Vaccination of sex reversed hybrid tilapia (Oreochromis niloticus × O. aureus) with an inactivated Vibrio vulnificus vaccine

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ABSTRACT

Vibrio vulnificus causes disease in economically important aquaculture raised fish and is an opportunistic human pathogen. This study reports on the isolation of V. vulnificus from diseased hybrid tilapia (Oreochromis niloticus × O. aureus) cultured in a North American water reuse facility. Our objectives were to characterize the isolate using biochemical and molecular methods, develop a disease challenge model, and determine the ability of a formalin inactivated whole-cell vaccine to protect against V. vulnificus. The V. vulnificus isolate recovered was biotype 1, 16S rRNA type B, vvcg type C, and vvhA type 2 and caused disease in tilapia held in static salt water (1.5 g/l sea salt). Fish vaccinated with the formalin inactivated whole-cell vaccine responded to vaccination with titers from vaccinated fish ranging from 32 to 64 and titers from non-vaccinated fish ranging from 4 to 8. In two trials, vaccinated tilapia exhibited relative percent survival (RPS) of 73 and 60% following homologous isolate challenge. In two additional trials, vaccinated tilapia exhibited RPS values of up to 88% following challenge with a heterologous isolate; the use of a mineral oil adjuvant enhanced protection. This vaccine may provide an effective means of preventing infections caused by biochemically and genetically diverse V. vulnificus.

Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.

1. Introduction

Vibrio vulnificus is a Gram-negative halophilic bacterium commonly associated with estuarine and marine environments worldwide [1]. This pathogen has the ability to cause severe infections in humans following the consumption of raw seafood contaminated with V. vulnificus, and thus presents a food safety issue. Wound infections can also occur through exposure of open wounds to the bacterium [2].

In addition to being an opportunistic human pathogen, V. vulnificus can cause disease in economically important aquaculture raised fish species [3]. Disease caused by V. vulnificus in fish is most often associated with aquaculture reared eels Anguilla anguilla and A. japonica [4,5]. However, there are also reports of this pathogen causing disease in pompano Trachinotus ovatus [6] and tilapia Oreochromis spp. under production conditions [7,8]. Vibrio vulnificus was re-isolated from dead rainbow trout (Oncorhynchus mykiss) reared in marine production systems; however, the role of the bacterium in causing disease was uncertain [9]. Laboratory studies have demonstrated the susceptibility of other species, such as turbot Psetta maxima, sea bass Dicentrarchus labrax, and rainbow trout [10,11]. With the ever increasing importance of cultured marine and freshwater fish as a source of protein, the potential for increased disease due to Vibrio spp. is recognized [12,13].

Vibrio vulnificus exhibits a large degree of phenotypic and genotypic heterogeneity. Isolates can be classified into three biotypes based on biochemical characteristics (reviewed in Jones and Oliver [2]). Although biochemical characteristics are commonly used to assign an isolate to a particular biotype, these can be variable and may not be adequate for typing [14]. Biotype 1 isolates are commonly associated with human infection; however, isolates from any of the three biotypes have the potential to cause disease in humans. Most isolates that cause disease in fish have been reported as biotype 2 [10,11,15].

The genetic heterogeneity observed among V. vulnificus isolates has allowed for the development of molecular methods to characterize isolates and these have been useful to discriminate between isolates with human-pathogenic potential (i.e., clinical isolates) and environmental isolates. Three such methods include restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene [16,17], polymerase chain reaction (PCR) analysis of the cytotoxin-hemolysin gene, vvhA [18], and PCR analysis of a virulence-correlated gene, vvcg [19,20]. The three methods were recently evaluated for their ability to distinguish between clinical and environmental isolates; based on these results the author’s proposed three genotypic profiles [14].

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In the present study, we report on the isolation of *V. vulnificus* from diseased hybrid tilapia (*O. niloticus × O. aureus*) under production in a North American water reuse aquaculture facility. The objectives of this study were to characterize the isolate using biochemical and molecular methods, develop a disease challenge model, and determine the ability of a formalin inactivated whole-cell vaccine to provide protection against *V. vulnificus* challenge.

