RESEARCH REPORT
SWINE HEALTH

Title: Development of a non-antibiotic selection vector for a live vaccine against PWD, **NPB #07-006**

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Industry Summary:
Porcine post-weaning diarrhea (PWD) continues causing substantial economic loss to swine producers. Vaccines strategy especially live attenuated vaccines could be the most effective and most affordable treatment against PWD. However, development of live attenuated vaccines typically needs antibiotic selection system (by using antibiotic resistance genes for selection). Release of large quantity of live vaccine strains carry antibiotic resistance genes could transfer antibiotic resistance genes to microorganisms in the environment. That causes major concerns on environment and medical practices using antibiotic drugs. In this study, we developed a non-antibiotic selection vector that can be used to express antigens against PWD. The experimental vaccine strains are selected by this non-antibiotic system, thus, we resolve problems regarding release of antibiotic resistance genes to the field. We used this vector to express enterotoxin antigens from enterotoxigenic *Escherichia coli* (ETEC), and to develop a live attenuated vaccine against PWD. For detailed information, please contact Dr. Weiping Zhang at 605-688-4317 or e-mail at weiping.zhang@sdstate.edu.

Scientific Abstract: Live vaccines are the most effective and the most affordable treatment for some infectious diseases, and current development of live vaccines is based on antibiotics selection system. But application of vaccines carrying antibiotic resistance genes has caused major concerns in environment and medical practice. Consequently, live vaccines using antibiotic selection vectors have been deemed as undesirable, unacceptable or even banned in some countries. Therefore, a non-antibiotic selection system must be developed. To construct a non-antibiotic selection vector, we isolated and amplified the *rtlt* operon from genomic DNA of an *E. coli* strain C using PCR with a reverse and a forward primers which were specifically designed to contain an *Ase*I and a *Spe*I restriction sites, respectively. The amplified PCR products were purified and digested with *Ase*I and *Spe*I restriction enzymes, so was the vector pBR322. The digested *rtlt* operon and pBR322 was ligated together with T4 DNA ligase. The resultant plasmid was introduced into an *E. coli* competent cell. Colonies selected by 2B minimal medium were initially screened with PCR to verify the presence of the *rtlt* operon, and the positive colonies were confirmed by DNA sequencing. Only *E. coli* cells transformed with the pBR322/*rtl*-operon can grow in the 2B minimal medium which contains ribitol as the only carbon source, thus it provided a non-antibiotics select marker. This 'pBR322/*rtl*-operon' vector still carried the tetracycline resistance gene. We replaced the *tet* region

with a fusion antigen from LT and STb, and constructed a non-antibiotic selection vector to express vaccine antigens. By transformation of a nonpathogenic but K88ac fimbrial porcine *E. coli* field isolate (8511), we developed a non-antibiotic selected living vaccine 8595 to protect young pigs against porcine post-weaning diarrhea. This non-antibiotic selection system can also be applied in live vaccine development against other infectious diseases.

Introduction:

Porcine post-weaning diarrheal disease (PWD) caused by enterotoxigenic *Escherichia coli* (ETEC) is economically one of the most important diseases in the North-American swine industry. ETEC strains are responsible for the death of 10.8% of all pre-weaning pigs and 1.5 – 2% of all weaned pigs (Tubb et al., 1993; Hampson, 1994). The key virulence factors of ETEC in diarrhea are: 1) bacterial adhesins which mediate the attachment of bacteria to the surface of host enterocytes and initiation of colonization; and 2) enterotoxins which are responsible for fluid secretion. Heat-labile toxin (LT) and heat-stable toxins (STa, STb and EAST1) are the main enterotoxins produced by ETEC strains. These enterotoxins are secreted into the porcine gut lumen, attach to specific intestinal receptors, directly disrupt fluid homeostasis and stimulate fluid secretion into the lumen of small intestine, which results in diarrhea. PWD is characterized by severe diarrhea, dehydration, weight reduction, and death. The weight loss and mortality are responsible for substantial economic loss for swine producers. Currently, there is no licensed vaccine available to effectively protect pigs from post-weaning diarrheal disease. Therefore, vaccine development against post-weaning diarrhea in swine is a great need.

