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Spontaneous haemolytic activity of rohu (*Labeo rohita*) serum

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Abstract

The spontaneous haemolytic activity has been used as an indicator of the effects of inherent or external factors on the immune system and the disease resistance of fish. The present study was undertaken to estimate the spontaneous haemolytic activity of healthy rohu, (Labeo rohita) fingerlings serum and to determine optimum temperature, capacity to lyse RBC from different donors, heat sensitivity, storage temperature and effect of different agents like EDTA, EGTA, on spontaneous haemolytic activity of rohu. Besides this, complement binding factors such as zymosan, Lipopolysacharide were tested for spontaneous haemolytic 50 % (SH 50%) activity. The effect of bacterial culture was also determined on SH 50% activity of rohu sera. The optimum lysis was obtained with rabbit RBC at 20 •C for rohu sera. The heat sensitivity of sera found to be 65 •C. The haemolytic activity of rohu sera maintained fairly good activity after short term storage at -80 •C as well as 5 months storage at -80 •C. The EGTA inhibited the SH 50% but EDTA enhanced the SH 50% activity. Zymosan significantly inhibited the haemolytic activity of rohu sera whereas bacterial LPS and Aeromonas hydrophila culture reduced the SH 50% activity to less extent.

Key words: Complement; spontaneous haemolytic activity; heat sensitivity; zymosan, A. hydrophila; Labeo rohita

I. INTRODUCTION

Immunity of a species has evolved from non-specific immune system which acts through pattern recognition receptor and eliminates pathogenic invaders by acting on pathogen associated molecular patterns which is conserved among microbes (1-3). Although the specific immune system is required for homeostasis of animals and for preventing recurrent infection. The innate immune system which acts as a first line of defense and provides immunity in non-specific manner without time lag (4). The innate immune system is very much efficient in invertebrates, which rely exclusively on it for surviving in diverse aquatic environmental condition. The innate system is also of primary importance in combating microbial infection in fish and also the fish contains many non-specific humoral defense mechanisms in order to compensate their reduced specific response (5). Among these, the spontaneous haemolytic activity of normal sera for untreated RBC is believed to be the function of alternative pathway of complement system, which has been evolved first in fish (6). Alternative complement pathway of complement system is very efficient and powerful humoral defense mechanism which provides

protection from a variety of potentially disease causing microorganisms such as bacteria, fungi, viruses or parasites (7). The higher ACP titer and lower optimal temperature (10-17°C) than mammals has been reported in fish, which makes fish complement the most effective immune mechanism (8). Complement system of fish is very efficient and play important role in innate immune response. Like mammalian complement system, the fish complement system is activated through three pathways viz. classical pathway-the antibody dependent, alternative complement pathway-the antibody independent and, activated directly by foreign microorganisms and lectin pathway (8, 9). All three pathways combine to form lytic pathway i.e., formation of membrane attack complex which leads to direct lysis of invading pathogens. The complement system is composed of numerous proteins and all pathways generate complement component (C3), which has been described and isolated from teleost species (10). A recent study on the molecular characterization and tissue level expression of complement component (C3) of *L. rohita* by Pushpa et al., (2014) has also been reported (11).

The SH activity has been used as an indicator of the effects of inherent or external factors on the immune system and the disease resistance of fish. The factors are genetic traits (12), seasonal factors (13), the environmental temperature (14), pollution (15), handling and crowding stress (16), diets and food additives (17), immunostimulants and probiotics (18) as well as the effects of disease and vaccination (19), etc. Not much information is available on spontaneous haemolytic activity of fish, particularly in Indian major carp, *Labeo rohita* which is a commercially important freshwater carp. Recently, SH activity of seabass and halibut in normal sera was investigated by Lange and Magnadottir, 2003 (20). The present study was undertaken to investigate the SH activity of rohu (*L. rohita*) normal sera and to determine the haemolytic activity as a result of complement activation. Thus, gaining insight into the defense mechanism of the species not only could provide useful information on the potential of immune response and at the same time, it could be a valuable tool for monitoring health status of the fish under aquaculture practices.

II. MATERIALS AND METHODS

A. The experimental fish

Rohu (*Labeo rohita*), weighing 50 ± 5 g, were obtained from the local fish farm, Mumbai, India. The fish were maintained in a cemented circular 500 L capacity tank and acclimatized under aerated condition for the period of one month at ambient water temperature, 28 ± 2°C. Water was changed every two days intervals and fishes were fed ad-libitum with commercially available feed.