2. Materials and methods

2.1. Bacteria

In December 2009, our laboratory was contacted by a North American hybrid tilapia producer experiencing a significant disease outbreak on a commercial water reuse aquaculture facility. Six dead fish were shipped to our laboratory and fish were necropsied using standard procedures. Aseptically acquired samples of the anterior kidney and brain were inoculated onto sheep blood agar (SBA) and incubated at 28 °C for 24–72 h. Both organs sampled from all six fish yielded pure cultures of a Gram-negative bacterium. The isolates were identified as *V. vulnificus* by fatty acid methyl ester analysis [21] and API 20E test (bioMérieux, Inc., Durham, NC, USA). One isolate, ARS-1Br-09 (deposited in the USDA Agricultural Research Service culture collection (NRRL) under accession number B-50360), was chosen for subsequent characterization and used in the infectivity and vaccine experiments.

Three additional *V. vulnificus* isolates (Spanish type culture collection (CETC) 529 = ATCC 27562, CETC 4601 and CETC 4608) were included as positive controls in the characterization of the ARS-1Br-09 isolate. These were cultured on SBA plates at 28 °C.

2.2. Molecular characterization

Total DNA was extracted from *V. vulnificus* isolates using a DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol for Gram-negative bacteria. The 16S rRNA genotype of each isolate was determined as described by Nilsson et al. [17], with minor modifications. A 492-bp segment of the 16S rRNA gene was amplified by PCR from each isolate using primers UFUL and URUL [17,22]. PCR was performed using HotStarTaq® Plus Master Mix kit (Qiagen) and the final concentration of each component in the 20-μl reactions were as follows: 1 × HotStarTaq Plus Master Mix, 0.3 μM each primer (UFUL, URUL), 1 × CoralLoad Concentrate, and 50 ng total DNA. PCR amplification was performed with a Primus HTD thermocycler (MWG AG Biotech) and the following cycling protocol was used: one cycle of 5 min at 95 °C; 30 cycles of 30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C; final cycle of 10 min at 72 °C. PCR products were separated by agarose gel electrophoresis (1.5% w/v) in TAE buffer and stained with ethidium bromide. Isolates were ascribed to vvhA genotype based on the size of PCR product obtained following the multiplex PCR; a vvhA type E isolate (environmental) is predicted to produce a 199 bp product and a vvhA type C isolate (clinical) is predicted produce a 97 bp product [20].

The vvhA (cytotaxin-hemolysin gene) genotype of each isolate was determined by PCR as described by Senoh et al. [18], with minor modifications. PCR was performed using HotStarTaq® Plus Master Mix kit (Qiagen) and the final concentration of each component in the 20-μl reactions were as follows: 1 × HotStarTaq Plus Master Mix, 0.1 μM each primer (vcgE F, vcgE R, vcgC F, vcgC R), 1 × CoralLoad Concentrate, and 50 ng total DNA. PCR amplification was performed with a Primus HTD thermocycler (MWG AG Biotech) and the following cycling protocol was used: one cycle of 5 min at 95 °C; 30 cycles of 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C; final cycle of 10 min at 72 °C. PCR products were separated by agarose gel electrophoresis (1.5% w/v) in TAE buffer and stained with ethidium bromide. Isolates were ascribed to vcg genotype based on the size of PCR product obtained following the multiplex PCR; a vcg type E isolate (environmental) is predicted to produce a 248, 202, and 42 bp and fragments of 204, 147, 120, and 21 bp when digested with HaeIII [17].