Efforts have been made in the past to develop vaccines against porcine diarrhea. Vaccination of pregnant sows with purified fimbriae confers protective immunity for ETEC on their suckling offspring (Rutter and Jones, 1973; Rutter et al., 1976). The antibodies specific to the fimbriae present in colostrum and milk of immunized sows prevent adhesion of *E. coli* strains expressing the same fimbria to the intestinal epithelium of the neonatal piglets, thereby preventing diarrhea (Smith, 1972). However, immunization of the piglet by the injection of purified fimbriae provides no or only partial protection to the weaned pigs, mostly likely due to the failure to induce mucosal immunity. Moreover, it is not only expensive to prepare purified fimbriae, and fimbria vaccines developed from one fimbria type fail to provide protection against *E. coli* strains expressing a different fimbria, thereby making fimbria vaccines impractical. Vaccines based on enterotoxins, especially, LT, LT-A or LT-B subunit, have also been attempted. Live *E. coli* vaccines expressing enterotoxin antigens as the only virulence antigen are not effective in protecting weaned pigs (Francis and Willogohs, 1991). Furthermore, LT toxoids inoculated into sows provides some protection of nursing piglets against LT-producing *E. coli*, but did not extend protection against ETEC strains that also produced ST toxins (Frantz and Mellencamp, 1983). In contrast, live oral vaccines containing both fimbria and enterotoxin antigens exhibited great protection. Early experimental live vaccines developed at South Dakota State University proved highly effective when both LT toxin (LT-B subunit) and K88 fimbria were included as vaccine components (Francis and Willgohs, 1991). However, the utility of enterotoxins and fimbriae as potential live vaccine candidates has not been effectively explored under field conditions, largely due to concerns over the potential for commercial success of the product, and the involvement of antibiotics selection markers in the vaccine strains.

Antibiotics are typically involved in construction of live vaccines when enterotoxins are selected as the main antigens. Most currently available gene cloning vectors for bacteria are based on multi-copy plasmids which carry antibiotic resistance genes as dominant markers to select positive transformats in vitro. Although plasmids can be maintained in bacteria cultivated as monocultures under control conditions, they are not stable when host organisms are grown in the absence of antibiotics. Such instability can be exacerbated when the host bacteria have to compete with the normal microbe flora

inside the host. Furthermore, as antibiotics are of major clinical importance in the treatment of bacterial infections, a major concern is that the antibiotics resistance genes may opportunistically spread to pathogenic microbes in the environment, especially when the release in large quantity of antibiotics resistance genes from live vaccine strains. Consequently, treatment to infections caused by these pathogens with antibiotics becomes therapeutically ineffective. Therefore, under the current regulatory environment, antibiotic-resistance based plasmid selection systems used in live vaccine development are undesirable or unacceptable, and non-antibiotic selection vectors must be developed.

Objectives:

