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Development and potential use of an *Edwardsiella ictaluri wzz* mutant as a live attenuated vaccine against enteric septicemia in *Pangasius hypophthalmus* (Tra catfish)

Tran H. Triet, Bui T.T. Tinh, Le V. Hau, Tran V. Huong, Nguyen-Quoc Binh[*]

Division of Aquacultural Biotechnology, Biotechnology Center of Ho Chi Minh City, 2374, Highway 1, Quarter 2, Trung My Tay Ward, District 12, Ho Chi Minh, Viet Nam

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ABSTRACT

Edwardsiella ictaluri is a causative agent of enteric septicemia of catfish (ESC), a seriously lethal disease in Vietnamese catfish (*Pangasius hypophthalmus*). A safe and effective vaccine against ESC is currently an urgent demand due to antibiotic overuse in pangasius farms has led to an alarming antimicrobial resistance. In this study, two *E. ictaluri wzz*E mutants (WzM-L3, deficient in a 1038bp-entire *wzz*E gene and WzM-S3, a 245bp-partial deletion of *wzz*E) were developed and their protection efficiancy was evaluated in hatched pangasius against ESC by immersion vaccination. As comparing to the high virulent wild-type strain who caused 73.33% of death on pangasius fingerlings immersed at 7.1×10^6 CFU ml^{-1}, both mutants showed extremely low mortality rates at 3.33% (WzM-S3) and 0% (WzM-L3) on pangasius fingerlings immersed at high concentration of 1.5×10^7 CFU mL^{-1} and 9.7×10^6 CFU ml^{-1}, respectively. Interestingly, both WzM-S3 and WzM-L3 had a remarkably high protection against ESC, as RPS % were found at 89.29% and 90%, respectively. The mutant WzM-L3 is a potential live attenuated vaccine against ESC in Vietnamese catfish farms with good protection and simple practice.

1. Introduction

Pangasius farming is one of the two key seafood factors in Vietnam, enormously contributing to national aquaculture export in recent years. The export is constantly increasing, recently reaching a peak of 1.78 billion USD in 2017, the highest value since 2011 [1]. However, intensive monoculture in local earthern ponds or river-based cages is facing challenges with infectious diseases. The most destructive damage, up to 50–90% mortality, for pangasius farming is from enteric septicemia of catfish (ESC), caused by *Edwardsiella ictaluri* [2,3]. This bacterium was initially confirmed to be responsible for ESC in channel catfish, *Ictalurus punctatus* in the US since 1979 [4,5] and later identified in cultured *Pangasius hypophthalmus* in Vietnam since 2002 [3,6]. Treatments depend heavily on antibiotics, however, leading to a severe multidrug resistance and declining antibiotic effectiveness, reported by Vietnamese farmers [3]. ALPHA JECT ® Panga 1 & 2 vaccines (Phamaq), the only killed-vaccine marketed in Vietnam and lisensed for injection vaccination of pangasius against ESC, showed a morderate success; however, the injection immunisation requires more time, labour and skilled personnel. An easier handling and more effective vaccine that could well adapt to Vietnamese pangasius industry is urgently required.

Bacterial cell surface's antigens such as O-polysaccharides (OPS, also known as O-antigen or O-chain) or enterbaterial common antigen (ECA) polysaccharides have been instensively studied for decades showed that loss or alternation of these structures can link to virulence reduction. The OPS is the external part that can be only found in lipopolysaccharides (LPS) [7–9], whose roles in adherence, colonisation and invasion [8], resistance to antibiotics [10] and cationic antimicrobial peptides (CAMPs), a host innate immune protection [11]. In case of ECA, the polysaccharides can appear in three forms: peripheral chains linked either to LPS (ECA^{LPS}) [12] or to phosphatidylglycerol (ECA^{PG}) [13,14] or cyclic form maintained in periplasm (ECA^{CYC}) [15,16]. While OPS has its length and composition varied specifically to each species and serotypes [10], ECA chains of all *Enterobacteria* are mostly conserve in their moiety, a repeating unit of trisaccharides: →3)-α-D-Fuc*p*4NAc-(1 → 4)-β-D-Man*p*NAcA-(1 → 4)-α-D-Glc*p*NAc-(1→ [17–19]. However, specific functions of ECA are still far from being fully understood. ECA^{LPS} is so far believed as the only immunogenic ECA like LPS [12], while ECA^{CYC} may involve in permeability barrier of cell envelope [20]. ECA^{PG} is supposed to regulate Shiga toxin of *E. coli* O157:H7 [21] and to have roles in bile salts resistance [22]. Despite those differences, ECA chains also share with OPS several similar

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[*] Corresponding author.
E-mail addresses: binhbiji@gmail.com, ttcnsh.snn@tphcm.gov.vn (N.-Q. Binh).

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biosynthesis mechanisms [23]. One of these assembly methods contains a chain length regulator protein WzzE, encoded by *wzz*E located in *wec* cluster [15,24]. Wzz protein is believed to act as a structural scaffold [25] modulating the length of the OPS or ECA by maintaining or terminating the polymerization controlled by *wzy* [9]. A *E. coli K-12* Δ*wzz*E mutant strain was found lost its entire ECA [24]. Null-ECA strains of *Salmonella enterica* Serovar *Typhimurium* or *Salmonella enterica,* lacking of *wec*A or *wec*D (in *wec* cluster), were both attenuated in oral mouse infection and had high protection against challenge [26,27].

Most studies on external polysaccharides of *E. ictaluri* mainly focused on OPS gene cluster, e.g. a mutant strain of *gne* (NT01EI_1311) had its OPS biosynthesis completely disrupted and showed a marked attenuation and high immune protection on catfish against virulent wild-type challenge [10,11]. However, there has been no published research on either *wzz* locus (proximal to OPS cluster) or *wzz*E (in *wec* cluster) of *E. ictaluri*. According the Genebank, *E. ictaluri* 93–146 (CP001600.2) contains *wzz*E locus (NT01EI_0091), hypothesized to regulate chain length of ECA. In this study, an *E. ictaluri* Δ*wzz*E mutant was developed and its potential use as a live attenuated vaccine against *E. ictaluri* infections in Tra catfish (from lavae to fingerlings) was investigated by immersion immunisation.

2. Materials and methods

2.1. Bacteria, plasmids, media and growth conditions

Bacteria strains and plasmids used in this study are presented in Table 1. Strains were selectively isolated in EIM (10 g Bactotryptone, 10 g yeast extract, 1.25 g phenylalanine, 1.20 g ferric ammonium citrate, 5 g sodium chloride, 0.03 g bromothymol blue, 17 g agar, and 990 mL distilled water) [28] and grown on LB broth/agar or BHI broth/agar. Colistin (20 μg ml^{-1}, Sigma), kanamycin (50 μg ml^{-1}, Sigma) and ampicilin (100 μg ml^{-1}, Sigma) were added to media as required. Optimal growth conditions were conducted for all *E. coli* strains at 37 °C (except for pCP20-included *E. coli*, grown at 28 °C), agitation speed at 250 rpm (broth) and incubation time at 16 h; and, for *E. ictaluri* strains at 28 °C, 200 rpm (broth) and 24 h/48 h (broth/agar).

