Expression fusion immunogen by live attenuated Escherichia coli against enterotoxins infection in mice

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Summary

Previous epidemiological studies have shown that enterotoxins from enterotoxigenic Escherichia coli (ETEC) appear to be the most important causes of neonatal piglet and porcine post-weaning diarrhoea (PWD). Thus, it is necessary to develop an effective vaccine against ETEC infection. In the present study, the Kil cassette was inserted into the pseudogene yaiT by homologous recombination to create an attenuated E. coli double selection platform O142 (yaiT-Kil). After that, PRPL-Kil was replaced with a fusion gene (LTA1-STa13-STb-LTA2-LTB-STa13-STb) to establish oral vaccines O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb) (ER-T). Subsequently, BALB/c mice were orally immunized with ER-T. Results showed that serum IgG and faecal sIgA responded against all ETEC enterotoxins and induced F41 antibody in BALB/c mice by orogastrically inoculation with recombinant E. coli ER-T. Moreover, the determination of cellular immune response demonstrated that the stimulation index (SI) was significantly higher in immunized mice than in control mice, and a clear trend in the helper T-cell (Th) response was Th2-cell (IL-4) exceed Th1-cell (IFN-γ). Our results indicated that recombinant E. coli ER-T provides effective protection against ETEC infection.

Introduction

Enterotoxigenic Escherichia coli (ETEC) is a bacterial pathogen responsible for severe diarrhoea diseases in animals and humans. Enterotoxigenic Escherichia coli leads to high morbidity and mortality both for neonatal and post-weaning pigs (Harvey et al., 2006). ETEC diarrhoea causes slow growth, weight loss and death, which result in considerable economic losses for hog producers worldwide (Trevisi et al., 2015). The major virulence factors of these bacteria are bacterial fimbriae/non-fimbrial adhesins and enterotoxins (Nataro and Kaper, 1998). Fimbriae/non-fimbrial adhesions mediate the attachment of bacteria to host intestinal villus and facilitate bacterial colonization. Then, signal peptide guides enterotoxins through the cell membrane, and causes epithelial cell chloride-ion secretion and preventing sodium chloride absorption, exacerbated secretory diarrhoea by simultaneous fluid movement into the lumen (Field et al., 1978). In the past decade, ETEC infection has been prevented using antibiotic agents (Smith et al., 2010). Recently, multi-resistance has been reported with increasing frequency in several countries worldwide, as hogs have frequently been treated as a group with excessive mass medication (Wang et al., 2010). Moreover, antibiotics could select resistant Escherichia coli to transfer their resistance plasmids to other bacteria that may include pathogens in the faecal flora (Nijsten et al., 1996). Thus, the objective of this study is the prevention of ETEC invasion by alternative methods (Bischoff et al., 2002). Immunization remains an effective approach for preventing infectious diseases. However, there is no broadly effective vaccine available for swine ETEC diarrhoea in China. An effective porcine ETEC vaccine should include all the enterotoxins antigens to lead to anti-heat-labile enterotoxin (anti-LT) and anti-heat-stable enterotoxins (anti-STa and anti-STb) immunity (Liu et al., 2014).

Recent studies showed that most commercial vaccines are administered by injection, stress response in newborn piglets is induced by repeated injection. Nevertheless, activation of secreted intestinal anti-ETEC responses is impossible to achieve by parenteral administration (Lasaro et al., 2005). Therefore, stimulating a protective immune response by colonization in the intestinal mucosa without causing inflammations is important for an ETEC vaccine. The gastrointestinal tract (GIT) is the animal’s largest immunological organ, with a daily production of more than 60% of antibodies (Tang and Li, 2009). Mucosal immune activity plays a major role in neutralizing ETEC upon entry into the
body (Kotton and Hohmann, 2004). Oral vaccination avoids the use of syringes, which evokes both local and systemic immune responses, and production of secreted immunoglobulin A (IgA) blocks bacterial attachment to the intestinal epithelial cells (Jertborn et al., 1998). Furthermore, attenuated strains express more heterologous antigens simultaneously, and safely deliver multiple expressed antigens to mucosal sites (Charles and Dougan, 1990). In addition, oral bacterial vaccine vectors are stable in storage, simple to administer and inexpensive to manufacture (Ascon et al., 1998).

In this study, we have established a recombinant E. coli strain in pseudogene positions yaiT expressing LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion immunogen. Using an oral vaccine, we have gained detailed insight into the immune responses in mouse models. The statistics show that oral immunization with ER-T can elicit more potent systemic and mucosal immune response in mice.

Results

Establish double selection platform and assess its performance

PCR was used to identify O142 (yaiT-Kil) using primers T1 and T4. The products showed that the E. coli O142: ΔSTa (yaiT gene, 800 bp), O142: ΔSTa/pKil-donor (yaiT gene, 800 bp; pKil-donor, 3800 bp) and O142 (yaiT-Kil) (yaiT with Kil cassette flanked, 3800 bp) were of expected size (Fig. 1, panel B). The double selection platforms were plated on MacConkey agar 18 h later, the platform strains grew normally at 30°C. Platform-expressed Kil gene caused cells to die at 43°C (Fig. 1, panel C).