The objectives of this research project are to develop a non-antibiotic selection vector and to apply the non-antibiotic selection vector in the development of a live vaccine against porcine post-weaning diarrhea (PWD). Enterotoxigenic *Escherichia coli (*ETEC) associated diarrhea is economically one of the most important diseases for swine industry in the North America. Currently, there is no vaccine available to protect weaned pigs from PWD. Vaccines developed previously from either purified fimbriae or heat-labile (LT) enterotoxin provide only partial or no protection against PWD. In contrast, live vaccines containing both fimbria and enterotoxin antigens showed high protection, and production of live vaccines are usually more cost effective than subunit vaccines. Introduction of virulence-associated antigens into a live *E. coli* strain requires the introduction of a vector expressing those antigens. Most currently available vectors for bacteria are based on antibiotic resistance genes as selection markers. However, those antibiotic selection vectors are usually not stable when the host *E. coli* strains grow in an environment absence of antibiotics. Moreover, as antibiotics are of major clinical importance in the treatment of bacterial infections, a major concern is that these resistance genes may opportunistically spread from vaccine strains to pathogenic microbes in the environment, resulting in antibiotic resistance and therapy failure. Therefore, there is a great need to develop a novel vector system with non-antibiotic selection markers. In this proposed study, we will clone an *rtl* operon from *E. coli* stain C into the plasmid pBR322, to construct a non-antibiotic selection vector. The transformation of a K88-poitive *E. coli* strain with the 'pLT192:STb/*rtl*-operon' vector would allow only the transformed cells, and not the parent cells, to use ribitol as the carbon source. The plasmid pLT192:STb encodes a non-toxic mutant of LT fused to another important E. coli enterotoxin heat-stable type b (STb). STb is a small peptide that by itself is non-immunogenic. Fusion of STb to LT mutant provides for a much larger molecule, which is expected to be highly immunogenic. Thus, anti-STb antibodies will be formed upon vaccination. The transformed *E. coli* carrying K88, non-toxic-LT and STb antigens but no antibiotic resistance genes, would be developed as a live vaccine to protect pigs against PWD.

Materials & Methods:

Procedures involved in **Aim 1** (*to isolate the rtl operon containing genes to control metabolism of ribitol from the E. coli strain C and to clone the operon into a pBR322 plasmid for constructing a vector with a non-antibiotic selection marker*) are typically gene isolation and gene cloning. The *rtl* operon consists of three genes: ribitol dehydrogenase (*rtl*D), ribitol kinase (*rtl*K) and ribitol transporter (*rbt*T) (Fig. 1). This operon is naturally carried by the C strains, and also has been cloned in vector pMECA.

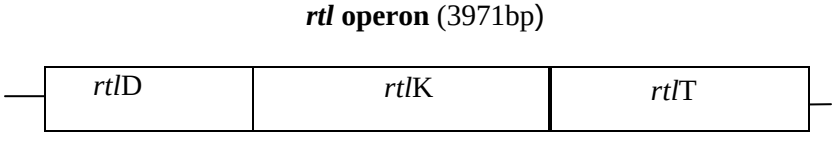

Fig. 1. The *rtl* operon structure. The operon is composed of three genes: *rtl*D (753 bp), *rtl*K (1605 bp) and *rbt*T (1257 bp).

We will PCR amplify the *rtl* operon from genomic DNA of *E. coli* strain C (gift from E. coli Genetic Resource Center, Yale University) or the pMECA-R plasmid (a gift from Dr. W.A. Parrott, Univ. of Georgia). Genomic DNA of *E. coli* strain C has been purified, and we also have the pMECA-R plasmid DNA. PCR primers to amplify the *rtl* operon have been designed in our lab: rtlDAatI-R (5'-tggtttcttagacgtcaggtggcac-'3) and rtlTBsaI-F (5'-cagtgaggcaccggtctcagcg-'3). The reverse and the forward primers contain unique restriction sites *Aat*I and *Bsa*I (*Eco*31I), respectively. Amplified PCR products will be purified and digested by *Aat*I and *Bsa*I restriction enzymes, so will be the pBR322 plasmid. The digested *rtl* operon and pBR322 will be ligated together by T4 DNA ligase. The ligated product will be introduced into *E. coli* competent cells. Positive colonies will be PCR screened first, and then will be confirmed by DNA sequencing. The minimal medium will be used to select positive colonies. Only *E. coli* cells transformed with the pBR322/*rtl*-operon can grow in the minimal medium which contains ribitol as the only carbon source. The pBR322/*rtl*-operon vector will still have the tetracycline resistance gene, but it will be replaced with vaccine antigen(s) of interested. Thus, a non-antibiotic selection vector will be constructed.

