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Development of a recombinant vaccine against foot and mouth disease utilizing mutant attenuated *Listeria ivanovii* strain as a live vector

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ABSTRACT

The drawbacks of conventional inactivated Foot and Mouth Disease (FMD) vaccine, such as escaping of the virus during manufacture processes prompted researchers to explore novel types of vaccine to overcome these disadvantages. *Listeria ivanovii* (LI) is an intracellular microorganism that possesses immune-stimulatory properties, making it appropriate for use as a live bacterial vaccine vector. The *Foot and mouth disease virus* (FMDV) VP1 protein is the most immunogenic part of FMDV capsid, it has most of the antigenic sites for viral neutralization.

The expression of antigen gene cassette *in vitro* was confirmed by Western blot analysis. Mice were able to eliminate LI△*actAplcB-vp1* from the liver and spleen within few days revealed a safety of the candidate vaccine. Two doses of LI△*actAplcB-vp1* with 14 days of interval were injected into mice. High levels of specific IgG antibodies and $CD8^+$ and $CD4^+$ T cells secreted cytokines including IFN-γ, TNF-α and IL-2 against FMDV-VP1 were achieved. Based on the obtained results, LI△*actAplcB-vp1* candidate vaccine utilizing *Listeria ivanovii* as a live vector-based vaccine could enhance a specific cellular and humoral immune responses against the inserted FMDV-*vp1* heterologous genes. LI△*actAplcB-vp1* candidate vaccine could be a modern tool to overcome the disadvantages of the traditional inactivated FMD vaccine.

1. Introduction

Unlike other diseases, Foot and Mouth Disease (FMD) is one of the most destructive diseases of domestic livestock due to its highly contagious, causing severe effects on a broad range of domestic animals (Phologane et al., 2008). FMD causes severe economic impact since it causes losses of production, reduced milk yields, abortions and hindering trade of animals locally and internationally (James and Rushton, 2002). *Foot and mouth disease virus* (FMDV) is a member of Family *Picornaviredea*, Genus *Aphthovirus*, has seven immunological serotypes with no cross-protection presented among the serotypes (Mason et al., 2003). FMDV has a positive sense, single-stranded RNA genome which encodes for twelve proteins, four of them are structural including VP1, VP2, VP3 and VP4 form the capsid of the virus and the other eight proteins are non-structural (Martinez-Salas et al., 2008). The FMDV-VP1 protein has the main role in the neutralization of the virus particle since it includes both B and T cell epitopes (Yang et al., 2005). Eradication and control of FMD mainly depended on vaccination of most susceptible domestic animals. The conventional inactivated FMDV vaccines still considered the preferred process for controlling this disease (Sutmoller et al., 2003). The inactivation FMD vaccines have several shortages such as improper viral inactivation; the vaccine antigen needs a sequential purification process to allow the differentiation between vaccinated and infected animals; in addition, it couldn't minimize the shedding of FMDV since it needs a long time to the outset of immunity (Doel, 2003). In order to address these shortcomings, alternative methods were used to perform a novel FMD vaccine, including subunit or peptide vaccines that are delivered directly or through a live vector (Taboga et al., 1997; Wong et al., 2000). The live vector delivery system is the preferable tool to construct an alternative vaccine. The main concept behind this method is utilizing the bacterial or viral vectors to get the benefits from its ability to induce infection and its

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immunological properties to induce an immune response to its own proteins and heterologous inserted protein (Shata et al., 2000). The live bacterial vectors have several advantages such as it more like adjuvant which make it appealing structures for heterologous antigen presentation. Besides, the bacterial vectors can elicit humoral and cellular immune response, also most of them are sensitive to the antibiotic, so allow antibiotic treatment in case of any adverse reaction (Perrie et al., 2008). Several bacterial strains were used as vectors, like *Mycobacterium bovis, Listeria monocytogenes* (LM), *Listeria ivanovii* (LI), and *Salmonellae*, to elicit immune responses against heterologous inserted genes (Bruhn et al., 2007; Bastos et al., 2009). LI is a facultative intracellular bacterium, it has numerous immunological advantages; such as its ability to multiply in phagocytic cells and induce specific IFN-γ secretion from the activated CD8 T cells that make it attractive to be a live bacterial vector (Alvarez-Ordonez et al., 2015; Zhou et al., 2016). Our research team conducted a previous study related to the role of both LM and LI as a vaccine vector platform for heterologous inserted bacterial genes, the obtained results revealed a promising new bacterial carrier platform (Wang et al., 2014; Lin et al., 2015a,Lin et al., 2015b).

In this study, we constructed a novel recombinant FMD candidate vaccine utilizing the most immunogenic coding region (*vp1*) and attenuated mutant LI strain (LI△*actAplcB-lacZ*) (Wang et al., 2014) to build a stable homologous recombinant strain through chromosomal integration in the site-specific direction in LI chromosome with evaluation to its safety and potency.

2. Materials and methods

2.1. Bacterial vector strain and plasmids

The bacterial vector strain and plasmids utilized in the current study were illustrated in Table 1. The experiment was designed as shown in (Fig. 1).

2.2. Construction of target gene cassette

The pCW203 plasmid was digested with *Hin*dIII and *Xho*I restriction enzymes (TaKaRa Biotechnology, China) to perform a linearized plasmid. In order to generate pCW203-*vp1*, distinctively designed primers were planned which included complementary sequences of both terminal ends of linearized pCW203 plasmid and *vp1* gene (sense primer 5′-GAGAAGTGAAAGACCACAAGCTTTGACAACAAGTACTGGTGAAT-3′ and anti-sense primer 5′-CGATGCGGCCGCTCGAGTTGTTTTACTGGAGCAACAATTT-3′).