The vcg (virulence-correlated gene) genotype of each isolate was determined by multiplex PCR as described by Warner and Oliver [20], with minor modifications. PCR was performed using HotStarTaq® Plus Master Mix kit (Qiagen) and the final concentration of each component in the 20-μl reactions were as follows: 1 × HotStarTaq Plus Master Mix, 0.1 μM each primer (vcgE F, vcgE R, vcgC F, vcgC R), 1 × Coraload Concentrate, and 50 ng total DNA. PCR amplification was performed with a Primus HTD thermocycler (MWG AG Biotech) and the following cycling protocol was used: one cycle of 5 min at 95 °C; 30 cycles of 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C; final cycle of 10 min at 72 °C. PCR products were separated by agarose gel electrophoresis (1.5% w/v) in TAE buffer and stained with ethidium bromide. Isolates were ascribed to vcg genotype based on the size of PCR product obtained following the multiplex PCR; a vcg type E isolate (environmental) is predicted to produce a 248, 202, and 42 bp and fragments of 204, 147, 120, and 21 bp when digested with HaeIII [17].

2.3. Fish

Sex reversed hybrid tilapia (mean weight of 6.7 g) were obtained as fry from AQUASAFRA, Inc. (Bradenton, FL, USA) and used as experimental animals. For trials 1 and 2, fish were stocked at approximately 60 fish in each of two 200 l aquarium at time of vaccination. For trials 3 and 4, four groups of 50 fish each were utilized (see vaccine and vaccination below for details). The fish were maintained in a flow through (0.5 l/min) system using dechlorinated municipal water heated to 28 ± 2 °C until challenge (see below). All procedures utilizing fish were approved by the USDA-ARS AAHRU Institutional Animal Care and Use Committee.

2.4. Infectivity

Infectivity trials were conducted with 20–31 hybrid tilapia held in 57 l aquaria maintained on flow-through freshwater at 28 ± 2 °C. Fish were challenged by intraperitoneal (IP) injection with *V. vulnificus* (ARS-1Br-09) grown in tryptic soy broth supplemented with 0.5% sodium chloride (TSB + NaCl) in 50 ml culture tubes at 28 °C for 24 h. The doses administered ranged from 2.2 × 108 to 3.0 × 109 cfu/fish in four experiments. A fifth experiment consisted of IP injection at a dose of 2.0 × 107 cfu/fish using 31 hybrid tilapia. Fish in this experiment were injected after being maintained in water containing 1.5 g/l sea salt for 4 days prior to and for the duration of the challenge (7 d post challenge).

2.5. Vaccine and vaccination — trial 1

*Vibrio vulnificus* (ARS-1Br-09) was grown at 28 °C in 500 ml TSB + NaCl for 24 h prior to adding 1% (v/v) formalin to inactivate the cells. The formalin inactivated whole-cell vaccine was administered via IP injection of 100 μl per fish. Cell counts of a sample
taken prior to inactivation indicated $1.5 \times 10^6$ cfu was given to each fish ($n = 50$). Sham vaccinated fish received $100 \, \mu l$ of sterile TSB + NaCl ($n = 50$).

2.6. Vaccine and vaccination — trial 2

*Vibrio vulnificus* (ARS-1Br-09) was grown, inactivated and administered as described above. Cell counts of a sample taken prior to the addition of the formalin indicated $2.8 \times 10^7$ cfu was injected per fish ($n = 57$). Sham vaccinated fish received $100 \, \mu l$ of sterile TSB + NaCl ($n = 55$).

2.7. Vaccine and vaccination — trial 3 and 4

*Vibrio vulnificus* (ARS-1Br-09) was grown at $28 \, ^\circ C$ in $500 \, ml$ TSB + NaCl for $24 \, h$ prior to adding 1% (v/v) formalin to inactivate the cells. Following inactivation, $20 \, ml$ of the culture was centrifuged at $4000 \times g$ and $10 \, ml$ of the supernatant was discarded. The cell pellet was resuspended into the remaining $10 \, ml$ and was mixed with an equal volume of mineral oil. Following mixing, the suspension was emulsified via an 18 gauge micro-emulsifying needle. Fifty tilapia were immunized with the inactivated vaccine without adjuvant via IP injection of $100 \, \mu l$ per fish. A second group of fish ($n = 50$) were IP injected with $100 \, \mu l$ per fish of the mineral oil adjuvanted vaccine. Cell counts of a sample taken prior to inactivation indicated $9 \times 10^6$ cfu was given to each fish. Two groups of fifty sham vaccinated fish were included and were IP injected with either $100 \, \mu l$ of sterile TSB + NaCl or $100 \, \mu l$ of TSB + NaCl emulsified with mineral oil.