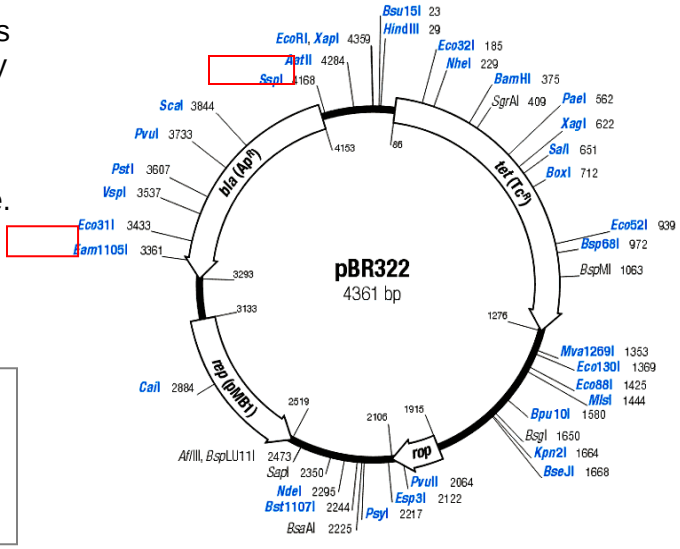

Fig. 2. Construction of a non-antibiotic selection vector. pBR322 will be digested with *Aat*I and *Bsa*I to remove the ampicillin resistance gene, which will be replaced by the *rtl* operon.

<u>*The rtl operon* isolation and cloning:</u> Genomic DNA from an *E. coli* strain C or plasmid pMECA-R will be used as template for PCR amplification. The designed forward and reverse PCR primers possess the *Aat*I and *Bsa*I restriction enzyme sites, respectively. PCR will be conducted in a reaction containing 1X DNA *Taq* polymerase buffer (with Mg^{++}), 250 nM each dNTP, 0.5uM each primer, 100 ng of template DNA, and 1 unit of *pfu* polymerase. The PCR program will consist of 1 cycle of 2 min at 94 ºC; 30 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC, 5 min at 72 ºC; and an extension of 15 min at 72 ºC. PCR products will be separated by gel electrophoresis, and purified using a QIAquick Gel Extraction Kit (QIAGEN). Purified *rtl*-operon-PCR products will be digested by *Aat*I and *Bsa*I restriction enzymes, as will be the vector pBR322. Digested *rtl*-operon-PCR products and vector will be purified by gel electrophoresis, and then will be ligated by T4 DNA ligase (New England BioLabs, MA). 1- 2 ul of ligated product will be used for transformation by electroperation at 2.5 kV with 25 uF of capacitance and 200 Ω of resistance. Positive transformats will be selected by their growth in the minimal medium.

<u>DNA sequencing:</u> Positive colonies will be sequenced to confirm the correct insertion of the *rtl* operon in the pBR322 vector. An ABI BigDye Terminator Sequence kit (Applied Biosystem, CA) will be used to prepare sequencing reactions by follow the manufacturer's instructions. Reaction samples will be sent to the DNA sequencing facilities at Iowa State University for assessment.

The procedures involved in **<u>Aim 2</u>** (*applying the non-antibiotic selection vector to the construction of a live vaccine expressing K88, non-toxic LT, and STb antigens against porcine PWD*) are quite similar to those in Aim 1. However, instead of using the pBR322 vector, Aim 2 will start with a pLT192:STb vector. Vector pLT192:STb was pBR322 derived, with the genetic fusion of LT192:STb cloned into pBR322 at the tetracycline resistance region. Thus the pLT192:STb vector has only the ampicillin resistance gene left. The pLT192:STb was proven to express both attenuated LT and STb antigens. Animal challenge studies showed that piglets did not develop diarrhea after inoculation with the K88/LT192:STb strain, and oral immunization with K88/LT192:STb strain appears to provide protection against an oral challenge with ETEC wild-type strain 3030-2 (K88/LT/STb). Replacement of the ampicillin resistance gene with the *rtl* operon will allow us to construct a non-antibiotic selection vector also expressing LT192 and STb antigens (Fig. 3). By transformation

of an *E. coli* field isolate 1836-2, which expresses K88 fimbriae, with the vector pLT192:STb/rtl-operon, we can construct a non-antibiotic
selection live vaccine strain expressing LT, STb, and K88 antigens.