The composed FMDV-*vp1* fragment attached to the complementary sequence of both terminal ends of linearized pCW203 plasmid was mixed with the linearized pCW203 plasmid at the restriction sites for ligation with overlapping manner utilizing Sosoo cloning kits (Tsingke company, China) at 50°C for 15 min. (Fig. 2a)

In order to generate the integrative plasmid (pCW154-*vp1* including the target gene cassette), pCW154 plasmid and pCW203-*vp1* plasmids were digested with *Xho*I, *Bam*HI and *Hin*dIII, *Xho*I restriction enzymes respectively to perform a linearized pCW154 plasmid and *vp1* fragment fused with (*HA*) gene. Amplification of *(vp1-HA)* fragment was achieved utilizing specially designed primers (forward primer: 5′-TATAATTTTGCTACTATGAAGGATCCATACCATATGATGTTCCAGATTATG-3′, and reverse primer: 5′-CTGGTCCATTTAATCCCTCGAGTTGTTTTACTGGAGCAACAATTT-3′). *(vp1-HA)* the fragment was ligated with pCW702 plasmid by overlapping utilizing Sosoo cloning kits at 50°C for 15 min. (Fig. 2b)

2.3. Chromosomal integration of gene cassette

The integrative pCW154-*vp1* plasmid was introduced to the mutant attenuated LI competent cells by electroporation following by cultivation on BHI agar. Presumptive positive blue colonies with erythromycin sensitive were collected. After 2 successive passages (42°C for 48 h) in BHI plates with erythromycin (3 μg/ml), putative integrates blue colonies were grown in BHI broth media for 6 generations (30°C for 24 h). Serial dilutions from the 6th generation were cultivated in BHI agar plates with IPTG and X-gal without erythromycin to monitor the successful constructs. The white colonies with erythromycin sensitivity were distinguished as positive, successful construction (LI△*actAplcB-vp1*). Then it was confirmed by PCR and gene sequencing. (Fig. 3)

2.4. Analysis of the expressed recombinant protein

LI△*actAplcB-vp1* recombinant strain was cultivated in BHI media overnight, then centrifuged at 13,000 rpm for 10 min at 4 °C. Trichloroacetic acid (TCA) was used for precipitation and concentration of secreted protein while sonication and TCA were used for non-secreted protein precipitation. For separation of proteins, SDS-PAGE and electrophoresis at 100v/90 min were used. Thereafter separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA) through transfer buffer at 200 mA for 1.5 h. After blocking, PVDF membrane was incubated with HA monoclonal primary antibodies (1:5000) (Sigma, USA), then incubated with horseradish peroxidase conjugated (HRP) secondary antibodies (1:1000) (Beyotime Institute of Biotechnology, China). The signals were developed by the enhanced chemiluminescent substrate (Super Signal West Pico Trial kit, Thermo Scientific).

2.5. Determination of bacterial load in internal organs

In order to evaluate the bacterial load of LI△*actAplcB-vp1* recombinant strain in the liver and spleen, groups of *C57BL/6 J* mice (30 mice in each group) were inoculated in the tail vein with $0.1 \times LD_{50}$ (10^6CFU) of LI△*actAplcB-vp1* in a volume of 100 μl of normal saline. LD_{50} was calculated by the improved Spearman-Karber method to estimate LD_{50} according to this equation: $\log_{10}$ 50% endpoint dilution = -[(total number of animals died/number of animals inoculated per dilution) + 0.5] × **log** dilution factor (Ramakrishnan, 2016). Six mice from each group were sacrificed at 1, 2, 3, 5 and 7 days after injection. Liver and spleen were homogenized in PBS containing 0.1% Triton-X100, then serial dilutions of this homogenate were cultivated on BHI agar plates at 37°C for 24–48 h following by the count of colonies.

Table 1
The utilized Bacterial vector strain and plasmids.

Bacterial strain or Plasmids	Relevant characteristics	Origin or Reference
L. ivanovii△*actAplcB-lacZ*	Recombinant LI, lacking *actA* and *plcB*, integrating with *lacZ* gene in the genome	Lab reserved (Wang et al., 2014)
L. ivanovii△*actAplcB-vp1*	Recombinant LI, lacking *actA* and *plcB*, integrating with *vp1* gene cassette in the genome	The current study
pUC57-*vp1*	FMDV-*vp1* gene serotype (O1) isolate of HKN/19/2010 (Accession No. JQ070305.1) was optimized to be expressed by LI (Accession No. MK050946). It provided by the company fused with the pUC57 plasmid.	Provided by GenScript company (China)
pCW203	Containing *Hin*dIII and *Xho*I single sites	(Wang et al., 2014)
pCW154	Containing expression cassette	(Wang et al., 2014)
pCW154-*vp1*	Derived from pCW154, with *vp1* gene inserted into the expression cassette	The current study