2.8. Challenge — trial 1

At 78 days post vaccination (dpv), triplicate groups of 15 randomly selected fish from the treatment and control groups were allocated to six $57 \, l$ aquaria for the challenge. A single tank of 15 sham vaccinated fish was included as mock infected controls. *Vibrio vulnificus* (ARS-1Br-09) was grown in TSB + NaCl for $16 \, h$ at $28 \, ^\circ C$ and used for the challenge. Fish were challenged by IP injection with $100 \, \mu l$ containing $1.26 \times 10^7$ cfu and mock infected controls were IP injected with $100 \, \mu l$ of sterile TSB + NaCl. Following challenge, fish were held in the $57 \, l$ aquaria filled with static water containing $1.5 \, g/l$ sea salt. Each day half of the tank water volume was drained, re-filled, and salt was added to maintain the concentration of sea salt at $1.5 \, g/l$. Microbial isolation from the brain of at least $50\%$ of the dead fish from each tank was accomplished in all trials.

2.9. Challenge — trial 2

At 40 dpv, triplicate groups of 15 randomly selected fish from the treatment and control groups were allocated to six $57 \, l$ aquaria for the challenge. A single tank of 15 sham vaccinated fish was included as mock infected controls. *Vibrio vulnificus* (ARS-1Br-09) was grown in TSB + NaCl for $6 \, h$ at $28 \, ^\circ C$ and used for the challenge. Fish were challenged and the water was maintained as described for trial 1, with the exception that each fish received $2.15 \times 10^6$ cfu.

2.10. Heterologous challenge — trial 3 and 4

At 81 dpv (Trial 3), duplicate groups of 10 randomly selected fish from the treatment and control groups were allocated to eight $57 \, l$ aquaria for the challenge. A single tank of 10 sham vaccinated fish was included as mock infected controls. *Vibrio vulnificus* isolate CECT 4601 was grown in TSB + NaCl for $6 \, h$ at $28 \, ^\circ C$ to a cell density of $7.4 \times 10^5$ cfu/ml and then diluted 1:100 prior to challenge. Fish were challenged and the water was maintained as described for trial 1, with the exception that each fish received $7.4 \times 10^5$ cfu/fish. The challenge in trial 4 was conducted at 88 dpv as described for trial 3, with the exception that each fish was injected with $5.1 \times 10^5$ cfu.

2.11. Blood collection and agglutination antibody titers

In trial 1, blood was collected from the caudal vasculature of 4 individual fish from each of the sham vaccinated and vaccinated fish prior to challenge using a non-heparinized syringe with 25-gauge needle to obtain serum for antibody titer determination. Following the challenge, blood was collected from the remaining fish in each aquarium to determine antibody titers post challenge (sham vaccinated, $n = 11$; vaccinated, $n = 36$). Serum was collected following centrifugation of clotted blood at 3000 g for 10 min. Agglutinating antibody titer was determined using a modified microagglutination method [23]. Briefly, *V. vulnificus* (ARS-1Br-09) was grown for $8 \, h$ in TSB + 0.5% NaCl at $28 \, ^\circ C$ and killed with 1% (v/v) formalin. The cells were then centrifuged ($5000 \, g$) for $15 \, min$ and the resulting pellet was resuspended and washed in phosphate buffered saline (PBS, pH 7.2). Following three washes, the bacteria were adjusted to an optical density of 0.6 at 540 nm in PBS. Round bottom 96-well microtiter plates were initially plated with $25 \, \mu l$ of PBS. In each well of the first row, $25 \, \mu l$ of serum was added and mixed. Following mixing, two-fold serial dilutions were made. After this, $25 \, \mu l$ of the cell suspension was added to each well. Positive and negative control sera were included on each plate as assay controls. Plates were covered and incubated overnight (about $16 \, h$) at $22 \pm 2 \, ^\circ C$. The agglutination endpoint was determined as the final dilution where visible cell agglutination was observed. Titers were reported as the reciprocal of the highest serum dilution showing visible agglutination.

