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Development of a trivalent vaccine for prevention of co-infection by *Miamiensis avidus* and *Tenacibaculum maritimum* in farmed olive flounder

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Abstract

Scuticociliatosis, caused by the parasitic pathogen *Miamiensis avidus*, poses a significant threat to olive flounder farms in South Korea. Infected fish suffer from severe systemic infections affecting various organs, with potential secondary bacterial diseases. This study investigated the emergence of different *M. avidus* serotypes in 20 olive flounder farms on Jeju island, South Korea, from 2015 to 2020. Additionally, we identified *Tenacibaculum maritimum* as a co-infecting bacteria. Based on serotyping and monitoring data, we developed a trivalent vaccine targeting two serotypes of *M. avidus* and one strain of *T. maritimum*. The efficacy of the vaccine was evaluated under laboratory conditions and demonstrated a relative percentage of survival (RPS) of 75%, 80%, and 93% for *M. avidus* serotype I, *M. avidus* serotype II and *T. maritimum*, respectively. Furthermore, successful field trials conducted on four different olive flounder farms resulted in significantly higher survival rates (52%–76% RPS) and weight gains in vaccinated fish. Overall, this study presents an effective vaccine against *M. avidus* and *T. maritimum* infections in farmed olive flounder, making a valuable contribution to sustainable aquaculture in South Korea.

Keywords: Olive flounder, Scuticociliatosis, *Miamiensis avidus*, *Tenacibaculum maritimum*, Vaccine

Introduction

Scuticociliatosis is the prevalent parasitic disease affecting finfish aquaculture worldwide. It is caused by various ciliate species, including *Uronema nigricans*, *Philasterides dicentrarchi*, *Uronema marinum*, *Pseudocohnilembus persalinus*, and *Miamiensis*

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avidus. Among these species, *M. avidus* is the primary agent responsible for scuticociliatosis in olive flounder (*Paralichthys olivaceus*) aquaculture (Kang & Kim, 2014; Song et al., 2009a). Olive flounder infected with *M. avidus* suffer from severe systemic infections, leading to organ-specific pathological changes from the skin to the brain (Kim et al., 2019). Moreover, *M. avidus* infection also increases the risk of secondary bacterial diseases, such as tenacibaculosis and vibriosis (Jung et al., 2007). Current control measures for scuticociliatosis include antibiotics and formalin treatments, although their efficacy is limited to the cases of mild infection (Iglesias et al., 2002; Jee & Jo, 2002). Furthermore, the government strictly regulates their usage due to concerns related to food safety and environmental impact. Therefore, immunization of fish with an effective vaccine against *M. avidus* infection is crucial for successfully preventing scuticociliatosis without compromising public health. Researchers have developed experimental vaccines that show promising preventive effects against scuticociliatosis under laboratory conditions (Lamas et al., 2008; León-Rodríguez et al., 2012). Nevertheless, there is a lack of comprehensive field data on the efficacy and safety of these vaccines.

In this study, we conducted a 5-year-investigation into the emergence pattern of *M. avidus* and the co-infecting bacteria, *Tenacibaculum maritimum,* in 20 olive flounder farms on Jeju island, South Korea. Through comprehensive disease monitoring, including *M. avidus* serotyping and analysis of *T. maritimum* co-infection, we developed a trivalent vaccine containing two different serotypes of *M. avidus* and one strain of *T. maritimum* to prevent the corresponding diseases in farmed olive flounder. The vaccine's efficacy has been assessed through experimental infection under laboratory conditions and further validated through a field test conducted on different fish farms.

Materials and Methods

Monitoring of *Miamiensis avidus* infection and co-infection with *Tenacibaculum maritimum*

The intensive monitoring was implemented at 20 different olive flounder farms located at Seongsan, Pyoseon, Namwon, Daejeong, Hangyeong, Jocheon, and Gujwa in Jeju island, Korea (Fig. 1A). Five fish were randomly collected from each farm every month. In total, 6,200 olive flounder samples were obtained between February 2015 and March 2020. The collected fish were examined using a light microscope to determine if infection by *M. avidus* was present.

T. maritimum was isolated from the liver, kidney, and spleen of olive flounder suffering from scuticociliatosis and incubated on Difco™ marine agar plates (BD, Franklin Lakes, NJ, USA). Following 48 hr-incubation at 25℃, the isolated strains were confirmed by microscopic and molecular analysis using polymerase chain reaction (PCR) with species-specific primers (forward: 5′-AATGGCATCGTTTTAAA-3′, reverse: 5′-CGCTCTCTGTTGCCAGA-3′; Avendaño-Herrera et al., 2004).

Serotyping of *Miamiensis avidus* using *cox*1 gene sequence analysis

M. avidus strains isolated from the infected fish were cultured by feeding the parasite with fathead minnow (FHM) cells. Genomic DNA was extracted using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The PCR was performed to amplify the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) genes of *M. avidus* using the specific primers (*cox*1 forward: 5′-GGTTCTAAAGATGTGGCTTACCCTAGAC-3′, *cox*1 reverse: 5′-CATACCAGGCATACATAAAGTACGTCTTGT-3′). The amplified PCR fragment was purified, cloned into the pMD20-T vector, and sent for sequencing (Macrogen, Seoul, Korea). The obtained *cox*1 nucleotide sequences of *M. avidus* were aligned with those reported in previous studies using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed using the neighbor-joining method using the *cox*1 sequences. The percentage of replicates was 1,000 replicates.