2.2. Construction of the E. ictaluri wzzE mutants

Two different lengths of *wzz*E gene, a short inner fragment (245 bp) and a whole gene (1038 bp) were deleted separately in this study by homologous recombination between the target gene in *E. ictaluri* wild-type strain (EIAG) and recombinant suicide vectors pGPHKT for partial *wzzE* gene deletion or pGPUKD for whole *wzzE* gene deletion. Both plasmids carried a kanamycin-resistant gene, which was separately flanked by head and tail sequences (5′ 388 bp and 3′ 405 bp) of *wzz*E in vector pGPHKT, or upstream and downstream sequences (5′ 461 bp and 3’ 369bp) of this gene in vector pGPUKD. Oligonucleotides were supplied by IDT (Singapore). The 388 bp-head sequence (H*wzz*E), 405 bp-tail sequence (T*wzz*E), 461 bp-upstream sequence (U*wzz*E) and 369 bp-downstream sequence (D*wzz*E) were amplified from genomic DNA of EIAG by primers FH*wzz* and RH*wzz*, FT*wzz* and RT*wzz*, FUwzz and RUwzz, and FDwzz and RDwzz, respectively (Table 2). In addition, primers used to amplify a kanamycin resistance gene (*Km*R) from vector pCampia1300 (Havard university) were designed to contain FRT sequences (Table 2) for later elimination of this *Km*R sequence. Restriction sites were included in all the primers for the arrangement of flanking regions. The PCR products of H*wzz*E, T*wzz*E, U*wzz*E, D*wzz*E and *Km*R sequences were seperatedly digested, ligated and cloned into the vector pJET1.2/blunt (Fermentas) to eventually generate pJETHKT and pJETUKD (Table 1), possessing a constructed sequence H*wzz*E-*Km*R-T*wzz*E and U*wzz*E-*Km*R-D*wzz*E respectively. These flanking sequences were then separately ligated into a suicide vector pGP704 (Havard university), creating pGPHKT and pGPUKD. These vectors were transformed into *E. coli* SM10 λ pir (Biomedal) and then individually transferred into wild-type *E. ictaluri* by conjugation method, as described by Maurer et al. (2001) [29]. The pGPHKT or pGPUKD-containing *E. coli* SM10 λ *pir* strains (donor, 1 ml) were mixed with wild-type EIAG strains (recipient, 10 ml) at 1:10 donor:recipient ratio, before being supplied with 10 mM $MgSO_4$, 2 mg mL^{-1} bovine serum albumin (BSA) and 10 μg mL^{-1} DnaseI (final concentration). As described by de Lorenzo and Timmis (1994), the conjugated mix was transferred to a 0.45 μm pore-size filter membrane, mounted on the surface of LB agar containing 10 mM $MgSO_4$ and 2 mg mL^{-1} BSA, and incubated at 28 °C for 4 h. After the incubation, bacterial suspension was spreaded on BHI agar containing 50 μg mL^{-1} kanamycin and 20 μg mL^{-1} colistin for selecting the *E. ictaluri* recombinants. Suspected colonies were confirmed as double crossover recombinant strains (WzM-S1 or WzM-L1, Table 1) by direct PCR using designed primers FH*wzz* and RT*wzz* (short deletion) or FU*wzz* and RD*wzz* (whole sequence deletion).

2.3. Elimination of the marker gene KmR

Kanamycin resistant gene (*Km*R) inside flanking regions H*wzz*E-*Km*R-T*wzz*E or U*wzz*E-*Km*R-D*wzz*E of WzM-S1 or WzM-L1 mutants respectively was eliminated by recombinase activity of FLP/FRT system

Table 1

Bacterial strains and plasmids.

Strains or plasmids	Characteristics	Reference
Escherichia coli		
DH5α	F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA) U169 (Φ80lac ΔM15)*	Invitrogen
SM10 λ pir	*KmR thi*-1 thr *leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*	Biomedal [41]
Edwardsiella ictaluri		
EIAG	Wild-type; ColR	Collected in An Giang, Vietnam
WzM-S1	*EIAG* derivative; *wzz*EΔ245::*Km*R; *Col*R; *Km*R	This study
WzM-S2 (pCP20)	WzM-S1 derivative *wzz*EΔ245; pCP20; *Amp*R; *Cm*R; *Col*R	This study
WzM-S3	WzM-S2 derivative; *wzz*EΔ245; *Col*R	This study
WzM-L1	*EIAG* derivative; Δ*wzz*E::*Km*R; *Col*R; *Km*R	This study
WzM-L2 (pCP20)	WzM-S1 derivative Δ*wzz*E; pCP20; *Amp*R; *Cm*R; *Col*R	This study
WzM-L3	WzM-S2 derivative; Δ*wzz*E; *Col*R	This study
Plasmids		
pCP20	FLP recombinase expression; ApmR; CmR	Yale University [42]
pJET1.2/blunt	Cloning vector; AmpR	Fermentas
pJETHKT	pJET1.2::*wzz*EΔ245::*Km*R; AmpR; KmR	This study
pJETUKD	pJET1.2::Δ*wzz*E::*Km*R; AmpR; KmR	This study
pGP704	*oriR6K mobRP4;* AmpR;	Harvard University [41]
pGPHKT	pGP704::*wzz*EΔ245::*Km*R; AmpR; KmR	This study
pGPUKD	pGP704::Δ*wzz*E::*Km*R; KmR	This study

Table 2
Primers.

Primers	Sequences (*)	Restriction site
FHwzz	5′-AAGTCGAC ATGGTGAGTCAAAATCCGATGC-3′	*Sal*I
RHwzz	5′-AAGGATCC GTGCACTCCCCTCTTTATGACG-3′	*Bam*HI
FTwzz	5′-AAGGATCC GTGGCACAGGCGATCTATCAGC-3′	*Bam*HI
RTwzz	5′-AATCTAGATCAGATCCGTGCACGACG-3′	*Xba*I
FUwzz	5‘-AAGGTACC GTACCCTGATCGGCTTTACC	*Kpn*I
RUwzz	5′- AAGGATCC GGTTTTCCCTTTATGGCCGA- 3′	*Bam*HI
FDwzz	5′- AAGGATCC GGATCGGCGGTAACAAAAGA-3′	*Bam*HI
RDwzz	5′- AAGAGCTC ACCGGAATTCGCTGGTAAAA-3′	*Sac*I
FFRTK	5′- AAGGATCC *GAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTC*CCAGCCAGCCAA -3′	*Bam*HI
RFRTK	5′- AAGGATCC *AGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTT*CCTAAAACAATTCATCCAGT -3′	*Bam*HI
M13F	5′- GTTTTCCCAGTCACGAC - 3′	
M13R	5’ – AACAGCTATGACCATG - 3′	
WFsq	5′-CTGCGTAGCCCCTATTATCAGC-3′	
WRsq	5′-TGAAACGCCGCGTCCAAAACC-3′	

(*) Restriction sites are underlined.
FRT sequences are showed in *Italic*.

