Effects of Hen Egg Yolk Immunoglobulin in Passive Protection of Rainbow Trout against *Yersinia ruckeri*

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Anti-*Yersinia ruckeri* egg yolk immunoglobulin (IgY) was transferred to egg yolk after immunization of White Leghorn hens with formalin-killed whole cells of serovar 1 (RS1154) and serovar 2 (RS1153)-*Y. ruckeri* and its lipopolysaccharide (LPS). The IgY was specific for its homologous LPS in western immunoblot, whereas some protein bands were commonly recognized, even by IgY from eggs of unimmunized hens. Purified LPS from both *Y. ruckeri* serovar types 1 and 2 had a very poor immunogenicity. The IgY activity was stable when processed into pellet form by a microbial transglutaminase treatment and showed a considerable resistance against acid pepsin for at least 2 h. Feeding specific anti-serovar 1 *Y. ruckeri* IgY to fish either before or after immersion infection produced marginal reductions in mortalities and in intestine infection. The same IgY did passively protect rainbow trout against infection when administered by intraperitoneal injection 4 h before an immersion challenge.

Keywords: *Passive immunization; rainbow trout; Yersinia ruckeri; egg yolk immunoglobulin; antibody stability; transglutaminase*

INTRODUCTION

Laying hens transfer large amounts of immunoglobulin (IgY) to the yolks of their eggs, where it serves as a means of passively protecting the developing chick. An egg may contain 100−150 mg of yolk immunoglobulin, and substantial amounts of specific antibody may be collected and purified from the eggs of immunized hens (Akita and Nakai, 1992). The availability of large amounts of relatively inexpensive immunoglobulin from egg yolks makes it feasible to use these antibodies for passive immunization by oral administration. The efficacy of this approach has been shown in human and veterinary medicine for rotavirus diarrhea in humans (Yolken et al., 1988), *Escherichia coli* infections in pigs (Hoblet et al., 1986; Yokoyama et al., 1992; Imberechts et al., 1997) and rabbits (O'Farrelly et al., 1992), and *Streptococcus mutans*-induced dental caries (Hamada et al., 1991). In aquatic species, IgY against *Edwardsiella tarda* was administered orally to passively immunize the Japanese eel, *Anguilla japonica* Temminick and Sclegel (Gutierrez et al., 1993; Hatta et al., 1993). In those studies, the challenge dose of live *E. tarda* was mixed into a paste of yolk powder containing 20% IgY and sterilized eel diet, and administered by cannulation into the stomach of each eel. The efficacy of the treatments was judged on the basis of reduced mortalities, clearance of the pathogen from the intestine, and reduced invasion of the liver and kidney (Hatta et al., 1993). A dose of 400 mg of egg yolk powder was effective in protecting a 100−130 g eel from infection with a concurrent 10^4−10^7 colony-forming unit (cfu) oral dose of *E. tarda* (Gutierrez et al., 1993). These studies illustrated that IgY antibodies could provide a barrier against edwardsiellosis infections established through the gastrointestinal tract. Mixing the IgY and bacteria together would optimize a protective effect, but in practical aquaculture, it would be difficult to anticipate precisely when a pathogen might infect fish, and the cost of continuous feeding of IgY to prevent initial infection would be prohibitive except for very small fish.

Nevertheless, we are interested in determining whether oral application of IgY could protect rainbow trout (*Oncorhynchus mykiss*) against infection with *Y. ruckeri*, the causative agent of enteric redmouth disease, a systemic bacterial septicemia of salmonid fish (Stevenson et al., 1993). *Y. ruckeri* can be isolated from the intestinal lining of fish (Busch and Lingg, 1974), and Evelyn (1996) has suggested that this bacterium may be resistant enough to use the gastrointestinal tract as one of its routes of infection. Persistence of *Y. ruckeri* for long periods in carrier fish and shedding of bacteria in feces can present a continuing source of infection. If a population of carrier fish could be substantially cleared by oral administration of anti-*Y. ruckeri* IgY treatments, it may be a cost-effective alternative to slaughtering a stock of fish that pose a health risk.

The objective of these studies was to determine if egg yolk IgY raised against *Y. ruckeri* could be effective against infections in salmonid fish. The first part of the present work was to determine if IgY antibodies raised against the fish pathogen *Y. ruckeri* could discriminate two serological varieties of this pathogen. Subsequently, the IgY was used in attempts to determine if infections could be reduced, particularly when administered orally.

