%, 40.0% and 50.0% test sera samples of *C. carpio, C. striata* and *A. testudineus* didn't agglutinate any of the *A. hydrophila* while that of *C. mrigala* and *P. brachypomus* was 28.72% and 18.23 %, respectively.

Anti- *A. hydrophila* sera raised against Ahm in rabbit was found to agglutinate all the three strains of *A. hydrophila* (Table 3). The antibody titre (log_2) against Ahm was found to be 2^9. Similarly the titre value (log_2) was found to be 2^6 and 2^4 against whole cell antigens of Ahv and Ahr, respectively when cross reacted with anti-*A. hydrophila* sera from immunized rabbit raised against Ahm. On the other hand, fish sera of naturally infected fish and disease survived fish exhibited either strong agglutination reaction with high titre value against one or more *A. hydrophila* strains. Similarly agglutination titre could not be detected against several test sera against certain strains of *A. hydrophila* (Table 4). Test sera samples from fish like *L. rohita, C. catla* and *A. testudineus* exhibited high agglutination titre against Ahv and Ahm while test sera of *P. brachypomus* and *C. striata* showed high mean (±SD) agglutination titre of 5.6 (±0.54) and 5.00 (±1.15) against Ahv only. Similarly, the mean (±SD) agglutination titre (Log_2) values of the test sera samples from *C. catla* were found to be 5.6 (±0.54) and 5.00 (±1.15) against Ahv and Ahm, respectively. Similarly, the values were 5.25 (±1.5) and 5.6 (±0.54) for *L. rohita* test sera against Ahv and Ahm respectively. Likewise for *A. testudineus* test sera the mean (±SD) agglutination titre (Log_2) against Ahv was 5.0 (±0.8) and Ahm was 4.33 (±0.5).

Earlier serosurveillance studies are successfully implemented to identify specific infection in several animal models (Al Dahouk *et al.*, 2005; Albina *et al.*, 2000; Blacksell *et al.*, 2008; Dias *et al.*, 2016; Esteve *et al.*, 1995; Ghosh *et al.*, 2017; Trott, 2013). This is possibly the first investigation on seromonitoring of *A. hydrophila* infection in several freshwater fishes which indicated the pre-exposure of this pathogen. The ubiquitous nature of *A. hydrophila* in freshwater environment and existence of cross reactivity among the pathogenic and non-pathogenic *A. hydrophila* strains as evidenced from the agglutination reaction with immunized rabbit sera and test fish sera as evidenced from more than 100% cumulative seropositivity could lead to false diagnosis of aeromoniasis. But the high percentage of seropositivity along with high antibody titre against pathogenic forms of *A. hydrophila* (Ahv & Ahm) as compared to non-pathogenic reference *A. hydrophila* (Ahr) confirmed their involvement either individual or combining in the disease outbreak. This findings along with the bacteriological findings, confirmed the fact that aeromoniasis is highly prevalent in several freshwater fishes. Hence, such types of studies can be used as a tool for routine seromonitoring and epizootiological study of *A. hydrophila* and other microbial infections in fish. Earlier Swain *et al.* (2003) also succeeded in detecting of *Edwardseilla tarda* infection in fish through serosurveillance studies.

Serosurveillance study indicates among different fish species *Catla* to be most susceptible to *A. hydrophila* infection irrespective of strain. *A. hydrophila* pathogen was successfully detected by using hyperimmune sera raised against *A. hydrophila* in rabbit. The serum agglutination test is very cheap, easy to perform as compared to more sensitive, costly and highly skilled assays like ELISA and most important aspect is that the assay can be performed at firm level by common farmers. Therefore, such gold standard, effective simple assay could also be very useful in detecting infection even though bacteriological studies failed to isolate that specific infectious agent.

