Bactericidal activity of grass carp *Ctenopharyngodon idella* C9-deficient serum against *Aeromonas hydrophila*

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Neutralizing complement C9 in grass carp *Ctenopharyngodon idella* sera with rabbit anti-C9 sera against fish complement C9, demonstrated that bactericidal activity against *Aeromonas hydrophila* of the C9-deficient fish sera was greatly impaired. These results indicated that the fish complement C9 plays a key role in pathogen killing through the lytic pathway.

Key words: bacterial % survival; innate immune system; membrane attack complex; rabbit anti-C9 sera.

It is widely accepted that the innate immune system is the first line and fundamental defence mechanism of fishes, contributing to the prevention of infection and diseases. The system involves the participation of many molecules, including growth inhibitors and various lytic enzymes, agglutinins and precipitins (opsonins, primarily lectins), cytokines and chemokines, antibacterial peptides and complement (Ellis, 1999; Magnadottir, 2006). The complement system comprises a number of small proteins found in the blood, generally synthesized by the liver, and normally circulating as inactive precursors. It is an indispensable part of the innate immune system (Holland & Lambris, 2002). Activated by potential pathogens or by antibody–antigen (Ab–Ag) complexes, the complement protein fragments play important roles in the classical complement pathway (CCP), alternative complement pathway (ACP) and also the lectin pathway (Holland & Lambris, 2002). These converge to a lytic pathway which causes the formation of the membrane attack complex (MAC), which creates a pore in the phospholipid bilayer of pathogenic bacteria to disrupt the target cell (Nonaka & Smith, 2000; Holland & Lambris, 2002). All pathways involve the participation of the ninth component of complement (C9), which is one of five constituents of the MAC. Although some gram-negative bacteria resist the bactericidal effect of serum and frequently cause diseases, the functions of fish complement are known...
to be important in the innate defence response of fishes against bacterial infections (Boshra et al., 2006).

Many studies on the bactericidal activity of fish serum have confirmed that ACP plays an important role in the serum bactericidal activity in the common carp *Cyprinus carpio* L.1758, crucian carp *Carassius carassius* (L.1758) (Sugita et al., 1989; Hajji et al., 1990) and gilthead seabream *Sparus aurata* L.1758 (Sunyer & Tort, 1995). In the present study, fish sera were treated with rabbit anti-C9 sera against the complement component to attenuate the functions of an individual complement protein, and such complement deficient sera have been used to assess the role of complement in fish serum in bacteria killing. Sera were treated with rabbit anti-serum against complement components C9 of grass carp *Ctenopharyngodon idella* (Valenciennes 1844); the present study aimed to confirm the role of the complement component in fish serum bactericidal activity.

Healthy *C. idella* (1200–1500 g) were obtained from local markets. The fish were kept in three 100 l plastic tanks separately and supplied with aerated water at 20–22° C for acclimatization. The laboratory strain of *Aeromonas hydrophila* 58-2-09 was isolated from *C. idella* and stored at –80° C. Before testing, the bacteria were removed from the deepfreeze, allowed to thaw to room temperature, cultured in broth and incubated at 25° C for 24 h to reach the log growth phase of the isolates. Then, the organism was streaked onto a brain heart infusion (BHI, Becton Dickinson and Company; www.bd.com) agar slant. *Aeromonas hydrophila* was washed with sterile phosphate buffered saline (PBS, pH 7.4) from the BHI slant after incubation at 28° C for 48 h. The optical density (OD) of the suspension, at 600 nm by spectrophotometer, was adjusted to 0.1–0.5, which equals 1 × 10⁸ to 5 × 10⁸ colony-forming units (CFU) ml⁻¹. This bacterial suspension was serial 10-fold diluted with sterile PBS to give a concentration of c. 1 × 10⁵ to 5 × 10⁵ CFU ml⁻¹. The CFU of the bacteria were determined by plating serial dilutions on to Luria-Bertani (LB) agar plates for determining initial CFU.

