CONSTRUCTION OF A MODIFIED LIVE HP-PRRS VIRUS VACCINE AND AN ATTENUATED LISTERIA VACCINE VECTOR USING REVERSE GENETICS

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Abstract

The development of reverse genetics systems for the manipulation of viral and bacterial genomes has provided platforms for identifying virulence genes, studying pathogenesis and developing vaccines. Replication-competent vaccines (e.g., modified live virus (MLV) vaccines and replicating viral/bacterial vectors) are considered the most efficacious approach for vaccine development. We constructed replication-competent candidate vaccines for two viral diseases in pigs via reverse genetics. The first vaccine we designed is to protect against highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV). HP-PRRSV can cause high mortality in pigs of all ages. Vaccines to protect pigs from HP-PRRSV are not commercially available in the US. According to previous studies, the non-structural protein (NSP) coding region of HP-PRRSV is closely related to the high mortality rate and the structural protein (SP) coding region contributes to the induction of broadly protective neutralizing antibodies. We created a chimeric PRRSV, of which the SP coding region was derived from HP-PRRSV and NSP coding region was derived from a low-pathogenic strain. This chimeric PRRSV caused similar CPE in cells as parental viruses, but had slower growth kinetics. We hypothesize that this chimeric virus will have a low pathogenicity and could serve as a candidate vaccine that can provide protection against HP-PRRV. The second vaccine vector is a modified *Listeria innocua* (*L.inn*), a non-pathogenic strain of *Listeria*. Genetically related *Listeria monocytogenes* (*L.m*) is a well-known intracellular pathogen that encodes specialized virulent determinants facilitating its intracellular growth and spread. Our goal is to make *L.inn* a vaccine vector that can deliver classical swine fever (CSF) viral antigen into intracellular environments by complementation of *L.inn* with selected *L.m* virulence genes necessary for intracellular survival and induction of a robust immune response. In this study, we constructed a shuttle vector pHT-E2 that can express

CSFV antigen E2 in *L.inn*. We cloned the *plcA-prfA* operon of *L.m* virulence gene cluster (vgc) into pHT-E2, which enhanced the expression of E2 in *L.inn*. In future studies, we plan to clone additional *L.m* virulence genes into the shuttle vector to increase immunogenicity of this recombinant *L.inn* and test its ability to protect pigs from CSFV.

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Chapter 1 - Use of reverse genetics to create an attenuated HP-PRRSV strain

Abstract: Porcine reproductive and respiratory syndrome (PRRS) is the most economically important disease of pigs worldwide, for which current vaccines provide limited protection due to the high genetic diversity and rapid mutation of circulating PRRSV strains. In 2006, highly pathogenic PPRS (HP-PRRS) characterized by high fever (>41 ℃) and high morbidity and mortality in pigs of all ages emerged in China and devastated the pig industry. Application of MLV vaccines based on an attenuated HP-PRRSV strain have been reported in China, but no commercial HP-PRRSV vaccine is available in the U.S.. The aim of this study is to use reverse genetics to recreate and attenuate HP-PRRS virus. Using the infectious clone of a low-pathogenic (LP) classical NA strain P129 as a backbone, we constructed a full-length HP-PRRSV clone JXA1 and a series of P129/JXA1 (LP/HP) chimeric clones. Interestingly, neither JXA1 nor P129/JXA1 chimeric clones could produce infectious virus in MARC-145 cells, possibly due to the existence of fatal mutations in the original JXA1 sequence; however, we managed to use the sequence of a third virus, HP-PRRSV HV strain, to restore infectivity and generated an infectious P129/JXA1/HV triple chimeric clone. The virus rescued from this triple chimeric clone caused similar CPE in cells as parental viruses, but had slower growth kinetics. The viral genome contains NSP coding region derived from P129 and SP coding region from HP-PRRSV. According to previous studies, the non-structural protein (NSP) coding region of HP-PRRSV is closely related to the high mortality rate and the structural protein (SP) coding region contributes to the induction of broadly protective neutralizing antibodies. We hypothesize that this P129/JXA1/HV chimeric clone will have a low pathogenicity but could serve as a candidate vaccine that can provide protection against both HP-PRRV and classical PRRSV.

1.1 Introduction

Porcine reproductive and respiratory syndrome (PRRS), characterized by respiratory problems, weight loss and poor growth performance, as well as reproductive failure in sows [1, 2] was first reported in North America in 1987 [3] and in Europe in 1990. To date, PRRS has become an endemic disease in almost all pork-producing areas of the world and has led to huge economic losses in the pork industry. A recent study shows that this economically important disease costs the U.S. pork industry $664 million annually, an increase from the $560 million estimated annual cost in 2005 [4].

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, was first isolated in the Netherlands in 1991 [5] and in the USA in 1992 [6]. PRRSV is an enveloped, positive-strand RNA virus which, together with lactate dehydrogenase-elevating virus of mice (LDEV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV), belongs to the family Arteriviridae within the order Nidovirales [7, 8]. Phylogenetic analysis divided PRRSV isolates into two distinct major genotypes: Type I (European) represented by Lelystad strain, and Type II (North American) with VR2332 as the prototype strain [9]. These two genotypes differ antigenically as well as genetically and only share an approximately 60% overall sequence identity [10, 11]. The PRRSV genome is approximately 15 kb and includes the 5' cap structure and 3' polyadenylated tail. The genome contains at least 10 overlapping open reading frames (ORFs) [12, 13]. The ORF1a and ORF1b, constituting 75% of the viral genome, are translated directly from the incoming genomic RNA into two large polyproteins pp1a and pp1ab and then autoproteolytically cleaved into at least 15 nonstructural proteins (NSPs) [14-16]. The NSPs participate in genome replication and subgenome transcription [17]. The viral

structural proteins are encoded by ORFs 2 to 7, locating downstream of ORFs 1a and 1b, which are involved in virus infectivity and neutralizing antibody elicitation [18-20].

Vaccination is currently the principal strategy for prevention the treatment of PRRSV [21, 22]. PRRSV neutralizing antibodies induced by vaccination are believed to prevent infection and transplacental infection of pregnant sows [23]. There are two types of PRRS vaccine that are commercially available, modified-live virus (MLV) vaccine and killed virus (KV) vaccine. PRRS MLV vaccines licensed for use in the US are derived from the North American (NA) PRRSV, which include Ingelvac PRRS MLV and ReproCyc PRRS-PLE (both from VR-2332; Boehringer Ingelheim), Ingelvac PRRS ATP (from JA142; Boehringer Ingelheim), and Fostera PRRS (from P129; Zoetis) [24-26]. PRRS KV vaccine is licensed for use in European countries and other parts of the world, but not in the US. PRRS MLV vaccine has been proved to be effective for its protection against genetically homologous PRRSV strains, but only partial protective against heterologous strains, which is due to the high genetic diversity and rapid mutation of circulating PRRSV strains [27]. PRRS KV vaccine only confers limited protection against either homologous or heterologous strains. Hence, a better PRRSV vaccine effective in providing protection against both homologous and heterologous PRRSV strains is still needed.

In 2006, atypical PRRS outbreaks were reported in China with the characteristics of high fever and high morbidity and mortality in pigs of all ages [8, 28]. The causative agent was identified as highly pathogenic PRRS virus (HP-PRRSV). Different from classical PRRSV strains, HP-PRRSV can induce neurological symptoms (e.g., shivering), prolonged high fever of above 41 ℃, and red discoloration of the ears and body; it has a morbidity of 50–100% and the highest mortality rate can reach 100% in nursery pigs [8, 28]. Complete genome sequencing and phylogenetic analysis demonstrated that Chinese HP-PRRSV is a newly emerged evolutionary

subgroup that belongs to type II genotype but has a unique molecular hallmark of a discontinuous deletion of 30 amino acids in the NSP2 coding region [8, 28, 29]. At first, this deletion was considered related to the high virulence of HP-PRRSV, but later study disproved this theory [30]. Until now, the location of virulence determining region in the HP-PRRSV genome still remains unknown. In a recent study, researchers exchanged different ORFs between a HP strain JXwn and classical NA train HB-1. Pathogenicity analyses showed that JXwn ORF1b region, but not the structural protein region, could confer fatal virulence to HB-1, suggesting that replicase genes play an important role in virulence of HP-PRRSV [31].

Currently used NA strain based MLV vaccines for classical PRRS have limited protection against HP-PRRSV infection [32, 33]. Application of MLV based on attenuated HP-PRRSV strains (JXA1-R, HuN4-F112 and TJM) have been reported in China and showed effective protection against challenge with wild-types HP viruses [33, 34]. According to vaccine efficacy studies conducted in our lab, JXA1-R can also protect pigs from challenge with current circulating North American PRRSV strain NADC-20 by inducing high titer of neutralizing antibodies to NADC-20 (unpublished data). However, there is no commercial HP-PRRSV vaccine available in the US. Although HP-PRRSV only exists in Asia at the present time, it may become a threat to the US swine industry in the future as the result of the increasing international transportation. This availability of a HP-PRRSV vaccine is of great importance in protecting the US swine industry from the devastating attack of HP-PRRSV.

Reverse genetics, in the opposite direction of classical genetics, is an approach to discover what phenotypes arise as a result of particular genetic sequences. It can be a powerful tool in analyzing the fatal virulent determinant of HP-PRRSV and seeking ways to effectively attenuation for MLV vaccine development. The aim of this study is to use reverse genetics to

recreate and attenuate HP-PRRS virus. Using the infectious clone of a low-pathogenic (LP) classical NA strain P129 as a backbone, we constructed a full-length HP-PRRSV clone JXA1 and a series of P129/JXA1 (LP/HP) chimeric clones. Interestingly, neither JXA1 nor P129/JXA1 chimeric clones could produce infectious virus in MARC-145 cells, possibly due to the existence of fatal mutations in the original JXA1 sequence; however, we managed to use the sequence of a third virus, HP-PRRSV HV strain, to restore infectivity and generated an infectious P129/JXA1/HV triple chimeric clone. The recombinant virus rescued from this triple chimeric clone contains NSP coding region derived from P129 and SP coding region from HP-PRRSV. According to previous studies, the non-structural protein (NSP) coding region of HP-PRRSV is closely related to the high mortality rate and the structural protein (SP) coding region contributes to the induction of broadly protective neutralizing antibodies. We hypothesize that this P129/JXA1/HV chimeric clone will have a low pathogenicity but could serve as a candidate vaccine that can provide protection against both HP-PRRV and classical PRRSV.