2.12. Statistical analysis

The $t$-test procedure of SAS (Cary, NC, USA) was used to compare the mean cumulative percent mortality between the vaccinated and sham vaccinated treatments (Trial 1 and 2) and was used to compare the mean agglutinating antibody titers of the vaccinated and sham vaccinated fish at different time points post immunization. The general linear models (GLM) procedure with Duncan’s multiple range test was used to determine differences between treatments in Trial 3 and 4. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Bacteria characterization

Initial identification of isolate ARS-1Br-09 as *V. vulnificus* was made by analysis of the fatty acid methyl ester profile using the microbial identification system that yielded a high percentage match (0.61 similarity index) in the rapid method RCLN50 database [24]. The API 20E test strip was used to biochemically characterize the *V. vulnificus* isolates in this study (Table 1). The profiles were 5146105 (ARS-1Br-09; CECT 4608), 5006005 (CECT 4601) and 5146005 (CECT 529). The results of the 16S rRNA genotyping demonstrated that ARS-1Br-09 and CECT 4608 isolates were type B (clinical) and isolates CECT 529 and CECT 4601 were type A (non-clinical) (Fig. 1; Table 1). The results of the vgc genotyping demonstrated that ARS-1Br-09 and CECT 4608 isolates were vgc type C (clinical) and isolates CECT 529 and CECT 4601 were vgc type E (environmental) (Fig. 2; Table 1). The results of vvHa genotyping demonstrated that isolates ARS-1Br-09, CECT 4601, and CECT 529...
isolates digested with 4608 (type B). See section 2.2 for expected DNA fragment sizes for each type.

Lane 1: ARS-1Br-09 (type B); Lane 2: CECT-529 (type A); Lane 3: CECT-4601 (type A); Lane 4: CECT-4608 (type B).

Restriction profiles of 16S rRNA gene amplified from four Vibrio vulnificus isolates digested with AluI (A) and HindIII (B). Lane M: 50 bp DNA ladder; Lane 1: ARS-1Br-09 (type B); Lane 2: CECT-529 (type A); Lane 3: CECT-4601 (type A); Lane 4: CECT-4608 (type B). See section 2.2 for expected DNA fragment sizes for each type.

3.2. Infectivity

Four separate experiments were conducted using flow through freshwater and IP challenge. One of 20 fish died (5%) in each of two

Table 1
Biochemical profile and molecular characterization of Vibrio vulnificus isolated from the dead tilapia and from known culture collection isolates.

<table>
<thead>
<tr>
<th>Test</th>
<th>ARS-1Br-09 (Biotype 1 from tilapia)</th>
<th>CECT 529* (Biotype 1 from human)</th>
<th>CECT 4601 (Biotype 2 from eel)</th>
<th>CECT 4608 (Biotype 3#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginidihydrolase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysinedecarboxylase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrosefermentation</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ornithinedecarboxylase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indoleproduction</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>α-mannitolfermentation</td>
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<td>α-sorbitolfertilization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CitrateONPG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16S rRNA genotype</td>
<td>B</td>
<td>Acd</td>
<td>Acd</td>
<td>Bcd</td>
</tr>
<tr>
<td>vcg genotype</td>
<td>Cc</td>
<td>Ec</td>
<td>Ed</td>
<td>Cc</td>
</tr>
<tr>
<td>vvhA genotype</td>
<td>2c</td>
<td>2c</td>
<td>1c</td>
<td>1c</td>
</tr>
</tbody>
</table>

* Type strain also referred to as ATCC 27562. Reference strains are sometimes variable for α-mannitol [29].

b Results based on Bisharat et al. [30].
c Results from current study.
d Based on published literature [14].

were vvhA type 2 (environmental) and isolate CECT 4608 was vvhA type 1 (clinical) (Fig. 3; Table 1).