Expression of the fusion protein by recombinant E. coli

ER-T was verified by PCR using the primers T1 with P2. The product of ER-T was of expected size (3000 bp) for LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion gene, meanwhile, there were no bands in O142: ΔSTa, O142

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After sequencing, we found that fusion genes (LTA1-STa13-STb-LTA2-LTB-STa13-STb cassette) were correct insertions of the E. coli O142: STa (data not shown). In addition, Western blot analysis of LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion protein, and the expected sizes (17 kDa and 35 kDa) were observed (Fig. 2, panel D).

Feasibility analysis

Mice were orally inoculated with ER-T, after 4 h gut/carcass mass ratios (G/C = 0.065 ± 0.006) remained normal, E. coli O142 and E. coli 344-C induced a significant increase of intestinal fluid accumulation in mice model (G/C = 0.125 ± 0.005, G/C = 0.107 ± 0.003), indicating that

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**Fig. 2.** The schematic outline of the recombinant strategy for constructing the recombinant E. coli O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb) for oral vaccine candidate.

Panel A. The full-length porcine LT192 operon was used conjugate with LT192, STa13, STb for generating LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion antigen and the native LT promoter was retained which expressed without induction. PCR primers P1 and P2 amplified the entire LT cassette including the native LT promoter and terminator. Primers P2 paired with P4 amplified the STa13-6His-terminator chimeric gene. Primers A1 and A7 mutated the LT gene for LT192.

Panel B. Construction of the recombinant E. coli O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb) according to Gene Doctoring method. The pl-S-donor plasmid and the recombinating plasmid pACBSCE are co-transformed into the double selection platform. Arabinose induction promotes expression of the λ-Red gene products and I-Scel. I-Scel cleaves the pl-S-donor plasmid resulting in generation of the linear DNA fragment for λ-Red mediated recombination to generate the recombinant E. coli O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb).

Panel C. PCR reaction for the verifying of the recombinant E. coli strain ER-T by using the primers yaiT-L-arm and P2. M: molecular size marker; N: PCR negative control; 1: PCR product of E. coli O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb); 2: PCR product of E. coli ER-T.

Panel D. Detection of the LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion protein in the Western blot assay. anti-His (ZSGB-BIO Co.; 1:500) were used as primary antibody and secondary antibody by (HRP)-conjugated goat anti-mouse IgG (ZSGB-BIO Co.; 1:2000), development following the manufacturer's instructions of ECL Plus Reagent Kit (7 Sea Biotech, China) M: Protein marker; 1: E. coli O142: STa as the negative control; 2: E. coli ER-T.
the toxicity of ER-T had been reduced or eliminated (Fig. 3, panel A). The results of in vitro cytotoxicity assay showed that supernatant of ER-T cannot cause ZYM-DIEC02 cells to die, but supernatant of E. coli O142 (STa), 274-A (LT) and 344-C (STb) induce cell death (Fig. 3, panel B). The tolerance test indicated that ER-T tolerates well gastric acid pH 2.5 to 4.5, intestinal juice, and bile 0.05–0.3% (Fig. 3, panels C–E). During the first 3 days of the rearing period, the feed intake decrease. Over the whole period, the feed intake and weight were similar in each group (Fig. 3, panel F). The stability curves of ER-T in the growth phases were similar and no significant differences between them were found, indicating that insertion of the chimeric gene did not impact the growth of the bacterial cell (Fig. 3, panel G). Large doses of ER-T did not affect the weight gain of mice (Fig. 3, panel H). We checked the stability of ER-T, and the results indicated that all the colonies analysed presented the expected bands. Sequencing showed that chimeric gene LTA1-STa13-STb-LTA2-LTB-STa13-STb is not lost after 100 generations (data not shown). EM images revealed that insertion of the LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion gene did not affect the E. coli O142: ΔSta fimbriae expression (Fig. 3, panel I). The colonization assay demonstrated that ER-T had a higher binding capacity in the intestinal tract of mice (Fig. 3, panel J).

Systemic and mucosal immunogenicity elicited by recombinant E. coli

After oral immunization recombinant, bacteria can enhance the serum IgG and faecal IgA. High levels of anti-F41, anti-LTA, anti-LTB, anti-STa and anti-STb IgG antibodies were detected after 7, 14, 14, 21 and 21 days in the serum samples respectively (Fig. 4A–E). Forty-two days after immunization, ER-T (Fig. 4F–I) specific IgG antibodies were revealed in milk, spleens, mesenteric lymph nodes and intestinal mucus samples. By contrast, in the control groups there was no enhancement of specific IgG antibody responses. Similar findings were revealed on the secreted IgA antibody responses in faeces of mice after being administered orally with ER-T (Fig. 5A–I). These results show that the recombinant E. coli has the capability to elicit both systemic and mucosal antibody responses.

