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Production of an anti-idiotypic antibody single chain variable fragment vaccine against *Edwardsiella tarda*

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Edwardsiella tarda **is the pathogen responsible for edwardsiellosis, a serious infectious disease of freshwater and marine fish species, and currently recognized to be the species pathogenic for human. An anti-idiotypic monoclonal antibody (mAb), 1E11, has been developed. It mimics the protective epitope of *E. tarda* and can prevent fish from infection of *E. tarda*. In this study, the correct variable heavy (V_H) and variable light (V_L) genes were obtained from 1E11 by using bioinformatics methods, and a 15 amino acid $(Gly_4Ser)_3$ linker was used to hold the two V domains together for the construction of V_L–linker–V_H form of single chain variable fragment (scFv) gene. Then, the scFv was subcloned into the vector pET-28a, expressed in the *Escherichia coli* BL21 cells, and identified by SDS–PAGE and western blotting. Red drum (*Sciaenops ocellatus* L.) weighing about 50 g was subjected to challenge with different *E. tarda* strains after 4 weeks followed by vaccination, the mortality rates and relative percentage survival were recorded and calculated, and the survival rate of fish in the scFv subgroups was obviously higher than that of control subgroups ($P < 0.01$). Enzyme-linked immunosorbent assay results show that after 4 weeks of post-vaccination, the level of specific antibody in fish sera of scFv groups was significantly higher than control groups. This study indicates that the recombinant antibody scFv was successfully developed, and it may serve as an effective vaccine candidate against *E. tarda*.**

Keywords bioinformatics; anti-idiotypic antibody; single chain variable fragment; *Edwardsiella tarda*

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Introduction

Edwardsiella tarda, first isolated from pond-cultured eel by Hoshina [1], is a Gram-negative, motile and flagellated rod bacterium. It is the etiological agent of edwardsiellosis, which leads to widespread mortalities among commercially important freshwater and marine fish [2–9]. *Edwardsiella tarda* is widely distributed and has been isolated from reptiles, birds, mammals, humans, and environmental water [10–14]. The antigen structure of this bacterium is very complex, its serotype comprising 61 O groups and 45 H antigens [15,16]. The information on genomic and phenotypic characteristics of *E. tarda* is scanty. Genes specific to pathogenic *E. tarda* have been identified [17], and researches have been carried out to understand the pathogenesis of *E. tarda* [18–22]. However, up to now, no effective vaccines against *E. tarda* have been obtained. Several attempts have been made to induce protection against *E. tarda*, such as the vaccines based on *E. tarda* outer membrane proteins [23–26] or glyceraldehyde-3-phosphate dehydrogenase [27,28]. An alternative approach for the treatment and prevention of this infection was *E. tarda* ghosts [29]. And a TTSS mutant strain of *E. tarda* by introducing a non-reverting mutation into the regulator gene *esrB* was also produced [30]. Most of these vaccines are immunogenic, but no vaccine is commercially available at present, so other approaches are urgently needed for treatment and prevention of this infection.

According to the network theory of immunoregulation [31–34], the anti-idiotypic antibody, which is termed Ab2β, bears the internal image of the antigen and thus has the potential to be a vaccine candidate. The anti-idiotypic antibody may serve as an ideal vaccine against *E. tarda* in fish since the vaccines can retain the immunogenicity of antigen and were much safer than the attenuated vaccines. Previously, a monoclonal anti-idiotypic antibody 1E11 against *E. tarda* from mouse hybridoma cells was generated, and it is Ab2β in identification [35]. In a challenge test, it was found that 1E11 could protect flounder from attack of *E. tarda*, which suggests that it could be used as a good vaccine candidate against *E. tarda* in flounder. However, the production of vaccine from hybridoma cells is expensive and time-consuming.