MATERIALS AND METHODS

Immunization of Hens. *Y. ruckeri* strains RS1154 (serovar 1) and RS1153 (serovar 2) were fish-passaged strains from the Fish Health Laboratory collection, Department of Microbiology, University of Guelph. They were grown in tryptic soy

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broth (Difco, Detroit, MI) at 18 °C for 48 h in flasks with shaking. The cultures were treated with 0.3% formalin for 24 h, tested for viability, and then collected by centrifugation (10000*g*, 10 min). After three washes with phosphate-buffered saline (PBS; 0.12 M phosphate, 0.04 M NaCl, pH 7.2), cells were freeze-dried and stored at −20 °C. Lipopolysaccharide (LPS) of *Y. ruckeri* was isolated according to the phenol−hot water extraction method of Westphal and Jann (1965).

A group of White Leghorn hens, 23−26 weeks old, were kept for immunization and egg production in the Central Animal Facility at the University of Guelph. The antigen suspensions consisted of 1 mg of freeze-dried *Y. ruckeri* cells or 1 mg of LPS, suspended in 1 mL of sterile PBS, and were emulsified in an equal volume of Freund's complete adjuvant (FCA) (Sigma, St. Louis, MO). One milliliter of the emulsion was injected intramuscularly at two sites on each hen (0.5 mL per site). Two hens were injected with each antigen. Booster injections were given intramuscularly at 2, 3, 4, 5, and 10 weeks after the first injection. Eggs were collected every day during the immunization period. The antibody from yolk was purified from the water-soluble fraction of egg yolk by precipitating the lipoprotein, as described by Akita and Nakai (1992).

Enzyme-Linked Immunosorbent Assay (ELISA). Each well of the 96 well polystyrene plates (Corning Costar, Cambridge, MA) was coated with 100 μL of *Y. ruckeri* whole cell suspension (10^8/well) or LPS solution (10 μg/well) in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. After the wells were washed with PBS containing 0.05% Tween 20 (PBS-Tween), the wells were blocked by incubation for 1 h at 37 °C with 150 μL of 2.0% (w/v) bovine serum albumin (BSA; Sigma grade V, Sigma) in carbonated buffer. After four washings with PBS-Tween, appropriately diluted IgY was added and the plates were incubated for 1 h at 37 °C. After five washings with PBS-Tween, 100 μL of alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) was added, and the mixture was incubated at 37 °C for 1 h. Plates were washed with PBS-Tween, and 100 μL of the substrate solution *p*-nitrophenyl phosphate (1 mg/mL) in 1 M diethanolamine (pH 9.8) was added. After 30 min of incubation at 37 °C, 25 μL of 2 N NaOH was added to stop the reaction, and the color was read on an ELISA plate reader (model 550, Bio-Rad, Mississauga, ON) with a 405 nm filter.

Agglutinating antibody titers for freeze-dried IgY preparations, resuspended at 10 mg/mL, were determined using microtiter dilution plates as previously described (Stevenson and Airdrie, 1984). The antigen was formalin-inactivated *Y. ruckeri*, absorbance 0.9 at 520 nm.

Electrophoresis and Immunoblotting. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out according to the method of Laemmli (1970) using 10% acrylamide gel. The protein was stained with a mixture of Coomassie brilliant blue R-250 (Sigma) in 10% acetic acid/30% methanol. Gels were transferred electrophoretically to a nitrocellulose membrane using a transblot apparatus (Bio-Rad) at 150 V for 2 h. The membrane was washed briefly in Tris-buffered saline (TBS) containing 20 mM Tris, 500 mM NaCl (pH 7.5), and blocked overnight with a 2.5% solution (w/v) of BSA in TBS. The membrane was then washed three times in a 0.05% solution of Tween-20 in TBS (TTBS) and exposed to hens anti-*Y. ruckeri* IgY diluted 1:100 in 2% BSA−TBS for 2 h with gentle shaking. The membrane was washed twice in TTBS and then exposed to a solution of alkaline phosphatase (AP) conjugated to rabbit anti-chicken IgG (Sigma) diluted 1:3000 in 2% BSA−TBS for 1 h with gentle shaking. The solution was then discarded, and the membranes were washed six times in TTBS and once in TBS before being developed by the addition of the 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt/nitroblue tetrazolium chloride (BCIP/NBT) substrate (Gibco BRL, Gaithersburg, MD).

