

Journal of Zhejiang University SCIENCE B
 ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
 www.zju.edu.cn/jzus; www.springerlink.com
 E-mail: jzus@zju.edu.cn



Protection of *Carassius auratus Gibelio* against infection by *Aeromonas hydrophila* using specific immunoglobulins from hen egg yolk*

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Received July 10, 2006; revision accepted Sept. 19, 2006

Abstract: Specific immunoglobulin (IgY) from egg yolk against *Aeromonas hydrophila* was produced by immunization of White Leghorn hens with formalin-killed whole cells of *A. hydrophila*. ELISA test using *A. hydrophila* as the coating antigen revealed that the specific antibody titer started to increase in the egg yolk at the 13th day post-immunization ($P/N=2.18$), reached the peak at the 56th day ($P/N=13.82$), and remained at high level until day 133 ($P/N=7.03$). The antibody was purified by saturated ammonium sulphate with a recovery rate of 63.5%. The specific IgY inhibited the growth of *A. hydrophila* at a concentration of 1.0 mg/ml during the 18 h incubation. Pre-treatment of polyploid gibel carps *Carassius auratus Gibelio* with specific IgY had a protection rate of 60% (6/10) against challenge with *A. hydrophila*, while none of the fishes in the control groups receiving sterile phosphate buffered saline (PBS) or non-specific IgY survived the challenge. Treatment of fishes with the specific IgY 4 h after the challenge also had lower mortality (70%, 7/10), a 30% reduction against the control PBS or non-specific IgY groups (10/10). These results indicate that specific IgY antibodies could be obtained easily from hens immunized with an inactivated *A. hydrophila* and could provide a novel alternative approach to control of diseases in fishes caused by this organism.

Key words: *Aeromonas hydrophila*, Immunoglobulins, Hen egg yolk, Polyploid gibel carps, *Carassius auratus Gibelio*

doi:10.1631/jzus.2006.B0922

Document code: A

CLC number: S85

INTRODUCTION

Aeromonas spp. are autochthonous inhabitants of aquatic, sewage and soil environments (Janda and Abbott, 1998; Chowdhury *et al.*, 1990) and consist of two major groups, the psychrophilic group and the mesophilic group. *Aeromonas hydrophila* is one of the most important representative species in the mesophilic group (Gonzalez *et al.*, 2001). The bacterium is of scientific and economic interest because of its pathogenicity to human and fishes (Elwitigala *et al.*, 2005; Austin *et al.*, 1998). *A. hydrophila* infection in fishes occurs from time to time in Asian countries including China, Philippines, Thailand and India (Chen and Lu, 1991). Freshwater aquatic species

infected by *A. hydrophila* could exhibit different symptoms such as hemorrhagic enteritis, festering neck disease or furuncles in turtles, red body disease or septicemia in shrimps, and festering gill or hemorrhagic enteritis in fish species such as *Hypophthalmichthys molitrix*, *Ctenopharyngodon idella*, *Aristichthys nobilis*, *Carassius auratus Gibelio*. Antibiotics are often used in closed and open waters for therapy of furunculosis of farmed aquatic species (Kampfer *et al.*, 1999). Vaccination is an important strategy in the control of this disease caused by *A. hydrophila* among farmed fish (Asha *et al.*, 2004). However, vaccination varies considerably in efficacy, and epizootic events occur frequently in fish farms. Therefore, it is important to search for effective methods as alternative to antibiotics for treatment of *A. hydrophila* infections in the intensively farmed aquatic species.