The main advantage of single chain variable fragment (scFv) over intact monoclonal antibody (mAb) is the small size (Mr 30,000, only one-sixth of intact mAb), allowing it to penetrate rapidly and evenly. In addition, the lack of Fc domain makes scFv less immunogenic responsive. These characteristics make scFv potentially useful in diagnosis and therapy [36,37]. The common flexible linker $(Gly_4Ser)_3$, which connects V_H and V_L, may substitute for constant region contacts in the Fab and thereby help recover the native binding properties in the scFv [38]. Therefore, the genetically engineered scFv vaccine of anti-idiotypic antibody was adopted as an alternative of 1E11 against *E. tarda*. However, the mouse genes of immunoglobins are highly diverse in their amino acid composition and nucleotide sequence, the amplification of variable coding regions of the 1E11 gene would be of a great challenge.

This study aimed to develop a recombinant antibody vaccine against *E. tarda* by using genetic engineering approach. The RT–PCR and bioinformatics methods were used to achieve the exactly variable fragments and then constructed the recombinant antibody vaccine against *E. tarda*.

Materials and Methods

Bacterial strains and plasmids

Edwardsiella tarda standard strain ATCC19106 was purchased from American Type Culture Collection (ATCC) (Manassas, USA); *E. tarda* CTE1 was a gift from Department of Marine Biotechnology, Ocean University of China (Qingdao, China). Plasmid pET-28a and *Escherichia coli* BL21(DE3) strain were purchased from Infinitychem Company (Shanghai, China). All strains were cultured in the Luria–Bertani (LB) broth medium at 28°C for *E. tarda* or at 37°C for *E. coli*, and kanamycin was added at 50 μg/ml when required.

Cloning of V_H and V_L

Total RNA was isolated from 2.8×10^7 hybridoma 1E11 cells and used as templates for cDNA synthesis using superscript reverse transcriptase kit (Invitrogen). According to the GenBank data, the highly degenerate primers of the variable genes were designed for PCR from the conserved regions of the signal peptide sequence to the constant region: $V_H$back, 5′-ATGAAATGCAGCTGGGGCAT(C/G)-TTCTTC-3′ and $V_H$for, 5′-CAAGGGATAGACAGATGG-GGC-3′; $V_L$back, 5′-ATGAGTGTGCTCACTCAGGTCCT-GG(C/G)GTTG-3′ and $V_L$for, 5′-ACTGGATGGTGGGAA-GATGG-3′. The PCR products were analyzed on 1% agarose gel and cloned into pMD18-T vectors for sequencing.

Table 1 Primers of V_L gene

Group	Primers
I	K1: 5′-ATGAGTGTGCTCACTCAGGTCCTGG(C/G)-GTTG-3′
	K2: 5′-ACTGGATGGTGGGAAGATGG-3′
II	K3: 5′-CCAGATGTGAGCTCGTGATGACCCAGAC-TCCA-3′
	K4: 5′-GCGCCGTCTAGAATTAACACTCATTCCTG-TTGAA-3′
III	K5: 5′-GACATTGAGCTCACCCAGTCTCCA-3′
	K6: 5′-CCGT TTGATTTCCAGCTTGGTGCC-3′
	K7: 5′-CCGTTTTATTTCCAGCTTGGTCCC-3′
	K8: 5′-CCGTTTTATTTCCAACTTTGTCCC-3′
	K9: 5′-CCGTTTCAGCTCC AGCTTGGTCCC-3′

According to the sequencing results, we compared the amplified V_L gene sequence with the highest homology in NCBI and designed three groups of primers for amplifying the correct gene (**Table 1**); these primers were designed at the different regions of the V_L gene for PCR. On the basis of the sequencing results, all the amplified sequences were analyzed using the multiple sequence alignment software (ClustalW version 1.7), and the correct and complete variable fragments of V_L gene were obtained (**Fig. 1**). Similarly, by using the above methods, the correct and complete variable fragments of V_H gene were also obtained (data not shown).

Construction and expression of scFv

The scFv with V_L–linker–V_H orientation was constructed. PCR primers are shown in **Table 2**. The scFv gene was constructed with the following protocol. Briefly, the V_H and V_L genes were amplified using the PCR primers (**Table 2**), then the scFv was assembled: 1 μl of 0.2 μM V_H, 1 μl of 0.2 μM V_L, 1 μl of Platinum Pfx DNA polymerase (Invitrogen), 1 μl of 50 mM $MgSO_4$, 1.5 μl of 10 mM dNTP mixture, 5 μl of 10× Pfx amplification buffer, and distilled water were added to 50 μl. PCR was performed as follows: denaturation at 94°C for 2 min, 30 cycles of amplification at 94°C for 15 s, 58°C for 1 min, and 68°C for 1 min. The PCR products were cloned into the pMD18-T vector and transformed into *E. coli* DH5α for propagation. Positive clones were identified by colony PCR and DNA sequencing. The positive clones were digested by restriction enzymes *Eco*RI/*Hin*dIII and subcloned into the vector pET-28a, then expressed in competent *E. coli* BL21(DE3).