Table 3
Mutant and wild-type E. ictaluri strains used in attenuated experiments.

No. fish/tank	Strains	Method	Concentration
10	EIAG	Injection	1.3×10^4 CFU/fish
			1.3×10^5 CFU/fish
			1.3×10^6 CFU/fish
10	WzM-S3	Injection	1.7×10^5 CFU/fish
			1.7×10^6 CFU/fish
			1.7×10^7 CFU/fish
20	EIAG	Immersion	7.1×10^4 CFU ml^{-1}
			7.1×10^5 CFU ml^{-1}
			7.1×10^6 CFU ml^{-1}
20	WzM-S3	Immersion	5×10^4 CFU ml^{-1}
			5×10^5 CFU ml^{-1}
			5×10^6 CFU ml^{-1}
20	WzM-L3	Immersion	1.2×10^5 CFU ml^{-1}
			1.2×10^6 CFU ml^{-1}
			1.2×10^7 CFU ml^{-1}

*Each group was replicated 3 times.

in pCP20 (Yale university). The heat-sentitive pCP20 vector was electroporated into the *E. ictaluri* mutants and the bacterial suspension was incubated on BHI agar containing 50 µg mL^{-1} ampicillin and 20 µg mL^{-1} colistin at 28 °C. Suspected colonies were confirmed to have *Km*R gene deletion by PCR using primers FH*wzz* and RT*wzz* (WzM-S2) or FU*wzz* and RD*wzz* (WzM-L2). Finally, the elimination of heat-sentitive pCP20 vector was performed by incubating the mutant strains at 37 °C/16–30 h on BHI agar with or without 50 µg mL^{-1} ampicillin and 20 µg mL^{-1} colistin added. PCR was used to examine the absence of pCP20 vector in the *E. ictaluri* mutants WzM-S3 and WzM-L3.

2.4. Sequence analysis

A whole putative *wzz*E gene was amplified and cloned into a cloning vector pGEMT (Promega). The constructed pGEMT::*wzz*E was sequenced using primers (IDT) M13F/M13R and WFsq/WRsq. The obtained sequence was compared to the *wzz*E gene of *E. ictaluri* strain 93-146 (CP001600.2) by Nucleotide Basic Local Alignment Search Tool (BLAST). A secondary structure of WzzE protein was predicted by SWISS-MODEL [30]. WzzE protein sequence was aligned with homogenous published structures by Clustal Omega and analysed by ESPript 3.0 [31].

2.5. Bacterial preparation

Each colony of *E. ictaluri* mutants (WzM-S3 and WzM-L3) and wild-type (EIAG) was inoculated overnight into 5 mL of BHI broth supplied with 20 µg mL^{-1} colistin in shaking condition of 200 rpm at 28 °C for 16 h. Then, 1 mL of the bacterial culture was transferred into a 100 mL of BHI broth and incubated at 28 °C, 200 rpm, overnight until inoculation reached an OD$_{600}$ of 0.8–1.0 (equivalent to 1×10^9 CFU mL^{-1}). Biomass was centrifuged at 2200 × *g* for 15 min at 4 °C before being resuspended in 0.65% NaCl [32] and diluted to the required concentrations.

2.6. Attenuation trial

Virulence of two *E. ictaluri* mutants at different concentrations (Table 3) was evaluated and compared to that of the wild-type strain by injection and immersion in ESC-free pangasius fingerlings (5–10 g) from individual sibling crosses. The ESC-free fish were evaluated by PCR with specific primers FH*wzz*/RT*wzz* of *E. ictaluri*. Qualified fish then acclimated for 1 week in 100 L tanks at 28 ± 2 °C and then starved 24 h prior to each experiment. Water quality (pH, NO2, NH3) was checked daily by Sera test kits (Sera GmbH, Germany).

In the injection experiment, triplicate groups of 10 pangasius fingerlings were separately injected subcutaneously (i.s) with 0.1 mL of bacteria suspension of EIAG at 3 different concentrations: 1.3×10^4, 1.3×10^5 and 1.3×10^6 CFU/fish; and WzM-S3 at 1.7×10^5, 1.7×10^6 and 1.7×10^7 CFU/fish (Table 3). Duplicate control groups were injected with saline buffer. After injection, fingerlings were transferred to 100 L-tanks and monitored during 14 days for signs of ESC disease.

In the immersion experiment, triplicate groups of 20 pangasius fingerlings were separately immersed for 30 min with aeration in 2 L of bacterial suspension. The bacteria dilutions ranged from 7.1×10^4, 7.1×10^5 and 7.1×10^6 CFU ml^{-1} for EIAG; 5×10^4, 5×10^5 and 5×10^6 CFU ml^{-1} for WzM-S3; and 1.2×10^5, 1.2×10^6 and 1.2×10^7 CFU ml^{-1} for WzM-L3 (Table 3). Immersed groups then were transferred into 100 L tank. Mortalities were recorded daily for 2 weeks.

Moribund fish and mortalities from both the experiments were sampled by streaking out livers and kidneys on BHI agar containing 20 µg mL^{-1} colistin. Typical colonies of *E. ictaluri* were later confirmed by colony PCR. The 50% lethal dose (LD$_{50}$) of each strains were identified by Reed-Muench formulation.

2.7. Vaccination trials

Mutant *E. ictaluri* strains showing significantly low virulence (with the mortality rate less than 10%) were selected for four vaccination trials by immersion using pangasius larvae (under 1-day-old fish), 42-day-old fry, and 3-month-old fingerling (Table 4). During experiment time, water temperature was maintained at of 28 ± 2 °C. Water quality

Table 4
Description of vaccine trials.