3.3. Vaccination and antibody response

Vaccinated fish in trial 1 had significantly (p < 0.05) lower mortality than sham vaccinated fish following challenge (Table 2).

Table 2
Mean cumulative percent mortality (CPM) and relative percent survival (RPS) among sex reversed hybrid tilapia challenged with the homologous Vibrio vulnificus isolate (ARS-1Br-09) at 78 (Trial 1) and 40 (Trial 2) days post vaccination. Within a trial, mean CPM values with different superscript symbols indicate a significant difference at p < 0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean CPM ± SEM (RPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1*</td>
<td>Trial 2*</td>
</tr>
<tr>
<td>Sham vaccine</td>
<td>V. vulnificus vaccine</td>
</tr>
<tr>
<td>75.6 ± 8.0*</td>
<td>20.0 ± 3.9* (73)</td>
</tr>
<tr>
<td>73.3 ± 0.0*</td>
<td>28.9 ± 4.4* (60)</td>
</tr>
</tbody>
</table>

* Tilapia were challenged with 1.26 × 10⁷ cfu/fish.
b Tilapia were challenged with 2.15 × 10⁸ cfu/fish.

d of the experiments; however, V. vulnificus was only recovered from one of the dead fish. No mortality was recorded in the other two experiments conducted in freshwater. In the fifth experiment where fish were maintained in water containing 1.5 g/l sea salt, 93% mortality was observed (29 of 31 fish died by 7 d post challenge). Pure cultures of V. vulnificus were recovered from the brain and kidney of fresh dead fish in the static salt water experiment.

V. vulnificus were recovered from 100% (18/18) of the mortalities examined from the sham vaccinated and vaccinated fish, respectively. There was no mortality in the mock infected control fish maintained under the same experimental conditions (Table 2). Similar results were obtained in trial 2 with vaccinated fish showing significantly (p < 0.05) lower mortality than sham vaccinated fish following challenge (Table 2).

Relative percent survival was 60%. Pure cultures of V. vulnificus were recovered from 100% (30/30) and 100% (11/11) of the mortalities examined from the sham vaccinated and vaccinated fish, respectively. There was no mortality in the mock infected control fish maintained under the same experimental conditions (Table 2).

Mean antibody titers were significantly higher (p < 0.05) in the vaccinated fish (mean titer = 56; range 32–64) at 78 dpv as compared to the sham vaccinated fish (mean titer = 5; range 4–8) in trial 1. At 12 days post challenge a similar result was obtained where mean titers were significantly higher (p < 0.05) in the vaccinated challenged fish (mean titer = 206; range 64–1024) as compared to the sham vaccinated challenged fish (mean titer = 45; range 16–128).

In trial 3, tilapia vaccinated with the mineral oil adjuvanted V. vulnificus vaccine and challenged with a heterologous isolate showed significantly less (p < 0.05) mortality than the sham

Fig. 2. Agarose gel electrophoresis of DNA products amplified from Vibrio vulnificus isolates by polymerase chain reaction (PCR) using the multiplex vcg primers. Lane M: 50 bp DNA ladder; Lane 1: no template control; Lane 2: ARS-1Br-09 (type C); Lane 3: CECT-529 (type D); Lane 4: CECT-4601 (type E); Lane 5: CECT-4608 (type C). See section 2.2 for expected PCR product sizes for each type.

<ref>Fig. 1. Restriction profiles of 16S rRNA gene amplified from four Vibrio vulnificus isolates digested with AluI (A) and HindIII (B). Lane M: 50 bp DNA ladder; Lane 1: ARS-1Br-09 (type B); Lane 2: CECT-529 (type A); Lane 3: CECT-4601 (type A); Lane 4: CECT-4608 (type B). See section 2.2 for expected DNA fragment sizes for each type.</ref>
vaccinated with adjuvant group (Table 3). Relative percent survival was 73% for the mineral oil adjuvanted killed V. vulnificus vaccine. There was no significant difference between the mortality of fish vaccinated with the non-adjuvanted V. vulnificus vaccine and sham vaccinated group (Table 3). All dead fish from trial 3 were culture positive for V. vulnificus. Results of trial 4 showed that significantly lower mortality \( (p < 0.05) \) occurred in both vaccinated groups than either of the sham vaccinated groups (Table 3). Relative percent survival was 63 and 88% for the killed vaccine and adjuvanted vaccine treatments, respectively. Seven of the 8 dead sham vaccinated fish in each sham group were culture positive for V. vulnificus. V. vulnificus was recovered from 100% (1/1) and 67% (2/3) of the mortalities examined from the adjuvanted V. vulnificus vaccine and V. vulnificus vaccine treatment groups, respectively.