Lymphocyte proliferation responses and cytokines assay

The results indicated that the splenic and mesenteric lymphocytes from mice immunized orally with ER-T showed significant responses compared with the PBS group by an MTT assay. For splenic lymphocyte proliferation, splenocyte, mesenteric lymphocyte from different groups of immunized mice were cultured in vitro. The higher level of proliferation was demonstrated in ER-T group for STa, LT192-STa13, STb, LT192-STb, LT with the mean SI of 2.38, 2.34, 2.40, 2.11 and 2.31 (Fig. 6, panel A). In addition, the group of ER-T (Fig. 6, panel B) induced higher mesenteric lymphocyte proliferation responses (SI of STa 2.59, LT192-STa13, STb, LT2.58, LT192-STb 2.93, LT 2.79) than control and O142:ΔSta.

Mice vaccinated with ER-T had splenic lymphocytes IFN-γ (mean 43.55 ± 12.63), and neither were different from those found in splenic lymphocytes from both control groups (Fig. 6, panel C). In contrast, the groups vaccinated with ER-T (mean 313.89 ± 33.21) had notably higher splenic lymphocytes IL-4 than the groups control (Fig. 6, panel D). When the IL-4 to IFN-γ ratio was analysed, it was found that the oral immunization groups had 7 times more IL-4 than IFN-γ, which is a clear indicator of a polarized Th2 immune response.

Toxin-neutralizing ability in vitro

We used ZYM-DIEC02 cells to detect the neutralizing efficacy of serum, intestinal mucus, splenocyte lysate and mesenteric lymphocyte lysate from orally inoculated mice. Results showed that the samples (from the immunized mice) had effective neutralization LT toxin, in serum (1:32), splenocyte lysate (1:16), intestinal mucus (1:32) and mesenteric lymphocytes lysate (1:32) (Fig. 7,

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Likewise, the immunized mice exhibited higher STa toxin-neutralizing activity in serum (1:16), splenocyte lysate (1:16), intestinal mucus (1:32) and mesenteric lymphocytes lysate (1:32) (Fig. 7, panel E–H). Moreover, samples from the immunized mice showed the STb toxin neutralization potential in serum (1:16), splenocyte lysate (1:16), intestinal mucus (1:32) and mesenteric lymphocytes lysate (1:32) (Fig. 7, panel I–L).

Toxin-neutralizing activity in vivo

Recombinant E. coli ER-T used as immunoprophylactic had the ability to induce neutralizing antibodies against STa and STb. When comparing the samples of ER-T for the neutralizing efficiency to STa toxins, results indicated that STa toxin antibodies were in serum (1:5), splenocyte lysate (1:5), intestinal mucus (1:2.5) and mesenteric lymphocyte lysate (1:5) (Fig. 8, panel A–D). Likewise,
the samples from the immunized mice group of \textit{ER-T} showed the neutralization ability to STb toxin in serum of (1:2.5), splenocyte lysate (1:7.5), intestinal mucus (1:2.5) and mesenteric lymphocyte lysate (1:2.5) (Fig. 8, panel E–H).

\textbf{Protection efficacy of maternal antibody}

The protective efficacy of suckling mice intaking milk from the immunized pregnant mice challenged with STa or STb toxin was investigated. After being administered orally with STa toxin (1:15 diluted) the \textit{ER-T} G/C ratios, outlined below (\textit{ER-T} G/C = 0.083 \pm 0.005), significantly below that of the mice of the control group (Fig. 9, panel A). Likewise, after challenge with STb toxin (1:15 diluted), \textit{ER-T} G/C ratios outlined below (\textit{ER-T} G/C = 0.080 \pm 0.005), which is lower than control (1:15 diluted G/C = 0.104 \pm 0.005) and \textit{O142ΔSTa} (1:15 diluted G/C = 0.094 \pm 0.006) (Fig. 9, panel B).

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Porcine ETEC-associated diarrhoea, especially PWD and neonatal piglet diarrhoea, remains a major problem for swine producers around the world, but an effective vaccine against neonatal piglet diarrhoea and PWD is lacking (Ruan and Zhang, 2013). Due to the vast majority of piglet diarrhoea being caused by ETEC expressing K88ac or F18 fimbriae in North America (Zhang et al., 2007), abundantly expressed CFA/I, 987P, K99, F18 and/or K88ac fimbriae vaccines were developed. However, in China, pregnant sows are often vaccinated with inactivated vaccines containing fimbriae antigens to protect their offspring from intestinal infection with ETEC. In a recent epidemiological survey, the fimbriae were not frequently associated with ETEC for suckling pigs with diarrhoea in China. Likewise, low proportions of adhesin-positive E. coli strains have been reported in previous studies in the Netherlands (Guinee and Jansen, 1979), Japan (Nakazawa et al., 1987) and Sweden (Soderlind et al., 1988). Therefore, efficient ETEC vaccines should work against enterotoxins (Ruan and Zhang, 2013).