Group	Total sample	Treatment (i.m 10^7 CFU/mL)				Challenge (i.m. 10^6 CFU/mL)	
		Once		Boosted		Strain	Fish growth stage
		Strain	Fish growth stage	Strain	Fish growth stage		
Preliminary test							
1	15	WzM-S3	Fingerling	–	–	EIAG	Fingerling
2	15	WzM-L3	Fingerling	–	–	EIAG	Fingerling
3 (Control +)	15	–	Fingerling	–	–	EIAG	Fingerling
4 (Control -)	15	–	Fingerling	–	–	–	Fingerling
Larvae hatching to 42-day-old fry (42d-fry)							
II.1	1500	WzM-S3	larvae	–	–	–	–
II.2	1500	–	larvae	–	–	–	–
II.1.1 [a]	200	WzM-S3	larvae	WzM-S3	21d-fry	EIAG	42d-fry
II.1.2 [a]	200	WzM-S3	larvae	–	–	EIAG	42d-fry
II.2.1 [b] (Control +)	200	–	–	–	–	EIAG	42d-fry
II.2.2 [b] (Control -)	200	–	–	–	–	–	42d-fry
Larvae hatching to 3-month-old fingerling (3m-finger)							
III.1	1500	WzM-L3	larvae	–	–	–	–
III.2	1500	–	larvae	–	–	–	–
III.1.1 [c]	30	WzM-L3	larvae	WzM-L3	3m-finger	EIAG	3m-finger
III.1.2 [c]	30	WzM-L3	larvae	–	–	EIAG	3m-finger
III.2.1 [d]	30	WzM-L3	3m-finger	–	–	EIAG	3m-finger
III.2.2 [d] (Control +)	30	–	–	–	–	EIAG	3m-finger
III.2.3 [d] (Control -)	30	–	–	–	–	–	3m-finger

a and b were derived from group II.1 and group II.2 of the test "Larvae to 42-day-old fry".
c and d were derived from group III.1 and group III.2 of the test "Larvae to 3-month-old fingerling".
"-" not conducted.
* Each group was replicated 3 times.

(pH, NO2, NO3) was checked daily by Sera test kits.

In the trial I, duplicate groups of 15 fingerlings (5–10 g) were immersed in 2 L of WzM-S3 suspension at dilution of 1.5×10^7 CFU mL^{-1} or WzM-L3 suspension at 9.7×10^6 CFU mL^{-1} for 30 min and then transferred to 100 L tanks. Duplicate control groups were immersed in tank water. At 21 days post-vaccination, the fish were challenged with the wild-type *E. ictaluri* strain EIAG at the LD_{50} concentration (equivalent to 6.1×10^5 CFU mL^{-1}) for 30 min.

In the trial II, a group of 1500 pangasius larvae (group II.1) was immersed in 2 L of aerated water containing WzM-S3 at a concentration of 0.9×10^7 CFU mL^{-1} for 30 min before being reared in 500 L tank until reaching 21-day-old fry. A control group of 1500 pangasius larvae (group II.2) was immersed in tank water. At 21 days post initial vaccination, 200 vaccinated fry (subgroup II.1.1) were booster vaccinated the same dose of the *E. ictaluri* mutant WzM-S3 at 1.7×10^7 CFU mL^{-1} for 30 min and then transferred into 100 L tank, while another 200 vaccinated fry (subgroup II.1.2) were not booster vaccinated and continued being reared until the challenge. At 21 days post booster vaccination, fry from the two subgroup II.1.1 and II.1.2, and the control group (group II.2) were challenged with the *E. ictaluri* strain EIAG at a concentration of 5.4×10^5 CFU mL^{-1} for 30 min by immersion. In the trial III, a group of 1500 larvae (group III.1) were immersed with the WzM-L3 mutant at the concentration of 2.3×10^7 CFU mL^{-1} for 30 min, then transferred into 500-L tank and reared until reaching 3-month-old fingerling. A control group of 1500 larvae (group III.2) was immersed in tank water. At 3 months post initial vaccination, 30 vaccinated fingerlings (subgroup III.1.1) were booster immunized with roughly similar dose of the WzM-L3 mutant (1.7×10^7 CFU mL^{-1}) for 30 min, while another 30 vaccinated fingerlings (subgroup III.1.2) were not booster vaccinated and continued being reared until the challenge. In the other hand, a subgroup of 30 3-month-old fingerlings (subgroup III.2.1) from group III.2 was immersed with WzM-L3 at the concentration of 1.7×10^7 CFU mL^{-1} for 30 min, while two other subgroups of 30 control fingerlings (III.2.2 and III.2.3) were immersed in tank water. At the 21 days post booster immunisation, the fish from the subgroups III.1.1, III.1.2, III.2.1 and III.2.2 were challenged with the strain EIAG (1.7×10^6 CFU mL^{-1}) by immersion.

Moribund fish and mortalities were sampled during the immunisation and challenge time. All the vaccination trials were terminated 14 days post challenge.

2.8. Statistical analysis

The SPSS Statistic v.23 (IBM Corporation) was used to generate graphs and analyse for statistical differences. The significant difference on the mortality rates between groups in the attenuation trial was analysed by one-way ANOVA with post-hoc Tukey test. Kaplan-Meier survival curves were generated and a log-rank test was used to compare the survival curves of vaccinated and control groups [33,34]. The RPS values of the vaccination trials were calculated using the following equation [35]:

$$RSP = \left[1 - \frac{\text{average \% mortality of vaccinated fish}}{\text{average \% mortality of unvaccinated fish}}\right] \times 100$$

3. Results

3.1. Sequence analysis

A BLAST result of DNA sequence showed a 100% identical between cloned DNA fragment in pGMET::*wzz*E and Genebank database of a gene (ID 7959810) which encodes for a putative lipopolysaccharide biosynthesis protein in *Edwardsiella ictaluri* 93–146 (CP001600.2), also known as *wzz*E. High identities of *wzz*E sequences were found only in multi-species of *Edwardsiella*, as top hits were identified for *E. piscicida* (95%), *E. anguillarum* and *E. tarda* (94%). However, lower levels of moderate similarity (67%) with low query covers (74%) between *wzz*E nucleotides were observed for other Enterobacteriaceae, eg. *Escherichia coli* O157:H7, *Shigella flexneri*, *Shigella sonnei*. An extraordinary high GC content (63%) of *wzz*E was limitedly shared by *E. tarda* (64%). It could