1.2 Materials and Methods

1.2.1 Cells and virus

MARC-145 cells were grown in Modified Eagle's medium (MEM, Gibco) containing 10% fetal bovine serum (FBS), penicillin (100 U /ml) and streptomycin (100 μg /ml). Cells were maintained at 37 ℃ with 5% CO_2. For viral titration, MEM containing 2% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) was used. The full-length infectious cDNA clone plasmid (pCMV-P129, pcDNA-HV) of PRRSV P129 (GenBank: AF494042) and of HP-PRRSV HV (GenBank: JX317648) used in this study have been previously described [35, 36]. pcDNA-HV was a kind gift from Dr. Wen-hai Feng (State Key Laboratory of Agro-biotechnology, China

Agricultural University, Beijing, China). pCMV-P129 was a kind gift from Dr. Bob Rowland (College of Veterinary Medicine, Kansas State University, US).

1.2.2 Construction of JXA1 and chimeric full-length cDNA clones

pCMV-P129 infectious clone was used as the backbone for the construction of new infectious clones. Six restriction enzymes, *Pac*I, *Mlu*I, *Pme*I, *BspD*I, *Afe*I, *BsrG*I and *Spe*I in the pCMV-P129 infectious clone were selected for replacing the P129 genome with corresponding sequences from HP-PRRSV. Five corresponding DNA fragments, PM (*Pac*I-*Mlu*I), MP (*Mlu*I-*Pme*I), PA (*Pme*I-*Afe*I), AB (*Afe*I-*BsrG*I) and BS (*BsrG*I-*Spe*I), were synthesized (GenScript) based on the sequence of JXA1 strain of HP-PRRSV (GenBank: EF112445). These five fragments cover the whole JXA1 genome and represent 1-3150 (PM), 3145-7605 (MP), 7597-11714 (PA), 11709-12623 (AB) and 12618-15341 (BS) of the genome respectively. *Mlu*I, *Afe*I, *BsrG*I enzyme sites were created by mutating the nucleic acid sequence of the JXA1 genome without any change in amino acid sequence. PA fragment was further digested by *BspD*I site to 2 parts (*Pme*I-*BspD*I and *BspD*I -*Afe*I). The six fragments were cloned via corresponding enzyme sites in the pCMV-P129 to create a full-length JXA1 and series of P129/JXA1chimeric clones.

BS fragment corresponding region in HP-PRRSV HV infectious clone was amplified by PCR using primers HV-BS-F: AGCTTCATGACTTTCAGCAATGG and HV-BS-R: gaacACTAGTTAGAAGGCACAGTCGAGG (*Spe*I site is underlined). After double-digestion with *BsrG*I and *Spe*I, HV BS fragment was purified with QIAquick Gel Extraction Kit (Qiagen) and cloned into *BsrG*I-*Spe*I sites in JXA1 and P129-JS to create JXA1-HBS and P129-JAB-HBS respectively.

The correctness of the exchanged regions in all full-length clones was verified by PCR with specific primers. The whole viral genome and 5' and 3' vector sequences in P129-JAB-HBS clones were sequenced to ensure no mutations exist.

1.2.3 Site-directed mutagenesis

An 11712 G deletion mutation in *Afe*I enzyme site was identified in JXA1 full-length clone by sequencing. 11712G deletion was added to the genome by Q5 Site-Directed Mutagenesis Kit (NEB) using JXA1-NEB-F: gCTTACCATGGTGAAATGC and JXA1-NEB-R: GCTAGACAGAATAATTTTGGC (*Afe*I site is underlined). Reverse mutant was identified by the recovery of the *Afe*I site. After site-directed mutagenesis, JXA1 full-length clone was sequenced to ensure no more mutations existed.

1.2.4 Recovery of viruses from full-length cDNA clones

All full-length cDNA clone plasmids were purified with EndoFree Plasmid Maxi Kit (Qiagen) and transfected with 90% confluent MARC-145 cells using Lipofectamine 2000 (Life sciences). MARC-145 cells were maintained in 10% MEM medium in 6-well plates. Cells were washed with PBS once and 750 μl Opti-MEM (Gibco) was added to each well immediately before transfection. Transfection was conducted according to the protocol supplied by the manufacturer (Life sciences). Transfection mixtures were removed after 4-6 hours incubation and 10% MEM was added to cells. Cells were observed daily after transfection for morphological observation and cytopathic effects (CPE). The culture supernatants were harvested at 4 days post-transfection, no matter whether CPE were observed or not. Two cycles of freeze-thaw were performed and cells were centrifuged for 5 min at 1200 rpm. Supernatants were collected and designated P1 (passage 1). P1 supernatants were added to 90% confluent MARC-145 cells in 2%

MEM in 1/50 volume of the cell culture. Cells were incubated for an additional 48 to 72 hours to allow the possible CPE formation. Supernatants were harvested in the same way as for P1 and designated P2. P3 were prepared in the same way as for P2. Each passage was aliquoted and stored at -80 ℃ until use.

1.2.5. Titration of rescued viruses

The titers of rescued P129 (P3 virus) and Hybrid PRRSV (P3 virus) were determined using a microtitration infectivity assay and expressed as 50% tissue culture infective dose per ml (TCID50/ml). Virus stocks were 10-fold serially diluted (10^{-1} to 10^{-8}) in MEM containing 2% FBS and incubated with 90% confluent monolayers of MARC-145 cells cultured in 96-well plates. Eight replicate wells were prepared for each dilution. After incubation for 48 to 72 h at 37 ℃, the virus titers were determined.

1.2.6 RNA extraction, RT-PCR and sequencing

Viral RNA of hybrid PRRSV was extracted using P3 virus stock using Viral Nucleic Acid Extraction Kit II (IBI scientific). First-strand cDNA synthesis was performed by ProtoScript II First Strand cDNA Synthesis Kit (NEB) using reverse primers PRRSVpart1-R: TTTGCCAGTTTTCTCGCACG and PRRSVpart2-R: AATTACGGCCGCATGGTTCT according to the standard protocol supplied by the manufacturer. The viral genome was amplified in 4 fragments by Q5 High-Fidelity DNA Polymerase (NEB) using primer pairs PRRSVpart1-F+Hybrid1a-R, Hybrid1b-F+Hybrid1b-R, PRRSVpart2-F+PRRSVpart2-R and PRRSVpart2-F+Hybrid2b-R (Table 1.1). Amplified products were purified using the PCR Purification Kit (Qiagen) and sequenced.

1.2.7 Bioinformatics analysis

Sequence alignments between ORFs in JXA1 BS and HV BS were conducted using NCBI Protein Blast. A phylogenetic tree of 10 different PRRSV strains was constructed based on complete genomes obtained from GenBank. Multiplex sequence alignments were generated by CLUSTAL X (version 2.1) [37]. Phylogenetic analysis was conducted using the MEGA software (version 4.0.2). Structure homology-modeling was conducted by SWISS-MODEL with automatic template search. SMTL ID of templates used for homology-modeling of GP4 ectodomain were 2q22.1.A and 4cmr.1.A. SignalP 4.1 and PredictProtein were used for prediction of potential signal peptide and transmembrane helices repetitively.

1.3 Results

1.3.1 Construction of full-length JXA1 and P129/JXA1 chimeric clones

JXA1 is the first HP-PRRSV isolate discovered in 2006 [8] and also serves as the reference strain of HP-PRRSV. PRRSV P129 is a lowpathogenic NA strain, originally isolated in the autumn of 1995 from a swine herd in southern Indiana [38]. P129 only shares 92% genome similarity with JXA1and pCMV-P129 is a full-length infectious clone of P129 (Fig. 1.1). The P129 genome in pCMV-P129 was replaced by JXA1 genome fragments, PM, MP, PA, AB and BS by unique enzyme sites in P129 genome (Fig. 1.2A). A full-length JXA1 clone and a series of P129/JXA1 chimeric clones were created (Fig. 1.2B). P129-JS contains 5’ UTR and non-structural protein encoding region from P129 and structural protein encoding region (SP) and 3’ UTR (AB+BS) from JXA1. JXA-P1b contains 5’ UTR, ORF1a and SP from JXA1 and ORF1b of P129. P129-JAB contains only the AB fragment from JXA1 with the rest of the sequence coming from the P129 genome. JXA1 clone is consisted of entire and only JXA1 genome. A

deletion mutation of 11712 G in *Afe*I enzyme site in JXA1 clone was identified after PA fragment was inserted. Site-Directed Mutagenesis was used to add this 11712 G back to the genome and recovered the *Afe*I enzyme site. The entire viral genome and the 5' and 3' junction region in vector in JXA1 clone were sequenced. The first nucleotide 'A' of the viral genome positioned behind the -25 TATA box of CMV promoter and unique *Pac*I enzyme site (Fig. 1.2C) and the genome ends with 21-residue polyadenosine tail.

1.3.2 Infectivity of the full-length JXA1 and P129/JXA1 chimeric clones in cell culture

Infectivity of full-length JXA1 and P129/JXA1 chimeric clones was determined in MARC-145 cells. MARC-145 cells were directly transfected with the same amount of plasmid DNA of JXA1, JXA-P1b, P129-JS, P129-JAB or P129 respectively. CPE appeared at 3 days post-transfection for P129-JAB and P129 (Fig. 1.3). CPE was not observed for other full-length clones. The culture supernatants of all transfected cells were collected at 4 days post-transfection no matter whether CPE appeared or not and designated P1 (passage 1). To examine whether the absence of visible CPE in cells transfected with JXA1, JXA-P1b and P129-JS was due to low and therefore undetectable levels of virus production and stability of infectivity of viruses rescued from P129-JAB clone, P1 supernatants were passaged two times in MARC-145 cells for infectivity amplification. No CPE was observed for all JXA1, JXA-P1b and P129-JS even in P3, while extensive CPE was evident in 2 days in cells infected with the P1 and P2 P129-JAB or P129 viruses. These results suggested lethal mutation(s) may exist in the genome of JXA1 and most likely were located in the BS fragment. As a consequence, no viable virus was generated from cells transfected with any clones that had the JXA1 BS region.