IgY Microencapsulation by Transglutaminase (TGase). A microbial TGase was purchased from Ajinomoto Co. Ltd. (Tokyo, Japan). A mixture of gelatin (100 mg/mL), starch (20 mg/mL), and anti-*Y. ruckeri* RS1154 IgY (20 mg/mL) was prepared in a total volume of 1 mL of 1 M sodium bicarbonate (pH 8.2). TGase was added to the mixture at 0.05% (w/v) and then incubated at 37 °C for 2 h. The treated samples were dried in an oven at 50 °C overnight and ground using a laboratory mill. The resulting IgY pellet contained 12.5 mg of specific anti-*Y. ruckeri* IgY in each 100 mg pellet. The IgY polymer (no gelatin or starch) was made by incubating anti-*Y. ruckeri* RS1154 IgY (100 mg/mL) with 0.05% (w/w) TGase at 37 °C for 2 h.

Effects of Proteolytic Enzymes and Digestion on IgY Activity. Pepsin from porcine stomach mucosa (Sigma) was added at a ratio of 1:80 (w/w) to a 10 mg/mL solution of anti-*Y. ruckeri* IgY solution and microencapsulated IgY. The mixtures were incubated at 37 °C at pH 2.0. After a given time, the residual antibody activity was measured by ELISA. To examine the activity of gastric secretions and enteric digestive enzymes of rainbow trout on the activity of anti-*Y. ruckeri* IgY, 20 mg of specific IgY and 3% gelatin (fc w/v) were administered into the stomach of anaesthetized 150 g rainbow trout by cannulation. The stomach and front and back sections of the intestine were sampled from each fish at 0, 2, 4, and 6 h after administration. Samples were put into 10 mL of cold PBS and homogenized. The suspension was centrifuged at 10000*g* for 10 min, and then the supernatant was filtered through a 0.45 μm pore size membrane filter and IgY activity measured by ELISA.

Fish Experiments. All fish experiments were conducted according to the guidelines of the Canadian Council on Animal Care and the guidelines for the care and use of animals in research and teaching of the University of Guelph. Rainbow trout were from pathogen-free stocks and were maintained in stock tanks supplied with flowing well water at 15 ± 2 °C and fed a diet of commercial fish food. Anesthesia for all injection or cannulation studies used a dose of 0.015% (w/v) 3-aminobenzoic acid ethyl ester (Sigma) in water. For terminal samples, fish were killed by an overdose of 3-aminobenzoic acid ethyl ester.

For intraperitoneal (ip) challenges, anesthetized rainbow trout of 270−300 g were injected with a 1:1 mixture of viable *Y. ruckeri* RS1154 (1.8×10^8 cfu/mL) and anti-*Y. ruckeri* RS1154 IgY, at a final concentration of either 4 or 40 mg per fish. After injection, fish were monitored for distress at frequent intervals, to 4 days postchallenge. Fish were then killed, necropsied, and tested for the presence of *Y. ruckeri* in the kidney and intestine. Tissue 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 suspension was further diluted, and 25 μL volumes of the dilutions were spotted onto TSA plates for enumeration and identification. Samples of kidney and intestine tissue were also directly streaked onto TSA plates. After incubation at 18 °C for 48 h, colonies on the plates were screened by colony morphology, the cytochrome *c* oxidase reaction (negative for *Y. ruckeri*), and slide agglutination with specific anti-*Y. ruckeri* IgY reagent.

For immersion challenges, groups of four to five rainbow trout (∼5 g) were assigned to 700 mL experimental tanks, to provide a biomass of ∼4% (w/v) in each tank. *Y. ruckeri* RS 1154 (serovar 1) was grown in TSB shake-flask cultures at 18 °C for 48 h for challenges. Dilution plate counts were prepared to measure the challenge dose. Water flow to the tanks was stopped, and 70 mL of the culture was added to each tank, for an approximate dose of 10^8 cfu mL^{-1}. After 1 h, water flow was resumed, with the tank effluent being collected for decontamination by chlorination. Fish were monitored at frequent intervals for 8 days postchallenge, and any mortalities were necropsied and cultured. At the termination of the experiment, fish were examined for the presence and level of *Y. ruckeri* in the organs and intestine by plating on TSA.