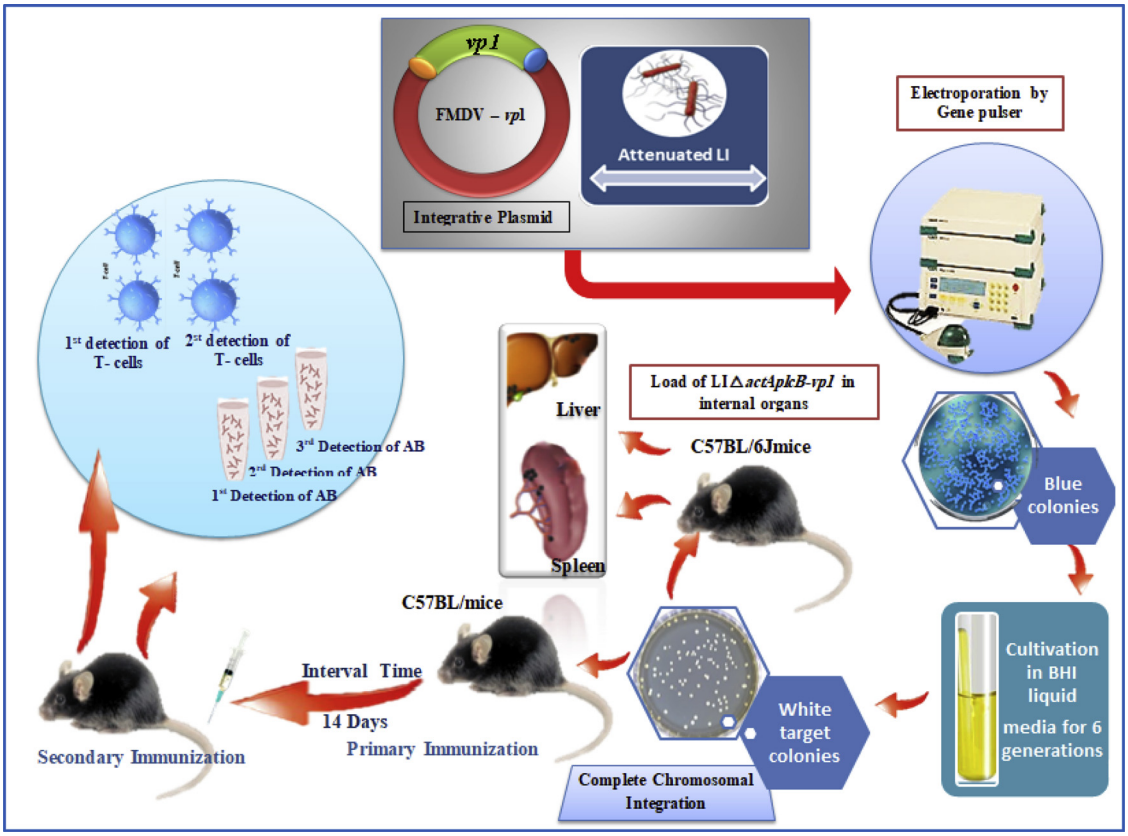


Fig. 1. Schematic diagram represents the current study. Numerous processes to construct LI△*actAplcB-vp1* recombinant strain as a novel vaccine against FMDV and assessment of its immunological value.

2.6. Immunization of mice

Groups of *C57BL/6 J* 6–8 weeks old mice were immunized intravenously. The injected dose containing 10^6 CFU of LI△*actAplcB-vp1* strain dissolved in 100 µl of normal saline. Groups of mice had been injected 2 successful doses with 14 days interval and control groups injected with 100 µl LI△*actAplcB-lacZ* or normal saline. Collection of the serum samples was done in 14 days after the 1st inoculation and at 14 or 28 days after the 2nd inoculation. Mice were kept under restricted hygienic conditions in Animal house in School of Public Health at Sichuan University.

2.7. The assessment of intracellular cytokine profiles

In order to detect the intracellular cytokines, the spleens of immunized and control mice were collected in 1 ml culture medium RPMI 1640 (Gibco, USA) following by mechanical disruption. Ex-vivo stimulation of splenocytes was achieved by 0.1 µg/ml GP-33 (KAVYNFATM) and 0.1 µg/ml GP 61 (GLKGPDIYKGVYQFKSVEFD) or without peptides (10% FBS-1640) at 37°C for 4 h. The cells were stained with PerCP-anti-mouse CD4 and APC-CyTM7-anti-mouse CD8 antibodies (BD PharMingen, China) at 4 °C for 30 min. Afterward, the cells were fixed by the Cytofix/Cytoperm Kit (BD PharMingen, China). Intracellular staining was performed with PE-anti-mouse IFN-γ, APC-anti-mouse IL-2, and PE-CyTM7 -anti-mouse TNF-α antibody (Biolegend, USA) at 4 °C for 45 min. The stained cells were tested with BD FACSverse flow cytometer with Flowjo software.

2.8. Capture of specific antibody by ELISA

Plates of 96 wells were coated with 100 µl of FMDV-VP1 peptide type "O1" diluted in $NaHCO_3$ buffer (10 mmol/L, pH 9.6) at a final concentration of 2 µg/ml, then incubated at 4 °C overnight. The plates were washed with washing buffer, followed by adding 200 µl of blocking buffer and incubated at 37 °C for 1 h. 100 µl of horseradish peroxidase labeled goat anti-mouse IgG (1:3000) (Sigma company, USA) were added to each well then incubated at 37 °C for 30 min. 50 µl substrate buffer was added to each well for 20 min followed by 50 µl of 1.2 mol/l H_2SO_4 to stop the reaction. The rate of absorbance was estimated by ELISA reader and the antibody levels were expressed as optical density values (OD_{490}) for the serum sample.

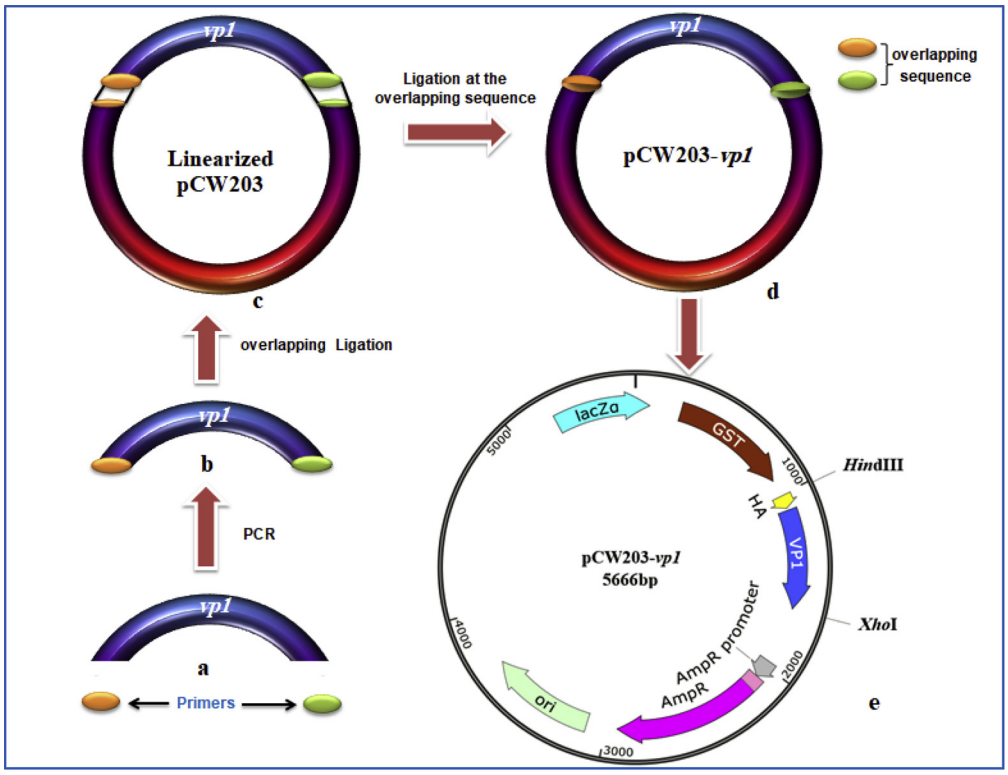
2.9. Statistical analysis and graph design

The obtained data were analyzed by Holm Sidak's multiple-comparison *t*-test. And GraphPad prism v 7.0. ($P < 0.05$) was considered statically significant. SnapGene software was used for plasmids design.

