Research in
ISSN : P-2409-0603, E-2409-9325
AGRICULTURE, LIVESTOCK and FISHERIES
An Open Access Peer Reviewed Journal

Open Access **Res. Agric. Livest. Fish.**
Research Article **Vol. 5, No. 1, April 2018 : 137-145.**

DEVELOPMENT OF ANTI-*Aeromonas hydrophila* SERUM FROM RABBITS

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ARTICLE INFO

Received
06 April, 2018

Accepted
26 April, 2018

Online
30 April, 2018

Key words
Serum
Aeromonas hydrophila
Rabbit
FKC
HKC
MAS

ABSTRACT

An experiment was conducted to prepare anti-*Aeromonas hydrophila* serum in two rabbits (*Oryctolagus cuniculus*). Formalin killed cell (FKC) and heat killed cell (HKC) were prepared by adding 0.5% formalin and 2.5 h heat treatment at 60°C of freshly cultured *Aeromonas hydrophila* (AQC810) isolated from naturally infected koi fish kidney respectively. Slide agglutination tests were performed for the determination of the presence of specific antibody against *Aeromonas hydrophila* in the serum collected from the blood of pre-immune rabbits or post immune rabbits. No agglutination of pre-immune serum with the homologous antigen proved that the rabbits had no previous *Aeromonas* contamination. Rabbits were injected subcutaneously with the FKC added with same amount of Freund's complete adjuvant. Three booster doses were given without the adjuvant at weekly intervals after second week of first injection. Agglutination titration of anti-*Aeromonas hydrophila* rabbit serum against HKC and FKC of homologous bacteria showed that the titers increased with the post inoculation days which rose at their peak during the fifth week of immunization and after three consecutive booster doses at weekly intervals starting from second week of first immunization. Rabbits were sacrificed after six weeks of immunization, whole blood, collected and centrifuged to accumulate the serum that were complement-inactivated by heating to 58º C for 30 min and stored at -20°C in appendorph tubes, with the aim of rapid diagnosis of *Aeromonas* infections in fishes. Result of this study would initiate the way of vaccine development against MAS (motile *Aeromonas* septicemia) in the aquaculture fishes of Bangladesh.

To cite this article: Hossen MN, SM Rakib, MA Rahman, S Sku, S Akter and MM Rashid, 2018. Development of anti-*Aeromonas hydrophila* serum from Rabbits. Res. Agric. Livest. Fish. 5 (1): 137-145.

INTRODUCTION

Motile *Aeromonas* septicemia (MAS) is an alarming problem now-a-days in Bangladesh Aquaculture caused by *Aeromonas hydrophila* (Rahman et al. 1996). Isolated it from an ulcer diseased farmed carp fishes of Mymensingh. Iqbal et al. (1998) Detected *A. hydrophila, A. veronii, A. sobria, and A. jandaei* as pathogenic bacteria from EUS affected mrigals. Studied effect of water temperature on the infectivity of *A. hydrophila* isolates. Detected the pathogen from naturally infected Thai pangas *Pangasius hypophthalmus* (Sarker et al., 2000). Experimentally infected Thai pangas with *A. hydrophila* successively (Alam, 2009 and Roshid, 2009). Anti-pathogenic rabbit serum is widely used in fish disease laboratories for identification of endemic fish disease and other serological research related in fish immunology. The only method to prevent disease in aquaculture is strengthening the defense mechanism of fish through prior administration of an antigen prepared for the homologous pathogen (Robertson et al. 2003). Rashid (1997) prepared anti-*Edwardsiella tarda* rabbit serum. Swain (2007) prepared Rabbit anti-rohu globulin conjugate from healthy adult rohu. Maji (2005) produced hyperimmune serum against the crude antigen of *A. hydrophila* from two New Zealand White male rabbits.Prepared antibody would be used to identify the *A. hydrophila* bacteria of any suspective sample by a rapid diagnostic method called “slide agglutination method” which will need only one minute. This antibody would also be used to serotype all *A. hydrophila* isolates in order to understand intra specific relationship/variation of the bacteria all over Bangladesh. This will also be helpful for the preparation of vaccine against the pathogen *A. hydrophila*. Thus, appropriate remedial measures can be taken against MAS within a very short time. So the present study was performed to attain the following objectives-

i. To prepare anti-*Aeromonas hydrophila* rabbit serum;
ii. To test the efficacy of the different booster doses in the production of the antiserum;
iii. To determine the titer of the antiserum against *Aeromonas hydrophila*; and
iv. To establish rapid diagnosis process of *Aeromonas* infection.

