Construction and characterization of a *Vibrio cholerae* serogroup O139 vaccine candidate by genetic engineering

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Abstract. The present study aimed to construct and evaluate the live attenuated *Vibrio cholerae* serogroup O139 vaccine candidate, in which genes encoding protective antigens were integrated into the chromosomal DNA. Using the initial strain, O139-ZJ9693, the toxin-linked cryptic (TLC) and cholera toxin (CTX) genetic elements and repeats in the toxin (RTX) gene cluster were deleted from its chromosomal DNA, and the cholera toxin genes, *ctxB* and *rstR,* were transferred into the chromosome to construct the candidate vaccine strain. The expression of *ctxB* and the vaccine virulence were then examined. Polymerase chain reaction (PCR), enzymatic digestion and electrophoresis were performed to confirm that TLC, CTX and RTX were deleted, and that *ctxB* and *rstR* were transferred into the vaccine candidate DNA. According to the preliminary evaluation, the *ctxB* gene exhibited cholera toxin subunit B expression, and no enterotoxigenic or cytotoxic effects were observed in this strain. In conclusion, a recombinant strain containing genes encoding protective antigens that replaced virulence-associated genes was successfully constructed in the present study; this candidate strain may have the potential to be utilized to further evaluate the immune response.

Introduction

Cholera has been widely studied over the past several decades (1). Following the development of gene recombination technology and awareness of the importance of intestinal immunity, orally recombinant live cholera vaccines have been investigated (2). A clinical episode of cholera induces long lasting protection (at least three years) (3). Therefore, an orally recombinant live vaccine that simulates natural cholera infection and, thus, protection is warranted.

At present, numerous orally administered cholera vaccines are being investigated in large-scale clinical studies worldwide (4,5). However, the residual virulence of the vaccine strains has affected the clinical application of these vaccines. The three key virulence factors of *Vibrio cholerae* are the cholera toxin (CTX) genetic element, the Vibrio pathogenicity island (VPI) and the repeats in toxin (RTX) cluster (6). CTX, RTX and the toxin-linked cryptic element (TLC) are the chief virulence gene clusters and are grouped together. Thus, they are able to be deleted together through homologous recombination in order to construct a suitable candidate vaccine strain.

The O1 or O139 groups of cholera vaccines do not exhibit cross-protection. Thus, it is necessary to construct a special or bivalent vaccine. In the present study, a *V. cholerae* serogroup O139 vaccine was constructed and evaluated using genetic engineering.

Materials and methods

Bacterial strains, plasmids and cultural conditions. The live attenuated cholera vaccine strain utilized in this study was based on the *V. cholerae* serogroup O139-ZJ9693, a virulent strain. The bacterial strains and plasmids used in this study are described in Table I (7,8). Sucrose medium (SM; 1% tryptone, 0.5% yeast extract, 1.5% agar powder and 10% sucrose) and AKI medium (1.5% peptone, 0.4% yeast extract, 0.5% NaCl and 0.3% $NaHCO_3$) were used in the study. Antibiotics were utilized as follows: Ampicillin (Amp), 100 μg/ml and chloromycetin (Cm), 30 μg/ml for *Escherichia. coli* and 15 μg/ml for *V. cholerae*. These reagents were purchased from Oxoid (Oxoid, Ltd., Basingstoke, Hampshire, UK).

Construction of the O139 vaccine candidate. The construction process for the O139 vaccine candidate consisted of two steps. Initially, recombinant suicide plasmids were constructed using genetic engineering (Fig. 1). Polymerase chain reaction (PCR) was performed using the primer pairs stated in Table II

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Table I. Bacterial strains and plasmids used in the present study.