1.3.3 Construction of JXA1/HV and P129/JXA1/HV chimeric clones

To further analyze if there was lethal mutation(s) in BS fragment, the homologous region of BS in the infectious clone of another Chinese HP-PRRSV strain HV was cloned and used to replace the BS in JXA1 clone and P129-JS clone. Two new chimeric clones JXA1-HBS and P129-JAB-HBS were generated (Fig. 1.4A). HV strain shares an overall sequence identity of 99.3% with JXA1 (Fig. 1.1). HV BS also shares an identity of 99.4% in nucleotide sequence with JXA1 BS. MARC-145 cells were transfected with the same amount of plasmid of JXA1-HBS P129-JAB-HBS, HV or P129 respectively. CPE was observed at 3 days post-transfection (Fig. 1.5) for P129-JAB-HBS, HV and P129. No CPE was observed for JXA1-HBS. Supernatant were collected at day 4 and passaged 2 more times in MARC-145 cells. The titer of 'Passgae-3' virus was 5.62×10^5 TCID50/ml for P129-JAB-HBS and 1.78×10^7 TCID50/ml for P129, indicating that the P129/JXA1/HV chimeric virus has slower growth kinetics compared to parental P129 virus. The plague morphology of progeny virus of P129-JAB-HBS was indistinguishable compared to the parental P129 virus (Fig. 1.5). No CPE appeared in any passage of JXA1-HBS. This result supports our hypothesis that fatal mutation(s) was harbored in JXA1 BS region, but also indicated that there are more fatal mutations existing in the remaining part of the JXA1 genome.

To rule out the possibility of contamination with parental viruses in P129-JAB-HBS samples, viral RNA was extracted from P129-JAB-HBS P3 virus and RT-PCR and sequencing of the whole viral genome were performed. Sequence of AB fragment and BS fragment of the virus derived from the infectious clone P129-JAB-HBS are the same as the AB of JXA1and BS of HV respectively. AB fragment derived from JXA1 contains most of the ORF2a and the intact ORF2b. BS fragment of HV contains the intact ORF3, ORF4, ORF5, ORF6, ORF7 and 3'UTR

and partial ORF2 (Fig. 1.4B).The rest of the non-structural proteins encoding region and 3' UTR are identical with P129. The pCMV-P129 infectious clone contains a silent mutation C to T at nucleotide position 1559 of the genome that is a genetic marker distinguishing the infectious clone from wild type P129. The P129-JAB-HBS also retained this 1559T in its genome.

1.3.4 Molecular analysis of JXA1 BS and HV BS

To further analyze the location of possible fatal mutations in JXA1 BS region, sequence alignment was conducted between JXA1 BS and HV BS region for all ORFs (protein blast) and 3' terminal untranslated region (nucleotide blast). Eight amino acid differences between these 2 strains in the BS region were identified (Fig. 1.6). No nucleotide differences were found in 3' UTR between these two strains. Two amino acid differences (F12L and F248V) distribute in GP3, 5 locate in GP4 (I66S, S132T, L172F, P173A and S174I) and 1(L196Q) was in GP5. Among them, F12L in GP3 locates in the predicted signal peptide region, I66S and S132T in GP4 locate in the predicted ectodomain, L172F, P173A and S174I in GP4 are predicted within transmembrane region, and F248V in GP3 and L196Q in GP5 are predicted being in C-terminal endodomain [18]. To analyze the possible effect of F12L mutation in GP3, prediction (SignalP) was conducted for signal peptide region in JXA1 GP3 and HV GP3, but no difference was found between these two proteins (data not shown). Structure homology-modeling (SWISS-MODEL Workspace) was conducted for ectodomain of JXA1 GP4 and HV GP4. No obvious difference in tertiary structure was found in I66S and S132T location in GP4 (Fig. 1.7). Structure homology-modeling could not be conducted for the transmembrane region of GP4 or endodomain of GP3 or GP5, because no templates were found matching the target regions. 172-174 LPS are within the alpha-helix structure of HV GP4 transmembrane region. If the change from LPS to FAI in JXA1 GP4 disturbed the helix structure, it may influence the anchor of JXA1 GP4 in the

membrane. However, analysis of transmembrane region using PredictProtein did not show any difference in the length of the helical transmembrane region in JXA1 and HV GP4. PRRSV GP5 has a large C-terminal endodomain, around 71 aa, and GP3 also has a 54 aa predicted endodomain. Both endodomain were predicted as disordered region in solution with no significant secondary structure (PredictProtein). There are no studies showing that these two proteins have any interaction with other intracellular proteins [18]. Therefore, it is difficult to predict that if the mutations in the endodomain (F248V in GP3 and L196Q in GP5) can affect the function of these two proteins. Interestingly, in a comparative genomic analysis of a HP-PRRSV strain, HBR, at the different passage levels, the same F248V mutation in GP3 and I66S mutation in GP4 were found in the 125th passage (F125) [39]. Therefore, these 2 mutations are less likely the cause of the loss of infectivity.

1.4 Discussion

Conventional approaches to live-attenuation virus vaccine development through passaging (forward genetics) or inactivation, though highly successful in the past, has been shown to be inefficient for generating broadly protective PRRSV vaccine and understanding PRRSV pathogenesis and attenuation mechanism. Reverse genetics, in the opposite direction of classical genetics, is an approach to discover what phenotypes arise as a result of particular genetic sequences. It allows introduction of changes at specific sites or regions of the viral genome in order to create modified infectious viruses [40], which can be a powerful tool for analyzing the fatal virulent determinant of HP-PRRSV and seeking ways to effective attenuation and developing MLV vaccines with expected characteristics (e.g., broad protection). Aimed at developing a reverse genetics system for studing of HP-PRRSV virulence and attenuation mechanism, we created a full-length HP-PRRSV clone and a series of full-length LP/HP PRRSV

chimeric clones. The full length cDNA clone of HP-PRRSV was generated by replacing all the viral genome in pCMV-P129 with sequence of JXA1 strain published in NCBI. Unfortunately, no virus with infectivity was rescued from this full-length clone. Fragments of JXA1 were synthesized according to the GenBank record EF112445. Although sequencing of the genome was modified to create *Mlu*I, *Afe*I, *BsrG*I enzyme sites, amino acid sequences of ORFs were not altered. Sequencing of the genome also showed that no mutations were introduced during the cloning procedure. Moreover, in all full-length JXA1/P129 chimeric clones created in this study, including JXA1-P1b, P129-JS and P129-JAB, only P129-JAB can produce progeny infectious virus. These data suggested that fatal mutation(s) may exist in the original JXA1 sequence in the GenBank record EF112445, and the BS fragment is the most likely region that harbors the mutation(s). To test this hypothesis, the BS region from another HP-PRRSV infectious clone pcDNA-HV was amplified by PCR and then cloned into the JXA1 and P129-JS to replace the JXA1 BS region. The new chimeric clones were named JXA1-HBS and P129-JAB-HBS. Significant CPE could be observed after 3 days post transfection of MARC-145 cells with P129-JAB-HBS and stably showed up in every further passage. However no CPE was observed in MARC-145 cells transfected with JXA1-HBS. The genome of P129-JAB-HBS derived recombinant viruses were extracted and sequenced to ensure no contamination with other viral strains.

The finding that replacement with HV BS region renders P129-JAB-HBS, but not JXA1-HBS, infectious supports our hypothesis that fatal mutation(s) was harbored in JXA1 BS region, and also indicated that there are more fatal mutations existing in the rest of the JXA1 genome. Sequence alignment between all ORFs locating in JXA1 BS and HV BS region indicated eight amino acid substitutions between these 2 strains, two of them in GP3, five in GP4 and one in

GP5 with a diverse distribution, covering the predicted signal peptide region, ectodomain, transmembrane region and endodomain (Fig. 1.6). To analyze the potential effect of these mutations in protein function, comparisons were conducted in secondary and tertiary structure level using different predict services. Prediction at the secondary structure level (SignalP and PredictProtein) did not show any mutations cause obvious difference in potential signal peptide and transmembrane region. Since no direct structure information is available for none of these 3 proteins, homology-modeling (SWISS-MODEL) was conducted for tertiary structure prediction. However, homology templates that can match the target regions were only found for the extracelluer region of GP4 and tertiary structure modeling did not show any difference in mutation region between JXA1 GP4 and HV GP4 either. There is not significant secondary structure predicted in endodomain in GP3 and GP5, of which the function still remain unknown. Therefore, the effect of mutations in endodomain could not be predicted. As the bioinformatics analysis was not conclusive in this study, site-directed mutagenesis may be used in the further studies for screening the fatal mutation(s) in JXA1 BS fragment.

The P129/JXA1/HV triple chimeric clones, P129-JAB-HBS, created in this study has structural protein coding region derived from HP-PRRSV and non-structural protein encoding region from a low pathogenic strain. This characteristic can be very useful in HP-PRRSV vaccine development and the study of pathogenicity of HP-PRRSV. First, structural protein contains the most neutralizing epitopes that are found in PRRSV; GP5 harbors the major neutralizing epitopes and GP2, GP3 and GP4 contain minor neutralizing epitopes[24]. Therefore, structural protein coding region of HP-PRRSV in P129-JAB-HBS may be able to induce effective humoral immunity against HP-PPRSV in pigs. Secondly, according to a vaccine efficacy study conducted in out lab, HP-PRRSV MLV vaccine can protect pigs from the challenge of heterologous North

American strain NADC-20 by inducing high titer of neutralizing antibodies to NADC-20 (unpublished data). These data suggest that neutralizing antibodies induced by HP-PRRSV can be broadly protective for heterologous challenge, and P129-JAB-HBS may preserve this characteristic in its SP coding region. Moreover, since P129-JAB-HBS only partially contains the HP-PRRSV genome, it can have an attenuated pathogenicity compared to the parental virus. It remains controversial as to which region(s) in the viral genome is the fatal virulence determinant; however recent studies [31, 41, 42] suggested non-structural proteins contribute more of the virulence of HP-PRRSV than structural proteins. Further study of the pathogenicity and immunogenicity of P129-JAB-HBS can provide new insights on the mechanism of virulence determination in PRRSV and on new PRRS vaccine development.