Laying hens transfer large amounts of immu-

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* Project (No. 2004C26026) supported by the Science and Technology Department of Zhejiang Province, China

noglobulin from serum to egg yolk of their eggs, where it serves as a means of passively protecting the developing chicks (Kariyawasam *et al.*, 2004; Rosenberger *et al.*, 1985). An average egg may contain 100~150 mg of yolk immunoglobulins (IgY), and substantial amounts of specific antibodies may be collected and purified from the eggs of immunized hens (Akita and Nakai, 1993). The availability of large amounts of relatively inexpensive IgY from egg yolks makes it feasible to use these antibodies for passive immunization by oral administration or injection (Carlander *et al.*, 2000). The efficacy of this approach has been shown in human and veterinary medicine for rotavirus diarrhea in humans (Ebina, 1996; Kuroki *et al.*, 1997; Sarker *et al.*, 2001), *Escherichia coli* infections in pigs (Yokoyama *et al.*, 1992; Ikemori *et al.*, 1992; Marquardt *et al.*, 1999; Hennig-Pauka *et al.*, 2003), and *Streptococcus mutans*-induced dental caries (Krüger *et al.*, 2004; Smith *et al.*, 2001). In aquatic species, IgY against *Edwardsiella tarda* was administered orally to passively immunize Japanese eels (Mine and Kovacs-Nolan, 2002). These studies demonstrated that IgY could serve as an effective means against bacterial and viral infections (van Nguyen *et al.*, 2006). Nevertheless, there has been no report so far on the use of IgY in the prevention and treatment of *A. hydrophila* infections. In this paper, we report the antibody response of laying hens to immunization with inactivated whole cells of *A. hydrophila* and the efficacy of the specific IgY preparation on control of fish diseases caused by *A. hydrophila* infection in *Carassius auratus Gibelio*.

MATERIALS AND METHODS

Immunization of hens

The *A. hydrophila* strain AS 1.927 was originally from China General Microbiological Culture Collection Center. The bacterium was grown in tryptic soy broth (Difco, Detroit, MI) at 35 °C for 24 h in a flask with shaking. After three washes with sterile 0.1 mol/L phosphate-buffered saline (pH 7.2), the number of cells was adjusted to 2×10^9 cfu/ml with sterile PBS. The culture was treated with 0.4% formalin for 24 h and tested for viability. This antigen suspension was mixed and emulsified with equal volume of Freund's complete adjuvant (FCA) or Freund's in-

complete adjuvant (FIA) (Sigma, St. Louis, MO). A group of 12 White Leghorn hens, 25 weeks old, were kept for immunization and egg production in the Central Animal Facility at Zhejiang University. Each hen received 1 ml of emulsified antigen in FCA intramuscularly at four sites (0.25 ml per site). Booster injections were given intramuscularly at 2, 4, and 6 weeks after the first injection with the emulsified antigen containing FIA. Eggs were collected from the time of first vaccination and then once a week during the experimental period (till day 133).

Purification of IgY

The crude antibody from yolk was extracted by the water-soluble fraction as described by Akita and Nakai (1993) with modification. Egg yolk was separated from the white, and the yolk preparation was diluted at 1 to 9 ratio with distilled water at pH 5.3. The mixtures were kept overnight at 4 °C. After centrifugation at 10000×g at 4 °C for 30 min, the water-soluble fraction (WSF) was carefully collected and further purified by 33% (v/v) saturated ammonium sulphate solution and ultrafiltration (UF) using a UF membrane (Millipore Corp., Bedford, Mass). Purified IgY was freeze-dried. The recovery rate of immunoreactive IgY was calculated using the formula $OD_{492\text{ nm}}(A)/OD_{492\text{ nm}}(B) \times 100$, where *A* is $OD_{492\text{ nm}}$ in the crude extract (WSF) or $(\text{NH}_4)_2\text{SO}_4$ -purified product at a given dilution and *B*, $OD_{492\text{ nm}}$ in the egg yolk pooled at the same dilution.