The overnight cultures of the LB medium with 50 μg/ml kanamycin, harboring recombinant plasmids pET-28a-scFv, were transferred into fresh LB and incubated at 37°C until the optical density at 600 nm reached 0.6 (OD_{600}).

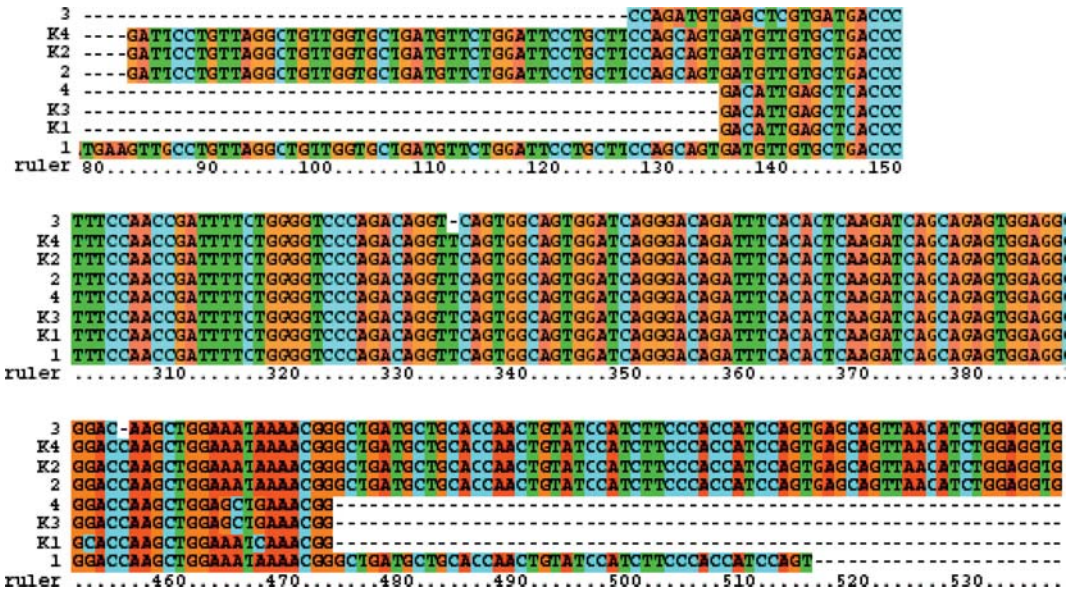

Figure 1 Results of the light chain gene sequence alignment

Table 2 Primers of scFv

Primer	Primer sequence
$V(H)_{scFv}$back	5′-ATCCGAATTCGTCTTGACCCAAACT-3′
$V(H)_{Link}$for	5′-*ACCGCCGGATCCACCGCCACCCGAGCCACCGCCACC*TTTTATTTCCAGCTTGGTCCC-3′
$V(L)_{Link}$back	5′-*GGCTCGGGTGGCGGTGGATCCGGCGGTGGCGGTTCG*GAGGTTCAGCTGCAG-3′
$V(L)_{scFv}$for	5′-GCAGGCGGCCGCAAGCTTTTATGAGGAGAGAGTGGC-3′

Underline indicates the *Eco*RI and *Hin*dIII restriction sites; italic indicates the linker sequence.