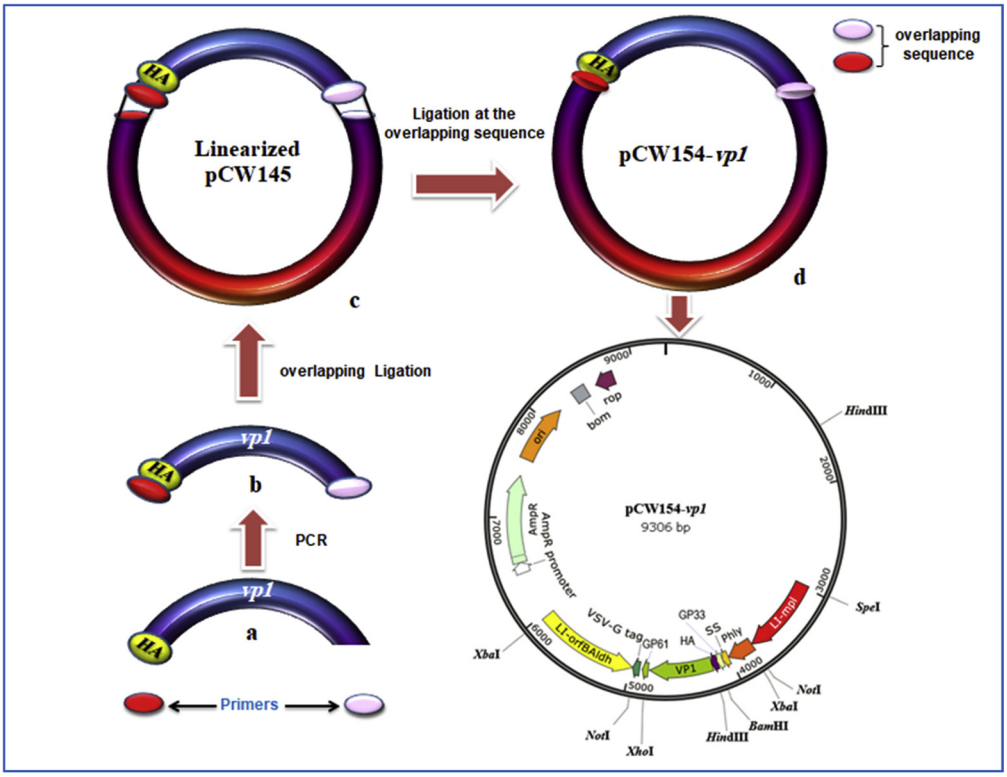
3. Results

3.1. Detection of the recombinant bacterial cells

The attenuated LI was generated before in the lab through deletion of the virulence genes *actA and plcB* to reduce its pathogenicity (Wang et al., 2014). In order to construct recombinant LI△*actAplcB-vp1*, the antigen cassette was inserted by the integrative plasmid pCW154-*vp1* into the bacterial genome during homologous recombination. Afterward, the cultivated bacterial colonies become **white** color and erythromycin sensitive since the decomposed plasmid lost erythromycin gene (*Ery*) as a successful construction. The results of PCR confirmed the successful integration of the heterologous gene cassette into LI△*actAplcB* genome as *vp1* gene was amplified from the bacterial genome and erythromycin resistance gene was undetectable. Moreover, the confirmation was achieved by gene sequencing and the required



a



b

Fig. 2. a Construction of pCW203-*vp1* plasmid. (a) Amplification of FMDV-*vp1* fragment with the distinctively designed primers. (b) The new fragment of *vp1* contains both complementary sequences of pCW203 plasmid restriction sites. (c, d) Insertion of the new portion of *vp1* with the linearized pCW203 plasmid at restriction site between HindIII and XhoI. (e) pCW203-*vp1* plasmid includes (*HA*) *human influenza* hemagglutinin epitope (YPYDVPDYA) for antigen marker. (GST) is glutathione S-transferase for visualization of the target protein. (AmpR) is Ampicillin resistance gene. (*lacZ α* gene) encodes the α fragment in the N terminal of β-galactosidase. b.Generation of integrative pCW154-*vp1* that possess the antigen cassette. To construct the target antigen cassette, the *vp1* fragment was ligated to pCW154 plasmid at *Xho*I and *Bam*HI restriction sites. (a) Amplification of *vp1-HA* fragment with the distinctively designed primers. (b) The created new fragment contained the complementary sequence of linearized pCW154 at *Xho*I and *BamH*I restriction sites. (c, d) Insertion of the new fragment by overlap ligation of the *vp1* fragment in the pCW154 plasmid to generate pCW154-*vp1*. (e) pCW154 include the antigen cassette contained (*Phly*) is the *hly* promoter of LI. (*ss*) is a secretory signal from the LI *hly* gene. (*GP33*) (KAVYNFATM) originated from LCMV for stimulation of CD8 + T cells. (*HA*) (YPYDVPDYA) as a western blot antigen marker. (*vp1*) is the main target gene of FMDV. (*GP61*) (GLKGPDIYKGVYQFKSVEFD) originated from LCMV stimulate CD4 + T cells. And (*VSV-G*) (YTDIEMNRLGK) western blot antigen marker. Upstream of the antigen cassette is a gene (LI-*mpl*), a homologous fragment for homologous recombination translates to metalloprotease, has a role in vacuole escape. Downstream of the antigen cassette is a gene (LI-*orfldh*), one of characterized open reading frames of bacterial genome coding L-lactate dehydrogenase, is also a homologous fragment for homologous recombination.