Virulence evaluation of isolated *Miamiensis avidus* and *Tenacibaculum maritimum* strains

Healthy olive flounders with an average body weight of 8 g were purchased from a local farm in Jeju island, South Korea. The fish were first diagnosed as free of pathogen infection and acclimated to experimental conditions at 20℃ for one week before the initiation of the experiment. The *M. avidus* parasite was cultured by feeding with FHM, which were grown in Leibovitz's L-15 (L-15) medium containing 100 U/mL penicillin and 100 μg/mL streptomycin supplemented with 10% fetal bovine serum at 20℃, as described by Dyková et al. (2010). The harvest of *M. avidus* was done by centrifugation at 500×g for 15 min at room temperature, and the resuspended cells were counted by hemocytometer under a light microscope. The *T. maritimum* bacteria were cultured in a marine broth medium at 25℃ for 24 h. The grown bacteria were centrifuged at 4,000×g for 30 min

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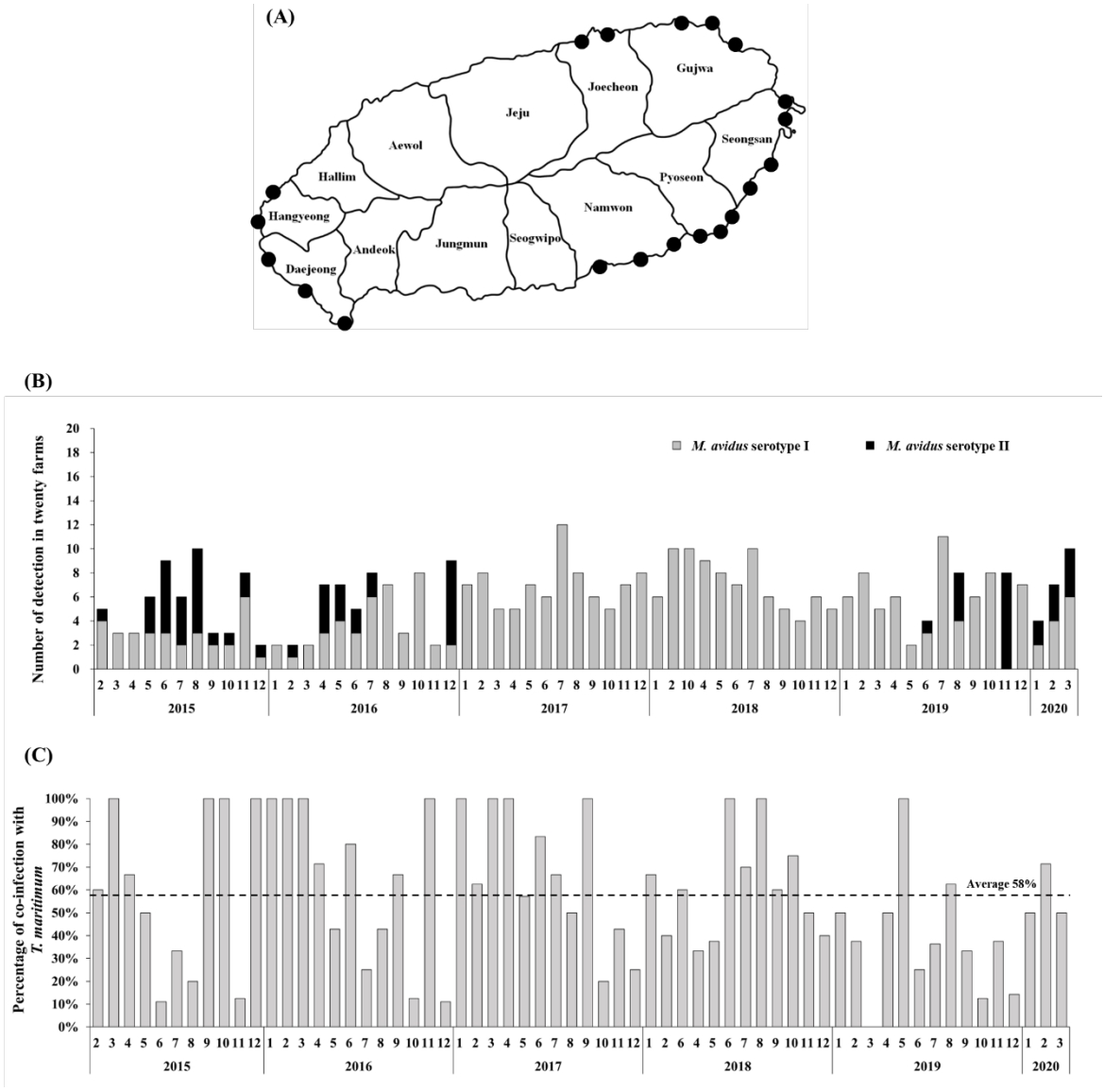

Fig. 1. Occurrence of *Miamiensis avidus* disease outbreak and *Tenacibaculum maritimum* co-infection in olive flounder farms on Jeju island. A: Twenty olive flounder farms on Jeju island where the intensive disease monitoring program was implemented. B: The number of *Miamiensis avidus* serotype I and *M. avidus* serotype II identified from February 2015 to March 2020. C: The percentage of *M. avidus* infection together with *Tenacibaculum maritimum* co-infection from February 2015 to March 2020.

and resuspended in phosphate-buffered saline (PBS). The *T. maritimum* cell number was determined using optical density measurements (OD) at a wavelength of 600 nm and the equation of 1OD = 2×10^8 CFU.

