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Passive transfer of immunity from *Tilapia antisera* enhanced *in-vitro* phagocytosis of *Aeromonas hydrophila* in naïve *Macrobrachium rosenbergii* haemocytes

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Abstract

Aim : To determine the effectiveness of antisera derived from a teleost to augment the phagocytic activity of prawn phagocytes.

Methodology: Antisera from tilapia, *Oreochromis niloticus*, immunised with methanol-killed *Aeromonas hydrophila* was added to the suspension of *A. hydrophila* prior to the incubation with the haemocytes isolated from *Macrobrachium rosenbergii* and examined for phagocytosis. The sera derived from the sterile saline-injected fish were used for comparison.

Results : *In vitro* phagocytosis assay showed that the supplementation of immunised sera significantly enhanced *Aeromonas* uptake by prawn haemocyte by increasing phagocytic index to 3.23 ± 0.44 , compared to control with phagocytic index of 1.35 ± 0.42 .

Interpretation : Tilapia antisera may potentially be adopted to provide passive immune-protection to crustaceans which lack adaptive immunity. Further studies should confirm the mechanisms involved in enhancement of prawn phagocytic uptake of bacteria by fish immunised sera.

Key words: Haemocytes, *Macrobrachium rosenbergii*, Passive immunity, Phagocytosis, *Tilapia antisera*

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Introduction

Giant freshwater prawn (*Macrobrachium rosenbergii*) is one of the largest freshwater prawns (Banu and Christianus, 2016) and most important cultured freshwater crustacean species in the world (Aziz et al., 2017). Today it holds high economic value in the aquaculture industry with global production exceeding 217,000 metric tons, valued at more than USD \$1.2 billion in 2014. Mass culture of crustacean species are often hampered due to occurrence of infectious diseases and spreading of pathogens (Oh et al., 2012), which are easily infected by pathogens such as virus, bacteria and fungi (Wang and Wang, 2013).

Adaptive immune system is only found in vertebrates. Like other invertebrates, prawns rely solely on innate immune defence mechanisms to protect their circulatory system. (Medzhitov and Janeway, 1997). Due to lack of adaptive immune system in invertebrate, immunoprophylactic approaches like vaccination are not plausible for preventing disease outbreaks in prawn and shrimp culture. Passive immunity is the process of providing pre-formed antibodies to confer immediate protection against infections.

To date, there is no study reported on passive immunity from teleost to crustacean species. However, passive immunity derived from chicken egg yolk IgY antibodies has been shown to elevate crustacean immune response against disease agents (Baloch et al., 2015; Lu et al., 2008). Teleost IgM antibodies are demonstrated to be stable within the pH range of 2.5–11 (Mochida et al., 1994), whereas chicken egg yolk IgY is only stable at pH 4–9 (Müller et al., 2015). Despite its relatively high stability, artificially acquired passive immunity derived from teleost IgM is poorly studied in prawns. To explore the potential transfer of passive immunity to enhance prawn immunity, the present study was conducted to determine the effectiveness of antisera derived from tilapia to augment the phagocytic activity of *Macrobrachium rosenbergii* phagocytes to *Aeromonas hydrophila*.

Materials and Methods

Immunisation of tilapia : A group of tilapia, *Oreochromis niloticus* consisting of 30 individuals with average weight of 380.0 ± 2.5 g were immunised intramuscularly with 200 µl of vaccine containing 1×10^6 CFU of methanol-killed *A. hydrophila* in order to acquire tilapia sera containing antibodies specific to *A. hydrophila*. Five boosters were given at an interval of 1 week. For the first and second injections, the vaccines were emulsified with equal volume of Complete Freund's adjuvant and Incomplete Freund's Adjuvant, respectively.

As control, another group of tilapia was injected with phosphate-buffered saline to acquire non-immunised sera. Haemocytes were isolated from haemolymph of the naïve healthy

prawn according to Acharya et al. (2004).

Detection of tilapia specific antibodies by tube agglutination test

The serum antibody titres of vaccinated and control fish were determined with a tube agglutination test as described by Gao et al. (2016). Three fish were randomly selected from each group for the test which was conducted in 15 ml sterile test tubes. The sera were subjected to serially two-fold dilution in a sterile saline solution, ranging from 1:2 to 1:4096. A 0.2 ml of *A. hydrophila* (10^9 CFU ml⁻¹) was added to each tube. The tubes were then shaken and incubated at room temperature ($27 \pm 2^\circ\text{C}$) for 24 hr. The reaction was observed visually and an umbrella-like white clump at the bottom of the tube indicated a positive reaction. The non-reactor tubes did not show any clump.