MATERIALS AND METHODS

Collection and acclimatization of experimental rabbits

Two male rabbits: one, white A of 1.0 Kg and another, white B of 0.90 Kg were bought from Market of Mymensingh district. The animals were apparently healthy with no detectable signs and symptoms of any disease.

Cleaning and sterilization of required glass wares and plastic wares

Experimental glasswares and plastic wares such as syringe, porcelain mortar, glass bottle eppendorph tubes, conical flask, measuring cylinder, pipettes, petridish, and screw-capped glass container were treated with 2% sodium hypochliride solution for cleaning. The cleaned glass wares were then dried in a drier at 70°C overnight and sterilized by a dry sterilizer at 160°C for 1.5 h. The tips for micropipettes were placed in a tip - dispensing box, autoclaved and dried as above. All sterile glasswares and plastic wares were placed in a clean place.

Preparation of physiological saline

Physiological saline (PS) was prepared by dissolving 0.85 g of chemically pure sodium chloride (NaCl) in 100 ml of distilled water in a conical flask. The PS was then sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the saline was cooled and kept at 4°C for future use.

Preparation of phosphate buffered saline

Phosphate buffered saline (PBS) was prepared by dissolving 8 grams of sodium chloride (NaCl), 0.2 grams of potassium chloride (KCl), 2.89 grams of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$) and 0.2 grams of potassium hydrogen phosphate (KH_2PO_4) in 1000 ml of distilled water. The pH of the solution was measured by pH meter and adjusted to 7.0-7.2. The solution was then sterilized and was stored by following the above method.

TSA (triptic soya agar) plate preparation

Required amount (40 g/L) of triptic soya agar (TSA) was weighed and placed in a clean conical flask. The conical flask was heated on hot plate and then sterilized by autoclaving at 121°C for 15 minutes. After solidification, the plates were put in upside down position for at least overnight and then kept at 4°C for future use.

Culture of *Aeromonas hydrophila* bacteria

Previously collected *Aeromonas hydrophila* bacteria stored in TSA slants with paraffin oil, were streaked onto TSA plates and incubated at 25°C for 48 h to observe characteristic colony appearance.Fresh TSA agar slants were then prepared as above for future use. *A. hydrophila* organisms were freshly cultured to test their morphological, biochemical and physiological characteristics for reconfirmation of their specific characters.

Confirmatory test of *A. hydrophila*

A fresh culture of test organism was obtained. A sterile swab was prepared from the pure culture. This swab was streaked onto a non-selective blood agar plate (containing 0.5% NaCl) in three directions to obtain a heavy, confluent growth. Aseptically one 150 µg 0129 disk was placed onto the agar surface. The plate was incubated aerobically at 35°C for 24 h. The zone of inhibition was observed.

Preparation of FKC (formalin killed cell)

For formalin killed cell preparation 100 mg colonies were collected from a freshly culture plate of *A. hydrophila* and suspended into sterile PS. One drop of formalin was added to the 10 ml of bacterial suspension (0.5%) washed twice in physiological saline and centrifuged. The formalin treated suspension was incubated at 37°C for 2 h and 100 µl was plated onto TSA plate, incubated at 37°C for 1day to observe no growth any bacteria.

Preparation of HKC (heat killed cell)

For heat killed cell preparation, 10ml of such bacterial suspension was allowed to kill by heat treatment keeping at 60°C for 2.5h in a water bath .After heat treatment; 100µl was incubated onto a TSA plate and kept at 37°C for 1day to observe no growth of bacteria.