B. Blood collection and serum separation

Blood was collected using syringe from the caudal peduncle of 20-25 rohu after anaesthetizing the fish by immersing in two lit of water containing 50 ppm clove oil (Himedia, India). The blood was allowed to clot for 30 min at room temperature and then kept at 4°C for 4 h. The serum was separated by centrifugation at 1500 × g for 10 min at 4 °C (Hareus, USA). Serum was stored in aliquots at -80° C until further use. Eight different serum samples from eight different fish was collected and evaluated for spontaneous hemolytic activity to see the variation among the fish serum with respect to the capacity to lyse rabbit RBCs.

C. Red blood cell (RBC) Preparation

The red blood cells from different donors (sheep, goat, rabbit, chicken and catfish, Pangasius sp.) were collected and mixed with equal volume of Alsevers anticoagulant solution (Sigma, USA) and were washed 2-3 times with normal saline (0.85% NaCl). The concentration of RBC was checked by adding 100 µl to 3.4 ml of distilled water and optical density read at 414 nm in ELISA plate reader (Biorad, USA). A 1% suspension should give a reading of 0.74 and the RBC dilution was adjusted to obtain this reading. The suspension was then diluted 1:1 in gelatin veronal buffer saline (GVB) to get a 0.5% suspension used for the haemolytic assay.

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D. The spontaneous haemolytic (SH) assay

The spontaneous haemolytic activity of rohu sera was estimated using a method described by Sakai (1992) 21, with some modifications Lange et al., (2001) 22. The target cells were RBC from sheep, goat, rabbit, chicken and Pangasius. The reaction buffer used was a GVB (Lonza, Switzerland) containing 10 mM barbital, and 145mM NaCl and 0.1% gelatin, pH 7.3.The test was carried out in 2 ml microcentrifuge tubes (Tarsons, India). A 2 fold dilution of rohu sera was made in GVB and 100 µl of 0.5% of respective RBC were added in all tubes and incubated at room temperature for 1 h with occasional shaking. For a negative control (0% lysis), GVB was used in the place of serum and positive control (100% lysis) distilled water replaced the serum. At the end of incubation period the tubes were centrifuged at 750 x g for 10 min, and 125 µl of the supernatant was collected from each well and transferred to a non-adsorbent flat bottom microtiter plate (Nunc, Denmark) and the absorbance read at 405 nm. Assay was done in triplicates for each dilution. A graph of OD values vs. serum dilution was drawn and the SH 50%, i.e., the serum dilution that gave 50% lysis was calculated. The 50% lysis point (SH 50%) was calculated by linear regression of each serum sample and expressed as the log dilution.

E. Sensitivity of rohu serum to mammalian, avian and fish RBC

Red blood cells from sheep, goat, rabbit, chicken and Pangasius were used as target cells in the Spontaneous Haemolytic test. All further assays were performed with sheep RBC at room temperature (28-30 °C). Eight individual fish sera were evaluated to see the difference of spontaneous haemolytic activity.

F. Estimation of optimum assay temperature for SH 50%

To estimate the optimum assay temperature for SH 50 % with sheep RBC, the assay was carried out by incubating samples at different temperatures viz. 10°C, 20°C and 30°C.

G. Heat sensitivity test

To determine the heat sensitivity of the complement factors in rohu sera, the serum was incubated at 18, 27, 45, 56 and 65°C for 30 min and the SH 50 % assay was carried out as described earlier.

H. The effects of EGTA and EDTA

The SH50% assay was carried out with GVB supplemented with 1 mM EGTA or 0.5 mM-5 mM EDTA.

I. Sensitivity of rohu sera with *Aeromonas hydrophila* culture

The *A. hydrophila* (ATCC7966) was grown for 24 h at 30°C in nutrient broth and equal volume of culture was incubated with rohu serum for 30 min and SH activity of serum was tested.

J. The effects of complement binding factors

Prior to carrying out the assay, the sera were incubated at room temperature for 30-60 min with zymosan and lipo-polysaccharide. Untreated control sera used for comparison was, incubated under the same condition with GV buffer replacing the different agents.