4. Discussion

In the present study, V. vulnificus was isolated from hybrid tilapia experiencing a significant disease outbreak on a commercial water reuse aquaculture facility. Biochemical characteristics indicated that the isolate was biotype 1, similar to human clinical isolates. The molecular characterization of the isolate provided mixed results in regards to the ability to classify it as a potential human clinical isolate. Genotyping the isolate using the 16S rRNA and vcg loci classified it as a clinical isolate; however, genotyping using the vvhA marker classified it as an environmental isolate. Recently, Sanjuan et al. [14] evaluated these three methods as tools for distinguishing between clinical and environmental isolates. The authors proposed three genotypic profiles: (1) vcg type C, 16S rRNA type B, and vvhA type 1, which included biotype 1 strains from human septicemia and oyster; (2) vcg type E, 16S rRNA type A, vvhA type 2, which included biotype 2 isolates and biotype 1 isolates from fish and water and some human isolates; and (3) vcg type E, 16S rRNA type AB, vvhA type 2, which included only biotype 3 isolates. They also reported atypical isolates which did not fall into these three genotypic profiles. The ARS-1Br-09 isolate, reported in the present study, appears to be an atypical isolate, similar to the isolate CECT 5164 that was obtained from human blood in the US and was biotype 1, vcg type C, 16S rRNA type B, and vvhA type 2 [14].

Most literature has suggested that V. vulnificus isolates pathogenic to fish, including tilapia, are biotype 2 [10,11,15]. Laboratory studies in five species of fish (warm and cold-water) with four biotype 1 isolates supported this because challenge with these isolates did not induce mortality [10]. However, there are a few reports of V. vulnificus isolates, exhibiting similarities to biotype 1, causing disease in finfish. Sakata and Hattori [7] isolated strains of V. vulnificus from diseased tilapia and suggested that they were similar to biotype 1 strains based on biochemical reactions. Chen et al. [8] characterized V. vulnificus isolates from diseased tilapia in Taiwan and suggested that the isolates were similar to biotype 1 isolates based on 16S rRNA gene sequences, but these isolates produced unique biochemical reactions compared to other human clinical isolates. Li et al. [6] characterized an isolate of V. vulnificus obtained from diseased pompano and it exhibited unique biochemical characteristics compared to all three biotypes, but 16S rRNA gene sequencing indicated that it was closely related to a biotype 1 isolate. Definitive genotyping was not carried out on the aforementioned isolates of V. vulnificus.

A recent study characterized V. vulnificus strains associated with tilapia aquaculture in Bangladesh and the results indicated that all the strains were biotype 1 and similar to human clinical isolates based on genotyping [13]. The authors hypothesized that environmental factors and aquaculture practices may contribute to the emergence of more virulent isolates [13]. The possibility of V. vulnificus (biotype 1 and human clinical genotype) emerging as a fish pathogen should not be overlooked as this may present a significant disease problem for aquaculture reared fish species. Additionally, V. vulnificus may present an increased food safety risk related to healthy individuals acquiring wound infections from handling whole fish harboring such isolates and/or susceptible individuals acquiring primary septicemias from handling and consuming raw fish.