Prior to the immersion challenges, anti-*Y. ruckeri* RS1154 IgY was provided either by feeding or by injection. In the injection trial, 0.1 mL of anti-*Y. ruckeri* IgY was injected intraperitoneally into each fish, at final concentrations of 4 or 20 mg/fish. Control fish received 4 mg of nonspecific, normal IgY. The immersion challenge in 1.8×10^8 cfu/mL *Y. ruckeri* was given 4 h after the injection. In the first feeding trial, each

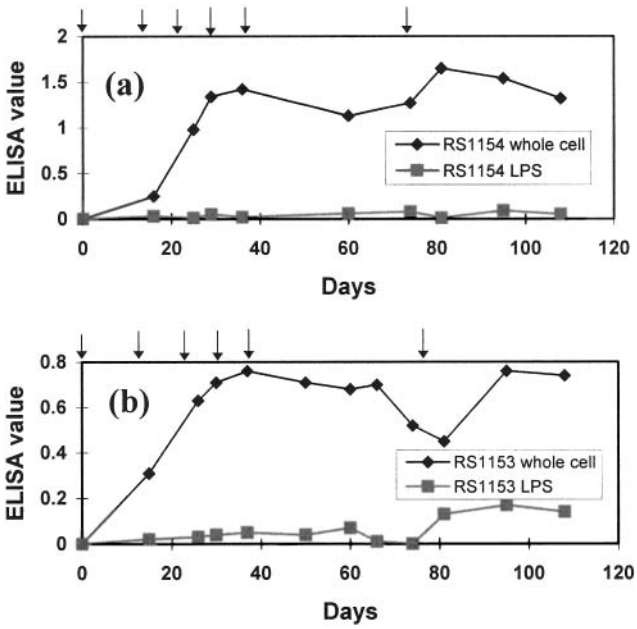

Figure 1. Antibody response in the egg yolk from hens injected with antigens of *Y. ruckeri*: (a) serovar 1, *Y. ruckeri* (RS1154) IgY; (b) serovar 2, *Y. ruckeri* LPS (RS1153). Antibody level in a 1000 dilution of egg yolk water-soluble fraction was measured by ELISA using the whole cell antigens or LPS antigens. Arrow indicates days of injection.

tank of fish was fed 400 mg of commercial rainbow trout diet mixed either with 400 mg of IgY pellet or with 400 mg of a control pellet lacking IgY. After 2 h, fish were exposed to 2×10^8 cfu/mL *Y. ruckeri.* In the second feeding trial, tanks were fed 400 mg of feed and 400 mg of IgY pellet, given either as a prefeed (once before challenge), a postfeed (once after challenge), or a multiple feed (four times after challenge). Control tanks received 400 mg of the control pellet lacking IgY. These fish were exposed to 4.1×10^8 cfu/mL viable *Y. ruckeri.*

RESULTS AND DISCUSSION

Antibody Production and Specificity. It is now well-known that a pathogen bacteria-specific antibody can be obtained in large quantities from eggs laid by hyperimmunized hens. In fact, a tremendous number of hens are immunized to protect them from several avian diseases and managed to lay eggs systematically. The eggs contain IgY and are consumed as food. Considering this fact, it is highly likely that it will be possible to safely apply IgY for passive immunization therapy through oral administration. Figure 1 shows the immune response of hens against *Y. ruckeri*. Thirty to 40 days after the first immunization, antibodies to whole cells of both serovars of *Y. ruckeri* were found at high titer in egg yolk. The maximum ELISA value against serovar 1 strain RS 1154 was twice as high as that for serovar 2 strain RS 1153, for equivalent concentrations of ELISA test antigens. Hens immunized with purified LPS of either serovar (in FCA) showed very poor antibody responses. This differs from the general experience with raising antisera in rabbits or fish, for which serovar 2 responses are usually higher (Raymond, 1991). After salting-out by 50% ammonium sulfate, the purified IgY from egg yolk was determined to be ~80% pure, based on SDS−PAGE electrophoresis (Figure 2). Some remaining protein from the water-soluble fraction was found as a distinct band of 35 kDa, corresponding to β-livetin. A 10 mg/mL solution of the purified IgY had an agglutinating titer of 1:32 for anti-

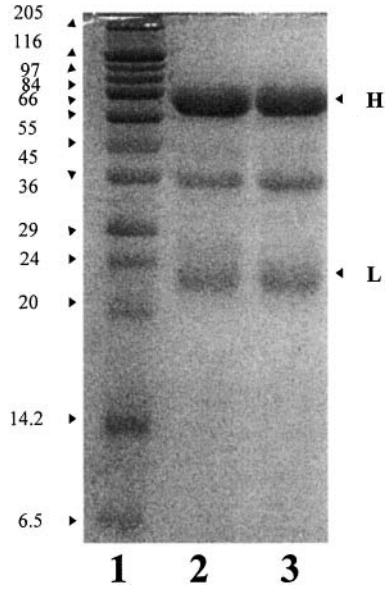

Figure 2. SDS−PAGE patterns of antibody (IgY) purified from egg yolk: (lane 1) standard protein (molecular masses are in kilodaltons); (lane 2) normal IgY before immunization; (lane 3) IgY after immunization. Heavy (H) and light (L) chains are indicated.