After anaesthetizing the fish, 18 blood samples were collected from the caudal vein into sterile 10 ml centrifuge tubes without anticoagulant and allowed to clot for 2 h at room temperature. The tubes were centrifuged at 1500g for 15 min, and the supernatant serum was collected. The serum was used the same day, or was stored at –20° C until use within 2 days. Complement was inactivated in *C. idella* serum and rabbit anti-C9 serum (Li et al., 2007) at 52° C for 30 min (Sakai, 1981).

The overnight bacterial suspension was adjusted to c. 10⁵ CFU ml⁻¹ in PBS (named as initial bacteria culture, IBC). A volume of 10 μl IBC was placed in drops on the surface of the TSA (tryptonic soya agar, Becton Dickinson and Company) plates in triplicate to count the number of bacteria in the IBC.

To determine what would happen after the C9 complement of *C. idella* complement system was neutralized using rabbit anti-C9 serum and to assess the role of complement in the bacteria killing, 60 μl heat-inactivated rabbit anti-C9 serum was mixed thoroughly with 120 μl *C. idella* serum at room temperature for 30 min, then 20 μl of *A. hydrophila* IBC was added and incubated at 25° C for 3 h. For controls, 60 μl of heated-inactivated normal rabbit serum or 60 μl normal *C. idella* serum was used to replace 60 μl heated-inactivated rabbit anti-C9 serum, and for blank control, 180 μl of PBS was mixed with 20 μl of *A. hydrophila* IBC without serum. After incubation at 28° C for 24 h, the CFU on agar plates were counted. The results were expressed as bacteria per cent survival (BP) which was defined as:
Bacterial survival (%) 100 80 60 40 20 0 1234

Fig. 1. Bactericidal activity of Ctenopharyngodon idella C9 deficient serum against Aeromonas hydrophila 58-2-09 1, C. idella serum; 2, C. idella serum with heat-inactivated rabbit serum; 3, C. idella serum with heat-inactivated rabbit anti-C9 serum (C. idella C9-deficient serum); 4, phosphate buffered saline (PBS). Bacteria per cent survival was defined as BPS = 100 [log\(_{10}\) colony-forming units (CFU) in the serum sample or control sample per log\(_{10}\) CFU in initial bacteria culture]. The values represent the mean ± s.e. (n = 6). Mean values with different lower case letters are significantly different (P < 0.01).

BPS = 100 (log\(_{10}\) CFU in the serum sample or control sample per log\(_{10}\) CFU in IBC) The SPSS software (www.ibm.com) was used to determine whether there was significant variation among treatments at the 1% level. The data were analysed by least-significant difference in (ANOVA). Six samples in each group were assayed. Treatment means and s.e.s were estimated.

The results showed that the bactericidal activity of C. idella C9-deficient serum against A. hydrophila was significantly lower than normal C. idella serum heat-inactivated normal rabbit serum in terms of BPS (P < 0.01) (Fig. 1). There were no significant differences between the C9-deficient serum group and PBS group. In the C9-deficient serum group, the BPS was 90.0%, and in the normal C. idella serum group, the heat-inactivated normal rabbit serum group and the PBS group, the BPS were 8.4, 23.5 and 78.5%, respectively.

The present study showed that bactericidal activity of C. idella C9-deficient serum against A. hydrophila was significantly lower than that of normal C. idella sera (P < 0.01), which indicated that complement plays a key role in bacterial killing through the lytic pathway. Participation of the lytic pathway is required for complement-mediated pathogen killing, and C9 is essential to the lytic pathway. This conclusion has a stronger theoretical basis than that drawn from experiments with heat-inactivated sera (Hajji et al., 1990). Traditional heat treatment not only inactivates the complement system but also may destroy other anti-microbial substances like lysozyme, C-reactive protein and antibacterial peptides in sera. This study demonstrated the key role of lytic pathway in the serum bactericidal activity using a specific antiserum against a complement component for the study of fish complement system.

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References