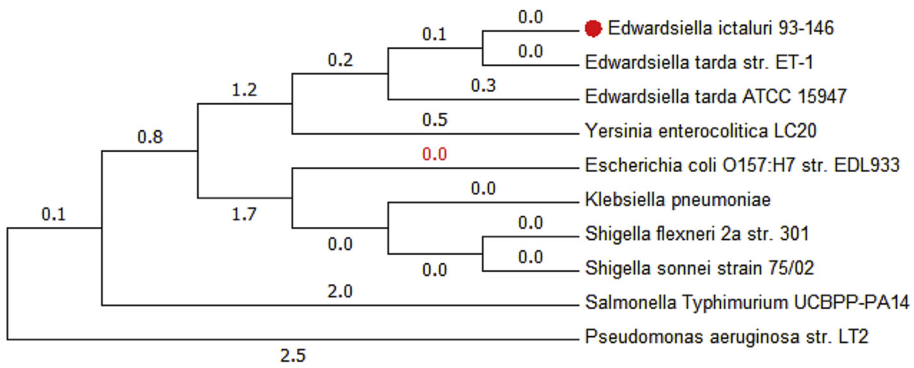

Fig. 1. Evolutionary relationships of *wzz*E. The evolutionary history was inferred using the Neighbour-Joining method [43]. The optimal tree with the sum of branch length = 9.21903024 is shown. (Next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method [44] and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 984 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [45].

be hypothesized that there is a widespread distribution of *wzz*E in multiple bacteria but with a considerable distant evolution, obviously confirmed by a phylogenetic analysis of *wzz*E (Fig. 1S) between *E. ictaluri* and other neighboring bacteria.

WzzE protein showed an insignificant similarity of amino acid sequence between *E. ictaluri* and other Enterobacteriaceae (Fig. 2) as precise homogeneity was discovered at only 59% (203/344 conserved amino acids). While these low scores stood at *E. coli* O157:H7, *Shigella flexneri*, *Shigella sonnei* and *Klebsiella pneumoniae*; higher matching levels were seen in *Yersinia enterocolitica* (63%) and *E. tarda* ATCC (82%). Relevant results of amino acid alignment and phylogenetic tree (Fig. 1S) revealed that WzzE proteins in *E. ictaluri*, *E. tarda* and *Yersinia enterocolitica* may derive from a near ancestor. Although there was a low identity in WzzE residues, predicted secondary structure of *E. ictaluri* WzzE showed a prominant similarity with a published 3D structure of *E. coli* O157:H7 WzzE including: the transmembrane domain from residues Leu330 to Leu339, Pfam family from Asn11 to Gln80 and the tail of periplasmic domain from Phe254 to Arg320 (Fig. 2). These results suggested an indispensable role of conserved head and tail structures of WzzE periplasmic domain among Enterobacteriaceae.

3.2. Construction of the *E. ictaluri wzzE* mutants

Suicide plasmid pGP704 was proved as an efficient DNA transfer tool for creating mutation by conjugation. Double crossing over recombinant was successfully attained to generate two *E. ictaluri* mutant strains WzM-S1 and WzM-L1, which a short *wzz*E 245bp-fragment and a whole sequence of *wzz*E was replaced by *Km*R, respectively. Genotype of WzM-S1 and WzM-L1 strains were confirmed by PCR results (Fig. 3A and B). Electroporation of pCP20 was also found remarkably efficient in eliminating *Km*R which was flanked by two FRT sequences. Therefore, mutant *E. ictaluri* strains WzM-S2 and WzM-L2 were simply created and verified by PCR (Fig. 3C and D).

Although pCP20 is a heat sensitive plasmid, WzM-S2 and WzM-L2 had to be incubated at 37 °C in at minimum 30 h to obtain a complete depletion of pCP20 at a low efficiency (Data not shown). Created colonies WzM-S3 and WzM-L3 which derived from WzM-S2 and WzM-L2 had a uniform appearance as their ancestors, implicating a stability of mutant phenotype.

3.3. Attenuation trials

Both mutant *E. ictaluri wzz*E strains WzM-S3 and WzM-L3 showed a remarkable attenuation as their mortality rates descended to a neglectably low figures of under 6.67% (Fig. 4). Complete attenuation could be observed in WzM-L3 as all immersed fingerlings were found alive and ESC free after 14 days of inoculation. Significant differences ($P < 0.05$) in mortality percentages between entire injected groups of WzM-S3 and EIAG (at 10^6 CFU/fish) or between all immersed mutant groups and wild-type group were illustrated in Table 1S.

Due to significantly higher standard deviations calculated in injection method than immersion (especially in WzM-S3 injection treatment at dose of 1.7×10^7 CFU ml^{-1} in Table 1S), we supposed that immerse administration was more controllable and reliable than injection. Furthermore, mortality rates of EIAG at 3 different immerse concentrations 7.1×10^4, 7.1×10^5, 7.1×10^6 CFU ml^{-1} were found almost equivalent to that of 3 different injection concentrations 1.3×10^4, 1.3×10^5, 1.3×10^6 CFU/fish. This result led us to a preliminary hypothesis that 30 min is an appropriate immersion time for a tenth of total number wild-type *E. ictaluri* which contact to external structures of fingerlings to invade into internal parts. Based on mortality figures, LD50 of the wild-type EIAG was identified at i.m dose of approximately 5.6×10^5 CFU ml^{-1}.

3.4. Vaccine trials

Preliminary vaccine test showed a crucial evidence that both mutant *E. ictaluri* strains had an extraordinarily high protection on fingerlings. After 14 days postchallenge with LD50 of EIAG, high survival rate of WzM-S3 and WzM-L3 were found at 95.24% ($P = 0.00016$) and 95.56% ($P = 0.00015$), respectively. Significant RPS index of these mutant strains were identified at 89.29%–90.00% (Table 2S). Decreased fish were confirmed to contain EIAG after being challenged (data not shown).

Larvae immunized with the mutant WzM-S3 (0.9×10^7 CFU mL^{-1}) showed no significant differences ($P = 0.428$) in survival percent as compared to non-immunized group (Fig. 5A). There was also morderate similarities in development of 21-day-old fry (weight, length) observed in these two groups (data not shown). Therefore, it was briefly suggested that the mutant WzM-S3 was safe for growth of larvae pangasius. Further boosed vaccination with WzM-S3 (1.7×10^7 CFU mL^{-1}) at 42-day-old fry presented considerable efficiacy in vaccine protection as compared to positive control group ($P = 0.039$), even though there was almost indifferent with non-boosted group ($P = 0.068$) (Fig. 5B, Table 2S).

At high challenged dose of LD70, mutant WzM-L3 was validated as a substantially better protection for 3-month-old fingerlings developed from larvae than non-vaccinated fingerlings or once-vaccinated larvae. Almost absolute survival rate and RPS of boosted vaccination group were found at 95.56% and 93.65%, respectively, which were significantly different from positive control group ($P = 0.001$) and once-vaccinated larvae group ($P = 0.009$) (Table 2S). A slightly lower protection level, however with statistical significance as compared to the positive control group, were the once-vaccinated group at 3-month-fingerlings ($P = 0.008$), whose survival rates and RPS were confirmed as high value 75.56% and 65.08%, respectively. Obvious distinction in survival function of these 2 groups was also depicted on survival plot (Fig. 5C).