K. Treatment of sera with zymosan

100 mg of zymosan (Sigma) was washed twice in 1-2 ml GVB by centrifuging it at 1200 x g for 5 min and the pellet was resuspended in 120 µl of serum and it was diluted 1:5 in GVB, incubated for 30 min at room temperature (28 °C). At the end of incubation period, it was centrifuged and the supernatant collected for testing the SH assay. The amount of zymosan used was equivalent to approximately 4 g ml^{-1} serum.

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L. LPS (Lipo-polysaccharide)

LPS, from E. coli (Sigma, USA) was used at 5mg ml^{-1}. 24 µl of undiluted serum was added to 50 µl of LPS (250 µg) and 46 µl GVB and incubated for 60 min at room temperature prior to carrying out the SH activity of treated serum.

M. To evaluate the effect of storage on SH activity

Rohu sera was pooled from 10 different fishes and one aliquot was stored at 4°C, and the SH activity was tested after 1 week. Two sera aliquot were stored at -80°C and SH activity was tested after 3 weeks and 5 months of storage. One sera aliquot was kept at -20°C and SH activity was tested after 1 year of storage.

N. Statistical analysis

For the statistical analysis of the results the one-way analysis of variance (ANOVA) and paired students t-test were used. $P<0.05$ was set as the critical value of significance. SPSS ver. 16 software was used for the statistical analysis.

III. RESULTS AND DISCUSSIONS

A. Optimum condition for spontaneous haemolytic activity of rohu serum

The assay temperature

The three different temperatures were used for incubation of sample and the optimum temperature was obtained as 20 °C, is the optimum temperature for maximum spontaneous haemolytic activity of rohu serum as shown in fig.1. Although there was little difference between SH 50% of rohu serum at 20 and 30 °C. Thus, for further experiments, room temperature was used for finding SH activity.

Red blood cell donors

The haemolytic activity of the sera showed variation with respect to the target cells used as shown in fig. 2. Rohu sera gave highest mean SH 50 % activity with rabbit RBC and lowest with Pangasius RBC and relatively lesser mean SH 50 % activity with goat and chicken RBC. There was variation in the spontaneous haemolytic activity among the eight individual fish sera tested (Fig. 3).

Heat sensitivity of rohu sera

The effects of different inactivation temperatures on SH 50 % activity of rohu sera is shown in fig. 4. The rohu sera showed a decreasing trend of mean SH 50 % activity with increasing temperature. There was a relatively small reduction in SH activity after inactivation at 22 and 37°C (about 11 % and 16 % reduction respectively) and at 45°C (about 23 % reduction). After inactivation at 56 °C the SH activity was about 25 % of control values but after 65 °C the mean SH 50 % reduced to approximately 90 % of control value.

The effect of EGTA, EDTA

The EGTA inhibited the mean SH 50 % activity of rohu sera whereas the EDTA enhanced the mean SH 50 % activity as evident from fig 4. The EDTA showed maximum mean SH 50 % activity at 1 mM, whereas the 0.5 mM, 2.5 mM and 5 mM concentrationsof EDTA also exhibited high SH 50 % activity than control value (Table 1, Fig.5). The mean SH 50 % activity reduced to approximately 40 % from 94 to 60 when assay was carried out at 1mM EGTA supplemented GVB (Table.1).

The effects of some complement binding factors on the SH activity of rohu sera

Zymosan and LPS showed reduction of SH 50 % activity of rohu sera, zymosan reduced the SH 50 % activity to a great extent as compared to control whereas LPS showed less reduction of SH 50 % activity of rohu sera as compared to control (Fig.6). The SH 50 % activity reduced to approximately 90 % when the sera was treated with zymosan, i.e., from 97 to 5. LPS reduced SH 50% activity by approximately 40 %, from 102 to 62.

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The effect of *Aeromonas hydrophila* culture on SH activity of rohu sera

The incubation of 24 h fresh *A. hydrophila* culture with rohu sera showed reduction in the mean SH 50 % activity (Fig.6). The reduction of SH 50 % activity was more than 63 % as compared to control.

The effect of storage on the SH activity of the sera

The effect of storage on the SH activity of the rohu sera is shown in fig.7. The storage of sera for 1 year at -20 °C diminished the SH 50 % activity, from 105 to 9, i.e., nearly 95 % reduction in SH 50% activity while storage at -80 °C for 3 weeks and 5 months showed slightly less reduction in SH 50 % activity than the control values. The storage at 4 °C for 1 week, reduced the SH 50 % activity to a certain extent than control.