Antigenic preparation

Antigen was prepared by mixing with 1.5 ml FKC (formalin killed cell) and 1.5 ml FCA (freund's adjuvant, complete) in a sterilized porcelain pot and stirred with a grinder until complete mixing. After, completion of proper mixing this antigenic preparation was stored for future use.

Preparing the rabbits for immunization

After collection, 1 ml blood from each rabbit was collected by cutting the ventral peripheral vein of ear by a new blade and simultaneously adding vaseline as an anti-coagulant and collecting the blood drops in a test tube. They were then fed with the antibiotic cotrim suspension at a dose of .5 tea spoon/kg body weight at 12 hours intervals for 5 consecutive days. After completion of antibiotic dose the rabbits were reared for 15 days to acclimatize with the animal house condition prior to immunization. The collected blood were centrifuged at 1000 rpm for 10 minutes, the supernatant sera were separated and subjected to agglutination tests.

Slide agglutination test

Slide agglutination tests were performed for the determination of the presence of specific antibody against *A. hydrophila* in the rabbit serum collected from the blood of pre-immune rabbit or immunized with FKC. One drop of PS and one drop of anti-serum were taken on the slide and kept at room temperature for two minutes to observe the agglutination reaction.

Immunization of rabbit

1.0 ml of adjuvant-mixed-antigen was taken into a sterile syringe for injecting into the white A rabbit (1.0 Kg body weight). The rabbit was then placed on the table for injecting, furs of its shoulder were cut out and the skin was disinfected with 70% alcohol cotton. The antigen was then injected subcutaneously in to five sites in the shoulder of the animal (0.20ml site^{-1}). The White B rabbit (0.9 Kg body weight) was also immunized with 1 ml antigen in the same way.

After 15 days 1 ml blood was collected from ear of each rabbit as above and subjected to agglutination titration of the serum with the FKC as previously done with pre-immune serum. Immediately after the collection of blood 1 ml FKC was injected in same way to each rabbit without mixing with the Freund's adjuvant, complete as the first booster dose. One week after the injection of the first booster dose, another 1 ml blood was collected from each rabbit and second booster doses were given to them. These 1 ml bloods were subjected to agglutination titration as above to read the efficacy of the sera.After 1 week of the second booster dose, blood collection, injection of third booster dose and agglutination titration were done in the same way.

Agglutination titration

Ninety six (96) well U-bottomed microtitre plates (Nunck, Japan) were used for the agglutination titration work to determine the antibody titres of the sera of rabbits. Each well of the microtiter plate was filled with one drop (10 µl) of sterile PBS. Diluters were heated to red heat, cooled in air, touched with the upper meniscus of the 20% diluted anti-serum with PBS and put into the first well of each line. They were mixed well with the PBS and then put in the second well and after mixing well, in the 3rd well. In this way the diluters were put into the 11th well to make serial dilutions of the 20% serum from the first well up to the 11th well. The 12th well of each line was kept untouched with the serum and designated as the negative control well. One drop of each of FKC or HKC was then put into each of the 12 wells. Duplicate lines were used for each antigen (FKC or HKC).

The microtiter plate was then wrapped with a plastic wrapper, shaked by a micro shaker for 5 minutes, incubated at 37°C for 2 h and kept at 4°C over night. Next morning the plate was observed upon a tube light to read the titer optically. The numerical expression of the titer was as follows. Strength of the agglutination in the first well was expressed as 20, a numerical expression of the titer of that well and doubling the number for each well up to the eleventh well. The right most well showing the agglutination appeared as power of the antibody (antibody titer). The lower value from the two duplicate lines was accepted.

Collection and preservation of the rabbit anti-serum

After one week of the third booster dose, when the titers rose sufficient enough, the rabbits were anaesthetized and sacrificed; total bloods were collected from their heart into a sterilized plastic syringe and kept in slanted position for one hour at room temperature. After the blood became clotted, the antiserum was collected by a pipette in centrifuge tubes. After centrifugation at 1000 rpm for 15 minutes, the serum was separated from the RBC and heated at 58°C for half an hour to inactivate the complements. Such prepared complement inactivated anti-*Aeromonas hydrophila* rabbit serum was then allocated to 1 ml in each sterile eppendorph tube and stored at 20°C for future use.