Oral subunit or killed vaccine antigen delivery through the digestive tract is subject to digestive degradation. Therefore, oral vaccination must maintain the integrity of the antigen during delivery to the intestinal tract. Recently, extensive work on a live vehicle vaccine system presented evidence that recombinant attenuated E. coli is an efficient carrier for antigens by the oral route (Liu et al., 2014). In our study, we build oral vaccine strains obtained from attenuated wild-type ETEC strain. Moreover, recombinant bacteria achieve ephemeral colonization in the host by F41 fimbriae to overcome host defences, such as the gastrointestinal mucus barrier and intestinal peristalsis cleaning mechanism (Pacheco et al., 2012). Orally administered ER-T can deliver antigens to the immune system for a prolonged period. In addition, we demonstrated that a single oral dose of recombinant E. coli was without significant reactogenicity or toxicity at dosages up to $10^9$ CFU. Recombinant E. coli containing LTA1-STa13-STb-LTA2-LTB-STa13-STb operon have been stably maintained for over 100 generations in wild-type E. coli. Another aspect of this research is that it is the first report of oral polyvalent vaccine fusion of all swine ETEC enterotoxins simultaneously. Enterotoxins are an important virulence factor of swine ETEC strains and have been frequently identified in pigs with ETEC diarrhoea disease (Zhang et al., 2006). The most significant contributors to piglet ETEC diarrhoea were LT, STa and STb.

STa is composed of only 19 amino acids (2 KDa), so the immunogenicity is poor, and it can cause diarrhoea unless inactivated or modified STa peptides pSTa (A13Q) reduction toxicity (Zhang et al., 2010). Recent studies indicate that STa (A13Q) fuses to LT became immunogenic (Zhang et al., 2013), resulting in LT-STb fusion antigen can stimulate body to produce antibodies against LT and STa toxins (Liu et al., 2011).

**Fig. 6.** Characterization of lymphocyte and cytokines proliferation responses in mice by orally administered ER-T. A. Splenocyte samples stimulated with different purified antigens by MTT method. B. Mesenteric lymphocyte samples stimulated with different purified antigens by MTT method. C. The levels of IFN-γ concentration in the culture supernatant were measured by ELISA. Error bars represent standard deviations, and mean values are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
STb positive isolates from diarrhoeic piglets were more prevalent than STa in Canada (Harel et al., 1991), Poland (Osek and Truszczynski, 1992) and Spain (Blanco et al., 1997) respectively. STb consisting of 40 amino acids (5.2 KDa) was too short for induced anti-STb immunity in hosts (Sears and Kaper, 1996). Moreover, a study demonstrated that STb coupled to an appropriate carrier molecule had the advantages of reducing toxicity and inducing neutralizing antibodies (Dubreuil et al., 1996).

LT consisted of a toxic A1 subunit (LTA1), non-toxic A2 subunit (LTA2) and five identical polypeptide chains of B subunit (LTB) (Spangler, 1992) and was capable of enhancing systemic and mucosal antibodies. Insertion of exogenous genes between LTA1 and LTA2 shows that reduce the toxicity of LTA1 and preserve the adjuvant function of LTA (Zhang and Sack, 2015). LTB is a potent immunogen and possess adjuvant properties (Millar et al., 2001; Sanchez and Holmgren, 2005), and binding to specific receptors on the host cell membrane then toxin entry into host cells (Salimian et al., 2010). In addition, LT facilitates the immunogenicity of STa and STb in vaccines immunization against bacterial infections (Norton et al., 2012). Therefore, using STa or STb fusion at the C terminus of LTB subunits would generate quadruple or quintuple anti-ST fusion antigens (Ruan et al., 2011). Thus, LT plays a key role in the prevention of enteric infection. Moreover, previous vaccines were mainly targeted at LT-STa or against LT-STb, so diarrhoea caused by enterotoxins (LTB, STa, STb) could not be completely prevented (Zhang et al., 2013; Ruan et al., 2014), to prevent enterotoxin-induced diarrhoea in piglets, two vaccines need to be used simultaneously. In this study, increasing immunogenicity of enterotoxins (STa, STb) a mature STb peptide fused to a full-length of porcine STA13Q toxoid generated STA13-STb fusion antigen. However, the preliminary results of this laboratory show that STA13 can poison its own cells after four