The isolate recovered from the dead tilapia in our study produced limited disease in sex reversed hybrid tilapia using a freshwater challenge model. However, if the challenge was carried out in tanks containing static water with 1.5 g/l sea salt, mortality and clinical signs of disease occurred (hemorrhaged skin and fins). The production facility, from which this isolate originated, did add salt to the system while purging the fish for transport to live markets. We hypothesize that over time the salinity increased in the system and may have been partly responsible for the disease outbreak. Alternatively, the bacterium may be similar to the isolate(s) recovered from the low saline or freshwater cultured tilapia in Taiwan [8].

Most vaccine studies to date have focused on killed vaccines against biotype 2 V. vulnificus isolated from eels [25,26]. The bivalent

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**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean CPM ± SEM (RPS)</th>
<th>Trial 3*</th>
<th>Trial 4†</th>
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<tbody>
<tr>
<td>Sham vaccine</td>
<td>40.0 ± 10.0**</td>
<td>40 ± 0.0*</td>
<td></td>
</tr>
<tr>
<td>Sham vaccine with adjuvant</td>
<td>75.0 ± 5.0*</td>
<td>40 ± 10.0*</td>
<td></td>
</tr>
<tr>
<td>V. vulnificus vaccine</td>
<td>35.0 ± 15.0* (13)</td>
<td>15.0 ± 5.0* (63)</td>
<td></td>
</tr>
<tr>
<td>V. vulnificus vaccine with adjuvant</td>
<td>20.0 ± 10.0* (73)</td>
<td>5 ± 5.0* (88)</td>
<td></td>
</tr>
<tr>
<td>Mock infected control</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

* Tilapia were challenged with \(7.4 \times 10^2\) cfu/fish.
† Tilapia were challenged with \(5.1 \times 10^2\) cfu/fish.
vaccine produced for use in eels is based on two biotype 2 V. vulnificus isolates [27], both of which exhibit the 16S rRNA type A profile [14]. There is a report in the literature of a vaccine developed for flounder (Paralichthys olivaceus) that utilized a human clinical isolate from Korea [28]. Unfortunately, the isolate was not molecularly characterized and no challenge data are presented in the results. The protective effect of the vaccine was inferred by in vitro immunological data, showing clearance of V. vulnificus. No work has been carried out on vaccine formulations against V. vulnificus in tilapia. Recent studies suggest V. vulnificus is emerging as an important pathogen of marine or brackish water finfish aquaculture [13]. Results of this study demonstrate that a formalin inactivated vaccine against an atypical V. vulnificus isolate was capable of stimulating a humoral antibody response in sex reversed hybrid tilapia. Protective immunity based on relative percent survival was demonstrated in the trials following IP administration of the inactivated vaccine. Although a higher level of protection was seen in trial 1, this may be due to a 10 fold higher challenge dose used in trial 2 and/or the fact that the fish were held for a greater duration prior to challenge (78 dpv vs 40 dpv). Heterologous isolate challenge using a V. vulnificus isolate obtained from a diseased eel (CECT 4601; biotype 2, 16S rRNA type A, vvhA type E, and adjuvanted vaccine formulations were effective and this may be a reflection of the overall challenge dose that was lower in trial 4 (5 × 10^5 cfu/fish vs 7 × 10^5 cfu/fish). This vaccine may provide an effective means of preventing infections caused by biochemically and genetically diverse isolates of V. vulnificus in commercial tilapia production. Further, use of the vaccine may reduce bacterial loads of V. vulnificus in tilapia or other species and reduce the risk of humans acquiring infections from consumption of raw fish and/or wound infections resulting from handling fish harboring the bacteria.

Acknowledgments

The authors thank Paige Mumma, Alberta Pink and Ning Qin of USDA-ARS for their technical assistance and Curtis Day of USDA-ARS for assistance with animal care. This research was funded by USDA-ARS CRIS Project No. 6420–32000–024–00D (Integrated Aquatic Animal Health Strategies). The authors thank Dr. Mark Strom (National Oceanic and Atmospheric Administration Fisheries Service, Northwest Fisheries Science Center, West Coast Center for Oceans and Human Health, Seattle, WA) and Dr. Dehai Xu (USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL) for providing critical review of the manuscript prior to submission. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

References