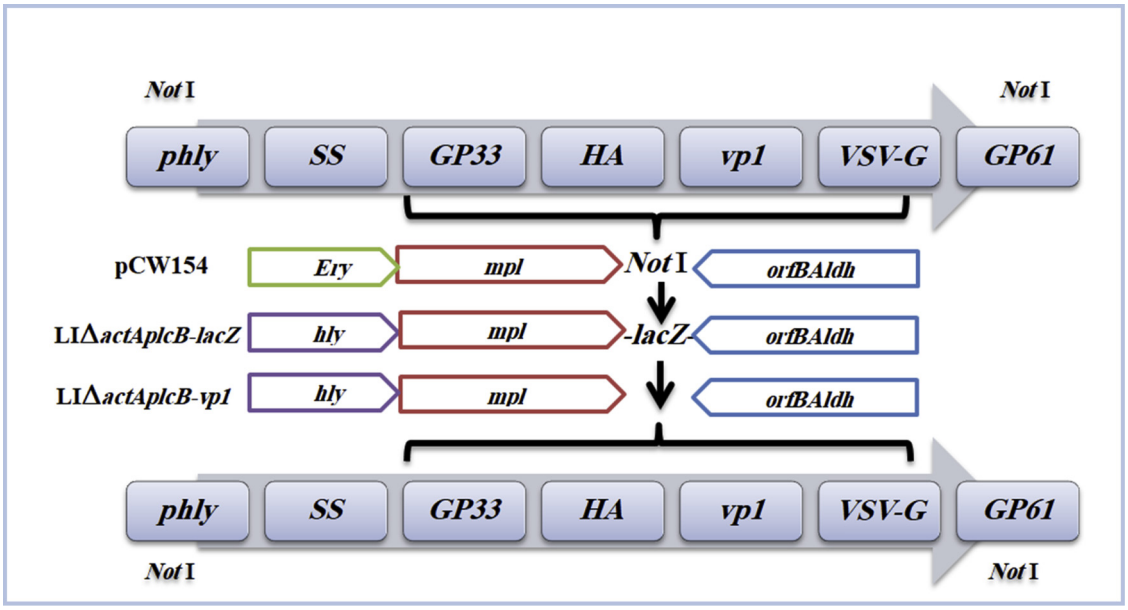


Fig. 3. Homologous recombination to generate a LIΔ*actAplcB-vp1* candidate vaccine. In order to establish the chromosomal integration, the antigen cassette between two *Not*I restriction sites in pCW154-*vp1* was inserted in LI△*actAplcB-lacZ* attenuated strain by electroporation to generate homologous recombination to construct stable LIΔ*actAplcB-vp1*.

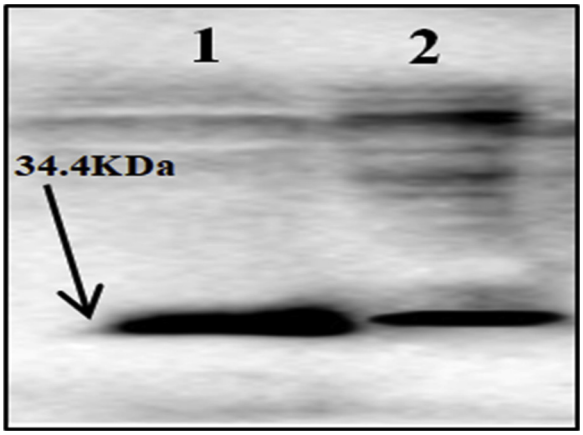


Fig. 4. Western blot analysis for protein expressed by LIΔ*actAplcB-vp1*. Bands of 34.4 kDa for secreted (lane 1) and non-secreted proteins (lane 2) of LIΔ*actAplcB-vp1* recombinant strain were detected.

result was achieved (data not shown).

3.2. Confirmation of protein expression

Western blot data confirmed the expression of FMDV-*vp1* gene in LI△*actAplcB-vp1* recombinant strain *in vitro*. After cultivation of LI△*actAplcB-vp1* recombinant strain, both secreted and non-secreted protein were collected and tested. We used monoclonal HA antibodies against the fused HA peptide with FMD-VP1 at its N terminus in LI△*actAplcB-vp1*. Bands of 34.4 kDa for secreted and non-secreted proteins of LI△*actAplcB-vp1* recombinant strain were detected while no specific band was detected with the negative control (non-recombinant strain) (Fig. 4).

3.3. The bacterial load of LI▵actAplcB-vp1 recombinant strain in the liver and spleen

To evaluate the safety of LI△*actAplcB-vp1* candidate vaccine in internal organs, especially the liver and spleen, *C57BL/6 J* mice groups were injected in the tail vein with $0.1 \times LD_{50}$ of LI△*actAplcB-vp1* (10^6 CFU). Data showed that the load of LI△*actAplcB-vp1* in liver was at the peak of more than 10^5CFU at the 1st day post-injection and continued for 3 successive days. However, on the 5th day post-injection, it notably reduced to average about 10 times lower than that in the first 3 days and decreased to an undetectable level at the 7th day post-injection. The growth rate of LI△*actAplcB-vp1* in the spleen showed the peak of the load (10^3CFU) in the 1st day post-injection. A moderate decrease in the load amount was observed on the 2nd day and reached to an undetectable level in the 3rd day post-injection (Fig. 5).

3.4. Specific humoral immune response

The assessment of the specific antibodies induced by vaccines is very necessary for the evaluation of its value. In the current experiment, groups of *C57BL/6 J* mice were vaccinated with 10^6 CFU LI▵*actAplcB-vp1* and LI▵*actAplcB-lacZ* or normal saline as a control groups. Blood samples were collected at 14 days post the 1st vaccine dose and at 14, 28 days post the 2nd dose. The indirect ELISA method evaluated the level of specific IgG antibodies against FMDV-VP1. The measured level of specific antibodies at 14 days after the 1st dose of LI▵*actAplcB*-VP1 were about (0.675 ± 0.0721) and the highest level was (1.0) while the control groups were (0.115 ± 0.01652). The level of antibodies raised to (1.17 ± 0.0536) after 14 days post the 2nd while the control groups were (0.119 ± 0.01480) and decline to (0.731 ± 0.0355) while the control groups were (0.105 ± 0.01763) at 28 days post the 2nd dose (Fig. 6).