A group of 20 fish were injected intraperitoneally (IP) with 100 μL of varying cell concentrations (1×10^4, 1×10^5, and 1×10^6 cells/fish) for *M. avidus* JJB1403 and JJC1404 strains, respectively. Similarly, the *T. maritimum* KOR-JJ strain was IP injected into fish at concentrations of 1×10^7, 1×10^8 and 1×10^9 CFU/fish. Fish injected with the same volumes of L-15 medium or PBS solution were used as negative controls. Injected fish were placed in 70-L tanks filled with 50-L aerated seawater treated by UV and filtered through 1-micron cartridge filters. Water change was done twice daily, and the mortalities were recorded daily for 21 days. Ethical clearance for animal experiments was obtained from the Institutional Animal Care and Use Committee of Jeju

National University, Republic of Korea (Approval no. 2018-0005).

Trivalent vaccine preparation

A trivalent vaccine was prepared using cells from the *M. avidus* JJB1403, JJB1404, and *T. maritimum* KOR-JJ strains that had been inactivated using formalin. *M. avidus* and *T. maritimum* were cultured using the method described in the virulence evaluation experiment above. The formalin fixation of *M. avidus* was performed using a final concentration of 0.3% formalin with continuous stirring for 1 h at 25℃, and *T. maritimum* was treated with 1% formalin for 72 h at 4℃. The adjuvant was prepared with 25% squalene (Sigma, S3626), 2.5% Tween 80 (Sigma, P1754), and 2.5% Span 85 (Sigma, S7135) in PBS via microfluidization at 12,000 psi as described in a previous study (Calabro et al., 2013). Finally, the *M. avidus* JJB 1403 (2.5×10^6 cells/mL), *M. avidus* JJC 1404 (2.5×10^6 cells/mL), *T. maritimum* KOR-JJ (2×10^9 CFU/mL) strains and 10% adjuvant were homogenized using a high shear mixer at a speed of 8,000 rpm for 30 min.

Fish vaccination and subsequent experimental infection under laboratory conditions

Healthy olive flounders with an average body weight of 15 g were purchased from a local olive flounder farm in Jeju island, Korea. Fish were cultured in three 2,000 L flow-through seawater tanks (n = 150 fish/group with three groups), with a 1,000% daily water exchange and continuous aeration at 18℃. After one week of acclimation, 100 μL of trivalent vaccine, adjuvant, and PBS were IP injected into the fish in each group, respectively. At four weeks post-vaccination, each experimental group (n = 20 fish/tank) was injected with 5×10^5 cells/fish of *M. avidus* (JJB1403, JJC1404) and 8×10^7 CFU/fish of *T. maritimum*. The mortalities were recorded for 21 days, and the efficacy of the vaccine was evaluated according to the formula below.

$$\text{The relative percentage of survival rate (RPS)} = 1 - [(\text{vaccine injected group mortality}/\text{control group mortality})] \times 100.$$

Antibody titration by enzyme-linked immunosorbent assay (ELISA)

Ten fish were sampled from the experimental groups at zero, two, four, six, and eight weeks post-vaccination. Blood was collected from the caudal vein of the olive flounders from each experimental group. Blood samples were kept at room temperature for 1 h, stored overnight at 4℃, and then centrifuged at 3,000×g for 10 min at 4℃ to obtain the serum. Serum samples were stored at –20℃ until use.

96-well flat-bottom culture plates were coated at 100 μL per well with *M. avidus* JJB1403 and JJC1404 (1×10^6 cells/mL) and *T. maritimum* (1×10^9 CFU/mL), along with a coating buffer (30 mM Na_2CO_3 and 70 mM $NaHCO_3$ at pH 9.0). The plates were incubated for 24 h at 4℃. After washing with tris buffered saline (TBS) containing 0.1% Tween 20 (TBS-T; 1%), plates were blocked with 200 μL skim milk solution (TBS-T with 5% skim milk) for 1 h at 4℃. Plates were then rinsed with TBS-T solution, and the wells were treated with serum (90 μL skim milk solution with 10 μL serum). After a 2 h incubation period at 4℃, the plates were washed three times with TBS-T solution and incubated with 100 μL of mouse anti-flounder IgM (1: 1,500) for 2 h at 4℃. Plates were rinsed again with TBS-T solution, and 100 μL horseradish peroxidase (1: 1,500) conjugated goat anti-mouse secondary antibody (YOUNG IN Frontier, Seoul, Korea) was added. After 2 h of incubation at 4℃, the plates were washed three times with TBS-T solution. Finally, 3,3′,5,5′-Tetramethylbenzidine liquid solution (Sigma-Aldrich, St. Louis, MO, USA) was added. The reaction was stopped with 2 N H_2SO_4, and the absorbance was measured at 450 nm using a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA).

Field trial of vaccine efficacy evaluation

Four olive flounder farms labeled A, B, C, and D, were selected to evaluate the efficacy of the vaccine under field conditions. The number of fish that were vaccinated is described in Table 1. Fish were subjected to fasting two days before vaccination. Following the fasting period, 0.1 mL of trivalent vaccine was IP injected into fish with an average size of 42.5 g at farm A, 65.2 g at farm B, 55.3 g at farm C, and 52.5 g at farm D, respectively. Mortality in each group was recorded daily. Dead fish were collected, and scrapes of the gills and skin were examined via microscopy to determine the level of infection by *M. avidus* and *T. maritimum*. Furthermore, bacterial strains were isolated from liver, kidney, and spleen smears and incubated on marine agar plates at 25℃ for 48 hours. The cultured bacteria were identified using *T. maritimum* specific PCR primers. Ethical clearance for animal experiments was obtained from the Institutional Animal Care and Use Committee of Jeju National University, Republic of Korea (Approval no. 2018-0006).