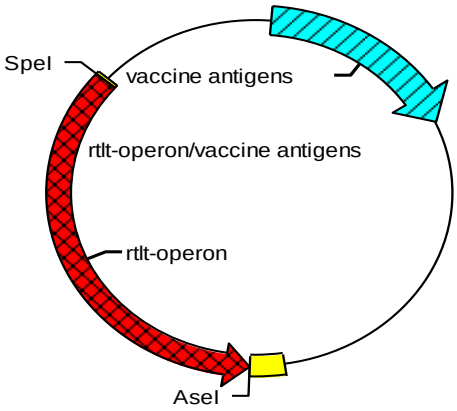

Fig. 3. Construction of a non-antibiotic selection vector for a live vaccine strain development. Vector pLT192:STb/rtl-operon expressing LT and STb antigens, and enzymes to metabolize ribitol.

The isolation and cloning of *rtl* operon into pLT192:STb will be identical to the procedures in Aim 1. The expression of LT192 and STb antigens will be verified by GM1 ELISA, and production of the K88 fimbriae will be tested by ELISA abd tge porcine brush border adherence assay.

GM1 ELISA: The expressed LT192 and STb from the fusion protein will be detected by GM1-ELISA by using anti-CT or anti-STb antibodies, respectively. Microtiter plates (Nalge NUNC, Denmark) will be coated with GM1 at room temperature overnight. Plates will be washed and blocked with 2% BSA-PBS. Plates will be incubated with concentrated supernatant of overnight grown culture or lysate of the pellet at 4°C overnight. After being washed, plates will be incubated with rabbit anti-CT or rabbit anti-STb sera, respectively. Then, plates will be incubated with goat anti rabbit immunoglobulin-horse radish peroxidase conjugate. Reaction activity will be visualized by the addition of peroxidase substrates, and measured by a plate reader at 405 nm.
Verification of K88 expression: K88 ELISA will be performed as described elsewhere (Sun et al., 2000). Porcine brush borders bacteria adherence assay will be conducted as briefly explained: brush borders freshly prepared from K88-receptor positive pigs will be tested for the binding of the constructed live vaccine strain. Bacterial and brush border samples will be mixed in the presence of mannose, incubated for 5 min on a shaker, then examined under a phase contrast microscope (Baker et al., 1997).

Results:

Isolation and cloning of the rtlt operon: The rtlt operon was PCR amplified, digested and cloned into vector pBR322 at the SpeI and AseI restriction sites (fig. 4). Cloned operon was verified by DNA sequencing.

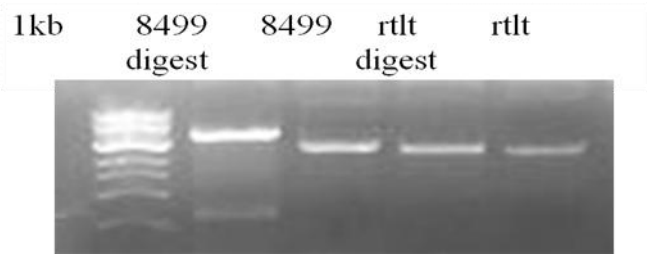

Figure 4. The rtlt operon was PCR amplified from genomic DNA of an E. coli C strain with PCR primers carrying SpeI and AseI restriction site nucleotides. Amplified PCR products were gel eletrophorezed and purified. Purified PCR products were digested with SpeI and AseI, so did vector pBR322. Digested products were purified and ligated with T4 DNA ligase.

Construction of a non-antibiotic selection vector: Plasmid with the cloned rtlt operon can be selected by 2B minimum medium (Fig. 5).