3.5. Evaluation of cellular immune response

In order to assess the cellular immune response as a second arm of the immune system against LI▵*actAplcB-vp1* candidate vaccine recombinant strain, detection of CD8+ and CD4 + T cell secreted cytokines levels, including IFN-γ, TNF-α, and IL-2 in immunized and control mice groups was conducted. After nine days post the primary immunization, the percentages of secreted CD4+ IFN-γ, TNF-α and IL-2 were 0.249%, 0.119% and 0.023% respectively in vaccinated mice, while the percentages of secreted CD8+ IFN-γ, TNF-α and IL-2 were 0.412%, 0.362% and 0.034% respectively. Following nine days post the

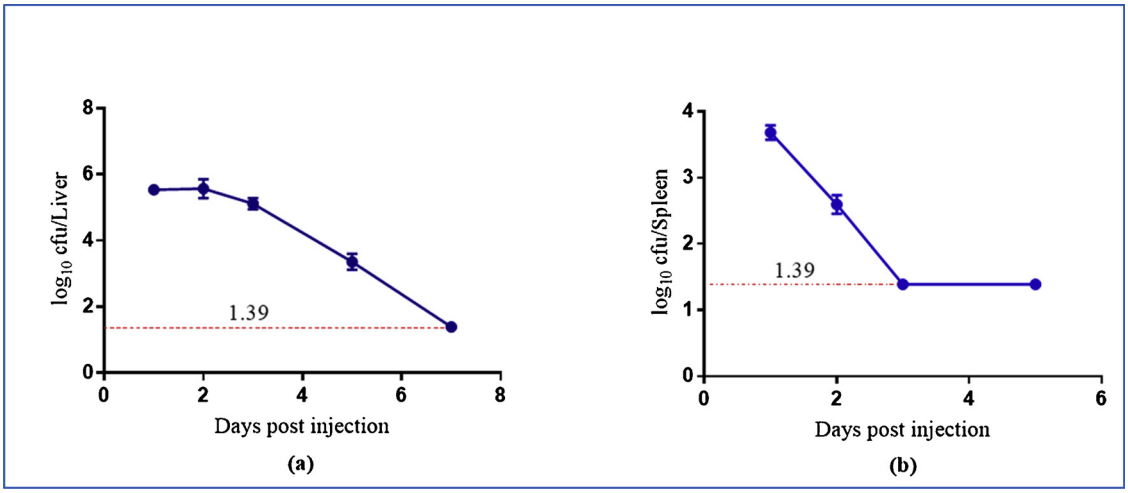


Fig. 5. The bacterial load of LIΔ*actAplcB-vp1* in liver and spleen of immunized mice. In order to detect LIΔ*actAplcB-vp1* load in the liver and spleen of mice, groups of *C57BL/6 J* mice were injected with 0.1 × LD_{50} (10^6 CFU) of LI△*actAplcB-vp1* and sacrificed at 1, 2, 3, 5 and 7 days after injection. (a) Bacterial load in the liver was at the peak in the 1st-day post-injection and continued for 3 successive days. However, at 5th¯day post-injection it notably reduced to average about 10 times lower than that in the first 3 days and decreased to an undetectable level at the 7th day post-injection (b) Spleen bacterial load showed the maximum level at the 1st day post-injection and slightly decrease was on the 2nd day and couldn't be detected at the 3rd day post-injection. Results are represented as means ± SEM per group. The interrupted line refers to the undetectable level of bacteria.

booster immunization, the percentages of secreted CD4+ IFN-γ, TNF-α and IL-2 were 0.454%, 0.218% and 0.230% respectively in vaccinated mice, while the percentages of secreted CD8+ IFN-γ, TNF-α and IL-2 were 0.845%, 0.872% and 0.213% respectively. The results showed a higher significance in the percentage of CD4+ and CD8+ secreted cytokines in vaccinated mice groups more than the control groups and the percentage level of CD8+ secreted cytokines were higher than CD4+ secreted cytokines. (Fig. 7).

4. Discussion

Inasmuch its contagious and highly economic losses, FMD infection control is the focus of attention of most immunologist in this field since they are trying to find a novel vaccine elicit humoral and cellular immune response, and avoiding the disadvantages of the conventional one which mentioned before. Live bacterial vectors have attracted much attention in the development of vaccines due to their emulation of a natural infection, its adjuvant properties and it is able to elicit both mucosal immunity and systemic immune responses, and could be genetically modified to express the heterologous antigens or diverse antitumor molecules for the purpose of preventing infectious diseases or cancer therapy (Lin et al., 2015a,Lin et al., 2015b). Numerous studies have described the attenuated LM strains as an antigens delivery vehicle due to its unique intracellular life cycle. LM can elicit both CD4+ and CD8 + T cell responses since it is able to infect phagocytic and non-phagocytic cells by either direct phagocytosis or internalization and can escape from phagosomes utilizing listeriolysin O (LLO) coded by hly gene, and multiply in the cytoplasm. LI has a similar intracellular life but lower toxicity (Wood and Paterson, 2014). In this current study, the main focus is to obtain a modern vaccine, which triggers humoral and cellular immune response using LI as a live vector for FMDV-*vp1* gene and evaluate its immunological role. In order to construct LI△actAplcB-*vp1*, bacterial chromosomal integration was done as it is more inherently stable than using replicative plasmids (Heap et al., 2012). Attenuation of LI has been achieved by deletion of the virulence factor genes *actA* and *plcB* since both genes are responsible for actin-based mobility and the spread of bacteria from cell to cell (Grundling et al., 2003). Our results confirmed the successful homologous recombination construction of a new candidate vaccine LI△*actAplcB-vp1* strain after several procedures. Upon western blot results, LI△*actAplcB-vp1* recombinant strain successfully expressed the fused VP1 protein *in vitro* and the protein secreted out of the cell. Some references refer to the ability of the secreted protein to trigger the immunity is more than non-secreted one (Drew et al., 2000). The safety of the vaccine is of great importance. Liver and spleen play an important role in clearance of systemic bacterial infections. After i.v infection, Listeria spp directly enters the blood circulation and rapidly arrives spleen and liver. So it is important to confirm the clearance of candidate vaccine strain from liver and spleen without causing any dangerous effect to these internal organs. Growth curve data *in vivo* revealed that attenuated mutant Li△*actAplcB-vp1* with a knockout of the virulence genes (*actA* and *plcB*) created a safe recombinant strain, since the load of LM△*actAplcB-vp1* in liver and spleen of immunized mice was cleared and eliminated during 3–5 days.