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Table 1. The number of experimental fish, average weight, number of dead fish, cumulative mortality rates and relative percent survival values in the four farms of field trial

Fish farm	Vaccine group					Control group					Relative percent survival (%)
	No. of total fish	No. of dead fish	Cumulative mortality (%)	Initial average weight (g)	Final average weight (g)	No. of total fish	No. of dead fish	Cumulative mortality (%)	Initial average weight (g)	Final average weight (g)	
A	11,700	499 (6[1)])	4.26 (0.05[1)])	42.5 ± 4.5	217.5 ±15.0	12,500	2,256 (2,133[1)])	18.04 (17.06[1)])	45.2 ± 3.8	215.2 ± 13.5	76.38 (99.70[1)])
B	8,500	2,468 (2,468[1)])	29.04 (29.04[1)])	65.2 ± 4.5	241.0 ± 9.9	10,000	6,920 (6,920[1)])	69.20 (69.20[1)])	71.4 ± 11.4	232.8 ± 7.0	58.04 (58.04[1)])
C	11,800	359 (317[1)])	3.04 (2.68[1)])	55.3 ± 3.5	171.1 ± 12.5	12,000	1,347 (1,301[1)])	11.22 (10.84[1)])	56.2 ± 4.8	160.4 ± 11.5	72.91 (75.18[1)])
D	4,000	1,308 (1,262[1)])	32.70 (31.55[1)])	52.5 ± 3.1	152.2 ± 8.5	6,500	4,488 (4,361[1)])	69.04 (67.09[1)])	51.4 ± 4.5	130.2 ± 7.9	52.64 (52.98[1)])

[1)] Fish mortality due to *Miamiensis avidus* and/or *Tenacibaculum maritimum* infection.

Results

Miamiensis avidus and *Tenacibaculum maritimum* infections in olive flounder farms

During the period from 2015 to 2020, a total of 390 *M. avidus* infections were identified. Serotyping analysis revealed the presence of two serotypes of *M. avidus* (serotype I and serotype II) on Jeju island, accounting for 83% and 17% of the total identified *M. avidus* infections, respectively (Fig. 1B). Notably, during the investigation, it was found that more than 50% of the *M. avidus* infections were accompanied by *T. maritimum* co-infections.

Serotyping of *Miamiensis avidus*

Sequence analysis revealed that *M. avidus* serotype I and II possess distinct sequence variations in the *cox*1 gene at 16 loci (Fig. 2). For phylogenetic analysis, the nucleotide sequences of the *cox*1 gene from *M. avidus* serotype I (*M. avidus* JJB1403) and *M. avidus* serotype II (*M. avidus* JJC1404) were compared with the *cox*1 sequences reported in previous studies (Jung et al., 2011). The results indicated that *M. avidus* JJB1403 was clustered together with 11 other strains (Nakajima, GJ01, WDS-0709, WDB-0708, SJF-06A, YS2, WD4, JJ4, JJ3, WS1, and SJF-03A) as serotype I, whereas *M. avidus* JJC1404 was clustered with seven other strains (SJF-03B, YK1, YK2, JF05To, RF05To, SK05Kyo, and Iyo1), confirming its classification as serotype II (Fig. 3).

Virulence of *Miamiensis avidus* and *Tenacibaculum maritimum* strains

In the virulence test, the cumulative mortality of *M. avidus* JJB1403 and *M. avidus* JJC1404 exceeded 70% across all three dosage levels (Fig. 4A and 4B). For *T. maritimum* KOR-JJ, fish injected with a concentration of 1×10^9 cells/fish exhibited 100% mortality within five days post-injection. At concentrations of 1×10^8 cells/fish and 1×10^7 cells/fish, the mortalities caused by *T. maritimum* KOR-JJ reached 55% and 30%, respectively (Fig. 4C). These results collectively demonstrate that the collected pathogen strains are virulent and pathogenic to olive flounder, making them suitable for vaccine development.

Vaccine efficacy in the laboratory

The final survival rates of the control group infected with *M. avidus* JJB1403, *M. avidus* JJC1404, and *T. maritimum* KOR-JJ were 20%, 15%, and 20%, respectively. In contrast, the vaccinated group showed significantly higher survivals, with rates of 80%, 75%, and 95% for *M. avidus* JJB1403, *M. avidus* JJC1404, and *T. maritimum* KOR-JJ, respectively. The group injected only with adjuvant exhibited a 20% survival rate after experimental infection with the three different strains (Fig. 5). The calculated RPS for the vaccinated groups were 75%, 80%, and 93% for *M. avidus* JJB1403, *M. avidus* JJC1404, and *T. maritimum* KOR-JJ, respectively (Fig. 5).

Serum antibody titer after vaccination

The vaccinated groups consistently exhibited higher levels of antibody compared to the control group against all three pathogens (Fig. 6). In the vaccinated group, antibody titers against *M. avidus* JJB1403, *M. avidus* JJC1404, and *T. maritimum* KOR-JJ steadily increased until four weeks post-vaccination (wpv) and subsequently began to decrease after six wpv. On the other hand, the antibody titers of the control group were maintained at a low level, showing no significant difference throughout the experiment.

Vaccine efficacy in a field test

The efficacy of the trivalent vaccine for olive flounder was evaluated at four farms (Table 1). On farm A, the vaccinated group had 499 dead fish compared to 2,256 in the control

Fig. 2. Sequence alignment of *cox*1 genes in two serotypes of *Miamiensis avidus*.

group. The control group experienced a substantial increase in mortality starting at 4 wpv, while the vaccinated group showed a delayed onset of significant mortality and significantly lower rates of weekly and cumulative mortality (Fig. 7A). On farm B, the vaccinated group had 2,468 dead fish compared to 6,920 dead fish in the control group. Both groups experienced mortality due to *M. avidus* and/or *T. maritimum* infections, but the severity of the disease outbreak was significantly lower in the vaccinated group (Fig. 7B). On farm C, the vaccinated group had 317 dead fish, and the control group had 1,301 dead fish diagnosed with *M. avidus* and/or *T. maritimum* infections. The control group showed a sudden increase in mortality starting at 9 weeks post-vaccination, while the vaccinated group maintained consistently low weekly mortality throughout the experiment (Fig. 7C). On farm D, the vaccinated group had 1,262 dead fish, and the control group had 4,361 dead fish. Both groups had considerable mortality between 4 and 6 weeks post-vaccination, but the vaccinated group had significantly lower mortality (Fig. 7D). Overall, the developed vaccine demonstrated a significant reduction in mortality caused by *M. avidus* and/or *T. maritimum* infections compared to the control group, with RPS values ranging from 52.98% to 99.70% across the four farms.