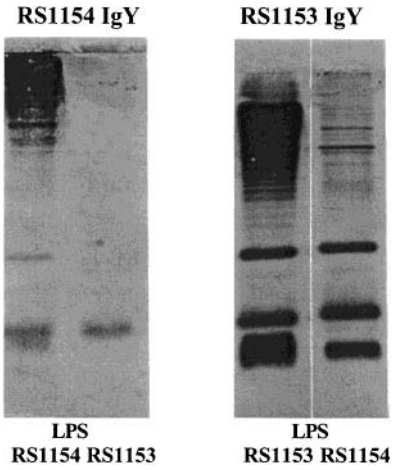

Figure 3. Western blotting of LPS of *Y. ruckeri* RS 1153 and RS1154 with anti-*Y. ruckeri* serovar 1 (RS1154) IgY or anti-*Y. ruckeri* serovar 2 (RS1153) IgY.

Y. ruckeri RS 1154 IgY and 1:32 for anti-*Y. ruckeri* RS 1153 IgY, respectively.

At least three of the immunodominant bands with low molecular weight that appeared with anti-*Y. ruckeri* IgY were also present when control IgY prepared from eggs of nonimmunized hens was used or with anti-*E. coli* IgY (data not shown). This indicates that those three components are common between *Y. ruckeri* and *E. coli* LPS. The anti-*Y. ruckeri* RS 1153 IgY also reacts well with some other bands in the RS 1154 LPS preparation (Figure 3), which also appeared in some immunoblots with anti-*E. coli* IgY and anti-*Y. ruckeri* RS 1154 IgY. The LPS of serovar 1 (RS1154) and 2 (RS1153) strains of *Y. ruckeri* were recognized by their homologous anti-whole-cell IgY preparations. The IgY against serovar 2 IgY reacted distinctly against its whole LPS, while the anti-serovar 1 IgY mainly reacted with higher molecular weight LPS. The *Y. ruckeri* RS1154 is the predominant isolate from enteric redmouth disease in rainbow trout culture and is the basis of the commercial bacterins used

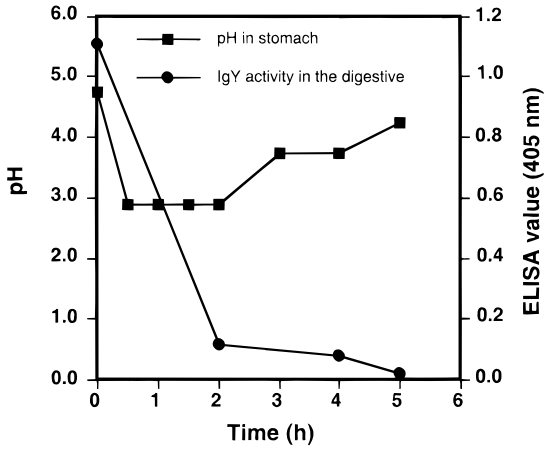

Figure 4. Changes of pH and antibody activity in rainbow trout stomach after feeding. Data are average of duplicate measurements. Stability of anti-*Y. ruckeri* (RS1154) IgY in the digestive tracts of rainbow trout after oral administration of IgY was measured by ELISA using the whole cells as an antigen.

in rainbow trout aquaculture. The LPS structure of *Y. ruckeri* RS1153 could be distinct from serovar 1.