Fig. 7. In vitro neutralization assays of samples from group ER-T neutralized to LT, STa and STb toxin. A–D. Serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate from immunized mice showed neutralization efficiency to LT toxin when compared with that from control mice. E–H. Serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate from immunized mice showed neutralization efficiency to STa toxin when compared with that from control mice. I–L. Serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate from immunized mice showed neutralization efficiency to STb toxin when compared with that from control mice. The ratios on the figure are the dilution gradient of toxins, the immune mice produced antibodies that protected ZYM-DIEC02 cells (cell death < 50%), the control group did not protect ZYM-DIEC02 cells.
times tandem repeat (STa$_{13}$-STa$_{13}$-STa$_{13}$-STa$_{13}$) (data not published). Therefore, to reduce the toxicity a point insertion was used. Connecting STa$_{13}$-STb to the 3’ end of the full-length porcine LT$_{R192G}$ operon, constitutes LTB-STa$_{13}$-STb. Between LTA1 and LTA2 inserted STa$_{13}$-STb then form LTA1-STa$_{13}$-STb-LTA2. Finally, LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb is formed. Since the native promoter of LT is preserved, the expression of the fusion protein does not require an inducer. Fusion antigen LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb was bicistronic like LT, LTA1-STa$_{13}$-STb and LTB-STa$_{13}$-STb were expressed in the cytosol of the bacteria. The A2 region of LT A subunit complexes with LT B subunit forming a structure of A:B5 when secreted to the external of live bacteria under the guidance of signal peptide. When SDS-PAGE carried out, subunits A and B were separated by heating. Previously, we inserted STa$_{13}$-STb-His after LTA1 and STa$_{13}$-STb-His downstream of LTB, respectively, therefore two bands in WB experiment 17 and 35 kDa.

Recombinant E. coli ER-T provides effective protection against ETEC infection
regions of the GM1 receptor; we also retained GM1 binding activity as suggested. Data from this study indicated that ER-T expression fusion gene LTA1-STa13-STb-LTA2-LTB-STa13-STb elicits specific immune response anti-LT, anti-STa and anti-STb in a mouse model by the oral route. We chose the location of pseudogene yaiT gene, inserted by IS element, in addition far away from the promoter and terminator. Homologous recombination systems were used to insert exogenous genes on pseudogenes. Then, the influence on ER-T of biological activity and growth was tested (Echols et al., 2002; Balakirev and Ayala, 2003).

IgA production exceeds all the other immunoglobulins at the mucosal surface. Thus, IgA plays a significant role in defending against the invasion of pathogens. Our results indicated faecal ER-T IgA levels against LTA (day 21, \( P < 0.001 \)), LTB (day 14, \( P < 0.001 \)), STa (day 21, \( P < 0.05 \)), STb (day 21, \( P < 0.01 \)) and F41 (day 21, \( P < 0.01 \)). Likewise, ELISA titres of IgG display statistically significant differences in spleens, milk, mesenteric lymph nodes and intestinal mucus of mice immunized with ER-T. In addition, serum IgG and faecal IgA titres from mice after immunization with the vaccine ER-T were conspicuously higher than those of the control group in both experiments (\( P < 0.05 \)). This indicates oral administration could trigger immune responses not only mucosa but also systemically. The lymphocyte proliferation assay indicated that SI ratios of splenocytes and mesenteric lymphocytes cell proliferative responses were significantly higher in the ER-T immunized groups than in controls (\( P < 0.05 \); Fig. 6, panels A and B). For this study, within the ER-T groups of mice immunized by oral administration, results suggested that LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion antigen can induce sufficient cellular immune responses. Immunized mice had higher levels of IL-4 than IFN-\( \gamma \). The IFN-\( \gamma \) effects are partially blocked by IL-4 in infectious diseases (Myers et al., 1992), suggesting that recombinant strains induced Th2-preferred responses. In vitro and in vivo neutralization assays were performed for evaluating the sufficient neutralizing antibody titres by ER-T. Results showed that after LT, STa and STb enterotoxin incubation with serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate samples from the immunized mice had the capability of preventing infection of ZYM-DIEC02 cells in vitro. Furthermore, these samples obviously neutralized the biological activity for STa and STb enterotoxins in suckling mice.

**Experimental procedures**

**Bacterial strains, plasmids and cells**

The strains used in this study are listed in Table 1. *E. coli* O142 was deposited in the Chinese Veterinary Culture Collection Center (Beijing, China) (CVCC accession no. C83920). *E. coli* C83903 was purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China). Strain LT192-STa13 fused LT192 (GenBank accession no.

| Strain       | Relevant properties                                                                 | Reference or source                  |
|--------------|-------------------------------------------------------------------------------------|--------------------------------------|
| O142         | Cattle ETEC field isolate, F41/Sta                                                  | CVCC: C83920                         |
| 274-A        | Porcine ETEC field isolate, LT/K88, harboring LT as the only toxin                  | This laboratory                      |
| 344-C        | Porcine ETEC field isolate, STb/paa, harbor the STb as the only toxin                | China Institute of Veterinary Drug Control |
| C83903       | Porcine ETEC field isolate, K88/LT/STb/EAST1                                       | This laboratory                      |
| ZYM-DIEC02   | Swine small intestine cell lines                                                    | This laboratory                      |
| O142: \( \Delta \)Sta | STa gene deleted in O142                                                               | This laboratory                      |
| O142 (yaiT: PRPL-Kil) | PRPL-Kil cassette inserted into the yaiT gene to construct the double selection platform: grows normally at 30°C but is killed at 43°C by the expression of Kil | This study                           |