The gene expression was induced with isopropyl-β-D-thiogalactopyranoside at the final concentration of 0.1 mM for 4 h at 15°C. After harvested by centrifugation, the whole-cell pellets were washed in the wash buffer [50 mM Tris–HCl, 10 mM EDTA, 0.5% (*v/v*) Triton X-100, and 100 mM NaCl, pH 8.0], and suspended in 20 ml washing buffer including 2 mg lysozyme, then incubated at 30°C for 20 min and disrupted by sonication in ice bath (400 W, 10 min × 6). After centrifuged at 12,000 *g* for 15 min at 4°C, the denatured proteins were suspended in the washing buffer and centrifuged for refolding process. The denatured proteins were dissolved in lysis buffer [50 mM Tris–HCl, 100 mM NaCl, 8 M urea, and 1% (*v/v*) glycerin, pH 8.0] at room temperature for 6 h, then centrifuged, collected the supernatant, and removed in dialysis tubing against refolding buffer [50 mM Tris–HCl, 100 mM NaCl, 7 M urea, and 1% (*v/v*) glycerin, pH 8.0] for 4 h at 4°C. Then the refolding buffer was replaced with urea concentration changing to 6, 5, 4, 3, 2, and 1 M, and other conditions in the refolding buffer were constancy. The dialysis in each refolding buffer with various concentration of urea was for 4 h at 4°C. Then, the refolding mixture was centrifuged at 12,000 *g* for 30 min at 4°C, and the proteins were further dialyzed with the phosphate-buffered saline (PBS, pH 8.0) at 4°C for 20 h. Finally, the recombinant proteins were purified using Ni^{2+}–nitrilotriacetic acid (Ni–NTA) column (Qiagen, Valencia, USA). The empty plasmid pET-28a was treated with the same procedure as a control.

SDS–PAGE and western blotting analysis

After gene expression, the collected *E. coli* cells were suspended in PBS (pH 8.0) and disrupted by sonication and centrifugation. The supernatant was added into the same volume of 2× SDS loading buffer, while the pellet fraction of *E. coli* was suspended in 1× SDS loading buffer, boiled for 10 min, centrifuged at 10,000 *g* for 10 min at 4°C, and collected the supernatant. The proteins obtained were subjected to SDS–PAGE with 12% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue R250 to visualize the protein bands.

The immunoactivity of the obtained protein was examined by western blotting. The purified proteins were electrophoretically transferred onto nitrocellulose membranes in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol), and blocked in PBST (25 mM Tris, 140 mM NaCl, 3 mM KCl, and 0.05% Tween 20, pH 8.0), with 2% (*w/v*) skimmed milk at 37°C for 1 h. Then, the

membranes were incubated with mouse mAb 3F7 against *E. tarda* (1:200 dilution) [35] for 8 h at 4°C. The membranes were incubated with mouse mAb 1D1 against *Vibrio anguillarum* [39] as a negative control. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Dingguo, Beijing, China) (1:1000 dilution) for 1 h at 37°C. The antibody-bound proteins were then visualized by using DAB–H_2O_2. The molecular weight marker (31.0–97.4 kDa) was from BIOGENRO (Beijing, China).

Vaccination of fish

Red drum (*Sciaenops ocellatus* L.), weighing about 50 g, was cultivated in Guangdong Daya Bay Fisheries Development Center (Huizhou, China). The fish were maintained in aerated tanks filled with filtered re-circulating fresh seawater at 22–25°C and were fed on the pelleted feeds twice daily. For vaccination, the red drum was divided randomly into three groups (150 fish per group): the scFv group, each injected intraperitoneally (i.p.) with purified scFv protein; control group 1, each injected i.p. with the same quantity of total proteins of empty plasmid pET-28a, both of them were emulsified with an equal volume of Freund's incomplete adjuvant (Sigma, St Louis, USA), separately; control group 2, each injected i.p. with 100 μl of 0.01 M PBS (pH 7.4). After vaccination, fish were maintained at the same condition as described above.

Enzyme-linked immunosorbent assay

Four weeks later, serum of fish (from 10 fish) in each group was collected and the specific antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, the sera were added in a 96-well ELISA plate (50 μl per well) in the dilution of 10^{-4} and immobilized by incubation at 4°C for 12 h. The plate was washed with PBS. The anti-idiotype mAb 1E11 was added onto the plates and incubation 1 h at 37°C and then washed three times again, followed by added the HRP-conjugated goat anti-mouse IgG at 1:1000 dilution. The color was developed with *O*-pheylenediamine for 30 min and the reaction was stopped with 2.0 M H_2SO_4. The absorbance at 492 nm was read with a microplate reader (Thermo Labsystems, Shanghai, China).