1.5 Figures and Tables

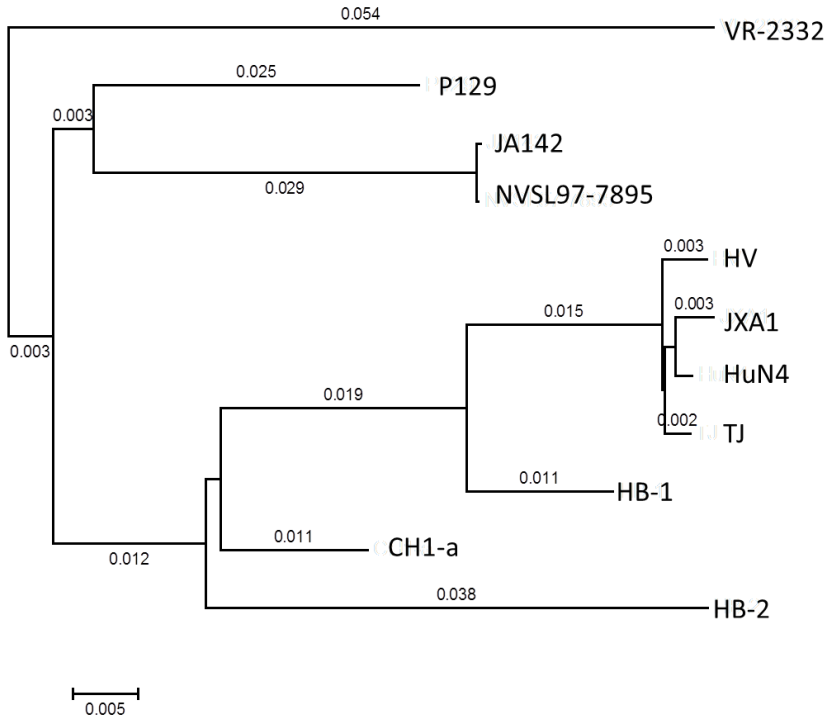


Figure 1.1 Phylogenetic relationship of PRRSVs. Phylogenetic tree based on complete genome demonstrated that Chinese HP-PRRSV is a new evolutionary subgroup in Type II North American genotype. VR-2332, prototype strain of Type II PRRSV; P129, JA142 (GenBank: AY424271) and NVSL97-7895 (GenBank: AY545985) are endemic strains in the US; CH1-a (GenBank: AY032626), HB-1 (GenBank: AY150312) and HB-2 (GenBank: AY262352) are classical PRRSV Chinese isolates. HV, TJ (GenBank: EU860248), JXA1 and HuN4 (GenBank: EF635006) are Chinese HP-PRRSV isolates.

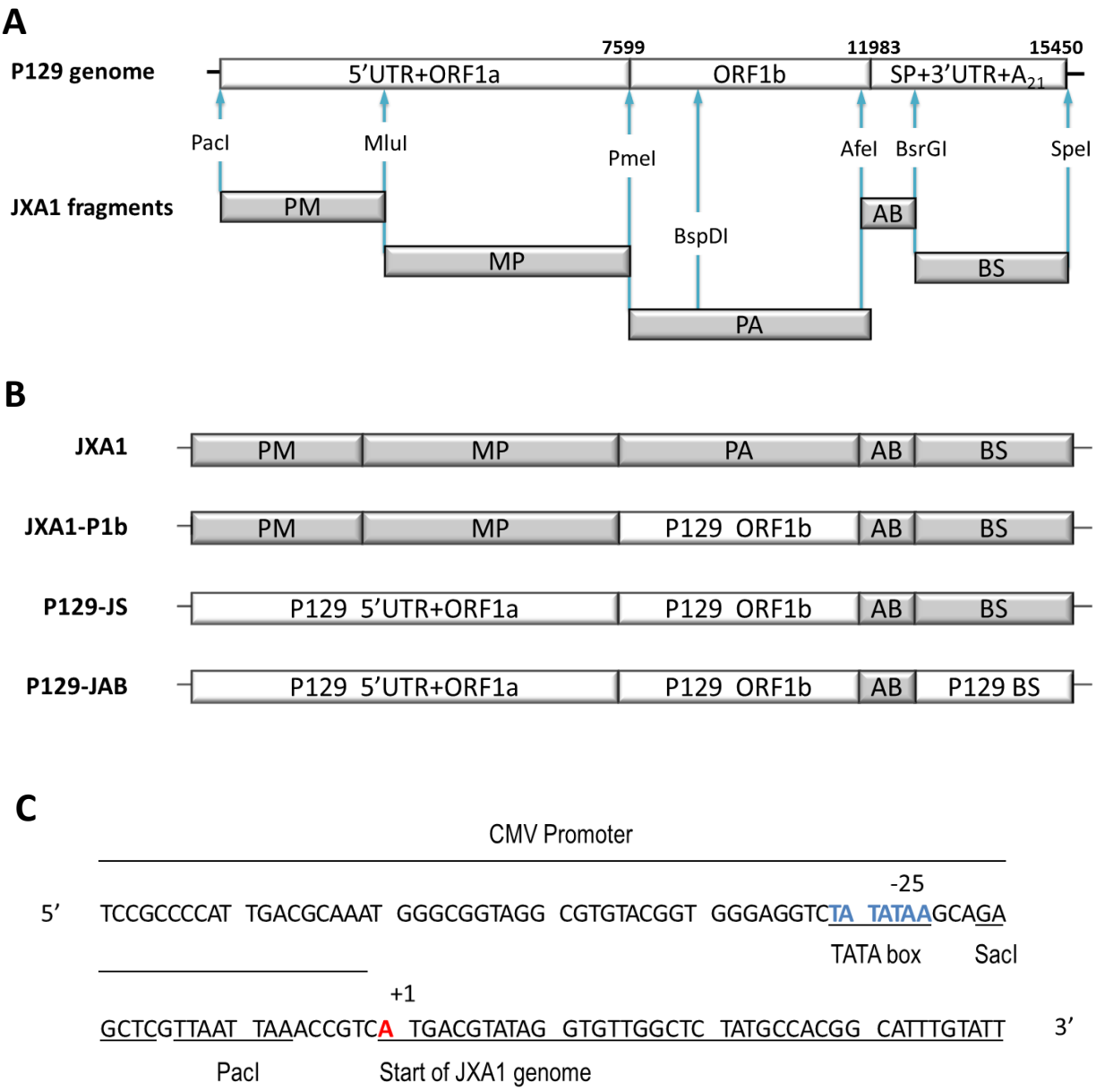


Figure 1.2 Strategies for construction of the full-length genomic cDNA clone of JXA1 and P129/JXA1 chimeric clones. (A) JXA1 genome was synthesized as five large DNA fragments with unique enzyme sites on the both ends. P129 genome in pCMV-P129 was replace by these five fragments one by one using corresponding enzyme sites. PA fragment was divided to 2 parts by BspDI for cloning due to the low ligation efficiency for long fragment. (B) Four new full-length genomic cDNA clones were created. JXA1 clone contain the entire and JXA1 genome;

JXA1-P1b is consisted of JXA1 sequence except the ORF1b that is derived from P129; P129-JS contains structural protein (SP) coding region and 3' UTR from JXA1, and 5' UTR and non-structural protein coding region from P129; P129-JAB contains only AB fragment from JXA1 and rest sequence are P129 genome. (C) Nucleotide sequence of the region between the human cytomegalovirus (CMV) immediate early promoter and the 5' terminal of the viral genome in JXA1 full-length clone. TATA box from the CMV promoter and the start of the JXA1 genome is underlined in bold face. The restriction sites are underlined.

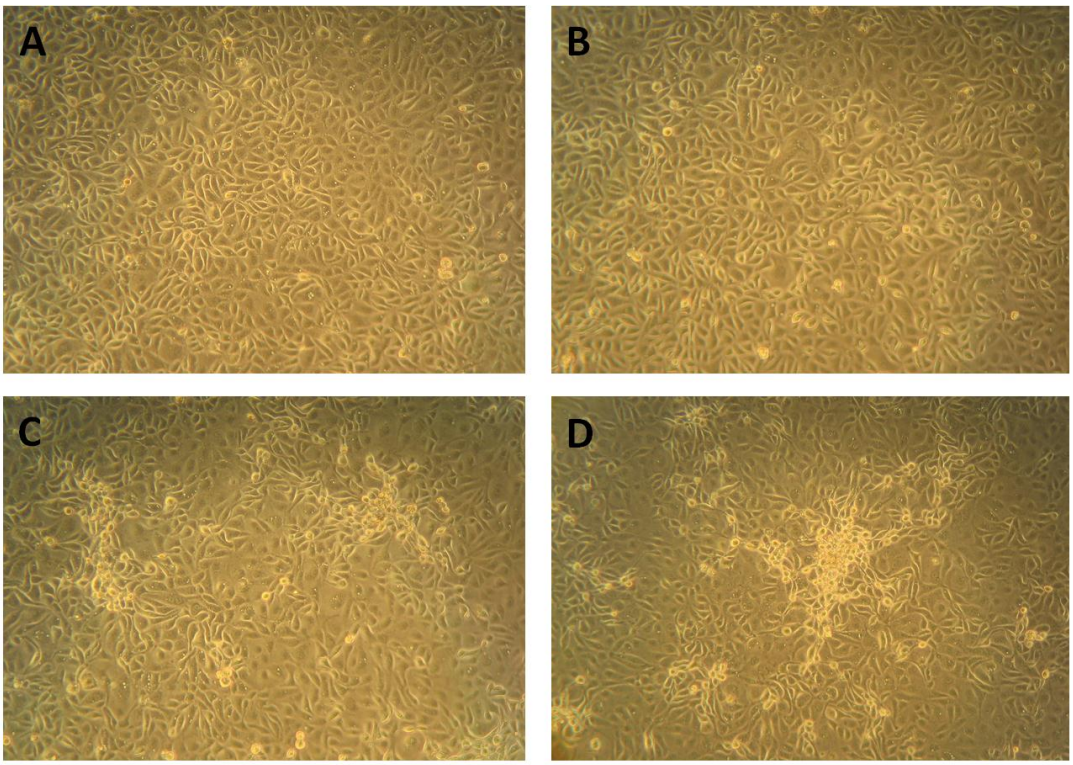


Figure 1.3 Transfection of full-length JXA1 and P129/JXA1 chimeric clones with MARC-145 cells. MARC-145 cells in a 6-well culture plate were transfected with (A) PBS or purified plasmid of (B) JXA1, (C) P129 or (D) P129-AB using Lipofectamine 2000 according to the protocol supplied by the manufacturer (Life Sciences). Cells were photographed at 3 days post-transfection. Cytopathic effect was visible for cells transfected with P129-AB or P129, but not for JXA1 or PBS control.