| Plasmid       | Relevant properties                                                                 | Reference or source                  |
|---------------|-------------------------------------------------------------------------------------|--------------------------------------|
| pDOC-K        | Kanamycin cassette flanked with FRT sites, MCS and I-SceI sites                     | Lee et al. (2009)                    |
| pDOC-C        | MCS flanked with I-SceI sites                                                       | Lee et al. (2009)                    |
| pACBSCE       | I-SceI and I-Red protease under control of arabinose promoter, p15A ori             | Lee et al. (2009)                    |
| pEASY-Blunt-Simple | TA cloning vector                                                                   | Beijing TransGen Biotech, China      |
| pKil         | PRPL-Kil cassette cloned into the MCS1 of pDOC-K at BamHI I and Kpn I sites        | This laboratory                      |
| pKil-donor     | The left side (yaiT-L) and right side (yaiT-R) of the insertion site cloned into pKil | This study                           |
| pL-S          | LTA1-STa13-STb-LTA2-LTB-STa13-STb cassette cloned into the MCS of pDOC-C at Sal I and Xho I | This study                           |
| pL-S-Donor     | yaiT-L-arm and yaiT-R-arm cloned into pL-S by BamHI l/Sal I, and Xho l/Spe I, respectively | This study                           |

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Table 3. PCR primers used for constructing the double selection platform

| Primer  | Sequence (5’–3’)                           | Description                                                                 |
|---------|--------------------------------------------|-----------------------------------------------------------------------------|
| yaiT1 (T1) | GGATCCTAACGGAAGCAAGTGGGTTGGTCAG          |Anneals to 5’ end of yaiT-L gene, with BamHI site                           |
| yaiT2 (T2) | GTCGAATCAAGCCACCCCCACCAGTAGATT           |Anneals to 3’ end of yaiT-L gene, with SalI site                             |
| yaiT3 (T3) | GTGCGGTAGACCTGAGTACGCCAATACTATCAC         |Anneals to 5’ end of yaiT-R gene, with XhoI site                            |
| yaiT4 (T4) | ACTAGTTGGTGTTGTCGATACGAACCTTGG           |Anneals to 3’ end of yaiT-R gene, with SpeI site                             |

CP002732.1) with (GenBank accession no. V00612.1) and Strain LT192-STb (GenBank accession no. V006790.1) have been previously described (Liu et al., 2015a, 2015b). The plasmids constructed and used are listed in Table 2. Plasmids pDOC-K, pDOC-C and pACBSCE were kindly provided by Prof. David J. Lee (School of Biosciences, University of Birmingham, UK).

Construction and characterization of the double selection platform

A double selection platform was established using gene doctoring method and λ-red method (Datsenko and Wanner, 2000; Lee et al., 2009). Restructuring strategy flow chart of E. coli O142: ΔSTa double selection platform is displayed in the online Resource Fig. 1, panel A.

Briefly, use primer T1 paired with T2 to clone the left homologous arms from E. coli O142: ΔSTa, use primer T3 paired with T4 to clone the right homologous arms from E. coli O142: ΔSTa (the primers T1, T2, T3 and T4 are listed in Table 3), construct pKil-donor plasmid, insert the left fragment and right fragment into the pKil plasmid. Transform pACBSCE plasmid and pKil-donor plasmid into E. coli O142: ΔSTa, subsequently, homologous recombination is induced by L-arabinose, then generated O142 (yai-T-Kil) double selection platform. Use PCR primers (T1 and T4) to verify that pKil-donor fragment was assembled on the platform, the platform screening capability validation by 30 and 43°C, as previously described (Liu et al., 2015a, 2015b).

Table 4. PCR primers used for constructing the recombinant E. coli

| Primer | Sequence (5’–3’) | Description                                                                 |
|--------|------------------|-----------------------------------------------------------------------------|
| P1     | GTCGACGCCGCTGATATCAGGATTAGCTT |Anneals to the left side of native LT promoter, with SalI sites, cloned into pDOC-C |
| P2     | CTCGAGAAGCTTGGGCCCACGCTTA |Anneals to the right side of native LT terminator, with XhoI sites, cloned into pDOC-C |
| P3     | CGGTTACCGGCTGATATCAGGATTAGCTT |3’ end of STa13 (26 bp, no stop codon) + 5’ linker (15 bp) |
| P4     | TGCTGATATCAGGATTAGCTT |3’ end of STa13 (13 bp, no stop codon) + 5’ linker (20 bp) |
| P5     | CAGATTTCGGTTCCTATCCTAAAGATCAGGATTAGCTT |5’ end of STa13 (26 bp, no stop codon) + 5’ linker (15 bp) |
| P6     | ATGATGATGATGATGGTGTCGATACGAACCATG |5’ end of LT-A2 (33 bp) + 6*his (13 bp) + 3’ end of STb (8 bp) |
| P7     | TGATGATGATGATGGTGTCGATACGAACCATG |3’ end of LT-A1 (30 bp) |
| P8     | CAGATTTCGGTTCCTATCCTAAAGATCAGGATTAGCTT |5’ end of LT-A2 (33 bp) |
| P9     | GGTGATGATGATGATGGTGTCGATACGAACCATG |5’ end of LT-A2 (33 bp) |