4. Discussion

Important findings about two avirulent *E. ictaluri wzz* strains could

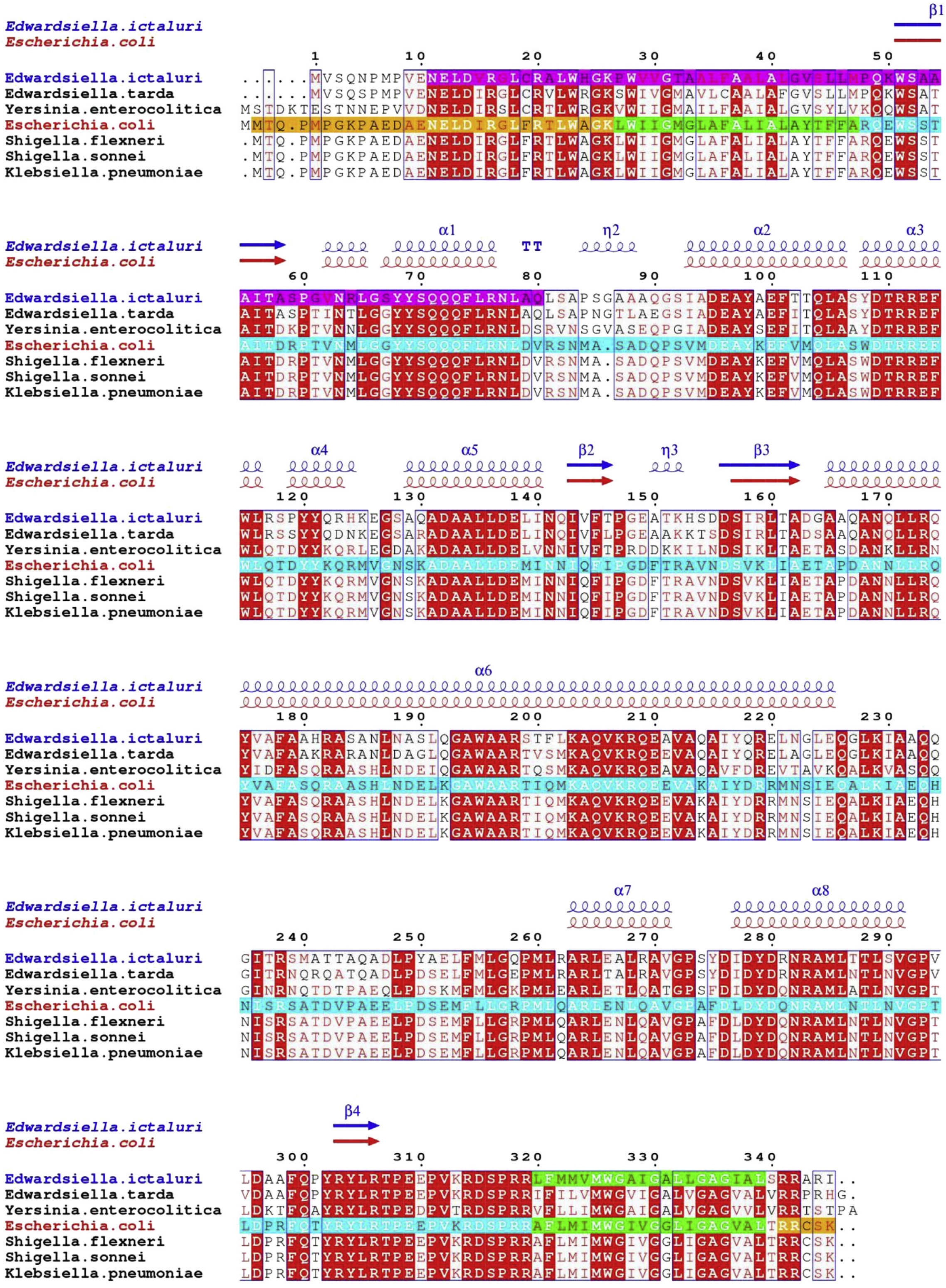

Fig. 2. Conservative domains in WzzE protein structures among multiple *Enterobacteriaceae* including *E. ictaluri*. Comparision between a predicted secondary structure of *E. ictaluri* by SWISS-MODEL [30] and a knowned structure of *E. coli* O157:H7 showed relative similarities in topological contribution (spiral indicates α-helix, arrow indicates β-sheet). Highlighted locations: Red – conserved amino acids, Green – Helical transmembrane, Orange – Cytoplasmic domain, Cyan – Periplasmic domain, Pink – Pfam signature structure. Alignment was constructed by Clustal Omega and web-based biotool ESPript 3.0 [31]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

be learnt from this study. The mutants were not only attenuated but also had a dramatically high protection against wild-type *E. ictaluri*. Homogeneity in attenuation and protection of these strains were witnessed in multiple development stages of pangasius, from larvae to fry and fingerlings.

Minor differences in attenuation and survival function were found between WzM-S3 and WzM-L3, which represented for short and long mutation of *wzz*E gene. In molecular analysis of WzM-S3, short deletion