RESULTS

Characteristics of *Aeromonas hydrophila*

Results of the morphological, bio-chemical, and physiological characters of *Aeromonas hydrophila* compared with the characters by Popoff et al. (1984) and Sabur (2006) are shown in Table 1.

Status of pre-immune serum

Results of slide agglutination tests of the pre-immune rabbit serum with FKC and HKC of *Aeromonas hydrophila* showed that no agglutination was occurred (Table 2).

Slide agglutination results of immune sera

Results of slide agglutination at 15 day-, 21 day-, 28 day- and 36 day- post immunization of the rabbit serum against FKC and HKC of *A. hydrophila* by 20 fold, 10 fold, and 100% of the serums are shown in Table 3. At 15 day post-immunization the undiluted antiserum agglutinated against both FKC and HKC of *A. hydrophila* but neither of the 10 fold and 20 fold diluted antisera agglutinated. At 21 day post-immunization and having first booster dose at 15 day post-immunization the undiluted as well as 10 fold diluted antiserum agglutinated against FKC as well as HKC. However, 20 fold diluted antiserum did not agglutinate. At 28 day and 36 day of post-immunization and after receiving 2nd and 3rd booster dose respectively, all the 10 fold and 20 fold diluted antisera agglutinated against both of the FKC and HKC.

Table 1. Characteristics of *Aeromonas hydrophila* bacteria in comparison to Popoff et al. (1984) and Sabur (2006)

Characters	Characterization by Popoff et al.(1984)	Characterization by Sabur (2006)	Present result
Gram stain	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
Sensitivity to 0129	ND	ND	-
Oxidase	+	+	+
Catalase	+	+	
OF test	F	F	F
Lactose	+	+	+
Acid & gas production from glucose	+	+	+
Acid production from			
Sucrose	+	+	+
Maltose	+	+	+
Manitol	+	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Esculin hydrolysis	ND	ND	+
Methyl-red test	-	-	-
Voges-Proskaur	+	+	+
Indole	+	+	+
H_2S production	+	+	+
Arginine decomposition	+	+	+
Lysine decomposition	-	-	-
Ornithine decarboxilation	-	-	-
Citrate utilization	+	+	+
Growth at: 4°C	-	-	-
5°C	+	+	+
37°C	+	+	+
40°C	-	-	-

-: Negative; +: Positive; F: Fermentative; ND: Not done

Table 2. Slide agglutination test results of pre-immune rabbit serum with FKC andHKC of *Aeromonas hydrophila*

Antigen	Agglutination of Serum		
	20-fold diluted	10-fold diluted	Undiluted serum
FKC	-	-	-
HKC	-	-	-

- : No agglutination

Table 3. Results of slide agglutination tests of immune serum after different days of post immunization with FKC and HKC of *Aeromonas hydrophila* bacteria

Post immunization days	Antiserum	Agglutination	
		FKC	HKC
15	20-fold	-	-
	10-fold	-	-
	100%	+	+
21	20-fold	-	-
	10-fold	+	+
	100%	+	+
28	20-fold	+	+
	10-fold	+	+
	100%	+	+
36	20-fold	+	+
	10-fold	+	+
	100%	+	+

- : No agglutination; + : Agglutination was observed

Agglutination titer of immune rabbit serum

The results of the agglutination titration of immune rabbit serum with FKC and HKC of *A.hydrophila*at 15 day-, 21 day-, 28 day-, and 36 day- post-immunization are shown in Table 4.At 15 day post-immunization the titers of the antiserum from white A rabbit were 80 with FKC and 40 with HKC of *A. hydrophila.* From white B rabbit, they were 80 with FKC and 40 with HKC. At 21 day post-immunization and after receiving 1st booster dose at 15 day post-immunization, the titers of the antiserum from the white A rabbit were 640 with FKC of *A.hydrophila*and 80, with HKC. From the white B rabbit, they were 320 with FKC and 160with HKC.

At 28 day post immunization and after receiving 2nd booster dose at 21 day post immunization the titers of the antiserum from the white A rabbit were 1280 and 160 with FKC and HKC respectively whereas, the titers from white B rabbit were 1280 with FKC and 320 with HKC.