The spontaneous haemolytic activity of serum is generally considered to be the function of alternative complement pathway of complement system, a humoral component in innate immune system of fish (17 Holland and Lambris, 2002; 23 Nikoskelainen et al., 2002; 24 Boesen et al., 1999; 25 Fock et al., 2001). In the present study, the spontaneous haemolytic activity of normal serum was evaluated with regard to different RBC donors, storage conditions, different assay temperature, and also the effects of different chemicals such as EDTA, EGTA, and complement binding factors.

The optimum temperature of rohu sera was obtained in our present study was 20°C while the earlier studies on seabass and halibut (20 Lange and Magnadottir, 2003) showed optimum assay temperature of 22°C in seabass and 16°C in the case of halibut. The spontaneous haemolytic activity of normal sera varies species to species.

The RBC donors used in our study were sheep, goat, rabbit, chicken and Pangasius fish. The SH activity of rohu sera was highest with rabbit RBC and was lowest with Pangasius fish. The result indicated that the rohu serum was more sensitive to rabbit RBC. Earlier study corroborated similar and different results, where the Seabass (*Dicentrarchuslabrax*) showed the highest activity with rabbit RBCs while halibut sera showed more sensitivity to sheep RBC (20). Usually rabbit RBC was found to be the good activators of complement system of fish (13, 26). The less SH activity of rohu serum with Pangasius RBC may be attributed to the closer phylogenetic relationship between the two species than between the fish and mammalian or avian species.

The SH activity of rohu serum showed decreasing trend with increase in the inactivation temperature. The 56 °C and 65 °C diminished the SH activity of rohu serum to a great extent indicating, that rohu serum was more sensitive to heat. However, there was little reduction in SH activity when incubated at 18 °C for 30 min, as reported earlier in sea bass and halibut sera (20), and in seabream sera (26).

EDTA, which binds with both Ca^{2+} and Mg^{2+}, inhibits both alternative and classical complement pathway, but in our present study, the lower as well as high concentration of EDTA enhanced the SH activity of rohu sera, when EDTA supplemented buffer was used. This unusual increase in the SH activity of rohu sera may be due to the increase in the ionic strength of buffer. Earlier study support our finding (13) was in cod (*Gadus* morhua) serum, there was also unusual increase in SH activity of cod serum when assayed with EDTA supplemented buffer. Lange and Magnadottir, (2003) (20) described that there was reduction in SH activity of seabass and halibut sera when assay was carried out using the buffer supplemented with 1 mM and 10 mM concentration of EDTA respectively.

EGTA, which binds with Ca^{2+} is a inhibitor of classical complement pathway. In the present study, there was slight reduction in the SH activity when EGTA supplemented buffer was used. Previous studies showed contrasting effects, an inhibitory effect of EGTA on SH activity was observed on seabass (*Dicentrarchus labrax*) and halibut (*Hippoglossushippoglossus*) sera (20), whereas, there was no effect of the EGTA on SH activity of cod sera (13).

The complement binding factors such as zymosan (fungal product) and LPS (bacterial cell wall component) reduced the SH activity of rohu sera. There was significant reduction of SH activity of rohu

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sera after treatment with zymosan whereas the LPS reduced the SH activity to less extent. The fungal and bacterial product was known activators of fish and mammalian complement system (27). Some studies (13, 20, 26, 27) described the reduction of SH activity of normal serum after treatment with these complement binding factors.

The rohu sera showed reduction in SH activity when incubated with fresh culture of *A. hydrophila indicating presence of* the LPS on the cell wall of bacteria. Similarly, it has been described that the bacterial preparation has reduced the SH activity of cod sera (13). Moreover, this study demonstrated the variation in haemolytic activity of sera among eight individual fish sera tested for this fish species. Similar feature has also been demonstrated in earlier studies on cod sera and on Atlantic halibut and sea bass sera (13, 20). Such variation at individual level may be due to unseen handling stress, which is unavoidable as for drawing of blood from fish.

IV. CONCLUSION

This study indicated that the mechanisms of serum complement activities for diverse fish species studied are similar for some characteristics and different for other characteristics as evident from the present work. These may highlight the species specific characteristics of the components participating in the SH activity in serum. The present data highlight the significant contribution of the complement system to immunological activities of this species. Rohu represent an extremely successful cultured Indian major carps that have good contribution for freshwater aquaculture production. The serum-complement system could be a very important innate immune component in the resistance to attack by microorganisms, and could be one of the reasons for their longevity.

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