Table 1: Table showing the samples collected from various freshwater fishes suffering and/or survived from typical aeromoniasis for bacteriological (only from disease affected fish) and serological studies using anti-*A. hydrophila* sera (Both from disease affected and survived fish)

Name of fish	Disease condition/ Symptoms (No. of sample)	Isolation and confirmation of *A. hydrophila*			No. of serum samples processed
		Medium (No.of isolates)	Identification	Confirmation	
Catla catla	Septicemia (1) Survived (7)	AIA(1)	Biochemical	Serological	8
Labeo rohita	Ulceration (2)/ Dropsy (1) Survived (17)	AIA(3)	Biochemical	Serological	20
Cirrhinus mrigala	Fin rot (2)/ Dropsy (1) Survived (4)	AIA (3) / TSA (3*)	Biochemical	Serological	7
Ctenopharyngodon idella	Survived (12)	Not Done	Not Done	Not Done	12
Labeo calbasu	Survived (8)	Not Done	Not Done	Not Done	8
Piaractus brachypomus	Septicemia (1) Survived (10)	AIA (1)	Biochemical	Serological	11
Hypophthalmichthys molitrix	Survived (7)	Not Done	Not Done	Not Done	7
Cyprinus carpio	Survived (6)	Not Done	Not Done	Not Done	6
Channa striata	Survived (5)	Not Done	Not Done	Not Done	5
Anabas testudineus	Tail rot (2)/ Dropsy (1) Survived (1)	AIA(3)/ TSA(3*)	Biochemical	Serological	4

AIA: Aeromonas Isolation Agar; TSA: Tryptone Soya Agar

*: One isolate each from both fish species was negative both serologically and biochemically

Table 2: Seroprevalence of antibody (expressed as agglutination reaction percentage) in test sera samples obtained from different freshwater fish species against different strains of *Aeromonas hydrophila*

Fish Name (Number of sample)	No Agglutination Reaction (%)	Agglutination reaction (%) against		
		Ahm	Ahv	Ahr
Catla catla	-	50.0	37.5	37.5
Labeo rohita	-	15.0	75.0	20.0
Cirrhinus mrigala	28.72	28.5	28.5	14.28
Ctenopharyngodon idella	-	100	0	0
Labeo calbasu	-	0	100	0
Piaractus brachypomus	18.23	54.5	9.09	18.18
Hypophthalmichthys molitrix	-	57.14	42.85	14.28
Cyprinus carpio	50.0	50.0	0	0
Channa striata	40.0	40.0	20.0	0
Anabas testudineus	50.0	25.0	25.0	0

Table 3: Agglutination reaction with titre value (Log_2) of the sera sample of *Aeromonas hydrophila* (Ahm) immununized rabbit against homologous Ahm and heterologous Ahv & Ahr antigens

Test	Ahv	Ahm	Ahr
Slide agglutination test	++	+++	+
Antibody Titre	2^6	2^9	2^4

Table 4: Agglutination titre (Log_2) of the sera samples from diseased and/or disease survived freshwater fishes against *Aeromonas hydrophila*

Fish	**Type of Sera**	**Ahv**	**Ahm**	**Ahr**
Catla catla	Control Sera	2.0±0.0	2.0±0.0	2.33±1.0
	Test Sera	5.6 ± 0.54	5.00±1.15	3.0±1.1
Labeo rohita	Control Sera	2.0±0.0	2.0±0.0	2.66±0.57
	Test Sera	5.25±1.5	5.6±0.54	4.33±0.57
Cirrhinus mrigala	Control Sera	2.0±0.0	2.0±0.0	2.33±1.0
	Test Sera	3.33±0.75	4.33±0.5	3.0±1.1
Ctenopharyngodon idella	Control Sera	3.0±1.1	ND	ND
	Test Sera	4.75±0.5	ND	ND

Labeo calbasu	Control Sera	ND	2.0 ±0.5	ND
	Test Sera	ND	3.33 ±0.75	ND
Piaractus brachypomus	Control Sera	2.66 ±0.57	2.0 ±0.5	2.0 ±0.0
	Test Sera	5.6 ±0.54	2.75 ±0.5	2.0 ±0.0
Hypophthalmichthys molitrix	Control Sera	3.0 ±1.1	ND	ND
	Test Sera	4.00 ±1.15	5.25^{b} ±1.5	3.33 ±0.75
Cyprinus carpio	Control Sera	2.33 ±1.0	ND	ND
	Test Sera	4.33 ±0.57	ND	ND
Channa striata	Control Sera	2.66 ±0.57	ND	ND
	Test Sera	5.00 ±1.15	2.5 ± 1.00	ND
Anabas testudineus	Control Sera	2.00 ±0.00	2. 0 ±0.0	ND
	Test Sera	5.0 ±0.8	4.33 ±0.5	ND

*ND- Not Detected

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