Stability of IgY. Figure 4 shows the antibody stability in the rainbow trout stomachs after feeding. The activity of anti-serovar 1 *Y. ruckeri* IgY decreased rapidly after 2 h of exposure in the stomach and was completely lost 5 h after oral administration (Figure 4). In parallel measurements, the pH of the rainbow trout stomach following feeding decreased rapidly from an initial pH of 4.5−5.0 to pH 2.6−3.0 after 30 min and then increased again after 3 h. However, it was reported that the ELISA value of anti-*E. tarda* IgY activity in the eel digestive tracts was found to be maintained for 6 h after oral administration (Gutierrez at al., 1993), results that are not coincident with our result during fish digestion. Although the change of eel stomach pH was not given in the paper, it was shown that the acidity in eel stomach increases after food intake (Mackary, 1929). It has been shown that the pH decreased to 2.0 in the stomach of tilapia a few hours after daily feeding (Moriarty, 1973). Shimizu et al. (1989) also report that the activity of IgY specific to *E. coli* examined by competitive ELISA was sensitive to pepsin, especially at low pH (<4.0). It was reported that the activity measured by neutralization titer of anti-human rotavirus IgY was completely lost by peptic digestion at pH 2.0, while a considerable extent of neutralization titer of the IgY (63% of the initial value) remained upon incubation with pepsin at pH 4.0; stability of IgY against pepsin seemed to be highly dependent on pH (Hatta et al., 1993). Ohtani et al. (1991) reported that IgY specific to α_{s1}-casein was relatively resistant to trypsin and chymotrypsin. From our study, it is obvious that anti-*Y. ruckeri* IgY administered orally to rainbow trout reached the small intestine with severe damage by gastric enzymes and low pH. If the IgY is introduced into the small intestine without a significant loss of activity caused by gastric enzymes and pH values, antibodies can reach into the intestine of rainbow trout after oral administration. Therefore, it is important to protect the IgY from degradation in the gastric enzyme and low pH. We used a new method of IgY microencapsulation using a microbial TGase. The IgY polymer was significantly more susceptible to inactivation by pepsin digestion than was the IgY pellet preparation (microencapsulation) (Figure 5). The polymerized IgY appears to break down after pepsin digestion for 1 h (data not shown). In contrast, the IgY pellet activity was stable after 2 h of pepsin digestion, and no cleavage of the IgY molecule was observed in SDS−PAGE gel (Figure 5b, lane 5). Also, the agglutination titer of IgY pellet was 1:16 per milliliter of 100 mg pellet solution.

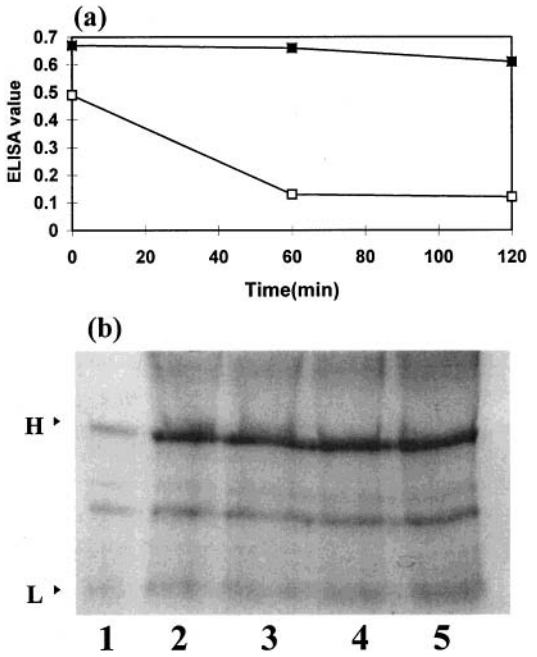

Figure 5. Antibody stability (a) and SDS−PAGE patterns (b) of anti-*Y. ruckeri* IgY following digestion with pepsin at 1:80 ratio (w/w) for 2 h at 37 °C: (a) anti-*Y. ruckeri* activity of IgY polymer (□) and IgY pellet (■) by ELISA; (b) SDS−PAGE patterns of IgY pellet following pepsin digestion [(lane 1) IgY solution, heavy chain (H), light chain (L); (lane 2) IgY pellet; (lanes 3−5) IgY pellet with pepsin digestion at pH 2.0 for 0 (lane 3), 1 (lane 4), and 2 h (lane 5), respectively].

Passive Protection of Fish. Groups of ~5 g rainbow trout that had been fed anti-*Y. ruckeri* IgY 2 h prior to an immersion challenge with *Y. ruckeri* RS 1154 had lower mortalities after 8 days compared with fish fed with normal food before the challenge. The group fed IgY appeared to have a lower number of infected fish after 8 days, on the basis of on organ and intestine culture. In a subsequent trial of the feeding procedures with triplicate replicates of the groups, IgY-fed groups showed lower mortalities than groups receiving normal feed (Table 1). The numbers of IgY-fed fish carrying *Y. ruckeri* in intestine samples appeared to be lower than the normal-feed controls, regardless of whether the feeding was given before or after the challenge. The groups fed IgY three times postinfection gave inconsistent results. Two groups of sampled fish, one in each of these treatments, showed no *Y. ruckeri* in individual intestinal samples or in a pool of kidney tissues. Although fish used in the trials above were actively feeding on the IgY mixtures, the doses taken up by the fish were uncertain because of uneven feeding and some discrimination between particles in the feed. Gutierrez et al. (1993) used the paste mixed with anti-*E. tarda* IgY powder, and viable *E. tarda* was orally administered by cannulation. However, our fish experimental design is a more natural anti-*Y. ruckeri* IgY pellet feeding in fish farm and immersion challenge. The immersion challenge method is similar to natural outbreaks of ERM disease by *Y. ruckeri* in fish farms.