Strains and plasmids	Characterization	Source
Vibrio cholerae		
N16961	*V. cholerae* O1$^{El\ Tor}$, CTXΦ^{+}	Laboratory Medicine Center
O139-ZJ9693-1	ZJ199693 ΔTLC-CTX-RTX: :*cat*	Present study
NFYY101	ZJ199693 ΔTLC-CTX-RTX: :*ctxB+rstR*	Present study
Escherichia coli		
JM109	*recA1supE44 endA1 hsdR17thi* Δ(*Lac-proAB*) F' (*traD36 proAB*$^{+}$ *LacIq LacZ* Δ*M*15)	Laboratory Medicine Center
SM10λ*pir*	*supE* , *recA*: :*RP4-2-Tc*: :*Mu*, Km^{r}λ*pir*	Reference (7)
Plasmids		
pUC18	Clone vector, *oriMB1*, *lacZ*$^{+}$, Amp^{r}	Laboratory Medicine Center
pUC18-TLCup	pUC18 : :TLCup, Amp^{r}	Present study
pUC18-TLCup-RTXdown	pUC18-TLCup: :RTXdown, Amp^{r}	Present study
pUC18-TLCup-*cat*-RTXdown	pUC18-TLCup-RTXdown: :*cat*, Amp^{r}, Cm^{r}	Present study
pDS132	Suicide plasmid, *mob*, *ori*, *sacB*, Cm^{r}	Reference (8)
pDS132-TLCup-*cat*-RTXdown	pDS132: :TLCup-*cat*-RTXdown, Cm^{r}	Present study
pUC18-TLCup-*rstR-ctxB*-RTXdown	pUC18-TLCup-RTXdown: : *rstR*, *ctxB*, Amp^{r}	Present study
pCVD442	Suicide plasmid, *mob*, *ori*, *bla*, *sacB*, IS1, Amp^{r}	Reference (8)
pCVD442-TLCup-*rstR-ctxB*-RTXdown	pCVD442: :TLCup-*rstR-ctxB*-RTXdown, Ampr^{r}	Present study

TLC, toxin-linked cryptic element; CTX, cholera toxin genetic element; RTX, repeats in the toxin gene cluster.

Table II. Primers used in the present study.

Primer	Primer sequence (5'-3')	RE site
TLCup-F	CAGGAGCTCATCCGCAACGTATTCCCACACC	*Sac*I
TLCup-R	TGGGGTACCTGCTCCGAGTTATTTCGAAACC	*Kpn*I
RTXdown-F	GCTCTAGATGACTCATGACCCAATG	*Xba*I
RTXdown-R	ACGCGTCGACATCACACGTCGTTTATC	*Sal*I
cat-F	CGTAGCACCAGGCGTTTAAG	-
cat-R	GATCGGCACGTAAGAGGTTC	-
rstR-F	CCGAATTCACTCACCTTGTATTCG	-
rstR-R	CGGAATTCTCGACATCAAATGGCATG	-
ctxB-F	AGTTCCATGGGGCAGATTCTAGACCTC	*Xba*I
ctxB-R	GATCTAGACGGTTGCTTCTCATCATCG	*Xba*I

RE sites are underlined. TLC, toxin-linked cryptic element; RTX, repeats in the toxin gene cluster; RE, restriction endonuclease.

to amplify the corresponding gene fragments, and plasmids treated with specific enzymes were utilized to construct recombinant plasmids. pDS132-TLCup-*cat*-RTXdown and pCVD442-TLCup-*rstR-ctxB*-RTXdown served as the recombinant suicide plasmids.

Following the construction of the recombinant suicide plasmids, a vaccine candidate was constructed by homologous recombination (Fig. 2). SM10λ*pir* carrying pDS132-TLCup-*cat*-RTXdown was utilized as the donor strain to transfect O139-ZJ9693. Transconjugants were propagated on lysogeny broth (LB) agar plates overnight at 37˚C, and then cultured on SM containing Cm. The recombinant strain, O139-ZJ9693-1, was thereby obtained.

A process similar to the aforementioned method was utilized to construct the NFYY101 candidate. O139-ZJ9693-1 was utilized as the recipient bacterium to transfect with SM10λ*pir* carrying pCVD442-TLCup-*rstR-ctxB*-RTXdown. The transconjugants were cultured on SM and then streaked