Collection of haemolymph

Haemolymph was collected from healthy adult prawns, *Macrobrachium rosenbergii*. Haemolymph was withdrawn from ventral sinus, located at the base of first abdominal segment of the prawn with 10 ml syringe along with 26 gauge needle. The syringe was coated with anticoagulant (30 mM trisodium citrate, 100 mM glucose, 510 mM NaCl, 10 mM EDTA, Na₂, pH = 4.6) in ratio 2:1 of haemolymph. Haemolymph was transferred to centrifuge tube and was mixed gently with anticoagulant and incubated for 5 – 10 min. Haemocytes were collected after 5 min of centrifugation at 1800 rpm.

Phagocytosis assay

One-hour *in-vitro* phagocytosis assay was carried out by mixing 2×10^6 cells ml⁻¹ of prawn haemocytes suspension with 2×10^7 cells of *Aeromonas* in 500 µl of PBS. *Aeromonas* were either pre-incubated for 30 min with 50% sera from immunised or non-immunised tilapia. In addition, the *in-vitro* phagocytosis assay was also carried out with *Aeromonas* not pre-incubated with any fish sera. The haemocyte suspension was centrifuged at 500 x g for min and incubated on poly-L-lysine-coated glass slide. To visualise phagocytosis, the slides were placed into methanol solution for 1 min, then slides were placed into May-Grunwald solution for 5 min and were placed into 5% Giemsa stain solution for 15 min. Finally, slides were rinsed with distilled water. Two phagocytic parameters mentioned below were calculated and compared: Phagocytosis Ratio (PR) = number of haemocytes ingesting *Aeromonas* / number of haemocytes observed. Phagocytosis Index (PI) = number of *Aeromonas* ingested / number of haemocytes observed (Zhou et al., 2009).

Results and Discussion

Antibody titre of the immunised tilapia: Tube agglutination test was conducted to determine the most optimum titre of tilapia serum antibodies that was specific against *A. hydrophila*. The result showed that injection with methanol-killed *A. hydrophila* could effectively result in specific antibody accumulation in tilapia serum. As shown in Fig. 1, the anti-*Aeromonas* titre showed a sharp increase of 16-fold (antibody titre increased from 2^4 to 2^8)

Fig. 1 : Mean anti-*Aeromonas* titre of tilapia serum deduced from tube agglutination assay, expressed as log₂ of serum serial dilution in tilapia with or without immunisation of 1×10^6 CFU of methanol-killed *A. hydrophila*. No agglutination was observed in the control group, which indicates the absence of anti-*Aeromonas* antibody in the serum. The immunised group showed seropositive against killed *A. hydrophila* after 1-week post-immunisation.

after 2nd week when booster with Incomplete Freund's Adjuvant was given to fish. At 4th week after first immunization, the titre of anti- *Aeromonas* reached a plateau at 512 ($2^9 = 512$). On the other hand, there was no anti- *Aeromonas* detected in the control group.

Three types of haemocytes were observed in *Macrobrachium rosenbergii* haemolymph, namely hyaline cell (HC), semigranular cell (SGC) and granular cell (GS). As shown in Fig. 2, the prawn HC were round, fusiform in shape with large nucleus and no granules in the cytoplasm whereas SGC were ovoid or fusiform in shape with small granules in the cytoplasm as compared to GC that harboured large granules in the cytoplasm.

Phagocytosis of *A. hydrophila* by the haemocytes in *M. rosenbergii* improved on supplementing with tilapia antiserum. The presence of *A. hydrophila* inside the cytoplasm of haemocytes indicated that phagocytosis had taken place. Haemocytes were able to phagocytise *Aeromonas hydrophila* in *in-vitro* condition (Fig. 3). The phagocytic ratio and phagocytic index are tabulated in Table 1.

Table 1 shows that there was no significant difference in phagocytosis ratio between haemocytes supplemented with antiserum as compared to haemocytes supplemented with non-immune serum or negative control. On the other hand, pre-incubation of *Aeromonas* with tilapia antisera recorded significant highest phagocytic index, i.e., 3.23 ± 0.44 , compared to control

with phagocytic index of 1.35 ± 0.42 .