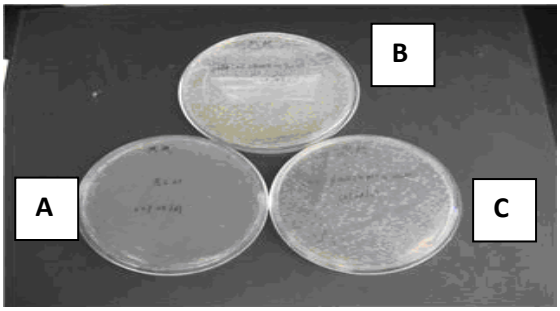

Figure 5. Demonstration of E. coli strains can be selected with rtlt operon on 2B minimum medium. A, an E. coli strain that does not carry the pBR/rtlt plasmid cannot grow on the 2B medium. B, an E. coli C strain can grow on the 2B medium. C, an E. coli strain that carries the pBR/rtlt plasmid can grow on 2B medium.

Construction of an experimental vaccine strain with non-antibiotic selection vector: A plasmid, p8488 that express LT and STb fusion antigen LT_{192}:STb, was used to construct an experimental vaccine strain that carries no antibiotic resistance gene (Fig. 6). By replacing the ampiciline resistance gene with the rtlt operon, this p8488 plasmid carries no antibiotic resistance gene, and

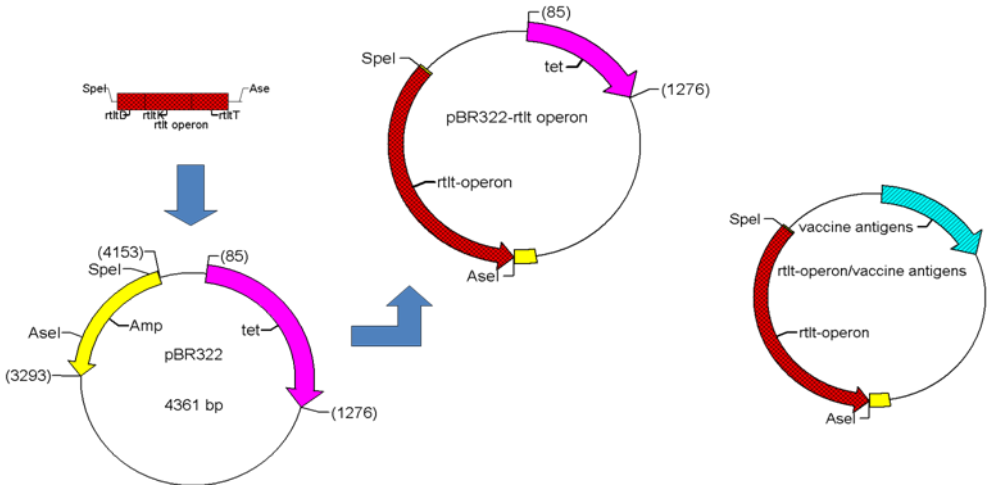

Figure 6. Construction of an experimental vaccine strain using the non-antibiotic selection vector. Plasmid p8488 expressing LT and STb antigens had its ampiciline resistance gene replaced by the rtlt operon.

The resultant E. coli strain (K88ac/LT/STb), an experimental strain, can be selected by 2B medium.

Discussion:

Porcine post-weaning diarrhea caused by enterotoxigenic *Escherichia coli* strains is responsible for the death of 1.5 – 2% of all weaned pigs and continues to be one of the most economically important diseases in the North American swine industry. Development of an effective vaccine to control or eradicate the PWD will directly reduce the production costs. A live vaccine strain with non-antibiotic selection vectors expressing K88, attenuated LT and STb antigens will be an excellent vaccine candidate to control PWD. Live vaccines are very cost effective, and a live vaccine with no antibiotic resistance genes is much more environmentally friendly and acceptable by regulatory agencies. The non-antibiotic selection vector developed from this study can be directly used in developing vaccines against PWD and vaccines against other porcine pathogens.