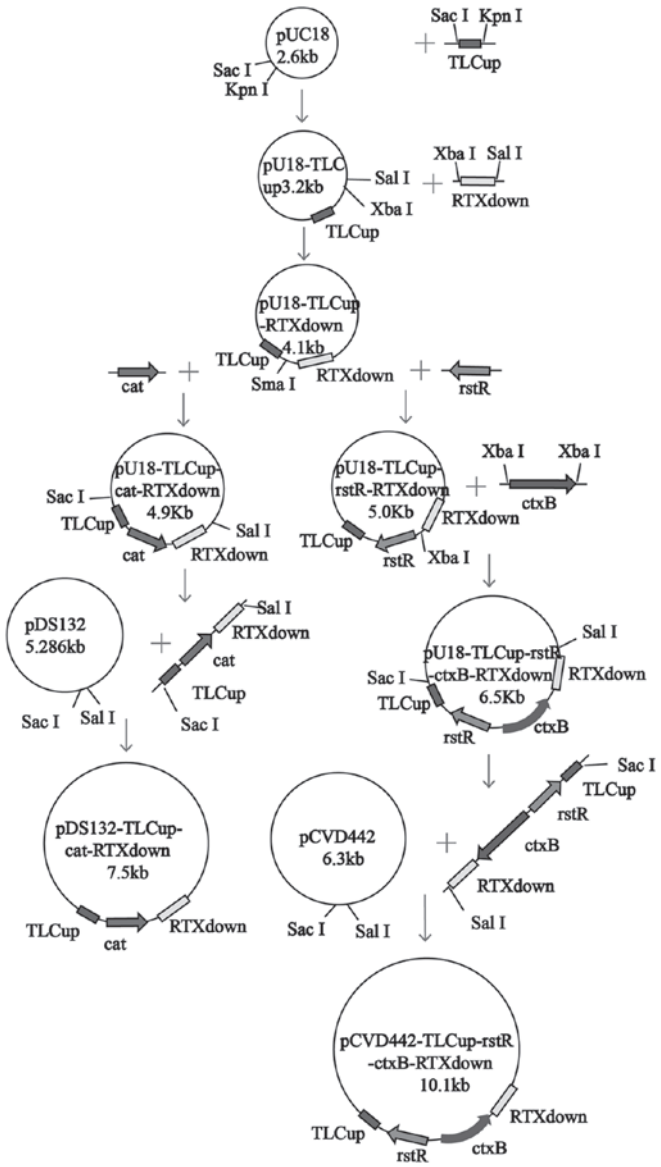

Figure 1. Schematic diagram of the construction of recombinant suicide plasmids. TLC, toxin-linked cryptic element; RTX, repeats in the toxin gene cluster.

on general and Cm-resistant medium, respectively. The colony that exhibited growth in general medium only, as identified by PCR, was the O139-ZJ9693-2 candidate and was then named NFYY101.

Monosialotetrahexosylganglioside (GM1)-ELISA. Cholera toxin B subunit (CTB) protein exhibits the ability to bind GM1. Thus, CTB expression of the recombinant strain that re-acquired *ctxB* can be detected by ELISA (R&D Systems Inc., Minneapolis, MN, USA). CTB extraction, preparation and analysis were conducted as described previously (9). A 96-well plate was coated with 100 μl GM1 (2 μg/ml; Sigma, St. Louis, MO, USA) per well, incubated overnight at 4˚C, and then washed three times with phosphate-buffered saline (PBS)-Tween-20. Each well was blocked with 3% bovine serum albumin (300 μl/well) for 1 h at 37˚C and then washed as described for the previous step. The supernatant of the cell lysates of JM109, O139-ZJ9693, O139-ZJ9693-1 and NFYY101

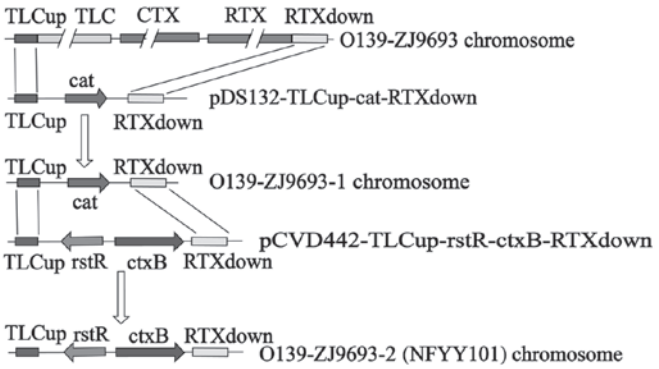

Figure 2. Schematic diagram of the construction of O139-45 by homologous recombination. O139-ZJ9693 carrying TLC, CTX and RTX was from the Laboratory Medicine Center, Nanfang Hospital, Southern Medical University. O139-ZJ9693-1 carried the *cat* gene with TLC, CTX and RTX clusters deleted. O139-ZJ9693-2 was the candidate vaccine named NFYY101. TLC, toxin-linked cryptic element; CTX, cholera toxin; RTX, repeats in the toxin gene cluster.

was added to triplicate wells (200 μl/well), and the plate was incubated at 37˚C for 1 h and then washed as described in the previous step.

