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Establishment of route of challenge and tissue level persistence study of *Aeromonas hydrophila* infection in rohu, *Labeo rohita* for running a selection programme

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Abstract
Selective breeding of rohu, *Labeo rohita* to *Aeromonas hydrophila* infection requires a challenge model to obtain LD_{50} dose and study of bacterial persistence in survivors' tissues. Fish were bath challenged with various routes viz., only immersion, after scale removal (with and without high stocking), after skin abrasion (with and without high stocking), under only high stocking density and after immunosuppression. Fixed dose of intramuscular and intraperitoneal injections with uniform and variable body weights and variable intraperitoneal dose g^{-1} body weight were attempted. Fish were challenged and tissues from internal organs were collected at different time periods. Bacterial load was detected by nested PCR-based screening using DNA extracted from various tissues. Results revealed intraperitoneal challenge g^{-1} body weight as the best mode to obtain LD_{50}. Survivors could be used as brood fish for next generation breeding to get better resistant offspring since they don't serve as carriers after 15 day post-challenge.

Keywords: *Aeromonas hydrophila*; challenge mode; *Labeo rohita*; bacterial load; selection programme.

1. Introduction
Rohu, *Labeo rohita* (H.) is one of the preferred carps in India with high consumer preference due to its fast growth and high quality flesh. Its global production surpassed 6,90,000 tonnes in 2007 and 3,70,000 tonnes was contributed by India [1]. However, like other carp species, rohu is seriously affected by *Aeromonas hydrophila* infection, which causes mass mortality in farms and hatcheries [2]. *A. hydrophila* ordinarily part of the normal gut flora [3] of fish become pathogenic under environmental and physiological stress. There is no permanent prevention available to get rid of this infection. Use of antibiotics and chemotherapeutics can lead to immunosuppression, tissue deposition and environmental pollution. Vaccination, probiotics and immunostimulants are also not completely effective. Therefore, selection of disease resistance stock could provide more reliability and long term prevention [4]. Selective breeding of *Jayanti* rohu for improved disease resistance to *A. hydrophila* is going on at the Central Institute of Freshwater Aquaculture, Bhubaneswar, India. A proper challenge mode to obtain a perfect LD_{50} value would be of immense help for choosing resistant stocks for breeding.

Challenge tests give valuable information about the disease resistance capacity of a stock with its percentage survival value. However, the survivors were not used as brood fish for the next generation breeding, since there is a chance of those being carriers of bacterial infection that will be more dangerous by causing both horizontal and vertical spread. The uses of naive fish from the same stock or their sibs are more practicable in disease selection programmes [5]. However, the survivors of challenge tests would have been the best candidates as their direct descendants would be more capable of defending infection. To achieve this, a compete study on tissue level persistence of each bacterial model is required, probably for individual fish species. It has been previously reported that *A. hydrophila* can affect both external and internal organs [6]. However, mortality, clinical signs, haemodynamic and tissue changes were more evident within 6 to 72 h post-infection [7]. Although several methods such as biochemical and histopathogical have been developed for detection of this bacterium; PCR-based detection was most widely accepted.

Out of the many virulence factors viz., extracellular products including aerolysins, α- and β-haemolysins [8, 9], enterotoxins, proteases, haemagglutinins and adhesins [10]; β-haemolysin was the most preferable from diagnostic point of view due to its abundance in all pathogenic strains in humans [11]. Xia et al. [12] first used the PCR technique to identify the β-haemolysin gene of pathogenic *A. hydrophila* strains from freshwater fish. Based on the above problems, the current work focused on the establishment of a challenge mode and study of tissue level persistence of *A. hydrophila* in *Jayanti* rohu that would able to answer a major issue in running selection programme for any fish species against this major pathogen of aquaculture importance.

2. Materials and methods

2.1. Fish

The rohu, *Labeo rohita* fingerlings were collected from selectively bred fish for increased growth (*Jayanti* rohu) generated under the on-going selective breeding programme for higher growth [13].