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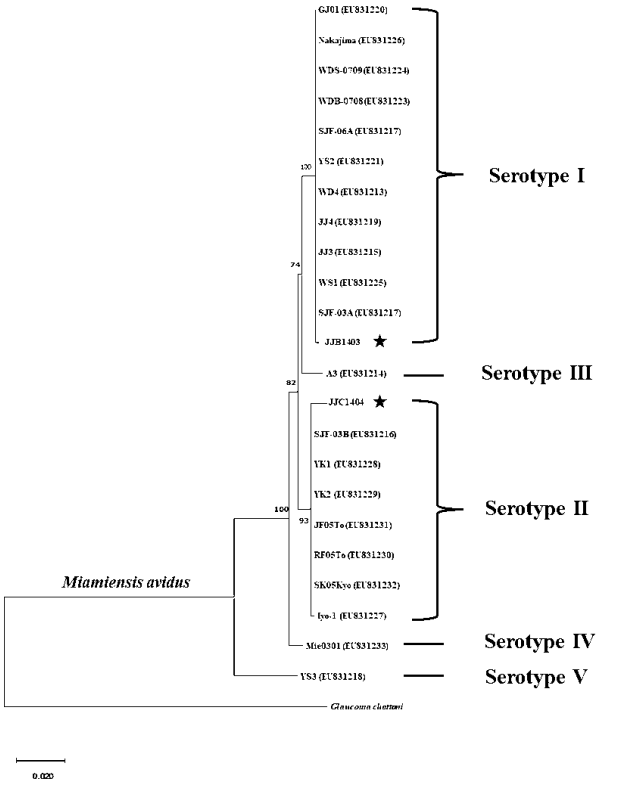

Fig. 3. Phylogenetic analysis of *Miamiensis avidus* JJB1403, JJC1404 with other *M. avidus* serotypes. Branch numbers indicated the bootstrap values based on 5,000 replications obtained using neighboer-joining method.

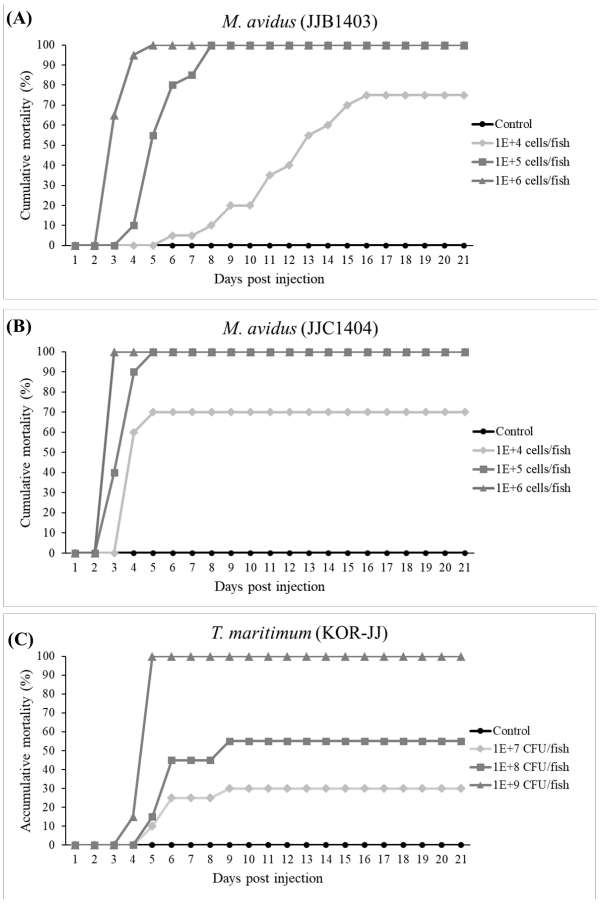

Fig. 4. The cumulative mortality of olive flounder groups IP injected with (A) *Miamiensis avidus* JJB1403 range of 1E+4 cells/fish to 1E+6 cells/fish, (B) *M. avidus* JJC1404 range of 1E+4 cells/fish to 1E+6 cells/fish, and (C) *Tenacibaculum maritimum* range of 1E+7 CFU/fish to 1E+9 CFU/fish. IP, intraperitoneally.

Discussion

The monitoring results revealed the presence of two serotypes of *M. avidus* on Jeju island as determined by *cox*1 gene sequence analysis. The *cox*1 gene has been widely recognized as a useful marker for species identification of animals due to its easy isolation, significant variation among species, minimal differences within species, and absence of introns (Rodrigues et al., 2017). Moreover, *cox*1 sequence analysis has been applied to the classification of ciliates to expand the utility of this technique within the phylum (Whang et al., 2013). Through comparison of the *cox*1 gene sequences, 21 *M. avidus* strains isolated from olive flounder (*P. olivaceus*), ridged-eye flounder (*Pleuronichthys cornutus*), and spotted knifejaw (*Oplegnathus fasciatus*) in Korea and Japan were classified into five serotypes (*cox*1 serotype I to V; Jung et al., 2011). Similarly, another study on *M. avidus* isolation during outbreaks of scuticociliatosis in Japan identified six isolates from different fish species and classified them into three serotypes through serological analysis (Song et al., 2009b). During the period between 2015 and 2020, we isolated a total of 390 *M. avidus* isolates from the disease olive flounder. Among them, serotype I (JJB1403) and serotype II (JJC1404) accounted for 83% and 17% of the total *M. avidus* isolates, respectively, indicating the new serotype distribution of *M. avidus* in Korean olive flounder aquaculture. There is a lack of research on the seasonal variation of *M. avidus* serotypes. Therefore, a specific investigation is warranted to understand these changes more comprehensively.