At 36 day post immunization and after receiving 3rd booster dose at 28 day post immunization the titers of the antiserum were 1280 and 320 with FKC and HKC of *A.hydrophila* respectively; and the titers of the antiserum from the white B rabbit were 1280and 640 with FKC and respectively. HKC showed lower titer than the FKC.

Table 4. Distribution of agglutination titer of the anti- *Aeromonas hydrophila* rabbit serum against HKC and FKC of homologous bacteria

Post-immunization days of blood collection from rabbit	Titer			
	FKC		HKC	
	White A	White B	White A	White B
15	80	80	40	40
21	640	320	80	160
28	1280	1280	160	320
36	1280	1280	320	640

FKC = formalin-killed cells; HKC = heat-killed cells

Anti *A. hydrophila* rabbit serum

In total 38 ml blood could be collected from two rabbits. After centrifugation, 17 ml serum could be collected from the rabbits.

DISCUSSION

Serology and serodiagnosis of bacterial fish pathogens have contributed a lot to fish health management in recent years. A wide variety of serological procedures have been increasingly used to provide diagnostic techniques for fish pathogens, detect antibodies against specific pathogens in fish sera, evaluate vaccines, and establish serological relatedness of strains of bacterial fish pathogens. From the view point of developing an effective vaccine against *A. hydrophila* infection, the more bacterial serotype present, both locally and internationally, the less the chance there is of developing an effective vaccine. Also, a vaccine containing many serotypes will be more expensive to produce. For the vaccine to be effective in the prevention of motile *Aeromonas* septicemia (MAS), more work is needed to clarify the antigenic types of *A. hydrophila* from different geographical regions. Most disease outbreaks caused by *A. hydrophila* are currently confirmed from bacterial culture after isolation of the pathogen from infected fish. This can be time consuming and is often unsuccessful since the bacterium is particularly fastidious.

The results of the characterization done by Popoff et al. (1984), Sabur (2006) and during the present study were same. No agglutination of pre-immune serum with the *A. hydrophila* antigen proved that the rabbits had no previous *A. hydrophila* contamination.

Slide agglutination test results at different days of post-immunization proved that the antibody started to be concentrated in the serum of experimental rabbits. At 15 days post-immunization little antibody production *in-vivo* in the rabbit's serum was evident by positive agglutination of 100% serum but negative results of 10 and 20 fold dilutions. From the 4th week 20 fold diluted antiserum started showing positive agglutination result explaining its considerable concentration in the blood of the immunized rabbit. Mamnur Rashid et al. (1994b) found the similar results during their immunization work on New Zealand white rabbit with *Edwardsiella tarda* bacteria. Majumder (2006) also found the similar result during his immunization work on locally available white and black rabbits with *Aeromonas hydrophila.* Results of the agglutination titration of presently prepared *Aeromonas hydrophila* rabbit serum against FKC and HKC cells of homologous bacteria showed that the titers increased with the post inoculation days which rose at their peaks during the 5th week of immunization and after three consecutive booster doses at weekly intervals starting from 2nd week of immunization. However, HKCs always showed lower titers than the FKCs of both the rabbit as also shown by Mamnur Rashid (1997) and Majumder (2006) which was in confirmation of the theory of Brock and Medigan (1994). FKC contained more antigen than HKC because heat destroyed some of the protein. Although some interesting serological works have been done for different pathogen in different countries like Mamnur Rashid et al., (1994a,b), Guz (2001), Hua et al. (2002), Okbi et al. (2005), Fei et al. (2007) and Mali et al. (2007), before Majumder (2006) no such work had ever been done with Aeromonas hydrophila isolates in Bangladesh

For a fruitful contribution towards the vaccination steps against *A. hydrophila*, serotyping of different isolates of the homologous bacteria from different environmental condition of Bangladesh aquaculture including those of Sabur (2006) has become an important step from the result of the present work. Moreover, simultaneous sero-diagnosis of suspected *Aeromonas* fish pathogen should also be done with the present anti *Aeromonas hydrophila* rabbit serum.

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