Antibody titration

Enzyme linked immunosorbent assay (ELISA) was used to check the titer of the specific antibody. Each well of the 96-well polystyrene plates (NUNC, Denmark) was coated overnight at 4 °C with 100 µl of *A. hydrophila* whole cell suspension (10^9 /well) in 0.05 mol/L carbonate buffer (pH 9.6). The wells were washed with phosphate-buffered saline (PBS)-Tween (0.05% Tween 20 in PBS at pH 7.2, PBST), and then blocked by incubation for 1 h at 37 °C with 150 µl of 1.0% (w/v) bovine serum albumin (BSA, Sigma) in carbonated buffer. After three washings with PBS-Tween, appropriately diluted IgY preparations were added to the wells and the plates were incubated for 1 h at 37 °C. One hundred microlitres of HRP conjugated rabbit anti-chicken IgG (BioChain Institute, Inc., Hayward, USA) at 500-fold dilution with PBST with 0.05 BSA was added to the wells after another

three washings. The plates were incubated at 37 °C for 1 h and washed with PBS-Tween for 3 times. The substrate solution (20 ml PBS+8 mg *o*-phenylenediamine+100 µl H₂O₂) was added. After 20 min of incubation at 37 °C, 50 µl of 2 mol/L H₂SO₄ was added to stop the reaction. The color reaction was read on an ELISA plate reader (Model Elx800, Bio-Tek, USA) at 492 nm. The antibody titer was expressed as *P/N* values, where *P* represents the *OD*_{492 nm} of IgY from immunized hens at a given dilution and *N*, the *OD*_{492 nm} of IgY from non-immunized hens at the same dilution.

SDS-PAGE electrophoresis

Purification of the antibody was visualized by SDS-PAGE at 6% gel concentration according to the method of Laemmli (1970) on a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Hercules, Calif). The protein concentration using the micro-BCA protein assay (Pierce, IL, USA) was adjusted to 1 mg/ml and mixed with the sample buffer (pH 6.8, 62.6 mmol/L Tris-HCl, 25% (v/v) glycine, 2% (w/v) SDS). The samples were heated for 3 min at 100 °C. Fifteen microlitres of the samples were loaded into corresponding wells for electrophoresis. The gel was stained with a mixture of Coomassie brilliant blue R-250 (Sigma) in 10% acetic acid and 30% methanol. The protein bands from the samples were compared with reference IgY from Jackson Immuno Research Laboratories.

Inhibition of *A. hydrophila* growth in vitro

The specific antibody from *A. hydrophila* vaccinated hen eggs and the non-specific control from those of non-immunized hens were purified with 33% (v/v) saturated ammonium sulphate solution, followed by ultrafiltration. The specific and control antibodies were adjusted to the level of 2 mg/ml and serial 2-fold dilutions were made. The antibody solutions were then added into corresponding wells of a 96-well polystyrene plate containing 10⁸ cfu/ml *A. hydrophila* in 100 µl of 2× sterilized tryptic soy broth. Inhibition of bacterial growth was continuously recorded as changes of *OD*_{560 nm} at the 30-min interval for 18 h at 35 °C on the SPECTRAMAX M2 spectrophotometer (Molecular Devices Corporation, California, USA) preset to shake the plate for 5 s before each measurement.

Fish experiments

All fish experiments were conducted according to the guidelines for the care and use of animals in research and teaching of Zhejiang University. Polyploid gibel carps *Carassius auratus Gibelio* were clinically healthy and free of any external abnormalities. The fishes were maintained at (24±2) °C in stock tanks supplied with flowing water and fed a diet of a commercial fish food. All experiments were done after fishes were acclimatized for one week.

For prevention experiments, three groups, each with 10 fishes ((27±1) g), were randomly assigned to the experimental tanks. Each fish was injected by intraperitoneal route either with 0.25 ml of 30 mg/ml specific IgY preparation (s-IgY group), 0.25 ml of 30 mg/ml non-specific IgY (n-IgY group) or sterile PBS (control). The fishes were kept for 4 h in the tanks and then challenged with 0.25 ml of AS 1.927 (1×10⁹ cfu/ml) via intraperitoneal route. Distress or death of the fishes was monitored at frequent intervals until day 8 post-challenge. For therapeutic experiments, the groups were assigned as above. Fishes in each group were challenged first with 0.25 ml with 1×10⁹ cfu/ml of AS 1.927. After 4 h in the tanks, the fishes were injected intraperitoneally with 0.25 ml of 30 mg/ml specific IgY, non-specific IgY or sterile PBS as above. They were monitored for distress or death at frequent intervals until the 8th day post-challenge.