T. maritimum is the major causative agent of tenacibaculosis, the most frequently occurring secondary infection following the onset of scuticociliatosis in olive flounder (Jin et al., 2007). Our

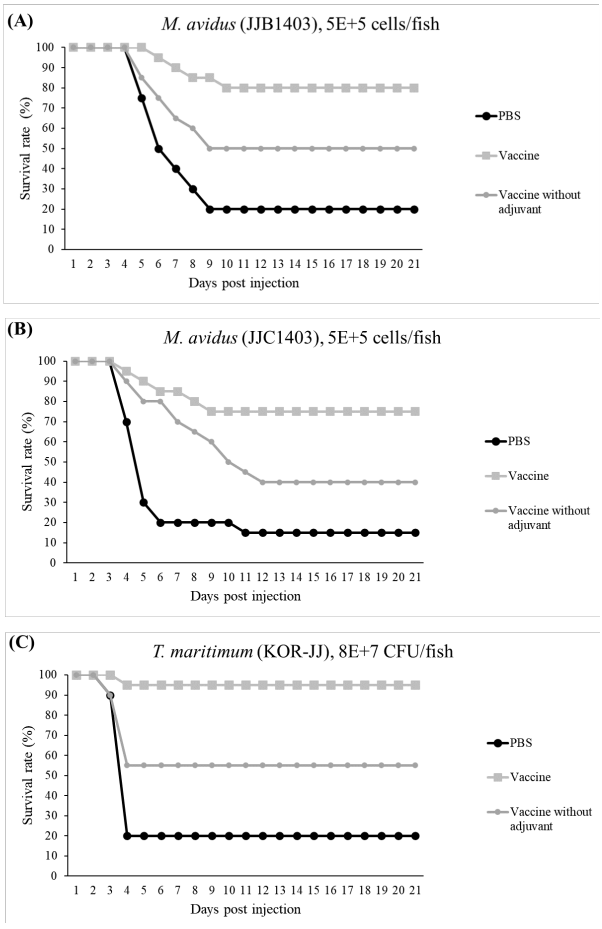

Fig. 5. The survival patterns of fish injected with complete vaccine (inactivated two *Miamiensis avidus* and one *Tenacibaculum maritimum* antigens containing squalene adjuvant), adjuvant only without antigen and PBS following experimental infection with (A) *M. avidus* JJB1403 5E+5 cells/fish, (B) *M. avidus* JJC1404 5E+5 cells/fish and, (C) *T. maritimum* 8E+7 CFU/fish. Experiment was conducted at four weeks post-vaccination. PBS, phosphate-buffered saline.

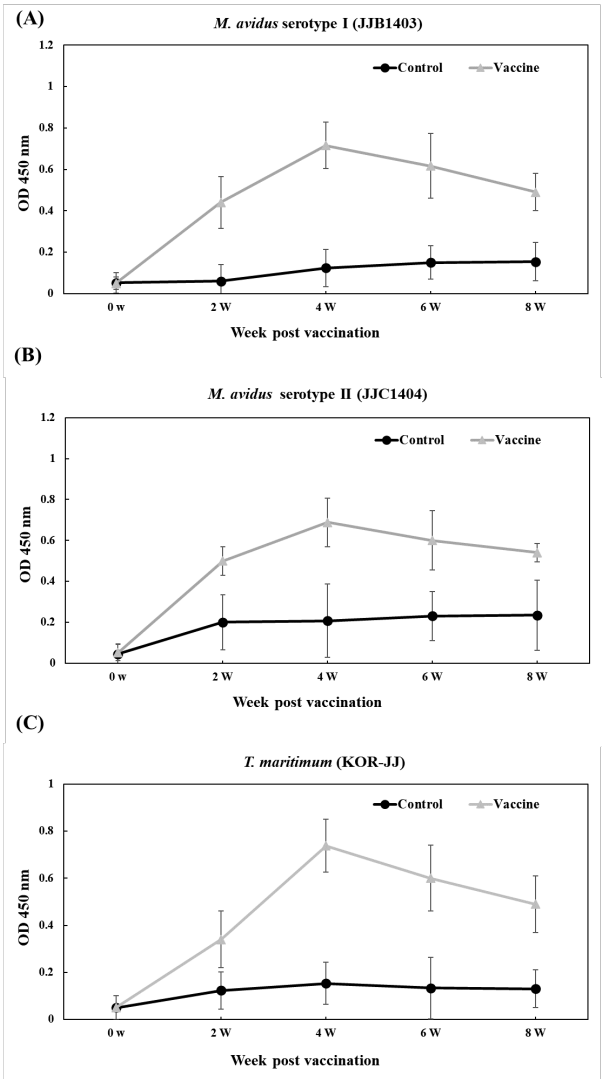

Fig. 6. The serum antibody levels in olive flounders after vaccination against (A) *Miamiensis avidus* JJB1403, (B) *Miamiensis avidus* JJC1404 and (C) *Tenacibaculum maritimum*. OD, optical density.