Challenge test with different strains of *E. tarda*

Four weeks after vaccination, the remaining fish in each group ($n = 120$) were then divided into two subgroups ($n = 60$) for challenge test. The fish in each subgroup were challenged by i.p. injection with different strains of *E. tarda*: one dose, 3×10^6 CFU per fish (equivalent to 5-fold LD50) for ATCC19106; the other dose, 1×10^7 CFU per fish (equivalent to 5-fold LD50) for CTE1, which were cultured in a separate tank with running fresh water at 25°C for 15 days. The mortality was recorded and relative percentage survival (RPS) was calculated from the cumulative mortalities using: RPS = [1 − (% mortality in vaccinated fish/% mortality in control fish)] × 100% [40]. Significant differences were calculated by using Student's *t*-test.

Results

Amplification of the V_H and V_L genes and generation of scFv

Sequence analysis showed that the V_H gene contained 339 bp and encoded 113 amino acid residues; there were four frameworks (FRs), three complementary determining regions (CDRs), and the two cysteines located at aa 22 and 96, respectively; the V_H gene was rearranged as V–D–J. The V_L gene contained 336 bp and encoded 112 amino acid residues; there were four FRs, three CDRs, and the two cysteines located at aa 23 and 93, respectively; the V_L gene was rearranged as V–J. The V_H and V_L had the typical features of mouse IgG variable regions [41,42]; both are new sequences when compared with the data from GenBank.

The scFv was amplified using the primers in **Table 2**. The complete nucleotide sequence and deduced amino acid sequence of the scFv are given in **Fig. 2**. The agarose gel electrophoresis result of scFv gene is shown in **Fig. 3**.

SDS–PAGE and western blotting analysis

A novel protein band corresponding to 31 kDa was detected by SDS–PAGE and existed in *E. coli* as an

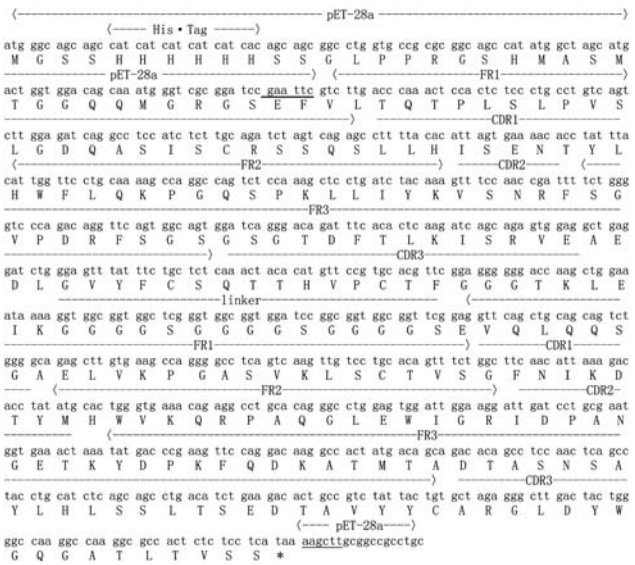

Figure 2 Nucleotide sequence and deduced amino acid sequence of scFv gene The CDRs and FRs are indicated on the sequences; the *Eco*RI and *Hin*dIII restriction sites are underlined.

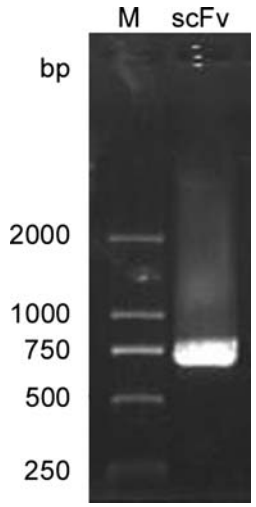

Figure 3 Agarose gel electrophoresis of scFv Lane M, DNA molecular marker.