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generated a fragment including the LTA2 subunit, the LTB subunit and the STA13-STb-6 × His chimeric gene, named A2-LTB-STa-STb-6 × His. Subsequently, the eighth SOE PCR connected the LTA1-STa-STb-6 × His and A2-LTB-STa-STb-6 × His fragment and produced fusion genes (LTA1-STa13-STb-LTA2-LTB-STa13-STb cassette). The primers are listed in Table 4.

The LTA1-STa13-STb-LTA2-LTB-STa13-STb cassette was inserted into the pDOC-C plasmid by restriction endonucleases, producing the pL-S plasmid, employing left side and right side PCR products ligated into pL-S plasmid and generating yaiT(pL-S-Donor) plasmid respectively. Then, for pACBSCE plasmid, pL-S-Donor plasmid co-transformation selection platforms, an analogous protocol was used for homologous recombination as mentioned above, to yield the recombinant E. coli O142(yaiT: LTA1-STa13-STb-LTA2-LTB-STa13-STb), named as ER-T (Fig. 2, panel B). Recombinant E. coli strains were identified by PCR, using primers L-arm paired with P2 respectively. The primers are listed in Table 3. The sequences of the respective fusion genes were confirmed by sequencing. Moreover, recombinant E. coli expressions of LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion protein separated by 12% SDS-PAGE, examined with anti-His, were used as primary antibody and secondary antibody by (HRP)-conjugated goat anti-mouse IgG, as previously described (Liu et al., 2015a, 2015b).

Feasibility analysis

Recombinant E. coli strains were further evaluated for feasibility as an oral vaccine by six different assays. First, the residual enterotoxinity in recombinant E. coli assay by suckling mouse and ZYM-DIEC02 cell. Briefly, 4-day-old suckling mice administered orally with Soybean trypsin inhibitor (2 mg ml⁻¹; Solarbio, Beijing, China), and 100 μl of culture supernatant was inoculated into the stomach. The mice were killed after 4 h, G/C (the weight ratio of gut to the remaining carcass) ratios of ≥ 0.090 were confirmed positive for toxicity of STa or STb as previously described (Frantz and Robertson, 1981). In the ZYM-DIEC02 cell assay, using 100 μl of culture supernatant incubation with ZYM-DIEC02 cells in 5% CO₂ at 37°C for 24 h, showed the change in the cells as previously described (Feng et al., 2013). Second, we analysed the survival characteristics of recombinant E. coli in the simuton environment of the gastrointestinal tract. Briefly, recombinant E. coli was cultured in lysogeny broth (LB) medium, supplemented with gastric acid (1.5, 2.5, 3.5 or 4.5 of pH), and intestinal juice and bile (0.05%, 0.1%, 0.2%, 0.3% and 0.4%). After shaking at 37°C for 8 h, bacteria survival rate was determined by the plate method of enumerated intermittently, as previously described (Liu et al., 2015a, 2015b). Third, we measured the influence of oral administration on feed intake and body weight. Briefly, groups of 10 six-week-old female BALB/c mice (Liaoning Changsheng Biotechnology Co., Ltd., China), received doses of 10⁹ CFU, 10¹⁰ CFU, 10¹¹ CFU and 10¹² CFU for 3 days, and were observed as previously described (You et al., 2011). Fourth, the growth curves of the recombinant E. coli were determined. Briefly, isolate single clone of ER-T from LB agar medium then inoculate into LB medium, at intervals of 2 h, take sample from LB medium, use the plate method to produce growth curves, as previously described (Yang et al., 2015). Growth curves of E. coli O142, O142: STa, ER-T. Fifth, analyse the structural stability of ER-T, continuous subcultures ER-T for 100 generations, then the 100 generations ER-T are plated on LB agar medium, randomly selected several colonies, and identified using PCR primers L-arm paired with P2, as described above (Nguyen et al., 2005). Sixth, recombinant E. coli was examined via electron microscope (EM). Briefly, recombinant E. coli was in static culture for 3 days, placed onto Formvar carbon-coated copper grids (200 mesh), then stained with 2% potassium-phosphotungstic acid (pH 6.8) and observed by EM as previously described (Torres et al., 2004). Seventh, recombinant E. coli intestinal colonization was evaluated. Briefly, six-week-old BALB/c mice were orally administered naldixic acid-resistant (Nal) recombinant E. coli ER-T 10⁹ CFU. Subsequently, faeces samples were collected at 7 day intervals and incubated on LB(Nal) agar plates, the assessed by PCR as described above (Huang et al., 2013).