At the end of each experiment, fishes that died during the experiment were necropsized for examination of the presence of *A. hydrophila* in the kidney. Kidney samples from each fish were placed into sterile Whirl-Pak bags and weighed. A volume of sterile saline (1:10 ratio, w/v) was added before processing with a stomacher (Lab-Blender 80) for 1 min. The suspensions were further diluted, and 100 µl each of the dilutions was spotted onto tryptic soy agar plates to screen *A. hydrophila*. After incubation at 35 °C for 24 h, colonies on the plates were tested for cytochrome *c* oxidase reaction (positive for *A. hydrophila*) and counted.

RESULTS AND DISCUSSION

Specific antibody production

It is well-known that a specific antibody against a particular pathogen can be obtained in large quan-

tities from yolk of eggs laid by hyper-immunized hens. In avian medicine, immunization of hens is often used to transfer maternal antibodies to chicks for preventive purposes. In the food or feed industry, the egg yolk could be utilized directly either for nutritional supplements or for therapeutic purposes because of its high IgY content (Larsson and Carlander, 2003; Camenisch *et al.*, 1999; Tini *et al.*, 2002). Therefore, the approach for use of yolk IgY in aquatic medicine seems to be plausible. ELISA test showed that the specific antibody started to increase in the egg yolk at day 13 from the 1st immunization ($P/N=2.18$), reached the peak at day 56 ($P/N=13.82$), and kept at high level until day 133 ($P/N=7.03$) (Fig.1). This is similar to the findings by Lee *et al.*(2002) and Kariyawasam *et al.*(2004) who reported that the specific IgY from egg yolk could be detectable at the 14th day post-immunization. The antibody response in the yolk is delayed as compared to occurrence in the serum after immunization (Patterson *et al.*, 1962). The recovery rate of IgY in the crude extract of the pooled egg yolks in the period of days 49~90 was 78.3%, and the rate was 63.5% upon further purification with 33% $(\text{NH}_4)_2\text{SO}_4$ precipitation of the crude extract (Fig.2). SDS-PAGE analysis showed substantial decrease of irrelevant proteins upon extraction with water or purification with saturated ammonium sulphate solution (Fig.3). A 1 mg/ml solution of the purified IgY had a titer of 1:64 as estimated by ELISA using *A. hydrophila* as the coating antigen. These results indicate that IgY is highly specific to *A. hydrophila*. The motility shift of purified IgY seen as its lower MW (Fig.3, lane 4) than those of lanes 1, 2 and 3 may be due to structural changes as a result of treatment with saturated ammonium sulphate.

In vitro inhibition of *A. hydrophila* growth

To determine the effect of the specific IgY against *A. hydrophila*, we compared its growth rate in the presence of specific IgY using the IgY from non-immunized hens as controls. Fig.4 indicates that the growth of *A. hydrophila* was completely inhibited at a concentration of 1.0 mg/ml specific IgY during the 18 h incubation. A concentration of 0.5 mg/ml was inhibitory to *A. hydrophila* as shown by a longer lag phase and less steep slope. However, the control IgY did not show any inhibitory effect at the level of 1 mg/ml. Sugita-Konishi *et al.*(1996) and Shin *et al.*

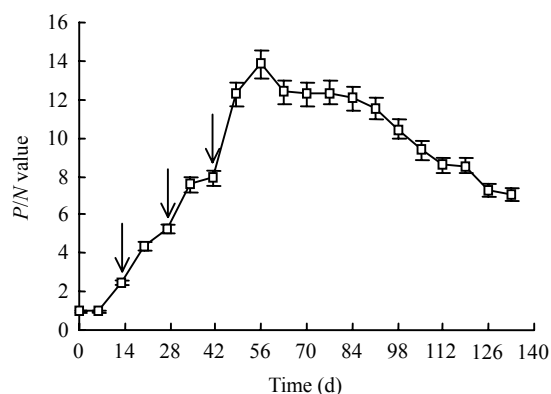


Fig.1 Changes of IgY titers over time following immunizations

The arrows (↓) indicate the booster injections. Each data point represents the average of three determinations of three eggs pooled and the error bars, the standard error of means