study observed an average co-infection ratio of 58% for *M. avidus* and *T. maritimum* in scuticociliatosis-affected olive flounder in the fish farms on Jeju island. Olive flounder infected with *T. maritimum* alone can be diagnosed by the presence of annular (ring-shaped) epidermal ulcerative lesions with dark-colored outer edges on the skin, along with frayed fins and a rotten tail (Mabrok et al., 2023). However, co-infection with *M. avidus* and *T. maritimum* leads to clinical signs closely resembling scuticociliatosis, characterized by multiple epidermal and dermal necrotic lesions on the skin and fins, which can extend deeply into the underlying muscles and cause severe inflammation at advanced infection stages (Moustafa et al., 2010). Co-infections between bacterial pathogens, viruses, and parasites are frequent in olive flounder aquaculture. However, due to the difficulty of distinguishing clinical signs, farmers often under-report or neglect the co-infections of *T. maritimum* during scuticociliatosis outbreaks, leading to a lack of associated information. Previous studies in other fish species indicate that the co-infection of bacterial pathogens may exert an immunosuppressive effect, which can potentiate the parasitic infection and significantly increase mortality (Wise et al., 2021). In our study, we developed

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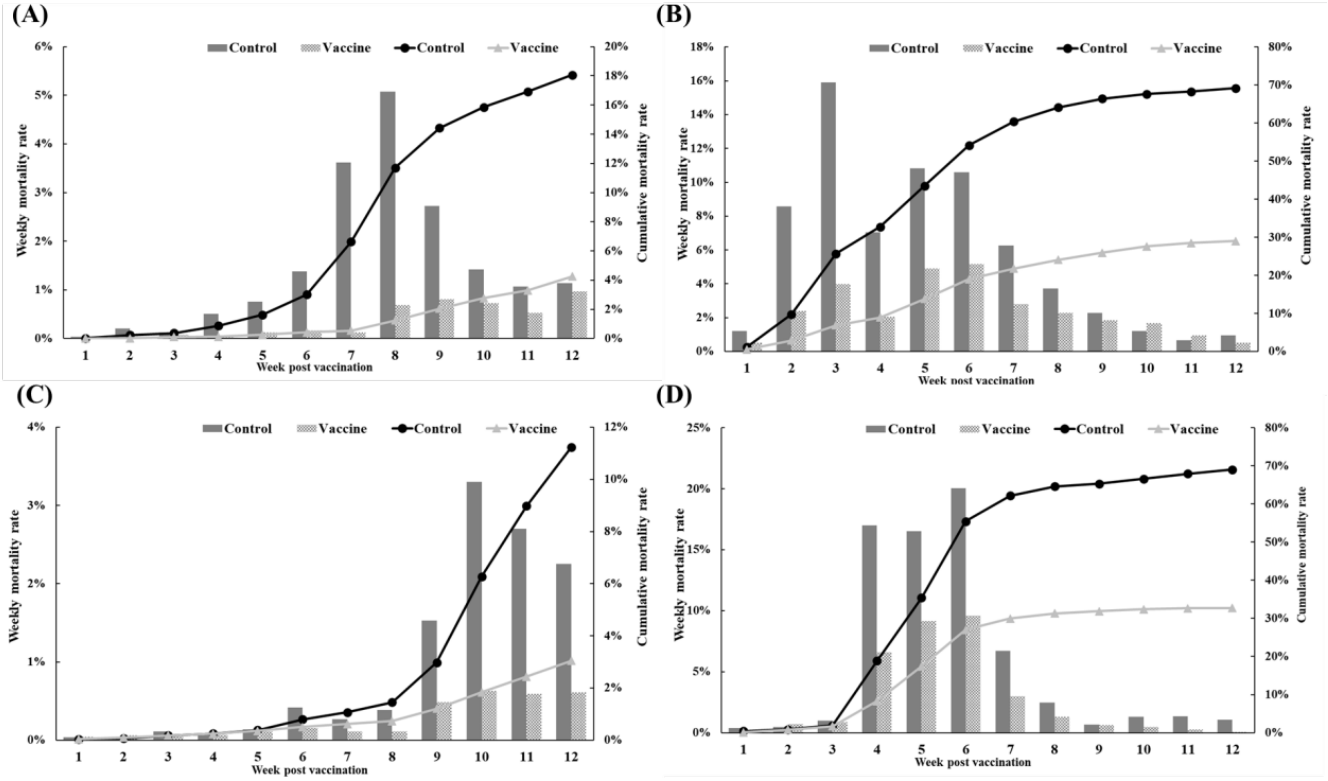

Fig. 7. The weekly and cumulative mortality rate of vaccinated and control group in (A) farm A, (B) farm B, (C) farm C, and (D) farm D.

a vaccine comprising two serotypes of *M. avidus* and *T. maritimum*, shedding light on its effectiveness in preventing sucuticociliatosis, especially in reducing co-infected bacterial disease burden and improving fish survival rates.

To enhance the vaccine efficacy, we employed a squalene-based emulsion (SE) adjuvant in the trivalent vaccine developed in this study. SE adjuvants, such as MF59, AS03, and AF03, are oil-in-water emulsions containing completely metabolizable oil droplets and surfactants. These adjuvants have been extensively studied and proven safe and effective, having received approval for human vaccine development in many countries (Wilson-Welder et al., 2009). The mechanism of SE adjuvant action is associated with promoting antigen uptake and recruiting immune cells, which leads to a stronger and more sustained activation of both the innate and adaptive immune systems (Nguyen-Contant et al., 2021). By incorporating the SE adjuvant, the trivalent vaccine developed in this study exhibited high immunogenicity, providing robust and long-lasting protection against scuticociliates and *T. maritimum*. Notably, several studies have also assessed vaccine efficacy and long-term effects associated with using oil-based adjuvants in fish. For instance, in turbot, vaccination with an antigen and mineral oil-based adjuvant resulted in significant protection against *P. dicentrarchi* and elicited high serum antibody titers (Sanmartín et al., 2008). Another study by Rahman et al. (2000) demonstrated that using a squalene adjuvant provided better protection than administrating the formalin-inactivated flavobacterium alone. Moreover, in olive flounder, a recent study evaluated the efficacy of the viral hemorrhagic septicemia virus (VHSV) vaccine and confirmed that adding a squalene adjuvant could increase both the efficacy and persistence of the vaccine (Vinay et al., 2013).