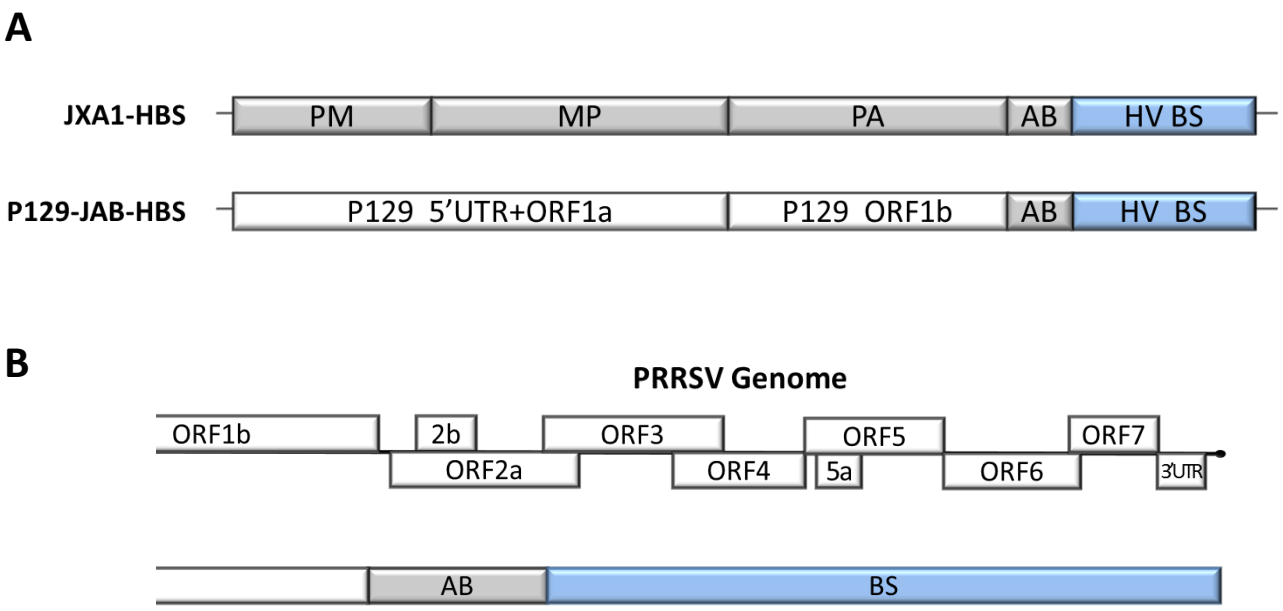


Figure 1.4 Construction of the JXA1/HV and P129/JXA1/HV chimeric clones. Homologous region of JXA1 BS in another Chinese HP-PRRSV strain HV was amplified by PCR and cloned into the *BsrG*I and *Spe*I sites in JXA1 and P129-JS clones. (A) Two new chimeric clones JXA1-HBS and P129-JAB-HBS were generated. P129-JAB-HBS is a triple chimeric clone, containing the structural protein coding region derived from HP-PRRSV and non-structural protein coding region of P129. (B) AB fragment contains most of the ORF2a and the intact ORF2b. BS fragment contains the intact ORF3, ORF4, ORF5, ORF6, ORF7 and 3'UTR and partially ORF2.

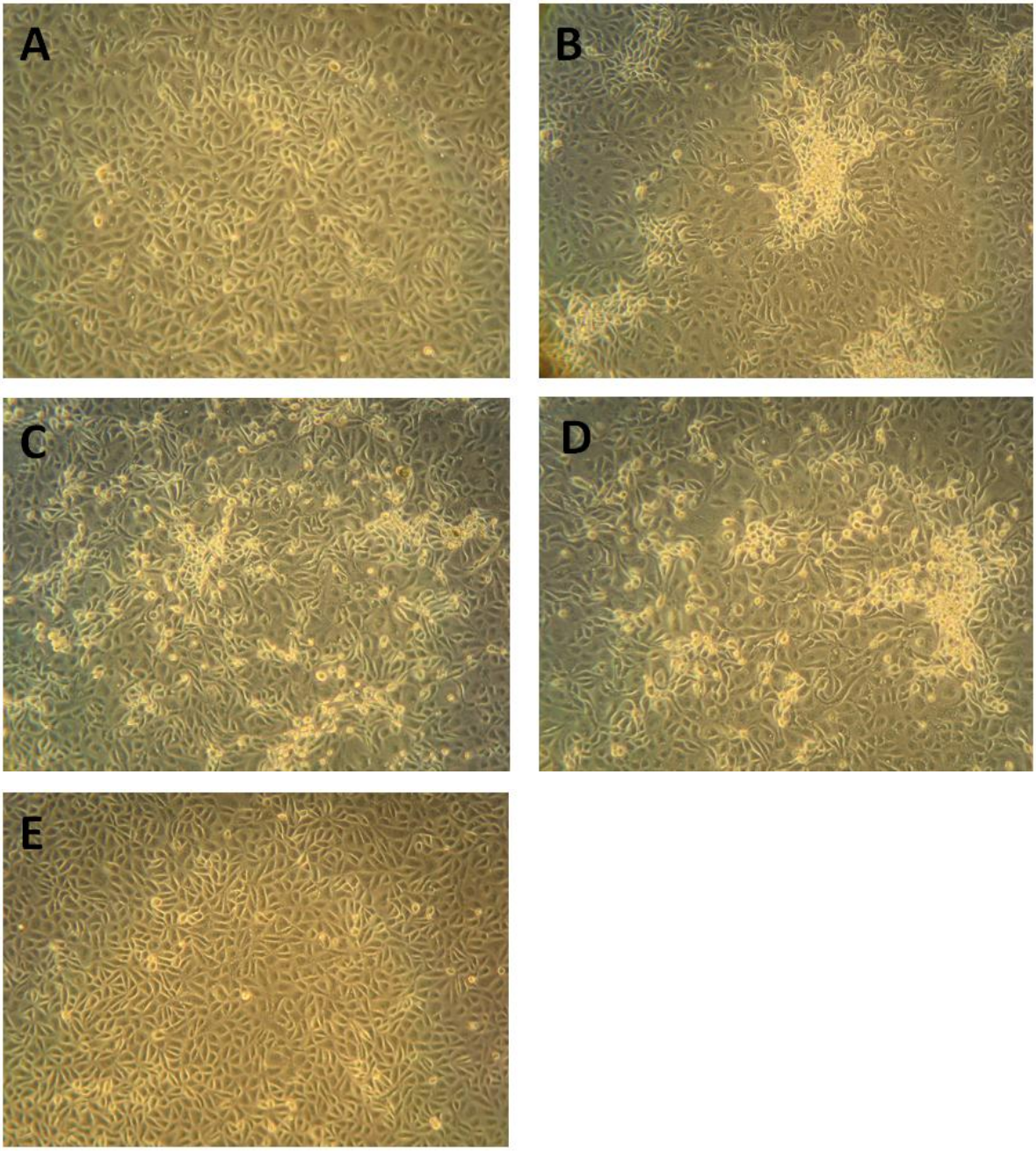


Figure 1.5 Transfection of JXA1-HBS and P129-JAB-HBS chimeric clones with MARC-145 cells. MARC-145 cells in a 6-well culture plate were transfected with (A) PBS or (B) purified plasmid of HV, (C) P129, (D) P129-JAB-HBS or (E) JXA1-HBS (E) using Lipofectamine 2000 according to the protocol supplied by the manufacturer (Life Sciences).

Cells were photographed at 4 days post-transfection. Cytopathic effect was significant for cells transfected with HV, P129 or P129-JAB-HBS, but not for JXA1-HBS or PBS control.

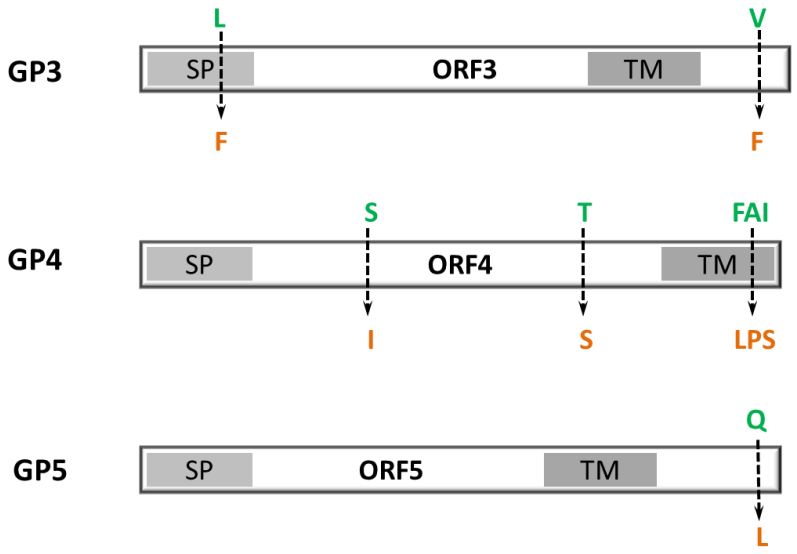


Figure 1.6 Differences in protein sequence between JXA1 BS and HV BS. HV BS has 8 amino acids differences that distribute in ORF3, ORF4 and ORF5 compared to JXA1 BS. Amino acids in BS fragment of JXA1are in green and amino acids in BS fragment of HV strain are in orange. Among them, F12L in GP3 locates in predicted signal peptide (SP) region, I66S and S132T in GP4 locate in predicted ectodomain, L172F, P173A and S174I in GP4 are predicted within transmembrane (TM) region, and F248V in GP3 and L196Q in GP5 are predicted being in C-terminal endodomain [18].