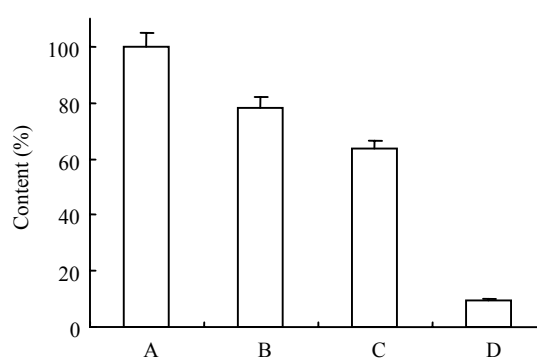


Fig.2 Recovery of immunoreactive IgY

A: Egg yolk pooled; B: Crude extract of IgY by water dilution; C: Purified IgY by 33% saturated ammonium sulfate; D: IgY from non-immunized hens

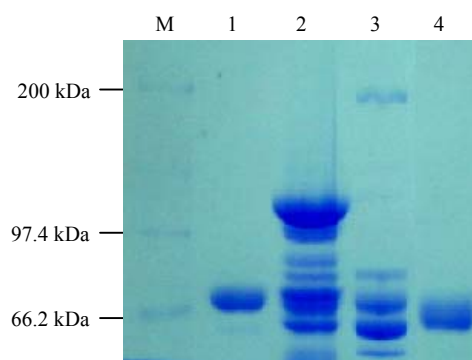


Fig.3 Coomassie blue stained SDS-PAGE gel of IgY extracted from egg yolk

M: Molecular weight marker; 1: Reference IgY; 2: Egg yolk in 10× dilution distilled water at pH 5.3; 3: Suspension of pellets from "2" after centrifugation; 4: Suspension from 33% saturated ammonium sulfate precipitates

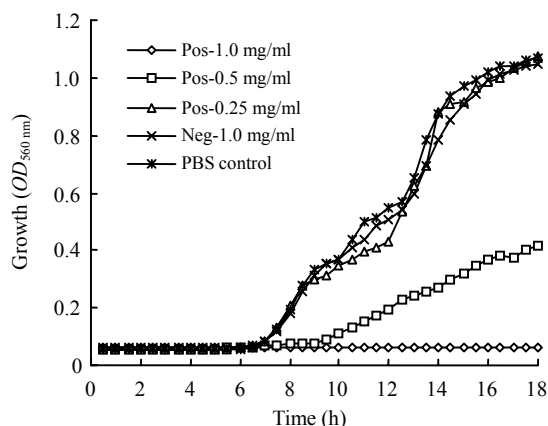


Fig.4 Effect of specific IgY (Pos) on growth of *A. hydrophila* in vitro using non-specific IgY (Neg) and phosphate buffered saline (PBS) as controls

(2002) reported that IgY obtained from hens immunized with a mixture of formalin-treated *Pseudomonas aeruginosa* and *Helicobacter pylori* inhibited the growth of both pathogens. Production of *Staphylococcus aureus* enterotoxin A and adhesion of *Salmonella typhimurium* serovar Enteritidis to cultured human intestinal cells were also inhibited in the presence of corresponding IgY. The IgY antibodies inhibited the colonization of teeth by *Streptococcus mutans*, thus preventing plaque formation in humans (Hatta et al., 1997). However, there is no report so far on the use of IgY in the prevention or treatment of *A. hydrophila* infections in aquatic species.