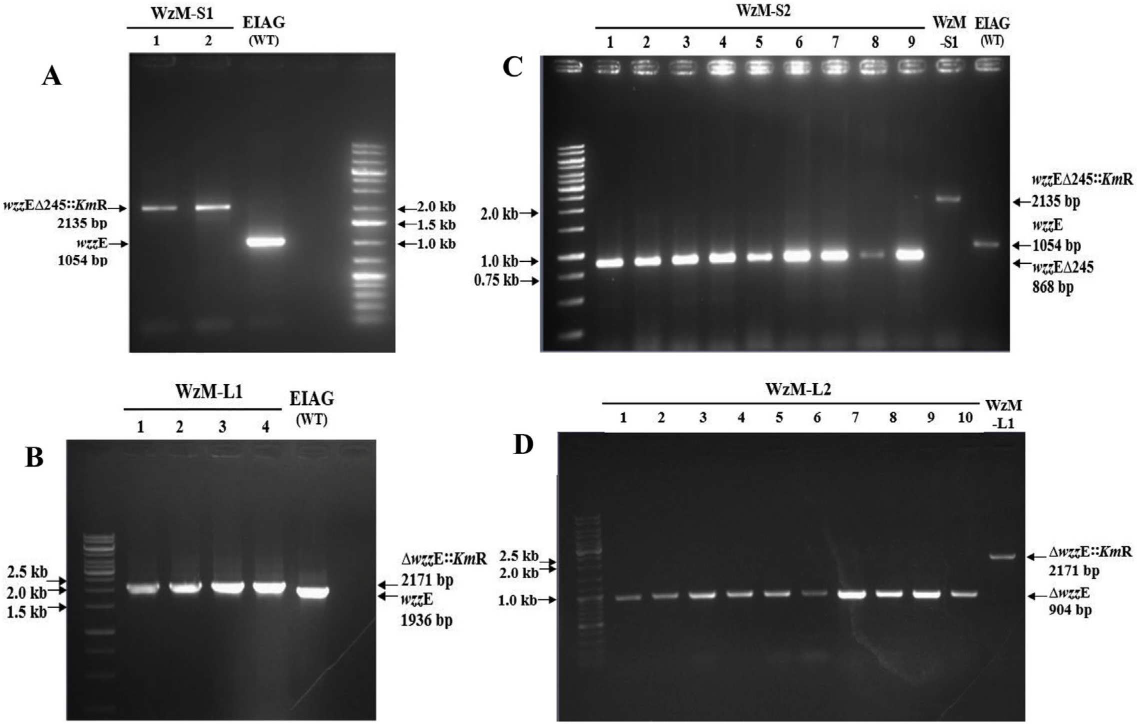

Fig. 3. Genotype confirmation of *E. ictaluri wzz*E. (A) Validation of WzM-S1 by PCR with primers FH*wzz* and RT*wzz*, (B) Validation of WzM-L1 by PCR with primers FU*wzz* and RD*wzz*, (C) Validation of WzM-S2 by PCR with primers FH*wzz* and RT*wzz*, (D) Validation of WzM-L2 by PCR with primers FU*wzz* and RD*wzz*.

of 245 bp which was equivalent to ~81 amino acids over total 345 residues located roughly from residues 130–210 of WzzE protein sequence shown in Fig. 2. The mutation not only eliminated a basic structure (including α5, β2, η3, β3, and α6) inside periplasmic domain, but also it interrupted the thereafter reading frames, completely modified the rest C-terminus domain (including α7 and α8). In addition, resembling results of fish trials between WzM-S3 and WzM-L3 obviously contributed to a suggestion that the removed fragment in the periplasmic domain and the C-terminus transmembrane domain played a fundamental role in the functions of WzzE. Moreover, the mechanism of WzzE was discovered sharing many similarities with Wzz, the regulator of OPS, whose periplasmic domains were previously studied in neighbour Enterobacteriaceae. For that reason, we suggest that both Wzz and WzzE also share related structures and functions. A middle part (residues 153–213) of periplasmic domain in *E. coli* O86:H2 Wzz was identified an olimerization site to create a tetramer of Wzz protein [9]. Oligomerization between periplasmic domains in multiple Wzz proteins was claimed highly crucial for the foundation of a barrel-shape which served as an acting periplasmic sites in Wzz scaffold structure [25]. The α-helix 6 and α-helix 8 at the end of periplasmic domain was considered an stabilizers for Wzz conformational structure of *E. coli* O157:H7 EDL933 and *S. enterica* serovar Typhimurium [36]. However, molecular interactions among WzzE with other proteins in WzyE-dependent pathways to regulate ECA length, or physical relationship between Wzz and Wzy-dependent pathways in the biosynthesis OPS, are not clearly understood.

Inactivation of *wzz*E showed an entire disappearance of ECA chains in *E. coli* K-12 [24]. Full recovery of ECA of this bacterium was only obtained in complementation of *wzz*E and immediately downstream loci including *wec*B, *wec*C and *rml*B located in *wec* cluster, which implied a shared transcriptional unit between these genes. We suppose that a similar manner may apply for *E. ictaluri* due to the same arrangement of *wzz*E and its downstream loci *wec*B and *wec*C. Nevertheless, this assumption need to be confirmed by complementation

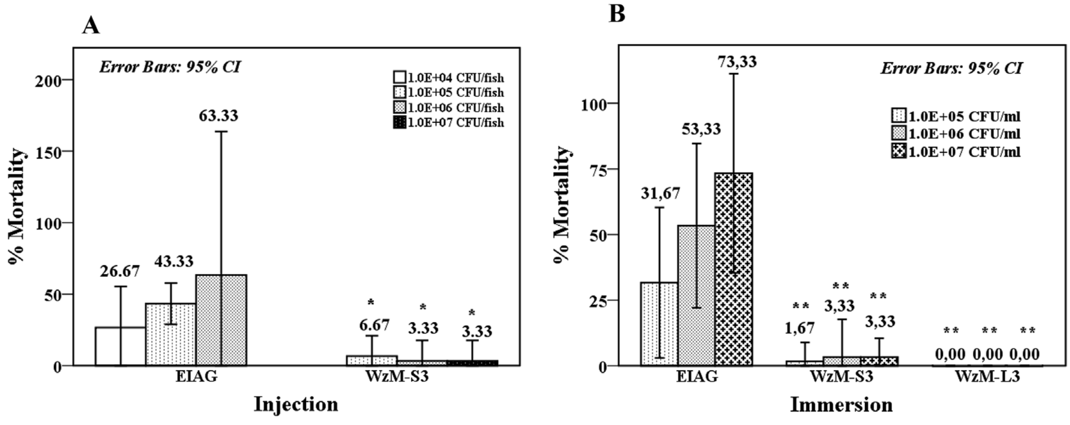

Fig. 4. Attuenuation of mutant *E. ictaluri wzz*E strains. (A) *Mortality percent of* WzM-S3 in comparision to wild-type EIAG by injection routes; (B) Mortality percent of WzM-S3 and WzM-L3 as compared to wild-type EIAG by immersion routes. (*$P < 0.05$, **$P < 0.001$).