Humoral immunity against FMDV is the predominant protective immune response since neutralizing antibodies are crucial for control of virus infection (Doel, 2003). Although the traditionally inactivated vaccines usually elicit high levels of neutralizing antibodies which correlated to protection against infection, a number of countries with huge livestock productions have neglected vaccination due to its disadvantages (Doel, 2005). In this study, LI▵*actAplcB-vp1* candidate vaccine could evoke the immune system to induce a specific immune response against FMDV. The obtained results revealed the level of specific IgG antibodies after 14 days post the 1st dose of LI▵*actAplcB-vp1* was elevated. Another elevation was observed following 14 days post the 2nd dose then the level decline following 4 weeks post the 2nd dose however still above that after the 1st dose. FMDV-VP1 protein has the ability to induce specific antibodies against FMD infection in the experimental and natural animals as it has the most immunogenic sites (DiMarchi et al., 1986). We supposed that during bacteremia, the immune system induces specific antibodies against released extracellular antigens of LI△*actAplcB-vp1* following the rapture of phagosome that can explain the detection of neutralizing antibodies against the VP1 protein (Bhunia, 1997; Edelson et al., 1999). While the decrease in the level of LI△*actAplcB-vp1* in the blood particularly after 28 days from the booster dose led to a decrease in the level of neutralizing specific antibodies.

The conventional FMD inactivated vaccine has the capability to induce specific CD4 + T helper cells and neutralizing antibodies, but unable to stimulate other T cells efficiently, such as the CD8 + T cells (Bautista et al., 2003). LI able to stimulate a strong cellular immune response as it can endure within macrophages after phagocytosis

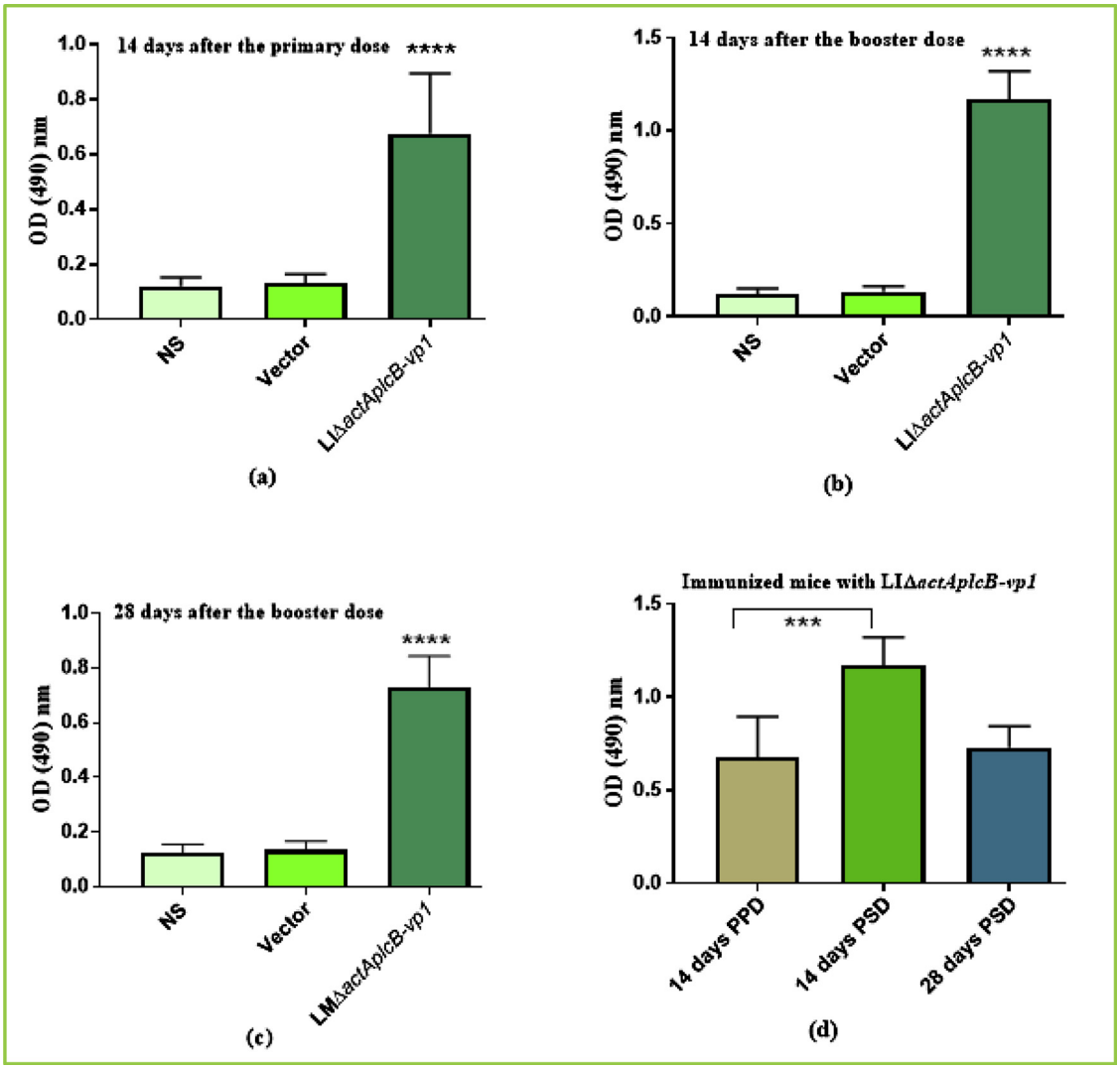


Fig. 6. Assessment the antibody level after immunizations with LIΔ*actAplcB-vp1*. ELISA was used to capture the specific IgG antibody against FMDV-*vp1* in mice sera. (a, b, c) Representative comparison between the levels of antibody in immunized mice at different days and the control groups. (d) Comparison between the Antibody level in immunized mice with LIΔ*actAplcB-vp1* after and before the booster dose. Data were expressed as means ± SEM per group and P values were determined by Holm Sidak's multiple-comparison ***t***-test. **** means $P < 0.0001$ and *** means $P < 0.001$.