The plate was then incubated with a 200 μl 1:1,000 dilution of polyclonal mouse anti-CT antiserum (Sigma) per well for 1 h at 37˚C and then washed as described for the previous step. This step was repeated following addition of a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Tetramethyl benzidine was added (100 μl per well). After 15-30 min, the reaction was blocked by the addition of 2 M H_2SO_4. The absorption was assessed at 492 nm. Results were considered positive when the ratio of the sample to the control was ≥2.

Cytotoxic effect assay. Fresh mammalian Hep-2 cells [10^5 colony forming units (CFU)/ml] were added to a 24-well cell culture plate, and fresh RPMI-1640 (Gibco) containing 10% fetal bovine serum (Gibco) without antibiotics was added. N16961, O139-ZJ9693 and NFYY101 were cultured overnight at 37˚C, washed twice with PBS and diluted to achieve 10^9 CFU/ml. This was followed by addition of 10 μl liquid containing bacteria to the wells of the cell culture plate (three wells per strain) and the plate was incubated at 37˚C with 5% CO_2 for 2 h. The cytotoxic effects were then observed and documented.

Ligated ileal loop toxin test in rabbits. N16961, O139-ZJ9693, O139-ZJ9693-1 and NFYY101 were cultured overnight in LB. In total, 10 adult New Zealand White rabbits (weight, 2 kg; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were fasted (water *ad libitum*) for 24 h, and then their abdomens were opened. The ilea of the rabbits were tied into 4-5-cm-long loops with 1-cm intervals, and the four above strains (10^6 CFU) and normal saline were injected into each loop, respectively. Saline solution was used for the negative control and N16961 was used as the positive control. The rabbits were sacrificed following closure of the abdomen for 16-18 h. Congestion and effusion in the ileum segments were then assessed. The present study was approved by the Medical Ethics Committee of Nanfang Hospital, Southern Medical

Table III. Test results by microplate reader.

Samples	OD_{492nm}	P/N
O139-ZJ9693	0.396±0.090	5.74
NFYY101	0.368±0.051	5.33
JM109-pUC18-TLCup-*rstR-ctxB*-RTXdown (1:5)	0.409±0.011	5.93
O139-ZJ9693-1	0.072±0.010	1.04
JM109	0.065±0.003	0.942
PBS	0.069±0.002	-

Results for OD_{492nm} are presented as the mean ± standard deviation. Positive results were determined if the ratio of the sample to the negative control [P/N, patient (sample)/negative] was ≥2. PBS buffer was detected as a negative control. TLC, toxin-linked cryptic element; RTX, repeats in the toxin gene cluster; PBS, phosphate-buffered saline; OD_{492nm}, optical density at 492 nm.

Figure 3. Toxin test results of the ligated ileal loop of rabbits: (1) N16961, 10^8 CFU/ml; (2) N16961, 10^6 CFU/ml; (3) O139-ZJ9693, 10^8 CFU/ml; (4) O139-ZJ9693, 10^6 CFU/ml; (5) O139-ZJ9693-1, 10^8 CFU/ml; (6) O139-ZJ9693-1, 10^6 CFU/ml; (7) NFYY101, 10^8 CFU/ml; (8) NFYY101, 10^6 CFU/ml; (9) Normal saline (solution of 0.9% w/v of NaCl). CFU, colony forming units.

University (Guangzhou, Guangdong, China) and conducted in compliance with the Declaration of Helsinki.

Results

Construction and characterization of O139-ZJ9693-1 and NFYY101. The O139-ZJ9693-1 and NFYY101 serum agglutination tests were positive and the biochemical reactions of each were identical to those of O139-ZJ9693. TLCup-*cat*-RTXdown was detected by PCR and confirmed by sequencing in O139-ZJ9693-1; *rstR* and *ctxAB* were not detected in the strain.

TLCup-*rstR-ctxB*-RTXdown of NFYY101 was detected by PCR and sequenced. The sequencing results were used to perform sequence alignment with *rstR* and *ctxB* of N16961; the open reading frame consistency was verified for both. These findings indicated that *rstR* and *ctxB* were successfully cloned and integrated into the chromosome of the vaccine candidate strain, NFYY101, as these genes are conserved in *V. cholerae*.