Passive protection of fishes

Table 1 indicates that 6 out of 10 fishes (60%) pretreated with *A. hydrophila* specific IgY 4 h were protected against challenge with *A. hydrophila* while no protection was seen in the control fishes injected with PBS or n-IgY during the 8-day period. The death

of fishes treated with s-IgY ceased at the 3rd day post-challenge. Moreover, the number of *A. hydrophila* in kidney samples of dead fishes treated with s-IgY was substantially lower (4.6×10^3 cfu/g) than the control dead fishes (2.9×10^6 cfu/g) and n-IgY group (3.1×10^6 cfu/g). These results suggest that s-IgY was protective against infection by agglutinating, and/or killing the bacteria in fishes. For treatment of fishes pre-exposed to *A. hydrophila*, 3 out of 10 fishes injected with s-IgY survived with an efficacy of 30% and none of the fishes in the PBS and n-IgY group survived (Table 1). Death of fishes in the s-IgY treated group ceased at the 4th day, and the count of *A. hydrophila* in kidney samples of fishes treated with s-IgY was also less than that from the PBS and n-IgY treated fishes. Lee et al. (2000) reported that intraperitoneal injection of anti-*Yersinia ruckeri* at a dose of 4 mg was effective against an immersion challenge with 10^8 cfu/ml of the homologous strain. However, Korbsrisate et al. (2002) found that the polyclonal antibodies against *A. hydrophila* strains 76 and 236 could agglutinate 21 different O antigen serogroups with no specific serogroups preference and rough strains as well as untypable *Aeromonas* strains.

The above findings reveal that specific IgY was more effective against *A. hydrophila* infections in fishes when used before challenge with the virulent strain than when applied after challenge. Direct inhibition of the challenged bacteria by specific IgY present in the body might have contributed to the efficacy as shown by the in vitro growth inhibition study (Fig.4). The antibody might also function to prevent the bacterial adhesion or attachment to the host cells, an essential step for the establishment of bacterial infections. However, the protection rate was generally low either in the prevention or treatment

Table 1 Efficacy of specific IgY (s-IgY) and non-specific IgY (n-IgY) against *A. hydrophila* infections when used before challenge (Prevention) or after challenge (Treatment) as evaluated by protection rate and average bacterial numbers in the kidney in the fishes died of the infections

Groups		Days post-challenge				Protection (%)	Average bacterial No. in died fishes' kidney (cfu/g)
		1	2	3	4~8		
Prevention	PBS	8	2	0	0	0/10 (0)	2.9×10^6
	s-IgY	3	1	0	0	6/10 (60)	4.6×10^3
	n-IgY	9	1	0	0	0/10 (0)	3.1×10^6
Treatment	PBS	8	2	0	0	0/10 (0)	3.2×10^6
	s-IgY	5	1	1	0	3/10 (30)	5.6×10^4
	n-IgY	9	1	0	0	0/10 (0)	4.5×10^6

experiments. This might be due to the fact that the challenge or pre-exposure dose with virulent *A. hydrophila* was too high with respect to the antibody concentrations used. Therefore, optimization of the challenge dose and specific IgY concentrations used for passive immunization is expected to generate dose- or concentration-dependent responses. Another factor may be due to the intrinsic bacteriostatic nature of the immunoglobulins, which might give the surviving bacteria chances of re-growth upon continuing decrease of the specific antibody.

In conclusion, the present study clearly indicates that the specific IgY obtained from hens immunized by inactivated *A. hydrophila* may provide a novel approach to the management of *A. hydrophila* including of various serotypes infections in aquatic species. However, there are still a number of issues remaining to be addressed before it can be used in the field, such as the mechanisms behind the effects of egg-yolk antibodies (Sugita-Konishi *et al.*, 1996; Carlander *et al.*, 2000), persistence of the antibodies with the body, its efficacy after oral administration and formulations suitable for oral application through feeds.

ACKNOWLEDGEMENT

We thank technicians Z.C. Zhou, W.D. Zhou, and Z.D. Jiang at School of Animal Science, Zhejiang University for their assistance during the experiments.

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