Through natural infections or immunization by vaccines, fish can generate specific antibodies against scuticociliates, which are directly associated with the defense against scuticociliatosis in various fish species (Piazzon et al., 2014). These antibodies agglutinate the ciliate cells to limit migration and exhibit direct cytolytic activity by activating the classical complement pathway (Palenzuela et al., 2009; Piazzon et al., 2014). Our study observed high antibody production in vaccinated fish throughout an eight-week experimental period, contributing to a high RPS during

the scuticociliate challenge. Previous studies evaluated antibody levels after vaccination towards different scuticociliate strains in different fish species. For instance, in turbot, fish immunized with formalin-killed *P. dicentrarchi* vaccines produced significantly higher levels of antibody in sera compared to the non-vaccinated group, which maintained the ciliate agglutinating activity for up to 6 months post-vaccination (Iglesias et al., 2003; Sanmartín et al., 2008). Moreover, several studies have demonstrated good correlations between the levels of specific antibody production and the survival rate under scuticciliatosis in immunized fish through experimental challenge tests in several studies (Lamas et al., 2008). The use of adjuvants, such as mineral oil and non-mineral Montanide ISA 763A, has been reported to be crucial for increasing specific antibody production in scuticociliate vaccines (Lamas et al., 2008; Mabrok et al., 2023). Additionally, in a study carried out in kelp grouper, encapsulation of *U. marinum* antigen with poly D, L-lactide-co-glycolic acid (PLGA) showed a promoting effect on the specific antibody production at 2 weeks and 4 weeks post-vaccination (Harikrishnan et al., 2012).

In previous studies, several scuticociliate vaccine trials have been performed solely under laboratory conditions, showing protective potency against various ciliate stains, including *P. dicentrarchi, U. marinum*, and *M. avidus*. In olive flounder, three different types of vaccines (injectable, immersion, and oral) have been developed against *M. avidus*, resulting in RPS values of over 60% (Jung & Jung, 2021; Kole et al., 2022; Piazzon et al., 2014; Wise et al., 2021). For turbot, two vaccines were developed using formalin-killed *P. dicentrarch* (Mabrok et al., 2023; Moustafa et al., 2010), and in one trial, the vaccine comprising antigen alone exhibited approximately 30% RPS. In another trial, when the vaccine containing Montanide ISA 763A adjuvant was combined with booster vaccination, the RPS increased to about 50%. In kelp grouper, immunization with the PLGA-encapsulated vaccine against *U. marinum* resulted in an impressive 80% RPS (Harikrishnan et al., 2012). Despite the high RPS values observed in the vaccines developed in the studies above, it remains uncertain whether these vaccines can effectively protect fish from infections caused by scuticociliates with multiple serotypes or bacterial co-infections frequently occurring in the field. To address this issue, we designed this trivalent vaccine targeting two serotypes of *M. avidus* and one co-infecting bacteria. We conducted a field test to evaluate its efficacy at four olive flounder farms on Jeju island. On farms B and D, where severe *M. avidus* and *T. maritimum* infections were experienced, a notable reduction in cumulative mortality rate was observed in the vaccinated groups. While the control groups exhibited cumulative mortality rates exceeding 65%, the vaccinated groups showed only approximately 30% mortality rate in both farms, demonstrating over 50% RPS values. On the other hand, farms A and C, which had relatively lower cumulative mortality rates below 20%, displayed even higher levels of protection in the vaccinated groups. The RPS values exceeded 70% in both farms, indicating a substantial reduction in mortality through vaccination during a moderate scuticociliatosis outbreak. To the best of our knowledge, this is the first time of field trial for the vaccine against scuticociliatosis. Our results underscore the potential of the developed vaccine as a valuable tool in managing and controlling *M. avidus* and *T. maritimum* infections in olive flounder farms. Furthermore, it is essential to assess the long-term effectiveness of the developed vaccine and investigate potential changes in pathogen strains and co-infection patterns over extended periods via continued monitoring and further research.

Conclusion

In conclusion, our study focused on the serotype distribution of *M. avidus* in the olive flounder farms on Jeju island, as determined through intensive disease monitoring between 2015 and 2020. The results revealed that *M. avidus* serotype I was predominant, accounting for 83% of cases, while *M. avidus* serotype II represented 17% of the observed serotypes. Additionally, we observed a significant co-infection rate of 58% with *T. maritimum* in fish affected by scuticociliatosis. Based on the monitoring data, we developed a trivalent vaccine against two serotypes of *M. avidus* and *T. maritimum*, which demonstrated remarkable efficacy with high RPS values under both laboratory conditions (ranging from 70% to 93%) and field trials (ranging from 52.64% to 76.38%). This vaccine could be a promising strategy for effectively managing and controlling *M. avidus* and *T. maritimum* infections, contributing to the overall health and sustainability of olive flounder farming.

Competing interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

Ethical clearance for animal experiments was obtained from the Institutional Animal Care and Use Committee of Jeju National University, Republic of Korea (Approval no. 2018-0005 & 2018-0006).

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