2.2. Experimental challenge tests

Rohu juveniles were collected from the nursery ponds and kept in 700 L ferro-cement tanks in an indoor wet laboratory for acclimatization. Water quality parameters were within optimum range (pH: 7.4 − 8.0, alkalinity: 171.3-197.3, nitrate: 0.0056 – 0.924 mg/L, nitrite: 0.560 – 0.632 mg/L, ammonia: 0.004 – 1.0842 mg/L, hardness: 3.4-3.7 mg/L, dissolved oxygen: 5.1 -6.4 mg/L). Fish were shifted to 500 L FRP tanks and divided into 11 groups with 40 fish in each group (one control and three experimental sub-groups containing 10 fish each). During bath challenge, water level was reduced to 40 L and overnight grown *A. hydrophila* culture was added to a final concentration of 1.4 x10^8 cfu mL^{-1}. The first group was challenged by only immersion for sixty minutes (A). Further challenge groups were made as follows: scale (5 scales $fish^{-1}$) removal (group B), skin abrasion of ~1 cm^2 with blunt sterile scalpel (group C), under high stocking (10 g fish L^{-1} water under aeration) density (group D), immunosuppression using 0.1 mg 0.1 mL^{-1} cyclophosphamide (CYP) intraperitonially 100 g^{-1} fish (group E) , under high stocking after scale removal (group F) and under high stocking after skin aberration (group G) and challenge of groups B to G after 3 days of procedure by immersion route. Intramuscular and intraperitoneal challenge modes were done with overnight grown *A. hydrophila* culture of 1.4 x10^8 cfu mL^{-1} (1.4 x10^7 cfu 100 μl^{-1}) with fixed (groups H and I, respectively) and varying (groups J and K, respectively) body weight of fish. Further, varying intraperitoneal doses (1.4 x10^7cfu 100 μl^{-1} 20 g^{-1} fish) with varying body weights (served as group L) was given to more number of individuals (200 fish with 50 fish in each sub-group). Mortalities were recorded up to 10 days post-challenge. The cause of mortality was determined and confirmed by isolating the bacteria from kidney tissues of 5% moribund fish. The control fish in each subgroup were given equal volume of PBS. During the experimental procedure (skin abrasion, de-scaling or injection), the fish were prior exposed to anaesthetization (MS222, Sigma, USA).

2.3. Detection of bacterial load

Rohu juveniles in another experiment were challenged with an optimum LD_{50} dose of *A. hydrophila* (1.4 x10^7 cfu 100 μl^{-1} 20 g^{-1} fish) intraperitoneally and tissue samples (skin, muscle, liver, spleen, anterior and posterior kidneys, intestine, caudal fin, gill, eye and brain) were collected in ethanol at different time periods such as 0, 0.5, 1, 3, 6, 12, 24 h, 3, 7, and 15 days post-challenge in triplicate (euthanized with an overdose of anesthesia). DNA was extracted following phenol-chloroform extraction method [14]. Purity and concentration of the extracted DNA were quantified using Nanodrop ND-1000 (Thermo Scientific, USA). First step PCR was carried out using primer pair of β haemolysin gene [12] by taking 2 µg of the DNA from each sample that will produce 1.5 kb fragment. Again, a second step PCR was run with a nested set of primers [12], by taking 5 µl of the first step PCR product to amplify 208 bp fragment. The amplified products (8 µl) were analysed in 1% agarose gel. The gel were visualized under UV-transilluminator and captured in Alpha Imager HP (Alpha Innotech Corp., USA).

3. Results and discussion

No mortality was observed (Table 1) in the first five groups (A, B, C, D and E) indicating the little effect of injury and/or overcrowding on bacterial pathogenesis when these factors act individually to cause infection. However, in groups F and G, where the scales were removed and abrasion was made prior to the bath and kept in comparatively high stock density, mortalities were recorded up to 16.67% and 66.67%, respectively. High stocking density plays a significant predisposing factor for bacterial pathogenesis since it promotes stress in fish. The gross clinical signs viz., necrosis, sloughing of caudal fin (erosion), development of deep ulcers beyond the abraded region exposing vertebral column, ventral congestion of body and congested operculum in group F revealed more stress effects and pathology especially during skin injury and crowding. Systemic infections were readily produced in channel catfish, *Ictalurus punctatus* having abraded skin prior to the bacterial exposure [15]. Fish treated with CYP prior to challenge reported a single mortality out of 30 fish challenged. There was a remarkable immunosuppressive action of CYP which can enhance *A. hydrophila* infection in freshwater catfish [16]. The immersion challenge may not be effective enough to initiate infection after the immunosuppressive action of CYP here in this case.