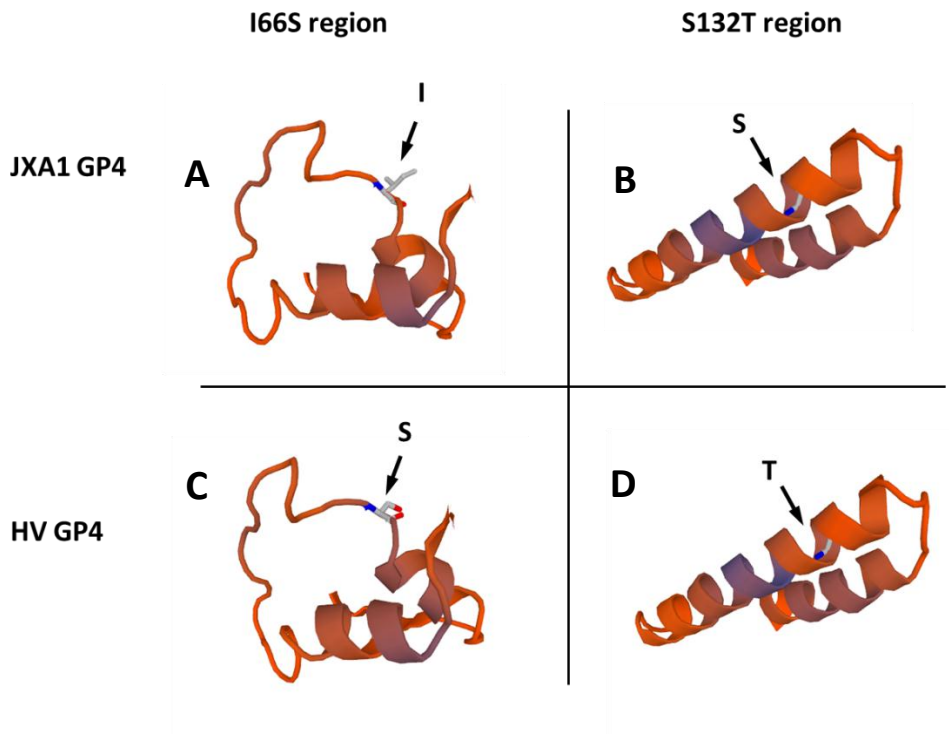


Figure 1.7 Structure homology-modeling for BS fragments of JXA1 and HV strains.

Structure homology-modeling (SWISS-MODEL, automatically template search) was conducted for regions in JXA1 GP4 containing (A) 66I and (B) 132S and regions in HV GP4 that contains (C) 66S and (D) 132T in GP4. Structure of amino acids that differ in JXA1 GP4 and HV GP4 were indicated in the predicted model by back arrows.

Table 1.1 Primers used in RT-PCR for amplifying the genome of P129-JAB-HBS chimeric virus.

No.	Name	Sequence
1	PRRSVpart1-F	TGACGTATAGGTGTTGGCTCT
2	Hybrid1a-R	CCTGCACTCGGGATCAACTT
3	Hybrid1b-F	TCGGTTTCGTTCCCCTCTTG
4	Hybrid1b-R	CTCAGGGTTGCCCCTAACAG
5	PRRSVpart2-F	GGGATGGGGACCGTTTGAAT
6	PRRSVpart2-R	AATTACGGCCGCATGGTTCT
7	Hybrid2b-R	AAAGCGGGCATACCGTGTAA

Chapter 2 - Construction of a recombinant *Listeria innocua* vaccine vector for the expression of a CSF viral antigen

Abstract: The use of bacterial vectors for the development of next-generation viral vaccines has proved beneficial in many studies. *L. monocytogenes* (*L.m*) is a well-known intracellular pathogen that encodes specialized virulent determinants to facilitate its intracellular growth and spread. It has been widely used as an experimental vaccine vector, given its capability to stimulate cell-mediated immunity. However, the safety of live *L.m* vector remains an important issue which limited its development in clinical applications. *L. innocua* (*L.inn*) is a non-pathogenic strain in the *Listeria* genus, closely related to *L. monocytogenes* (*L.m*). Our goal is to make *L.inn* a vaccine vector that can deliver target viral antigen into intracellular environments by complementation of *L.inn* with selected *L.m* virulence genes necessary for intracellular survival and induction of a robust immune response. In this study, we constructed a shuttle vector pHT-E2 that can express classical swine fever (CSF) viral antigen E2 in *L.inn*. At the same time, we cloned the *plcA-prfA* operon of *L.m* virulence gene cluster (vgc) into the pHT-E2. The presence of this operon enhanced the expression of E2 in *L.inn*. In future studies, we plan to clone additional *L.m* virulence genes into the shuttle vector to increase immunogenicity of this recombinant *L.inn* and test its ability to protect pigs from CSFV.

2.1 Introduction

Currently, vaccines developed to protect against viral pathogens fall into three major classes 1) modified live virus (MLV) vaccines [22, 43, 44] 2) antigen delivery systems based on replicating viral/bacterial vector [45], and 3) vaccines developed using non-replicating strategies like inactivated virus, subunit vaccines and nucleic acid vaccines [44, 46]. Although MLV vaccines

are highly efficacious, they are associated with potential risk for reversion to virulence [47]. Vaccines that are not based on replication-competent materials are considered to be safer, but they usually require a strong adjuvant, which can often induce detrimental tissue reactions, or require repeat injections [44, 48]. Bacterial vectors permit targeted delivery of viral antigens to mammalian cells or tissues in a way that closely mimics a natural infection to effectively stimulate the immune system [49, 50]. Therefore, bacterial vectors provide a vaccine technology for the development of next-generation viral vaccines. Over the past 15 years, experimental bacteria vectors based on Bacille Calmette-Guerin (BCG), *Listeria monocytogenes*, *Salmonellae* and *Shigellae* have been developed and tested in animal models [45, 49]. The ability of *L.m* to induce major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell responses makes it an attractive option for a viral vector [51, 52].

Listeria monocytogenes (L.m) is a gram-positive, food-borne opportunistic intracellular human and animal pathogen. It causes the disease listeriosis, which culminates in a gastroenteritic infection in healthy adults but can lead to serious diseases (e.g., meningitis and death) in immunocompromised individuals, pregnant women and neonates [53-55]. *L. monocytogenes* has the extraordinary capacity to survive within mammalian hosts. It employs a whole set of specialized molecules to facilitate adhesion and internalization into the host cells, escape from vacuoles, actin-based bacterial motility and cell-to-cell spread without being decimated by the host during the process. Many of those bacterial virulence-associated molecules have been identified, the most notable of which is the PrfA-regulated virulence genes that cluster on an approximately 9.8kb- region of the chromosome, termed the virulence gene cluster (*vgc*). The vgc locus encodes the *prfA, plcA, hly, mpl, actA, plcB* and 3 other genes ORFx, y and z, which are organized in three operons [56] (Fig 2.1A). The *hly* encoded listeria hemolysis, listeriolysin

O (LLO) is the most essential component for the *L. monocytogenes* intracellular life cycle. In the bacterial infection, *L.m* are initially phagocytosed by macrophages and hepatocytes and thereby enclosed within membrane-bound vacuoles [57]. With the help of phosphatidylinositol-phospholipase C (PI-PLC, encoded by *plcA*), LLO disrupts the membrane-bound vacuoles and release the engulfed *L.m* to the cytoplasm. Strains that contain mutated *hly* gene would be trapped and remain inside the vacuoles and become absolutely avirulent [58, 59]. Actin nucleator ActA (encoded by *actA*) promote formation of actin structures and propel the bacterium movement toward the cytoplasmic membrane, where *L.m* is then enveloped in filopodium-like structures and engulfed by adjacent cells in double-layer membrane vacuoles [55]. Phosphatidylcholine-phospholipase C (PC-PLC) activated by Mpl (a metalloprotease encoded by *mpl*) works in collaboration with LLO to disrupt the double-layer membrane vacuoles and initiate a new infection cycle of *L.m* [60, 61]. PrfA (encoded by *prfA*) is a positive regulator that is required for the expression of *hly* and other genes in vgc locus [55, 62]. Besides vgc encoding determinants, bacterial surface proteins internalins (e.g., InlA and InlB) also play an important role in *L.m* pathogenesis by facilitating the active entrance of *L.m* to non-phagocytic cells (e.g., epithelial cells) [63].