Oral immunization of mice

A total of 80 six-week-old BALB/c mice were split into eight groups (A, B, C, D, G1 female BALB/c mice, G2, G3 and G4 male BALB/c mice), with 10 mice per group. ER-T was grown in improved MINCA medium for 24 h at 37°C and harvested by centrifugation. Resuspension of ER-T centrifugal sedimentation in milk arrived at concentration of approximately 1 × 10¹⁰ CFU ml⁻¹. Mice in group A and B were orally administered with 10⁹ CFUs of ER-T with a 1.5-inch, 20 gauge ball-tip needle. Mice in group C and D received 10⁹ CFUs of O142: STa, G1, G2, G3 and G4 as control groups received doses of 0.1 ml of milk. All groups were inoculated on a single day (Time 0); then, identical booster doses were administered on 14 and 28 days later. At 21 days, groups B coupled with G2, D coupled with G4, G1 coupled with G3, as previously described (Liu et al., 2015a, 2015b).

ELISA analysis for antibody levels

IgG and IgA antibodies were measured by enzyme-linked immunosorbent assay (ELISA). On days 0, 7, 14,
lysate samples were collected from mice. Filter-sterilized mucus, splenocyte lysate and mesenteric lymphocyte of mice inoculated intragastrically with E. coli ER-T provided effective protection against ETEC infection.

Forty-two days after the first immunization, mice mesenteric lymphocytes and splenic lymphocytes were collected and measured by MTT assay, as previously described (Liu et al., 2015b). Briefly, the lymphocytes were incubated in 96-well plates at 5 × 10^5 lymphocytes cells/well for 100 μl then stimulated in vitro with LT (10 μg ml⁻¹), STa (10 μg ml⁻¹), STb (10 μg ml⁻¹), LT192-STa13 (10 μg ml⁻¹) proteins and LT192-STb (10 μg ml⁻¹) proteins for 100 μl respectively. Meanwhile, concanavalin A (con A) (10 μg ml⁻¹) was used as a positive control and a blank control were without proteins. The plates were kept at 37°C in 5% CO₂ for 72 h and then pulsed with MTT (10 mg ml⁻¹) per well for 4 h. The absorbance was measured with a spectrophotometer at 570 nm. The stimulation indices (SI) were calculated by the following formula: SI proliferation = ODsample/ODnormal. Th1 cells produce IFN-γ, and Th2 cells produce IL-4. We used mice cytokine ELISA-kits (IFN-γ and IL-4 kits; JRDUN, Shanghai, China) following the manufacturer’s instructions to investigate the role of IFN-γ (Th1) and IL-4 (Th2) cytokines in the spleen of mice.

In vitro neutralization assays
We used ZYM-DIEC02 cell to assay the neutralizing ability of mice inoculated intragastrically with E. coli ER-T. Serum, intestinal mucus, splenocyte lysate and mesenteric lymphocyte lysate samples were collected from mice. Filter-sterilized supernatant of enterotoxins LT, STa, STb, then serial dilutions respectively. Further, incubated enterotoxins with equal volume of mice samples at 37°C for 2 h, after that toxin-antibody mixture was added to the ZYM-DIEC02 cells culture plate 0.1 ml per well. After 24 h, crystal violet staining was carried out and normal cells were counted, as previously described (Guan et al., 2015).

In vivo neutralization test
To assess the neutralization activity of antibodies, we used suckling mice (suckling mice from G1 coupled with G3, aged 4 days). Serial dilutions of enterotoxins STa, STb, LT192-STa13, LT192-STb recombinant proteins for 100 μl in vivo neutralization of STa: fist orally soybean trypsin inhibitor, then received equal volume of STa toxin-antibody mixture respectively. Four hours after oral inoculation, the mice were killed and samples harested, as previously described (You et al., 2011).

Challenge for maternal antibody in suckling mice
The protective efficacy evaluation of maternal antibody uses suckling mice with milk from pregnant mice. Briefly, four 4-day-old mice per group received serial dilutions enterotoxins (STa or STb) 100 μl. After 4 h, the animals were killed and the G/C ratio was calculated as previously described (Norton et al., 2012).

Statistical analysis
The aim of this study was to estimate the recombinant E. coli ER-T, statistical analysis (ANOVA) was performed by SPSS version 19.0 (SPSS Statistics, Chicago, IL, USA) and Graphpad Prism 5.0 (Graphpad software, San Diego, CA, USA), and are represented by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001). P values of < 0.05 were considered to be statistically significant.

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Weikun Guan conceived the experiment(s), Ni Feng conducted the experiment(s) and Weikun Guan analysed the results. All authors reviewed the manuscript.

Conflict of interest
None declared.

Ethics statement
The present study did not involve endangered or protected species. The animal study complied with the Animal Welfare Act by following the NIH guidelines (NIH Pub. No. 85-23, revised 1996), and the protocols were approved and supervised by the Animal Care and Use Committee of Yichun University.

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