(Carryn et al., 2004) which make it an attractive vaccine carrier. The results of specific cellular immune response showed higher significance in the percentage of secreted CD4+ and CD8 + T cell cytokines in vaccinated mice groups more than control groups after 1st and 2nd immunization dose. Numerous studies referred to that cellular immunity in equal to humoral immunity, has an important role in protection against FMDV, especially type O (Sáiz et al., 2002). "Cross-presentation" can explain the cellular immunity results, since antigen presenting cells (APCs) capture LI△*actAplcB-vp1* by direct phagocytosis that important for stimulating T lymphocytes (Heath and Carbone, 2001; Goldfine and Shen, 2007). Besides, the role of MHC classIand classIIin presenting the LI△*actAplcB-vp1* to CD8+ and CD4 + T cells through internal and external pathways, respectively led to more stimulation for a specific T cell immune response (Szalay et al., 1994).

In conclusion, results indicated that construction of LI△*actAplcB-vp1* candidate vaccine was successfully achieved, and the assessment of its safety revealed a well-tolerated in the internal organs within 3 days. To evaluate the immunological value of the candidate vaccine, neutralizing antibodies and specific T cells secreted cytokines were measured. High levels of specific IgG antibodies and CD8+ and CD4 + T cells secreted cytokines including IFN-γ, TNF-α and IL-2 were obtained. According to our knowledge, it is the first report reveal to utilize LI as a bacterial vector for FMDV-vp1 gene.

Author contributions

Chuan Wang, Mahdy S.E and Xiaofang Pei designed the study, conceptualized and drafted the manuscript, designed and conducted the data collection, planned the data analysis, wrote the manuscript and revised the manuscript; Sijing Liu, Lin Su, Xiang Zhang, and Hao-tai Chen analyzed the data collection.

Ethical approval

All Female C57BL/6 J mice of 6–8 weeks old were purchased from Institute of Laboratory Animals of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. Mice were kept under restricted hygienic conditions during the experiments at the Animal Centre of School of Public Health at Sichuan University. Mouse experiments were performed according to the guidelines of the Animal Care and Use Committee of Sichuan University.

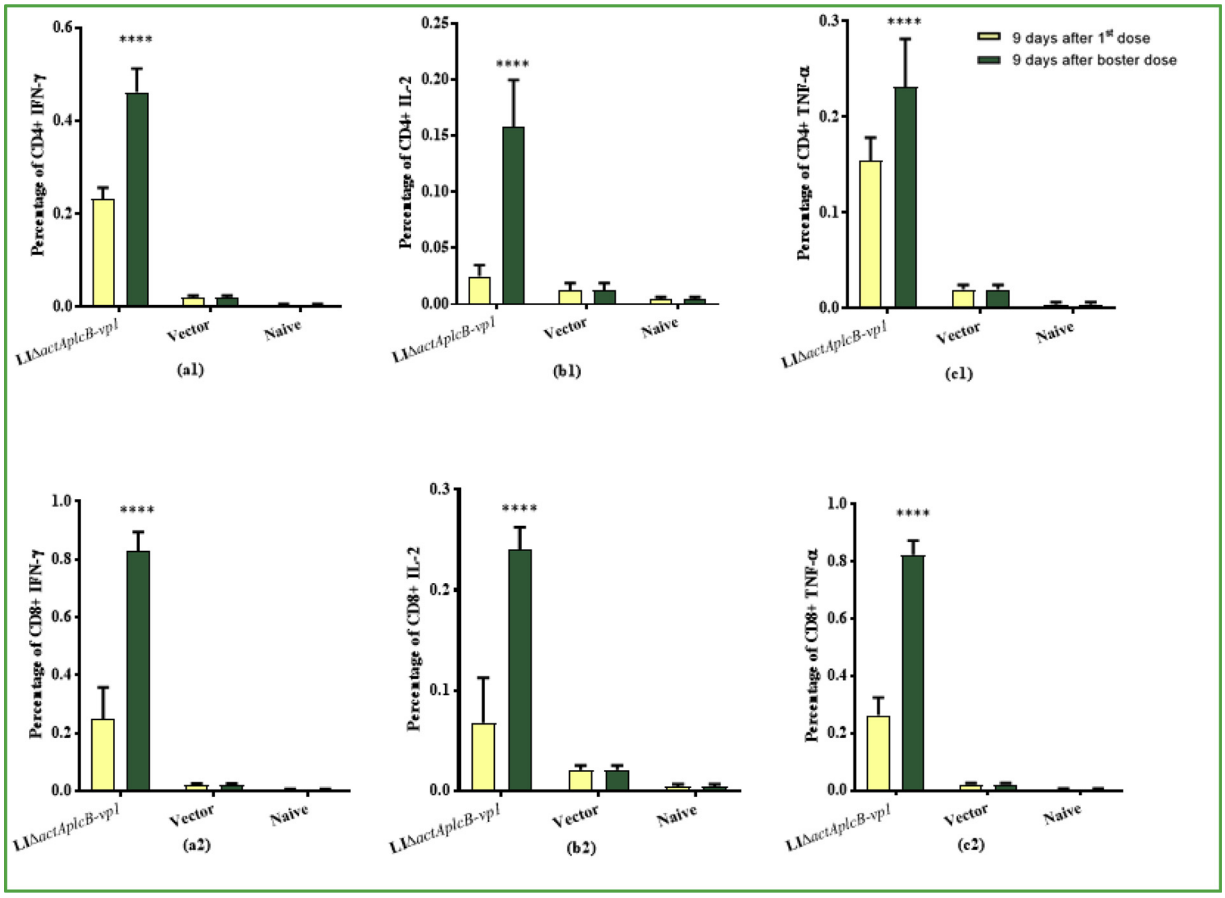


Fig. 7. The percentage of specific CD4 + and CD8 + T cell secreted cytokine. CD4 + and CD8 + T cells secreted cytokines level were evaluated in both immunized and control mice groups at 9 days post the primer and booster dose. (a1, a2) revealed the percentage of CD4 + and CD8 + secreted IFN-γ. (b1, b2) showed the percentage of CD4 + and CD8 + secreted IL-2. (c1, c2) Indicated the percentage of CD4 + and CD8 + secreted TNF-α. There was a significant increase in the percentage level of CD4 + and CD8 + secreted cytokines in immunized mice than control groups and a significant elevation was observed in the level of CD4 + and CD8 + secreted cytokines after the 2nd immunization. **** means $P < 0.0001$.

Declaration of Competing Interest

The authors declare no conflict of interest.

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