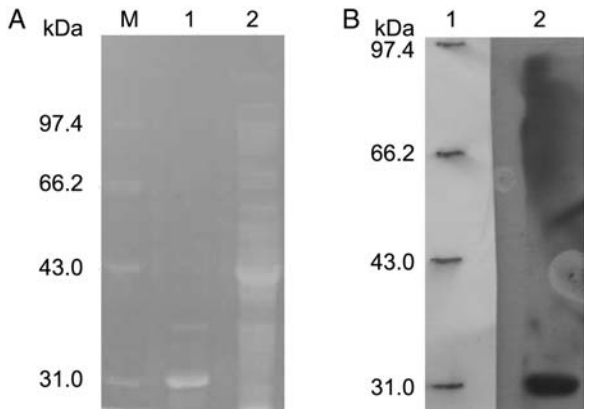

Figure 4 SDS–PAGE and western blot analysis of pET-28a-scFv expression in *E. coli* BL(DE3) (A) SDS–PAGE gel stained with Coomassie brilliant blue R250. Lane M, the low range of protein marker; lane 1, the inclusion body proteins of pET-28a-scFv; lane 2, the protein supernatant of pET-28a-scFv. (B) Western blotting. Lane 1, the low range of protein marker; lane 2, the purified proteins of scFv.

insoluble fraction [**Fig. 4(A)**, lane 1]. Western blotting showed that the purified scFv can react to the mouse mAb 3F7 against *E. tarda* [**Fig. 4(B)**], and the negative control can not, which suggests that the purified scFv has the same epitope as the anti-idiotypic mAb 1E11.

Evaluation of vaccine efficacy

Four weeks after vaccination, blood was taken from 10 fish in each group and the sera were used for specific antibody lever detection. Absorbance at serum dilution of 1:10,000 was employed in the ELISA test. The scFv group showed significantly higher absorbance than those of the control groups 1 and 2 (**Fig. 5**).

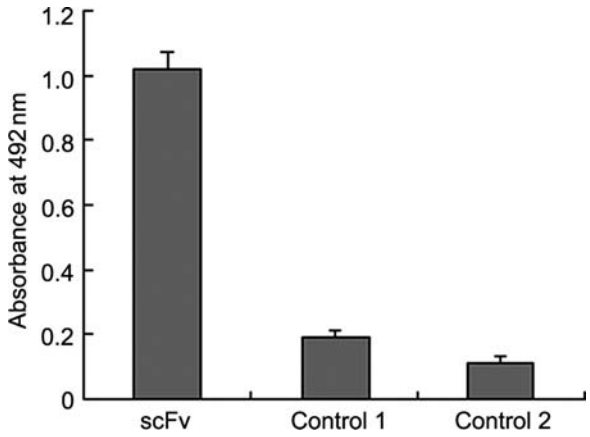

Figure 5 Absorbance at serum dilution of 10^{-4} was determined by ELISA Red drum were immunized with scFv group, control group 1 (empty plasmid pET-28a) and control group2 (PBS), at 4 weeks after immunization, absorbance of fish serum at dilution of 1:10,000 was employed in the ELISA test. Reactive antigen was the anti-idiotypic mAb 1E11. The level of specific antibody in fish sera of scFv group was significantly higher than control groups.

Table 3 shows the cumulative mortalities and RPS after challenge with different strains of *E. tarda*. One was ATCC19106 and another was CTE1. Fish began to die on the second day after challenge; from Days 3 to 7, the death of fish increased; and from Days 7 to 15, almost no fish died. Finally, the cumulative mortalities of vaccinated with scFv subgroups were 11.7% (ATCC19106) and 13.8% (CTE1), but those of the control subgroups were up to >96.7%. The RPS of scFv subgroups were calculated compared with those of the control subgroups 1 and 2; they were 88.1% and 87.9% (ATCC19106), and 86.7% and 86.4% (CTE1), separately. The fish vaccinated with scFv subgroups had a significantly higher survival rate compared with the unvaccinated fish (control subgroup 1, $P < 0.01$; control subgroup 2, $P < 0.01$, Student's *t*-test).