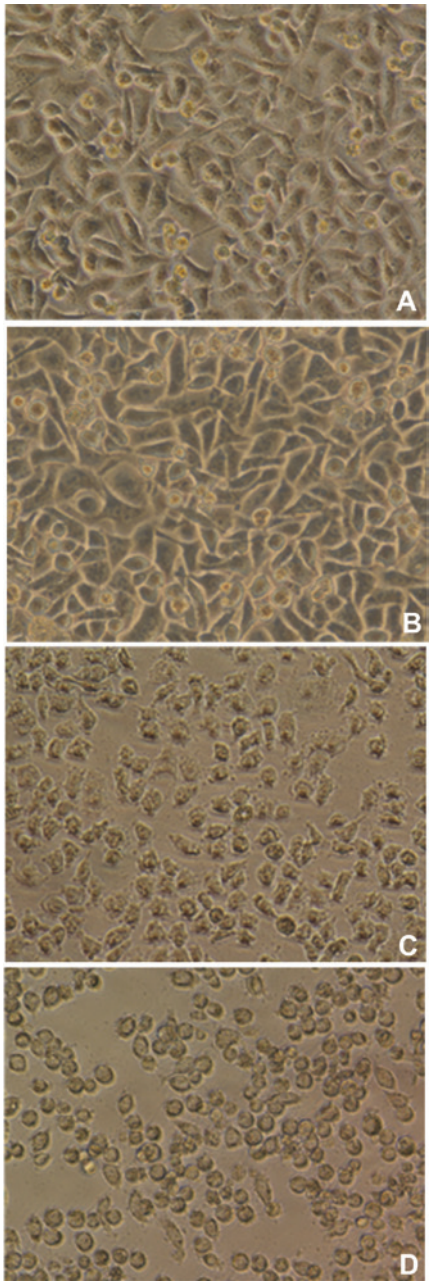

Figure 4. Cytotoxicity of NFYY101, O139-ZJ9693 and N16961 strains to Hep-2 cells (magnification, x10). Cells were incubated with (A) PBS, (B) NFYY101, (C) O139-ZJ9693 and (D) N16961 in cell culture wells. PBS, phosphate-buffered saline.

Results of ligated ileal loop toxin test. CT is encoded by the *ctxAB* gene and is the most important factor to cause cholera. The presented study demonstrated that the ileal loop containing N16961 and O139-ZJ9693 strains exhibited marked congestion and effusion, whereas no similar effect appeared in any of the other ileal loop specimens (Fig. 3). This phenomenon indicated that the deletion mutant O139-ZJ9693-1 and vaccine candidate NFYY101 were attenuated and that the chief toxin *ctxAB* gene was deleted.

Cytotoxic effect assay. Cell rounding was observed following co-incubation of Hep-2 cells with N16961 and O139-ZJ9693, whereas no similar phenomena were observed following addition of NFYY101 and PBS (Fig 4). This finding correlates

with the theoretical cytotoxicity of *rtxA* and toxin activation of *rtxC*.

Determination of ctxB expression by GM1-ELISA. GM1-ELISA analysis revealed that CTB was expressed in the O139-ZJ9693, JM109 carrying pUC18-TLCup-*rstR*-*ctxB*-RTXdown and NFYY101 strains, and absent in O139-ZJ9693-1 and JM109 (Table III). Results of the GM1-ELISA analysis indicated that *ctxB* was expressed in the NFYY101 vaccine candidate.

Discussion

Gene-knockout technology was first developed in the late 1980s and has exhibited the potential for broad application in the life sciences (10). Homologous recombination is one method of gene-knockout. Factors that affect the efficiency of homologous recombination include the length of the two end fragments of the target gene, the presence of the vector linearization-knockout and the position and structure of the target genes (11). The length of TLCup (562 bp) and RTXdown (888 bp) facilitates PCR and successful fusions. Using the 'upstream fragment-inserted gene-downstream fragment' model allows for the avoidance of the polar effects of strain and inactivates the target genes completely (12).

The suicide plasmid is an effective carrier that builds a seamless mutant through homologous recombination, precisely positioning the integration site using homologous fragments at the two ends of the target gene. The pDS132 in the present study was from pCVD442, with the insertion sequence 1 element removed and the *bla* gene replaced with the *cat* gene. The recombination efficiency of pDS132 is higher than that of pCVD442. The *sacB* gene contained in pDS132 encodes fructosan-sucrase, which induces sucrose to hydrolyze into fructosan, a toxic compound fatal to a number of Gram-negative bacteria. Thus, the deletion mutant was selected in the present study using SM supplemented with chlormycetin. This strain is able to grow on SM with chlormycetin as *cat* specifically replaces the target gene by homologous recombination and the pDS132 vector is then ablated.