As a facultative intracellular bacterium, *L.m* infection can induce robust cell-mediated immune responses by eliciting both $CD8^+$ cytotoxic T-lymphocytes (CTL) and type I $CD4^+$ helper T-lymphocyte (Th1) responses [64]. This feature makes *L.m* an attractive antiviral vaccine vector that can deliver target viral antigen into intracellular environs to strengthen the host's $CD8^+$ and $CD4^+$ T cell responses as well as innate immune response against the virus. Recombinant *L.m* vaccines have been developed for LCMV, murine influenza virus and HIV [51, 65, 66]. However, the safety of recombinant *L.m* remains an important issue, given the possibility of the

spread of the *L.m* vector from a recipient to immunocompromised individuals [45]. Over replication of *L.m* vector in such subpopulations can cause severe and life-threatening infections [67, 68]. In addition, infection of *L.m* can also cause undesired $CD4^+$ T-cell mediated delayed type hypersensitivity (DTH) responses and granulomatous inflammation [69, 70]. Therefore, the risk associated with live *L.m* may significantly hinder the vaccine development of this vector. The possibility of using naturally nonpathogenic strains as candidate vaccines has also been explored [71, 72]. A previous study showed that the transfer of the whole set of *L.m* vgc locus into a non-pathogenic strain *Listeria innocua* (*L.inn*), significantly increased its *in vivo* survival rate in mice and conferred the capability to induce a protective immune response against wild type *L.m* [72]. However, over complementation of a non-pathogenic strain with virulent determinants can turn it in to a pathogenic strain and raise safety concerns. Hence, a satisfactory balance between attenuation and immunogenicity is the key factor in the clinical development of *Listeria* vaccine vectors.

The goal of this study is to generate a *L.inn* vaccine vector by transforming it with the minimal amount of *L.m* virulent determinants that is enough for inducing an effective immune response towards expressed viral antigens. To explore this approach, we constructed a shuttle vector pHT-E2 that can express classical swine fever (CSF) viral antigen E2 in *L.inn*. At the same time the *plcA-prfA* operon of *L.m* virulence gene cluster (vgc) was cloned into the pHT-E2 to enhance the expression of E2 in *L.inn*. In further studies we plan to clone additional *L.m* virulence genes into this shuttle vector and evaluate vaccine efficacy of *rL.inn* carrying different *L.m* determinants in their ability to protect pigs from CSFV challenge.

2.2 Materials and Methods

2.2.1 Bacterial strains.

Bacteria strains used in this study includes a wild type *Listeria innocua* Seeliger Serotype 6a (ATCC 33090), a recombinant *L. monocytogenes* strain [72], *E. coli* One Shot OmniMAX 2 T1 R chemically competent cells (Life Technology) and *E. coli* 10-beta chemically competent cells (NEB). *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates supplemented with100 μg/ml ampicillin. Listeria strains were grown in Brain Heart Infusion (BHI) Broth (BD 237500) or on BHI agar plates (BD 211065) in presence or absence of 5 μg/ml chloramphenicol. Chloramphenicol was used as a selective antibiotic for growth of *L.inn* harboring the empty or recombinant pHT-01 vector.

2.2.2 Codon optimization and synthesis of CSF E2 gene

E2 is an envelope glycoprotein of classical swine fever virus (CSFV). The signal peptide, ectodomain and transmembrane region of CSF E2 gene (GenBank: AJ704817) were predicted by SignalP 4.1 and PredictProtein online analysis tools. Condon of ectodomain (24aa-364aa) was optimized for the preference of *L.inn* using Codon Usage Database (http://www.kazusa.or.jp/codon/) (Condon usage table: *Listeria innocua* [gbbct]: 3137) and codon adaptation tools, Optimizer (http://genomes.urv.es/OPTIMIZER/) and Jcat (http://www.jcat.de/). Optimized E2 ectodomain sequence has a percentage content of GC of 36%, close to the average value of 37% of the *L.inn* genome. Optimized ectodomain was synthesized (GenScript) with modified 5' terminal *BbvC*I and a 3' terminal *Avr*II enzyme sites for future cloning.

2.2.3 Construction of pHT01-E2 vector

An expression vector for heterologous expression of CSFV E2 in *L.inn* was constructed. A *Bacillus*/*E. coli* shuttle vector pHT01 (MoBiTec) was used in this study. pHT01 contains replication elements from *Bacillus subtilis* plasmid pBS72 [73] and ColE1 derivative replicon for replication in *E. coli*. A synthesized Multiple Cloning Sites (MCS) with the promoter (P) and signal sequences (SS) of the *hly* gene in it was cloned into the pHT01 vector using *Sac*I and *Xma*I enzyme sites. Synthesized E2 ectodomain was cloned into the MCS in pHT01using *BbvC*I and *Avr*II enzyme sites right behind the P_{hly}. The recombinant vector, pHT01- E2, was chemically transfected into *E. coli* One Shot OmniMAX 2. Recombinant clones were examined for correctness of insertion by PCR with specific primers E2test-F: AGGTGGTAACTGGACTTGTG and pHT01test-R: TATCGCCTTGTGGCTTTCTA. Recombinant plasmid was purified with QIAprep Spin Miniprep Kit (Qiagen) and verified by restriction digests with *BbvC*I and *Avr*II.

2.2.4 Construction of pHT01- VGCpart1-E2 vector

VGCpart1 was PCR amplified from *L. monocytogenes* genome DNA by using primer pair VGCpart1-F: CACCggtaccCGTACGCGTTCATGAAAATGC (*Kpn*I site was underlined) and VGCpart1-R: GTCACTGCATCTCCGTGGTA. VGCpart1a PCR fragment was purified by QIAquick Gel Extraction Kit (Qiagen). pHT01- E2 and VGCpart1 fragment were digested with *Kpn*I and *BstZ*17I and then purified with QIAquick PCR Purification Kit (Qiagen). Ligation between digested pHT01- E2 and VGCpart1a fragment was carried out using T4 ligase (NEB) according to manufacturer's instructions. After overnight incubation at 16 ℃, the ligation mixture was transfected into One Shot OmniMAX 2 competent *E. coli*. Recombinant clones were examined for correctness of insertion by PCR with specific primers M13-F: TGTAAAACGACG

GCCAGT and VGCtestP1a-R: TACAGAAACATCGGTTGGCT. Recombinant plasmid was purified with QIAprep Spin Miniprep Kit (Qiagen) and confirmed by sequencing of the junction between VGCpart1a insert and vector.

2.2.5 Preparation of electrocompetent L. innocua cells

L. innocua was made electrocompetent using methods previously described [74]. Briefly, overnight cultures of *L. innocua*, in BHI containing 0.5 M sucrose were diluted (1:100) into fresh media (BHI + 0.5 M sucrose) and grown at 37 ℃ with shaking at 250 rpm until reaching an OD_{600} of 0.2. Penicillin G was added to a final concentration of 10 μg /ml and the incubation continued for an additional 2 hours. Cells were harvested by centrifugation (8,000 ×g, 10min, at 4 ℃) and washed 3 times in ice-cold washing solution (1 mM HEPES (pH 7.0), 0.5 M sucrose), once with equal volume and twice with ½ volumes. Cell pellet was re-suspended in 0.0025 amount of the same buffer.

2.2.6 Transformation of L. innocua

Transformation was performed using 100 μl electrocompetent *L. innocua* cells and 1μl sterile water or 1 μg purified plasmid of pHT-VGCpart1a- E2, pHT- E2 or pHT01. The mixture of electrocompetent cells and plasmid were transferred to a 1mm disposable cuvette (Bio-Rad) and stored on ice for 5 min. Electroporation was conducted in a Gene Pulser (Bio-Rad) at a field strength of 1000V with pulse duration of 5ms. Transformed cells were mixed with 1 ml BHI supplemented with 0.5 M sucrose immediately and incubated at 37 ℃ for one hour. After, the transformation mixture was diluted and placed onto BHI plates containing 5 μg/ml chloramphenicol (Cm). Plates were incubated overnight at 37 ℃ for 3-4 days to allow the recombinant colonies to form. Recombinant colonies were then inoculated to fresh BHI broth

containing 5 μg/ml Cm for 1-2 days. Total DNA was extracted from 1.5ml bacterial culture with DNeasy Blood & Tissue Kit (Qiagen) and PCR was conducted to determine the transformation efficiency. Specific primer pairs used in PCR include 1. E2test-F: AGGTGGTAACTGGACTTGTG and pHT01test-R: TATCGCCTTGTGGCTTTCTA (for detecting E2 gene); and 2. M13-F: TGTAAAACGACGGCCAGT and VGCtestP1a-R: TACAGAAACATCGGTTGGCT (for detecting VGCpart1a).

2.2.7 SDS-PAGE and western blots

Recombinant bacteria were grown in BHI supplemented with 5 μg/ml Cm to an optical density at 600 nm of 1.0. After centrifugation, culture supernatant (3ml) was precipitated with 10% trichloroacetic acid, washed twice with acetone, and dissolved in 180 μl of 20 mM Tris-HCL (pH 8.0). Bacterial pellets were suspended in 180 μl enzymatic lysis buffer (2 mg/ml lysosome in 20 mM Tris-HCL, pH 8.0 containing 2 mM sodium EDTA, 1.2% Triton X-100 and 1% protein inhibitor cocktail), incubated for 30 min at 37 ℃ and disrupted by ultrasonication on ice. Protein extracts normalized to 0.5 ml of original culture were electrophoresed in SDS-% PAGE gel in duplicate for staining or for immunoblotting with Precision Plus Protein Kaleidoscope standards (Bio-Rad) as molecular weight markers. Proteins were transferred to nitrocellulose membrane and probed with a monoclonal anti-E2 antibody (Animal Health & Veterinary Laboratories Agency), (diluted 1: 2500 in 1% milk in PBST) overnight at 4 ℃ with gentle rocking. Subsequently the membrane was washed twice with PBST, incubated with goat anti-mouse HRP secondary antibody 0.8μg/ml in 1% milk in PBST for 1 hour at room temperature and then washed thrice with PBST. Finally, the immunoblotting was performed with the ECL reagent (Percie) as recommended by the manufacturer.

2.3 Results

2.3.1 Construction of a shuttle vector that can stably maintain foreign genes in Listeria.

pHT01 is an expression vector that can replicate in both *Bacillus and E.coli.* The original promoter that is used for foreign gene expression in *Bacillus* in the pHT01 was replaced by a new synthesized MCS with promoter (P) and signal sequences (SS) of *L.m hly* gene in it (Fig. 2.1). A synthesized E2 ectodomain was inserted into the *Bbv*CI and *Avr*II sites right behind the *hly* SS, and the new construct was named pHT- E2 (Fig. 2.1). pBS72 replicon in pHT01 vector was isolated from *Bacillus subtilis.* It is a low-copy replicon using theta-mode replication mechanism that can stably maintain large plasmids (~90kb) in *Bacillus.* The use of pBS72 replicon in *Listeria* has not been reported before. To test the replication ability of pHT-E2 in *L.inn,* electroporation was conducted to transformed wild type *L.inn* with pHT-E2 and the resulting strain was named *L.inn::e2*. pHT-E2 was detected in total DNA extracts purified from *L.inn::e2* by PCR using specific primers (Fig 2.2). This result suggested that pBS72 replicon can work properly in *L.inn*.