Table 1. Effects of Anti-*Y. ruckeri* IgY Pellet Feeding on Prevention of *Y. ruckeri* Infection of Kidney and Intestine after Immersion Challenge

treatment	IgY pellet[a] IgY	IgY pellet[a] additive	mortality[b]	no. of fish in each group infected with *Y. ruckeri* in the intestine	no. of groups that have *Y. ruckeri* in a kidney pool[c]
no challenge normal feed	−	−	0/5	0	0/1
challenge normal feed	−	−	6/15	T1 5/5 T2 5/5 T3 5/5	3/3
challenge prefed IgY pellet	+	+	1/15	T1 2/5 T2 2/5 T3 2/4	3/3
challenge postfed IgY pellet	+	+	2/15	T1 2/3 T2 2/5 T3 2/5	3/3
challenge multiply fed IgY pellet	+	+	2/14[d]	T1 1/3 T2 0/5 T3 3/4	2/3

[a] IgY pellet was mixed IgY, gelatin, and starch and polymerized with TGase for 2 h. Also, the 400 mg of IgY pellet contained the 50 mg of anti-*Y. ruckeri* (RS1154) IgY. Additive was polymerized gelatin and starch with TGase for 2 h. [b] Number of dead fish/number of tested group fish. [c] Kidney pool: groups were divided T1, T2, and T3; each group has five fish. Kidney pool was collected from three to five fish kidneys. Dead fish were not used in the kidney pool. [d] One fish that died 1 day postchallenge before the first feeding of IgY is not included in the total.

Table 2. Effects on Rainbow Trout by Passive Immunization[a] of Anti-*Y. ruckeri* IgY against Immersion Challenge[b]

treatment	CFU g^{-1} in group[c] intestine	CFU g^{-1} in group[c] kidney	no. of infected *Y. ruckeri* in group[d] intestine	no. of infected *Y. ruckeri* in group[d] kidney	no. of fish	*Y. ruckeri* positive intestine	*Y. ruckeri* positive kidney
challenge high-dose specific IgY	5.6×10^4	0	0/5	0/5	1	−	−
					2	−	−
					3	−	−
					4	−	−
					5	−	−
challenge low-dose IgY	7.2×10^3	0	0/5	0/5	1	−	−
					3	−	−
					4	−	−
					5	−	−
challenge nonspecific IgY	7.4×10^6	6.2×10^7	4/5	4/5	1	−	−
					2	+	+
					3	+	+
					4	+	+
					5	+	+
challenge saline	3.6×10^5	3.8×10^6	3/5	3/5	1	−	−
					2	−	−
					3	+	+
					4	+	+
					5	+	+

[a] Passive immunization: fish are infected with 0.1 mL of anti-*Y. ruckeri* (RS1154) IgY (high dose = 20 mg, low dose = 4 mg) and nonspecific IgY (4 mg). [b] Immersion challenge with viable *Y. ruckeri* (1.8×10^8 cfu mL^{-1}). [c] CFU g^{-1} of group in intestine and kidney 7 days after immersion challenge. [d] Number of *Y. ruckeri* positive/number of tested fish.

Table 2 shows the passive immunization of anti-*Y. ruckeri* IgY against immersion challenge. Four days after a challenge dose of 10^8 cfu *Y. ruckeri* was injected ip into three rainbow trout (~285 g), all fish had *Y. ruckeri* associated with the intestinal tract tissue, but none was found in the kidney tissue by direct streak. If the dose was mixed with either 4 or 40 mg of the IgY preparation immediately prior to injection, only one fish in each group had *Y. ruckeri* after 4 days, indicating some direct passive immunization effect of IgY but no carrier material. When groups of ~5 g fish were passively immunized by ip injection with IgY and subsequently immersion challenged, no *Y. ruckeri* were found in the intestine or kidney after 7 days, whereas fish groups injected with a nonspecific IgY or saline had the bacteria in both kidney and intestinal tissue. Therefore, the ip injection of anti-*Y. ruckeri* at a dose of 4 mg is effective against an immersion challenge of *Y. ruckeri* of 10^8 cfu/mL. Many pathogens of fish have been reported to spread by infection through intestinal mucosa. The naturally oral feeding of specific IgY against fish pathogens with feed will be an alternative to the method with antibiotics and chemotherapy for prevention of fish diseases in fish farms. Moreover, the oral feeding of active IgY would be a novel approach for preventing viral infection diseases of fish because no medicine has been reported to be effective.