The *cat* gene replaced the long segment (39 kb) between TLC and RTX; thus, the TLC, CTX and RTX clusters were deleted from O139-ZJ9693 through homologous recombination. O139-ZJ9693-1 was obtained using the aforementioned process. According to previous studies of O1, the CTX element is derived from the bacteriophage CTXΦ and is likely to be transferred among diverse *V. cholerae* strains in the environment (13). CTXΦ is composed of two parts, a core region of 4.6 kb and a repeat sequence region. The core region harbors six genes, including the *ctxAB* gene, which encodes the CT. TLC is located upstream of the CTX element, and its function is associated with the acquisition and duplication of CTXΦ. RTX is located downstream of the CTX element and is associated with cytotoxicity; its activity is independent of CTX, as it belongs to the RTX toxin family.

The construction of the suicide plasmid was continued by replacing the *cat* gene with the *rstR* and *ctxB* genes in O139-ZJ9693-1. O139-ZJ9693-1 carries Cm resistance and pCVD442 exhibits ampicillin resistance, whereas pDS132 exhibits Cm resistance, therefore the recombinant suicide plasmid pCVD442-TLCup-*rstR*-*ctxB*-RTXdown was constructed and then conjugated with O139-ZJ9693-1. The conjugant was streaked onto SM, and then the strains were transferred to the general medium and to the plate with Cm. The colony growing exclusively in the general medium was the candidate strain, NFYY101; it was identified and confirmed by PCR.

The *rstR* gene in the RS2 region of the CTX element encodes RstR, a repressed protein that mediates CTXΦ immunity via repression of *rstA* and *rstB* expression. The immunity maintains the lysogenized state of the strain; however, it represses the duplication of DNA in superinfection with CTXΦ (14-16). This type of CTXΦ-immunity is biotype-specific (14), therefore the present study utilized the *rstR* gene of El Tor type to prevent El Tor-derived CTXΦ (CTXETΦ) in the globally epidemic OEl Tor strain from potentially infecting the novel vaccine strain that was developed. This step enhanced the biological safety of the candidate vaccine strain.

The virulent CTB subunit encoded by *ctxB* conjugates ganglioside GM1 with close affinity in cellular membranes of karyocytes, including epithelial cells, lympholeukocytes and antigen-presenting cells (APCs). CTB is able to adjust the APC reaction, the T-cell reaction, antibody production and immune activation, and it also exhibits high immunogenicity that can induce the production of antibodies against CTB to efficiently prevent cholera. Therefore, it is necessary to integrate *ctxB* into the cholera vaccine (17).

Virulent *V. cholerae* carrying CT can cause congestion and effusion in the ileal loop of rabbits (18). The toxin test was used to investigate the effect of homologous recombination. The reaction of the ileal loop to CT is highly variable among individuals; thus, a minimum of two rabbits was necessary. A control in the ligated ileal loop of every rabbit was also used. The test results indicated that O139-ZJ9693-1 and NFYY101 were markedly attenuated, and that the chief toxin gene *ctxAB* was deleted from the two strains.

In the TLC-CTX-RTX clusters, the independent-activity RTX toxin encoded by the RTX gene, one of the virulence factors, causes actin cross-linking and cell rounding (19). In the cytotoxic effect assay, N16961 and O139-ZJ9693 caused Hep-2 rounding, whereas O139-ZJ9693-1 did not. This result indicated that O139-ZJ9693-1 without the RTX cluster loses cytotoxicity against Hep-2.

It has been previously reported that RTX in *V. cholerae* is only excreted by the type I secretory system during the growth phase, but not in the dormant phase (20). Thus, the cytotoxic effect assay in the present study required fresh strains able to react with Hep-2. Additionally, as N16961 grows faster than O139-ZJ9693, the cell rounding is more obvious in N16961 during the same period of culture.

In the present study, NFYY101, a novel *V. cholerae* O139 live attenuated vaccine candidate strain was constructed and characterized. Together, the results demonstrate that NFYY101 is attenuated and *ctxB* is highly expressed in the recombinant chromosomal DNA. No enterotoxigenic or cytotoxic effects were observed in this strain. A recombinant strain with the protective antigen genes that replaced virulence-associated genes was successfully constructed; this candidate strain could potentially be utilized to further evaluate immune response. Further evaluation of the stability of the vaccine is warranted,

as well as further investigation regarding its ability to protect against CTXΦ infection *in vivo* (21,22).

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