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Development of a serology-based assay for efficacy evaluation of a lactococcicosis vaccine in *Seriola* fish

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ABSTRACT

Lactococcicosis is an infection caused by the bacterium *Lactococcus garvieae* and creates serious economic damage to cultured marine and fresh water fish industries. The use of the assay currently applied to evaluate the potency of the lactococcicosis vaccine is contingent upon meeting specific parameters after statistical analysis of the percent survival of the vaccinated yellowtail or greater amberjack fish after challenge with a virulent strain of *L. garvieae*. We found that measuring the serological response with a quantitative agglutinating antibody against the *L. garvieae* antigen (phenotype KG+) was an effective method of monitoring the potency of lactococcicosis vaccines. Vaccinated fish had significantly higher antibody titers than control fish when the *L. garvieae* Lg2-S strain was used as an antigen. Furthermore, the titer of the KG + agglutinating antibody was correlated with vaccine potency, and the cut-off titer was determined by comparing the data with those from the challenge test. An advantage of the proposed serology-based potency assay is that it will contribute to reduced numbers of animal deaths during vaccine potency evaluations.

1. Introduction

Lactococcus garvieae is a gram-positive bacterial pathogen that causes septicemia and meningoencephalitis in several species of marine and fresh water fish [1–4] and mammals [5]. Lactococcicosis causes serious economic damage to *Seriola* fish farming in Japan. The injection vaccine for lactococcicosis has been licensed in Japan since 2000, and the use of the vaccine has been spreading among fish farms due to its excellent effectiveness. The increase in lactococcicosis vaccine distribution has led to an increase in the number of vaccinated fish and a concomitant decrease in the use of antibiotics.

Prior to the official release of each vaccine lot into the Japanese market, the Pharmaceutical Affairs Law requires a quality control evaluation that is performed by National Veterinary Assay Laboratory (NVAL); in addition, the manufacturer performs in-house tests for ensuring the product quality for each lot. Models to measure the effectiveness of fish vaccines are mostly based on an immunization-challenge procedure in laboratory fish. These models have proven to be extremely instrumental in scientifically underpinning the correlation between the protection conferred by the selected vaccine antigens and their efficacy. However, the 3Rs strategy (replacement, reduction, and refinement) recommends alternatives to using animal testing in vaccine potency measurement [6]. Furthermore, since challenge tests do not always yield reproducible results due to differences in fish size and bacterial pathogenicity, 2 or 3 bacterial challenge-dose groups are usually required. In addition, a number of vaccines have been developed recently, which requires testing of a large number of experimental fish. Larger facilities with more tanks are required to perform the tests, resulting in a considerable cost increase.

Experimental challenge tests have previously been used as efficacy assays for lactococcicosis vaccine release. They remain the only method of evaluating vaccines because as of yet, no serological parameter corresponding to vaccination has been detected. We previously conducted large-scale immune-related gene expression profiling in vaccinated yellowtail using microarray analysis so as to understand the basic mechanism of fish immunity triggered by vaccination. The results showed that vaccination elicited an

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immune response in yellowtail fish, activating both nonspecific cellular immunity and humoral immunity [7]. Based on this evidence, we hypothesized that there would be a serological marker that could be used to detect *L. garvieae* and focused on developing an assay to detect KG + agglutinating antibody titers.

Serologically, *L. garvieae* is categorized into 2 serotypes, KG+ and KG-, wherein the rabbit anti KG- serum agglutinates with both KG- and KG + serotypes, whereas the anti KG + serum agglutinates only with the KG + serotype [1]. In principle, KG- type isolates have a capsule that is virulent for fish, while KG + isolates, which have no capsule, remain nonvirulent for fish [8,9]. All the vaccine strains isolated from diseased yellowtail fish in Japan have had the KG- serotype. However, studies have demonstrated a lack of correlation between vaccine efficacy and KG- agglutinating antibody titer [7,10,11]. Formalin-killed KG- and KG + serotype cells induce high protection in yellowtail against artificial infection with virulent KG- and elicit high agglutinating titers of KG+ [11,12]. This suggests that antigens associated with immunity against *L. garvieae* are common in KG+ and KG- cells. In order to fulfill the recommendations of the 3Rs strategy, a study was carried out by the NVAL to assess the feasibility of replacing the challenge assay with a serology-based assay measuring anti-KG + titers for a lot-release test.

2. Material and methods

2.1. Fish

Healthy artificially hatched yellowtail and greater amberjack fish, with weights ranging from 50 to 300 g, were purchased from the Fisheries Research Agency (Kanagawa, Japan) and A-marine Kindai Co (Wakayama, Japan), respectively. The fish were maintained in 200-L or 500-L circular tanks, the water temperature was kept at 20 °C or 25 °C, and the salinity of rearing seawater was checked every day. The fish were fed the standard commercial diet for marine fish throughout the experiment. They were handled according to NVAL's animal management guidelines.