To develop a challenge model, it's difficult to generate an equivalent stressful environment every time where a large number of fish needs to be challenged in a short period of time. Additionally, the infection response can also be affected by resistance[17] and the size of the animal [8]. In group H (intramuscular challenge), 20-30% mortality was observed within 48-72 h post-infection. However, the results varied (10-20% mortality) when a range of 10-95 g fish were challenged (group J) with the same dose. Though a mortality of 50-60% was observed in group I (intraperitoneal challenge) within 6-48 h post-challenge, whereas the mortality varied between 30-40% when variable weights of 10-95 g fish were challenged (group K) with the same dose. However, intraperitoneal challenge given g^{-1} body weight (group L) with varying weights of fish gave a perfect 50% mortality which was confirmed by a number of individuals. An hourly record of mortality revealed maximum mortality within 6 to 12 h post challenge (Fig. 1). This indicated the peak of bacterial infectivity during those time periods.

Again to study bacterial load in each tissue as it happens in case of systemic infection, intraperitoneal injection seems to be a useful mode since it could infect all the internal organs. In

case of intramuscular challenge of *A. hydrophila* in Nile tilapia, *Oreochromis niloticus* (L.) infections were noticed only in skin and muscle, whereas intraperitoneal injection established infections in visceral organs like liver and kidney [19]. In the present study, detection of bacteria in various organs was evident from 30 min post challenge onwards, especially in liver, posterior kidney, muscle and skin tissues (Fig. 2). Other tissues were devoid of this bacterium up to 3 h post challenge (hpc) except for spleen where infection was noticed at 1 hpc. This indicates that bacteria start infecting its target organs; liver and kidney [20] of host body as soon as it enters. The presence of bacterium in skin and muscle at early periods might be responsible for causing haemorrhagic septicaemia in this infection, a prominent clinical signs and also it indicates the quick release and spread of this organism from infected host to cause acute horizontal transmission. However, after 6 hpc, the infection was quite evident in anterior kidney, gill, caudal fin and eye in addition to the previous described tissues. Further, at 12 hpc, infection could be detected in intestine and brain along with other tissues. Initiation of bacterial clearance started at 24 hpc from eye tissue. Along with the structural complexity, fish eye is equipped with many cellular and leukocytic defense molecules which protect it from infection [21]. At 3 dpc bacteria were cleared off from kidney, caudal fin and skin. Kidney serves as the main organ of defense in bacterial infection by producing many hydrolytic enzymes such as myeloperoxidase, lysozymes along with other antimicrobial peptides [22]. The cutaneous layer of fish skin contains many innate immune molecules which are involved in the bacterial defense mechanism [23]. In tilapia hybrids and white cachama as well tissue changes in liver, kidney, spleen, intestine, muscle, stomach and brain were noticed up to 3 d post-infection to *A. hydrophila* [7]. At the end of 7 days post-challenge (dpc) the bacteria got eliminated from other major tissues such as liver, spleen, intestine and gill tissues. The haemato-biochemical changes such as activities of alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine amino transferase (ALT) were the highest on 3 d post-infection in liver tissue of rohu during *A. hydrophila* infection [24]. Spleen being one of the main lymphoid organs of fish plays an essential role in antigen trapping [25]. Spleenic macrophages and neutrophils counts increase at 24-48 h post infection [26]. Absence of bacteria in gill and intestine may be due their cutaneous mucosal layer [23]. However, in the present study infection persisted up to 7 dpc in brain and muscle tissues. Sometimes bacterial infections carried asymptomatically in brain of fish without showing any signs of disease [27]. Probably, these two organs are not competent enough to produce large amount of innate immune molecules to handle the pathogen. Bacterial infection might have been detected in the muscle tissue of survivors at the site of intraperitoneal injection as in case of rainbow trout [28]. Moreover, at 15 dpc no infection was detected in any of the tissue examined. Similarly, bacteria were not detected in water samples after day 9 of bath challenge in rainbow trout when mortality ceased and dead fish were removed from the aquaria [28]. Therefore, the survivors of the challenge test would not serve as a carrier of infection and hence, those fish might serve as a potential stock for developing broodstock especially in the case of selective breeding programs for disease resistance.