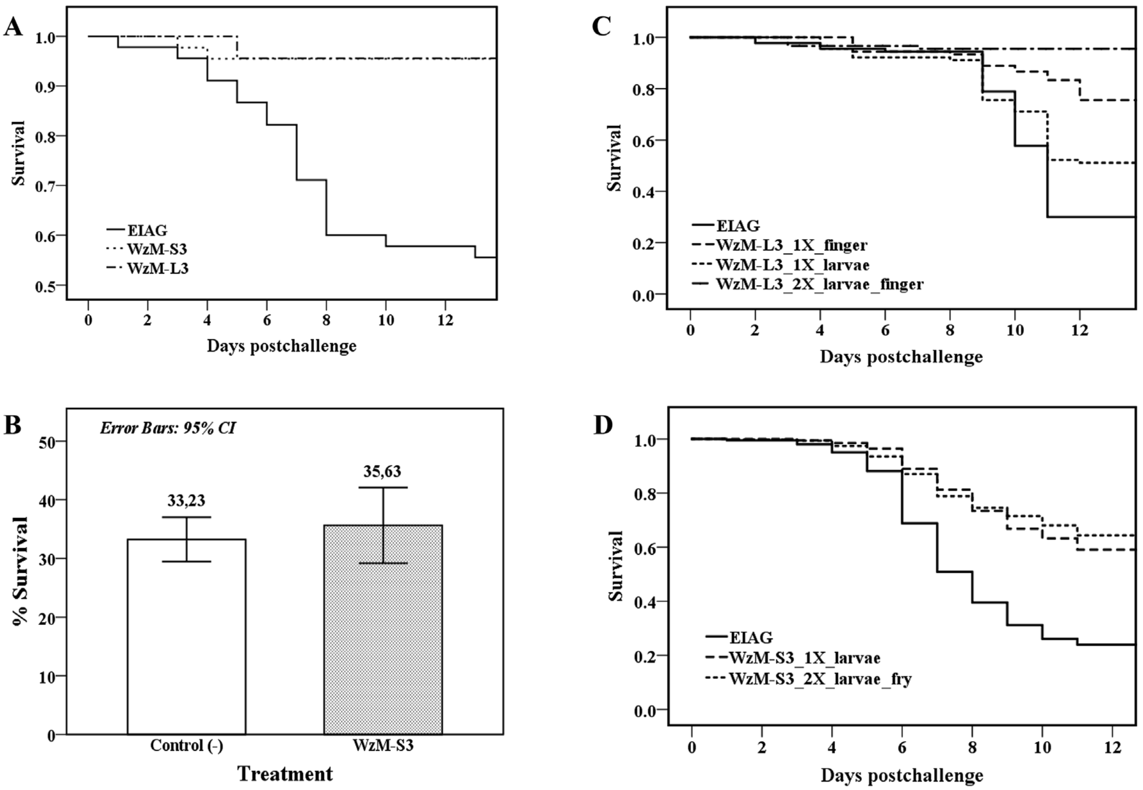

Fig. 5. Survival function of mutant E. ictaluri strains on pangasius from larvae to fingerlings. (A) Preliminary vaccine trial of WzM-S3 and WzM-L3 on fingerlings, n = 43–45. (B) Impact of WzM-S3 on survival of larvae developing to 21-day-old fry, n = 1500, repeated 3 times ($P > 0.05$). (C) Comparision of survival among 3 treatments: once i.m WzM-L3 on larvae (WzM-L3_1X_larvae), once i.m WzM-L3 on 3-month-old fingerlings (WzM-L3_1X_finger), and boosted i.m. WzM-L3 on larvae and 3-month-old fingerlings (WzM-L3_2X_larvae_finger), n = 90. (D) Comparision of survival between 2 treatments: once i.m WzM-S3 on larvae (WzM-S3_1X_larvae), and boosted i.m. WzM-S3 on larvae and 42-day-old fry (WzM-S3_2X_larvae_fry), n = 463–564. Survival curves of all treatments were compared by using log-rank test (Mantel-Cox).

studies of *wzz*E in future research.

Mutant *E. ictaluri wzz*E strains showed an ultimate attenuation but remained high protection for pangasius. Both WzM-S3 and WzM-L3 was avirulent (mortalities under 3.33%) on fingerlings at immersion dose 5×10^6 and 1.2×10^7 CFU ml^{-1}, respectively. On the other hand, survival rates in postchallenge of these mutants were surprisingly high. RPS values of WzM-S3 and WzM-L3 were also as high as 89.29% and 90%, respectively. It can be supposed that the loss of ECA due to the non-functional WzzE protein did not interfer the abilities of invading and triggering immune response of the *E. ictaluri*. Contributing to this hypothesis was related results of a mutant non-ECA *Salmonella enterica* Serovar Typhimurium, who was not only attenuated in oral and intraperitoneal routes, but also remained colonisation in liver and spleen and showed good protection in mouse model [26]. Similar results were also found in inactivated *wzz* regulating OPS chain length. In an experiment on rabbits, less virulent mutant *Y. enterocolitica* O:8 *wzz* showed a dramatically enhanced lymphocytic inflamation and immuno morphological evidences than wild-type strain, [37]. Similarly, LD50 of *P. aeruginosa wzz*1, infected in mouse, was found increased 4.5 times as compared to wild-type [38]. However, an inconsistent result was discovered in *S. typhimurium* when a single-mutant wzz_{ST} strain showed unchanged virulence, while double-mutant wzz_{ST} wzz_{febE} appeared significantly attenuated [17].

Vaccination booster with WzM-L3 at immersion dose of 1.7×10^7 CFU ml^{-1} clearly proved as the most effective protection on hatched fingerlings (RPS = 93.65%, P = 0.001). Furthermore, lower protection in single vaccinated on fingerlings (RPS = 65.08%, P = 0.008) but insignificant protection in single vaccinated on larvae (RPS = 30.16%, P = 0.228) was observed. Indifferent survival rates could also be found between boosted vaccination and single vaccinated on fingerlings (P = 0.264). These evidences gave us to a suggestion that effective vaccination with mutant *wzz* strains may only attain on fingerlings. Protection efficiacy of *wzz* vaccine may decrease substantially after 3 months of hatching from larvae to fingerlings. Similarly, mid-term evaluation on 42-day-fry exhibited meaningful protection of WzM-S3 on boosted-vaccinated fry (P = 0.039) but not on single-vaccinated at larvae (P = 0.068). It was convincible that WzM-S3 vaccination on larvae had limited protective efficiacy regardless the vaccine was safe for larvae hatched into 21-day-old fry, as indifferent survival rates were discovered between vaccinated and non-vaccinated group (P = 0.428). Partly supporting to this opinion was previous research on boosted vaccination. Fry boosted immunized with commercial live vaccine AQUAVAC-ESC™ (RE-33), containing attenuated mutant *E. ictaluri*, was reported to have lower RPS than single vaccination [39]. Other reports of RE-33 oral booster on immersed fingerlings as well as LSU-S1, an attenuated mutant *E. ictaluri aro*A, immersed booster on fingerings both achieved more significantly protection than once vaccination [32,40]. Although our bath immunisation experiments on pangasius showed significantly potential results, further antibody titer examination should be conducted to confirm the link between immersion of *E. ictaluri* Δ*wzz*E and the boosted immune system of pangasius against ESC. Besides, immunized pangasius with WzM-S3 or WzM-L3 are also needed to be challenged with heterologous wild-type strains of *E. ictaluri* to evaluate the protection range of the *wzz*E mutant strains.

In conclusion, WzM-S3 and WzM-L3, two *E. ictaluri wzz*E mutants, were obviously avirulent strains, which is safe for different developmental stages from larvae to fingerings pagansius. At immersion dose of 10^7 CFU mL^{-1}, both strains showed dramatically protection on fingerlings. Due to the full knock-out wzz gene, WzM-L3 was more considerably potential to be an effective and safe vaccine.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.005.

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