2.2. Vaccines

All the vaccines used in this study were inactivated injection-type mono or polyvalent lactococcicosis vaccines produced by 6 different manufacturers (A to F, Table 1). The quality control of all these lots had been previously confirmed by challenge tests by NVAL under the framework of Japanese lot-release tests.

2.3. Reduced-potency vaccine samples

To obtain reduced-potency vaccine samples, a vaccine lot from manufacturer B was diluted with phosphate-buffered saline (PBS). Fish (n = 140) were injected intraperitoneally (i.p.) with either 0.1 ml of the diluted vaccine or PBS. At 14 days post vaccination (dpv), blood samples were obtained from 6 groups of 10 fish vaccinated with either × 1, × 10, × 100, × 1000, × 10,000, or × 100,000-diluted vaccines (total of 60 fish) and 10 unvaccinated control fish. The other 70 fish were challenged i.p with 10^3 CFU of *L. garvieae* strain KG9502 (KG-), which is normally used in lot-release tests in Japan [13]. At 14 days post challenge (dpc), the mortality rates were determined.

2.4. Bacteria

Six *L. garvieae* strains were used in this study (Table 2). Two strains had the KG- serotype: Lg2 [14] and KG9502 [11]. The other 4 strains had the KG + serotype: Lg2-S [15], NSS9310 [16], ATCC49156 (American Type Culture Collection, Manassas, VA, USA), and ATCC43921 (American Type Culture Collection, Manassas, VA, USA). *L. garvieae* Lg2-S (KG+) was obtained after subculturing *L. garvieae* Lg2 (KG-) on Todd-Hewitt broth (THB; Becton, Dickinson and Company, Franklin Lakes, NJ USA) with 1.5% agar (THA) and supplemented with TCC, according to Ooyama et al. (1999) [12], and the change in serotype from KG- to KG+ was confirmed [15]. ATCC43921 isolated from cows is a type strain of *L. garvieae*; ATCC49156 is also a type strain that was previously the type strain of *Enterococcus seriolicida* but was reclassified as a type strain of *L. garvieae* [5,17].

Table 1
Vaccines used in this study.

Vaccine	Manufacturer/ Lactococcicosis vaccine strain	Lot	Antigen	Minimum required volume of Lactococcus antigen
A2-1	A	1	Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
A2-2		2	Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
B2-1	B	1	Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
B2-2		2	Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
B2-3		3	Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
B3-1		1	Iridovirus infection, Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
B3-2		2	Iridovirus infection, Vibriosis, Lactococcicosis, inactivated	10^9 CFU/ml
C3	C	1	Iridovirus infection, Vibriosis, Lactococcicosis, inactivated	1.58×10^8 CFU/ml
D3	D	1	Iridovirus Infection, Vibriosis, Lactococcicosis, Inactivated	1.58×10^8 CFU/ml
E3	E	1	Vibriosis, Lactococcicosis, Pseudotuberculosis (Oil adjuvant), Inactivated	6.8×10^8 CFU/ml
F1	F	1	Lactococcosis, Inactivated	5×10^9 CFU/ml

2.5. Agglutinating antibody measurement

The bacteria were cultured in THB for 20 h at 25 °C, suspended in 10% skimmed milk (Becton, Dickinson and Company) solution, and then stored at −80 °C until further use. Initially, 0.1 ml of frozen stocks of *L. garvieae* was used to inoculate 10 ml of THB, which was

Table 2
L. garvieae strains used in this study.

Strain	Phenotype	Source	Country	Year of isolation	Reference
Lg 2	KG-	Yellowtail	Japan	2002	Kawanishi et al., 2005
KG9502	KG-	Yellowtail	Japan	1995	Yoshida et al., 1996
Lg 2 -S[a]	KG+	–	–	–	Kawanishi et al., 2007
NSS9310	KG+	ND	Japan	1993	Ooyama et al., 2002
ATCC49156 T[b]	KG+	Yellowtail	Japan	1974	
ATCC43921 T	KG+	Cow	United Kingdom	1984	

ND: No data.
[a] Lg2-S (KG+) was spontaneously generated from a subculture of Lg2 (KG−) on Todd-Hewitt agar supplemented with 2,3,5-triphenyltetrazolium chloride (TTC).
[b] ATCC49156 was previously the type strain of *Enterococcus seriolicida* and is now reclassified in *Lactococcus garvieae*.