To date, there is no study reported on passive immunity derived from teleost species could provide protection to the crustacean species. However, passive immunity artificially acquired from chicken egg yolk IgY antibodies has been demonstrated to enhance crustacean defence against pathogens (Baloch et al., 2015; Lu et al., 2008). Piscine IgM antibodies have been found to be more thermal and pH stable than avian antibodies (Mochida et al., 1994, Müller et al., 2015). Despite relatively high stability, immunoprotection derived from teleost IgM is poorly studied in prawn.

In the present study, tilapia was selected as a model for the teleost antiserum because tilapia is one of the most commonly cultured freshwater teleost species and the presence of adaptive immune responses upon vaccination has been well reported in this fish (Li et al., 2015, Zhu et al., 2017). Furthermore, tilapia blood can be easily obtained by puncturing caudal vein.

In the current study, approximately 44%-54% of haemocytes were reactive phagocytes performing phagocytosis on *Aeromonas*. This indicated that not all the haemocytes can perform phagocytosis. Kakoolaki et al. (2010) demonstrated that only haemocyte subtypes of semi granular cell and granular cells can perform phagocytosis, as these cells can ingest microorganisms *in vitro*, such as yeast particles.

No significant difference in phagocytosis ratio was observed between haemocytes supplemented with antiserum as compared to haemocytes supplemented with non-immune serum or negative control. This showed that the presence of tilapia antibodies in antisera did not increase the number of reactive haemocytes performing phagocytosis.

On the other hand, surprisingly, there was significant difference in phagocytic index between haemocytes supplemented with immunized serum as compared to haemocytes supplemented with non-immunised serum or negative control. This indicated that the presence of tilapia antibodies in antisera may have enhanced the uptake of *Aeromonas* by phagocytic haemocytes in giant freshwater prawn. Due to presence of fish antisera, each of the reactive phagocytes in prawn was able to phagocytise 3.23 units of *Aeromonas* as compared to non-immune serum, where each reactive phagocytes was only able to phagocytise 1.35 units of *Aeromonas*.

Inferred by the mechanism adopted by hen yolk IgY to protect the crustacean host immunity (Gao et al., 2016), the increased bacterial uptake by haemocytes supplemented with teleost antisera is possibly due to several underlying mechanisms. First, the anti-*Aeromonas* antibodies of tilapia bound to a group of *Aeromonas* bacteria and clump them to form

Fig. 2 : May-Grundwald with Giemsa staining of haemocytes smear. A-C, Hyaline cells (HC), D-F, Semigranular cells (SGC) and G-I, Granular cells (GC). Scale bar = 10 μ m, $\times 400$

Table 1 : Comparisons of phagocytic ratio and phagocytic index in haemocytes treated with different fish sera.

Experimental group	Phagocytic ratio	Phagocytic index
Antisera Non-immunised sera	0.54 ± 0.07^a	3.23 ± 0.44^b
Negative control (without fish sera)	0.46 ± 0.11^a	1.35 ± 0.42^a
	0.44 ± 0.15^a	1.26 ± 0.57^a

*Phagocytic ratio and phagocytic index expressed as mean $\pm$ standard error. Difference in superscript indicates significant differences ($P < 0.05$) among data within column. N=30

antibody-antigen complexes and this in turn facilitates the uptake of *Aeromonas* by prawn haemocytes. Secondly, binding of tilapia

anti-*Aeromonas* antibodies to *Aeromonas* bacteria opsonises bacteria making them prove to prawn phagocytes. Further, prawn

Fig. 3 : *In vitro* comparison of phagocytosis of *Aeromonas* by giant freshwater prawn haemocytes, treated with different fish sera. A-C, Non-immune sera, and D-F, antisera. Scale bar = 10 µm, ×1000. Arrow indicates phagocytised bacteria by prawn haemocytes.

phagocytic haemocytes express molecules that can recognise fish antibodies, such as Fc receptors. It seems unlikely that crustacean possess Fc receptors for vertebrate antibodies.

In the *in-vitro* condition, tilapia anti-*Aeromonas* sera was shown to enhance the uptake of *Aeromonas* by phagocytic haemocytes in giant freshwater prawn. Tilapia antisera can be potentially used to provide passive immune-protection to the crustacean lacking adaptive immunity. Further studies are required that can confirm the mechanisms involved in enhanced prawn phagocytic uptake of bacteria by fish immunised sera.

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