Table 3 Cumulative mortality and RPS of red drum were calculated after challenge with different strains of *E. tarda* at 4 weeks post-vaccination

Strain	Group	Cumulative mortality (%)	RPS (%)
E. tarda ATCC19106	scFv subgroup	11.7	
	Control subgroup 1	98.3	88.1
	Control subgroup 2	96.7	87.9
E. tarda CTE1	scFv subgroup	13.8	
	Control subgroup 1	100	86.7
	Control subgroup 2	98.3	86.4

RPS, relative percentage survival. $n = 60$ for each group.

Discussion

Developing a vaccine against *E. tarda* has been a focus of study for many years; until now, there is no effective vaccine available against *E. tarda* infection; one major reason is the variation of its serotypes. Some researches about DNA or protein vaccines have been published recently [23–29], but the variable RPS values of these vaccines make it difficult for future development; currently, there are no practical and commercially available vaccine against *E. tarda*.

In a previous study, a monoclonal anti-idiotypic antibody 1E11 from mouse hybridoma cells was produced using the standard strain of *E. tarda* (ATCC19106), which carries an 'internal image' of the original antigen. And, an ELISA assay found that the mAb 3F7, which the 1E11 produced from, can recognize both strains *E. tarda* ATCC19106 and CTE1, and in western blotting assay, 3F7 shows positive reaction at 37 kDa to both *E. tarda* ATCC19106 and CTE1. The 1E11 as a surrogate antigen for the vaccine is one of the most promising approaches for active immunotherapy [35]. In this study, a new genetic engineering anti-idiotypic antibody scFv from 1E11 was developed as an effective vaccine against *E. tarda*, which is an ideal approach for prevention of fish disease.

The scFv is the smallest functional modules of antibody molecule; the lack of Fc domains makes it less immunogenic responsive [36,37], which may result in efficient protection against infection with different serotypes of *E. tarda*. Additionally, studies demonstrated that when the linker length was <25 amino acids, scFvs in the V_L–linker–V_H format had stronger binding activity than those with the V_H–linker–V_L format [43–45]. So, in the present study, the scFv in the V_L–linker–V_H orientation was constructed with the most commonly used $(Gly_4Ser)_3$ linker to obtain greater binding activity.

Previously, the anti-idiotypic mAb and genetically engineered vaccine of dsFv against *V. anguillarum* were successfully produced in our lab [39,46], which was an effective vaccine against *V. anguillarum* with identification. Based on these, this study generated recombinant anti-idiotypic antibody vaccine scFv against *E. tarda*. Because mouse Ig genes are highly diverse in their nucleotide sequence, it is very difficult to obtain the correct variable region (Fv) of immunoglobulin (Ig) by RT–PCR with degenerate primers [47–49]. So, a new method employing bioinformatics was used to achieve the aims: in the first step, the highly degenerate primers were employed for amplification of the Fv genes by RT–PCR from 1E11. In the second step, according to the sequencing results, compared with the homology sequences of NCBI, the several groups of primers for amplifying the variable genes were designed. In the last step, all the sequences of the Fv genes from the results of PCR were aligned with the bioinformation software and the complete and correct sequences were determined. Therefore, the scFv was constructed and expressed in *E. coli* BL21. The SDS–PAGE and western blotting results showed that the relative molecular mass of expressed scFv was 31 kDa. Red drum weighing about 50 g was vaccinated and subjected to challenge with different strains of *E. tarda*. The data showed that the scFv subgroups have significant protection over control subgroups, with 11.7% and 13.8% cumulative mortalities and RPS were up to $>86.4\%$ ($P < 0.01$). Moreover, ELISA showed that the specific antibody titer in fish sera were significantly higher in the scFv groups than in the control groups.

Taken together, this study is approached with the aim to produce vaccine in a large scale and as a practical candidate for the aquaculture industrialization. In addition, the recombinant anti-idiotypic antibody scFv, which lacks Fc domain, is the smallest functional module of antibody molecule, which can result in efficient protection against infection by different serotypes of *E. tarda*. It is demonstrated that the prepared anti-idiotypic antibody scFv vaccine is effective in protection against *E. tarda* pathogen infection. The recombinant scFv vaccine can be taken as a vaccine candidate and contribute to the development of an effective vaccine against *E. tarda*.

Funding

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