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LITERATURE CITED

Akita, E. M.; Nakai, S. Immunoglobulin from egg yolk; isolation and purification. *J. Food Sci.* **1992**, 57, 629−634.

Busch, R. A.; Lingg, A. J. Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rain-

bow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* **1974**, *32*, 2429−2432.

Evelyn, T. P. T. Infection and disease. In *The Fish Immune System: Organism, Pathogen and Environment*; Iwama, G., Nakanishi, T., Eds.; Academic Press: San Diego, CA, 1996; pp 339−362.

Gutierrez, M. A.; Miyazaki, T.; Hatta, H.; Kim, M. Protective properties of egg yolk IgY containing anti-*Edwardsiella tarda* antibody against paracolo disease in the Japanese eel, *Anguilla japonica* Temminck & Schlegel. *J. Fish Dis.* **1993**, *16*, 113−122.

Hamada, S.; Horikoshi, T.; Minami, T.; Kawabata, S.; Hiraoka, J.; Fujiwara, T.; Ooshima, T. Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of *Streptococcus mutans*. *Infect. Immun.* **1991**, *59*, 4161−4167

Hatta, H.; Tsuda, K.; Akachi, S.; Kim, M.; Yamamoto, T.; Ebina, T. Oral passive immunization effect of anti-human rotavirus IgY and its behavior against proteolytic enzyme. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1077−1081.

Hoblet, K. H.; Kohler, E. M.; Saif, L. J.; Theil, K.W.; Ingalls, W. L. Study of porcine postweaning diarrhea involving K88-(−) hemolytic *Escherichia coli*. *Am. J. Vet. Res.* **1986**, *47*, 1910−1912.

Imberechts, H.; Deprez, P.; Van Driessche, E.; Pohl, P. Chicken egg yolk antibodies against F18ab fimbriae of *Escherichia coli* inhibit shedding of F18 positive *E. coli* by experimentally infected pigs. *Vet. Microbiol.* **1997**, *54*, 329−341.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Mackay, M. E. The digestive system of eel-pout *Zoarces anguillaris*. *Biol. Bull.* **1929**, *56*, 8−23.

Moriarty, D. J. W. The physiology of digestion of blue-green algae in the cichlid fish *Tilapia nilotica*. *J. Zool.* **1973**, *171*, 25−39.

O'Farrelly, C.; Branton, D.; Wanke, C. A. Oral ingestion of egg yolk immunoglobulin from hens immunized with an enterotoxigenic *Escherichia coli* strain prevents diarrhea in rabbits challenged with the same strain. *Infect. Immun.* **1992**, *60*, 2593−2597.

Ohtani, H.; Matsumoto, K.; Saeki, A.; Hosono, A. Comparative studies on properties of hen egg yolk IgY and rabbit serum IgG antibodies. *Lebensm. Wiss. Technol.* **1991**, *24*, 152−158.

Raymond, B. T. Immune responses in fish to *Yersinia ruckeri*. M.Sc. Thesis, University of Guelph, 1991.

Shimizu, M.; Fitzsimmons, C. R.; Nakai, S. Serum and egg antibody response in chicken to *Escherichia coli*. *Agric. Biol. Chem.* **1989**, *53*, 3233−3288.

Stevenson R. M. W.; Airdrie, D. W. Serological variation among *Yersinia ruckeri* strains. *J. Fish Dis.* **1984**, *7*, 247−254.

Stevenson, R. M. W.; Flett, D.; Raymond, B. T. Enteric redmouth (ERM) and other enterobacterial infections of fish. In *Bacterial Diseases of Fish*; Inglis, V., Roberts, R. J., Bromage, N. R., Eds.; Blackwell Scientific Publications: Oxford, U.K., 1993; pp 80−105.

Westphal, O.; Jann, K. Bacterial lipopolysaccharides. *Methods Carbohydr. Chem.* **1965**, *5*, 83−91.

Yokoyama, H.; Peralta, R. C.; Diaz, R.; Sendo, S.; Ikemori, Y.; Kodama, Y. Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic *Escherichia coli* infection in neonatal piglets. *Infect. Immun.* **1992**, *60*, 998−1007.

Yolken, R. H.; Leister, Wee S. B.; Miskuff, R.; Vonderiecht, S. Antibodies to rotaviruses in chickens' eggs: a potential source of antiviral immunoglobulins suitable for human consumption. *Pediatrics* **1988**, *81*, 291−295.

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