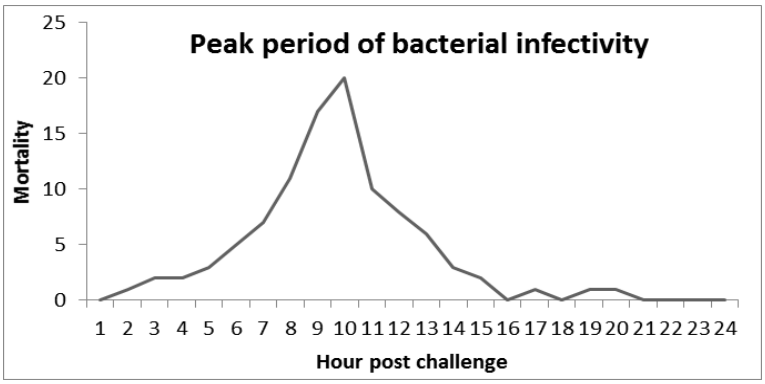

Fig 1: Hourly mortality records of challenge test where *Aeromonas hydrophila* was injected intraperitoneally g^{-1} weight (group L) of rohu juveniles

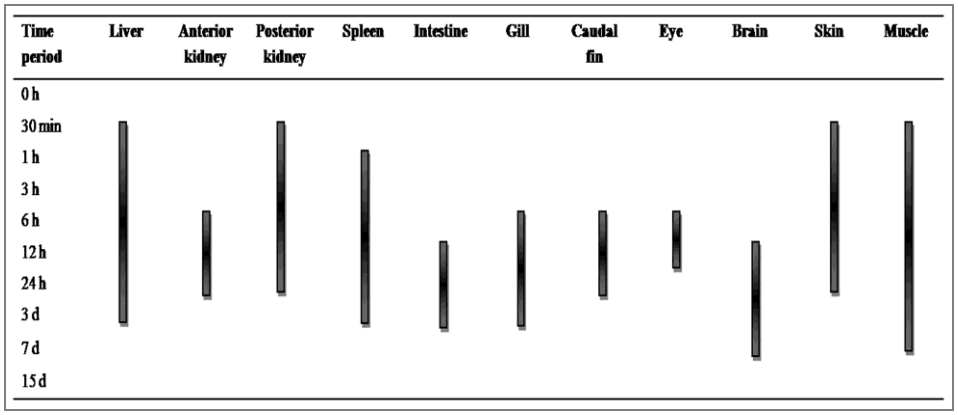

Fig 2: Appearance of bacteria (denoted by black bars) from infection to clearance at different time periods in various tissues of rohu post-infection to *Aeromonas hydrophila* detected through nested PCR based on β-haemolysin gene primers[12]

4. Conclusion

The study established that *A. hydrophila* is an opportunistic secondary invader in rohu that may flare up infection under stressful conditions or may arise due to high stocking density associated with other stresses. It further adds that for a suitable experimental challenge model, intraperitoneal injection g^{-1} body weight may serve as a reliable method to get a perfect LD_{50} value even with varying weight ranges. Once infected, it can affect almost all the internal organs within 12 h of infection and can cause maximum mortality. The survivors of infection after two weeks do not act as carriers of this infection and hence, would be a better brood stock for selective breeding programs for increased disease resistance than their sibs.

Table: 1 Mortality records (no. of dead fish/ no. of challenged fish) in twelve routes of bacterial exposure such as only immersion (A), bath after scale removal (B), bath after skin abrasion (C), bath under high stocking density (D), bath after immunosuppression (E), bath under high stocking after scale removal (F), bath under high stocking after skin aberration (G), intramuscular (H), intraperitoneal (I), fixed intramuscular dose with varying body weight (J), fixed intraperitoneal dose with varying body weight (K) and variable intraperitoneal dose as per gram body weight (L)

Replicate/Group	A	B	C	D	E	F	G	H	I	J	K	L
1	0/10	0/11	0/10	0/10	0/10	1/10	6/10	2/10	5/10	1/10	3/10	24/50
2	0/10	0/11	0/10	0/10	0/10	4/10	8/10	3/10	5/10	2/11	4/10	26/50
3	0/10	0/11	0/9	0/10	1/10	0/10	6/10	3/10	6/11	1/11	4/10	25/50
% mortality (Average)	0	0	0	0	3.3	16.7	66.7	60.0	51.6	12.5	36.7	50.0
Control for each group	0/10	0/10	0/10	0/10	0/11	0/10	0/10	0/10	0/10	0/10	0/10	0/50

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