2.3.2 Cloning of the plcA-prfA operons into the pHT- E2 vector.

L.m VGC locus consists of three operons, the *plcA-prfA* operon, the *hly* operon and the lecithinase operon [56]. We planned to clone these three operons separately into the pHT- E2 vector. In this way, we can evaluate the influence of each operon on the vaccine efficacy of the recombinant *L.inn* vector and identify the best balance between immunogenicity and pathogenesis. PrfA encoded by *prfA* gene in the *plcA-prfA* operon is a positive regulatory factor for most of *L.m* virulent genes, including *hly*. Transcription of *prfA* can be initiated from two different premotor region, one in front of the *prfA* gene and the other one in front of the *plcA*

gene [75]. *L.m* strain with interrupted *prfA* gene, expressed barely detectable levels of hly mRNA [56]. We expected the presence of *plcA-prfA* operon can enhance the expression level of E2 gene driven by *hly* promoter in the *L.inn*. The *plcA-prfA* operon (VGCpart1) was amplified by PCR from purified *L.m* genome and cloned into the pHT- E2 vector by *Kpn*I and *BstZ*17I sites. The new recombinant vector was referred to pHT- VGCpart1-E2. pHT-VGCpart1-E2 was transformed into wild type *L.inn* by electroporation and the resulting strain was named *L.inn::vgc1+e2*. Both VGCpart1 and E2 were detected in total DNA extracted from recombinant *L.inn* by PCR using specific primers (Fig 2.2).

2.3.3 Expression of the CSF E2 in recombinant L.inn.

Listeriolysin O (LLO) encoded by *hly* gene is a secretory hemolysin in *L.monocytogenes*. We anticipate the expression and secretion under control of the promoter and signal sequences of LLO would channel the foreign E2 protein to the host cell cytoplasm for antigen processing and presentation. To test the expression and compartmentalization of E2 in *L.inn* vector, *L.inn::e2* and *L.inn::vgc1+e2* were grown under conditions in which the *prfA* gene and *hly* promoter is maximally expressed (37 ℃ and neutral pH). Proteins in both supernatants and total cell lysates of recombinant *L.inn* cultures were extracted and subjected to immunoblotting test (Fig 2.3). Western blot using monoclonal anti-E2 antibodies detected E2 (40kDa) expression in total cell lysates of both *L.inn::e2 and L.inn::vgc+e2* cultures. Results show that E2 expression level was barely detectable in *L.inn::e2* cell lysates. This may be the result of the low expression efficiency of Phly in the absence of its positive regulatory factor PrfA in *L.inn::e2.* Unexpectedly, E2 expression was not detected in supernatants of either *L.inn::e2 or L.inn::vgc+e2* cell culture. A possible reason for this is that the E2 did not form the correct conformation due to the inability of *Listeria* to form disulfide bonds.

2.4 Discussion

To introduce heterologous genes into *Listeria,* genes may be carried on plasmids or integrated in the listeria genome. Integration-based methods may confer better genetic stability but usually can only maintain one copy of the foreign gene in the bacterial genome and these results in low expression levels. Plasmids, on the other hand, can maintain higher copies and facilitate an increase in antigen expression. pHT01 is a shuttle vector that can replicate in both *Bacillus and E. coli.* It carries an *E. coli* replicon ColE1 and a theta-mode replication element pBS72 derived from natural plasmids in *Bacillus subtilis* [73]. Natural plasmids using pBS72 or pBS72 derived replicons have an average size around 90kb. The vector constructed based on pB72 replicon has been proved that it can be stably inherited in *B. subtilis* and maintain a low copy number, around 6 units per chromosome [73]. There are other theta-mode replication origins like pAMβ1 isolated from *Enterococcus faecalis* [52, 76] and pIP501 isolated from *Streptococcus agalactiae* [77, 78] that have been used in contracting replicating plasmid in *Listeria*. However, the pB72 replicon that adopts an original initiation mechanism independent of DNA polymerase I has not been used in *Listeria* before [73, 79]. In this study, we constructed a pHT01-based CSFV E2 expression vector, pHT-E2. Wild type *L.inn* was transferred with pHT-E2 by electroporation and E2 gene could be detected by PCR in the progeny cells, suggesting that pB72 can work properly in *Listeria*.

Non-pathogenic *L.inn* is genetically the closest species to *L. monocytogenes* in the *Listeria* genus. Comparative genomic studies show that the most notable difference between these two species is the lack of vgc locus and certain internalin genes of *L.m* in the genome of *L.inn*. Previous studies also showed that the transfer of the full-length *v*gc locus into *L.inn* significantly increased its *in vivo* survival rate in mice and conferred its ability to induce protective immune responses against

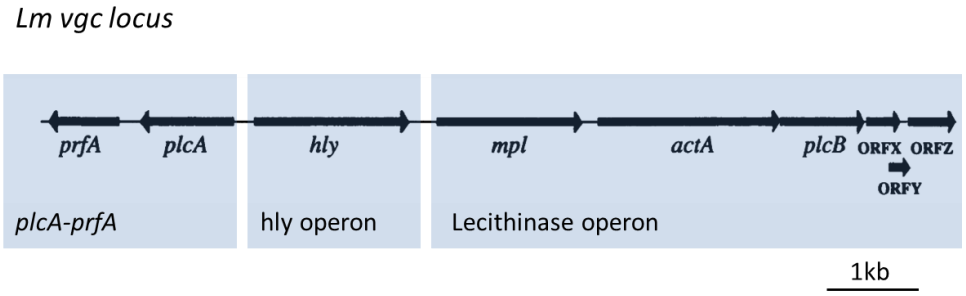
wild type *L.m* [72]. The vgc locus encoding proteins are involved in every stage of *L.m* intracellular life cycles. *hly* and *plcA* enable the escape of bacteria from the vacuole; *actA* facilitates the actin-based bacterial motility; *plcB* facilitate the cell-to-cell spread; and *prfA*, as a transcription enhancer, up-regulated the expression of virulent genes in host. *L.inn* transferred with the entire vgc is capable of escaping from the phagolysosome and surviving inside phagocytic cells. This raises the concern that over complementation with virulent determinants could lead to over-replication of *L.inn* vector, especially in immunocompromised hosts. Previous studies found that infection with *L.m* mutants that lack the ability for cell-to-cell spread or to multiply in cytosol can also elicit protective immunity by inducing robust expansion of epitope-specific $CD8^+$ T cells [80-82]. Hamilton and colleagues showed that *hly* deficient *L.m* that expression an additional CD8+ T cell epitope derived from LCMV induced sizable priming of epitope-specific $CD8^+$ T cells and the development of a functional memory cell population in mice after injection and offered protection against challenge with wild-type *L.m* and LCMV [83]. Those studies suggest that an intact intracellular life cycle of *listeria* vector is not indispensable for evoking a desirable immune response.

Our goal of was to develop a strategy to generate an *L.inn* vaccine vector by complementing it with a minimal number of *L.m* virulence determinants sufficient to elicit an effective immune response against expressed viral antigens. To explore this approach, we planned to clone the three operons in *L.m* vgc separately into the pHT- E2 vector. In this way, we can evaluate the influence of each operon on the vaccine efficacy of recombinant *L.inn* vector and identify the best balance between immunogenicity and pathogenesis. The *plcA-prfA* was the first operon we cloned into the pHT-E2 to enhance the expression of E2 in *L.inn*. In the immunoblotting test, E2 was detected in the cell lysates of *L.inn::e2* but in a lower level than its expression in cell lysates

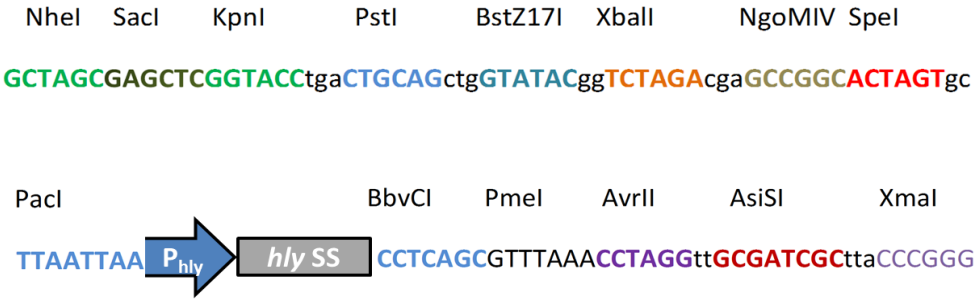
of *L.inn::e2*. This result suggests that the presence of PrfA can up-regulate the foreign gene expression driven by *hly* promotor. Unexpectedly, the expression of E2, which was supposed to be under the control of the secretory signal sequences of LLO, was not detected in supernatants of either *L.inn::e2 or L.inn::vgc+e2* cell culture. The possible reason for this is that *Listeria* may not be effective in forming disulfide bonds. The E2 ectodomain contains at least four disulfide bonds and they are key structural elements that stabilize the native conformation of E2 [84, 85]. The lack the correct disulfide bonds may reduce the solubility and secretion of E2 in *L.inn*. However, a previous study suggests that the compartmentalization of carried antigen in Listeria vector would not affect the antigen presentation by MHC class I pathway to prime $CD8^+$ T cells [86]. In future studies, we plan to clone additional *L.m* virulence genes into the shuttle vector to increase immunogenicity of this recombinant *L.inn,* and test the vaccine efficacy of *rL.inn* carrying different *L.m* determinants in their ability to protect pigs from CSFV.

2.5 Figures and Tables

A



B



C

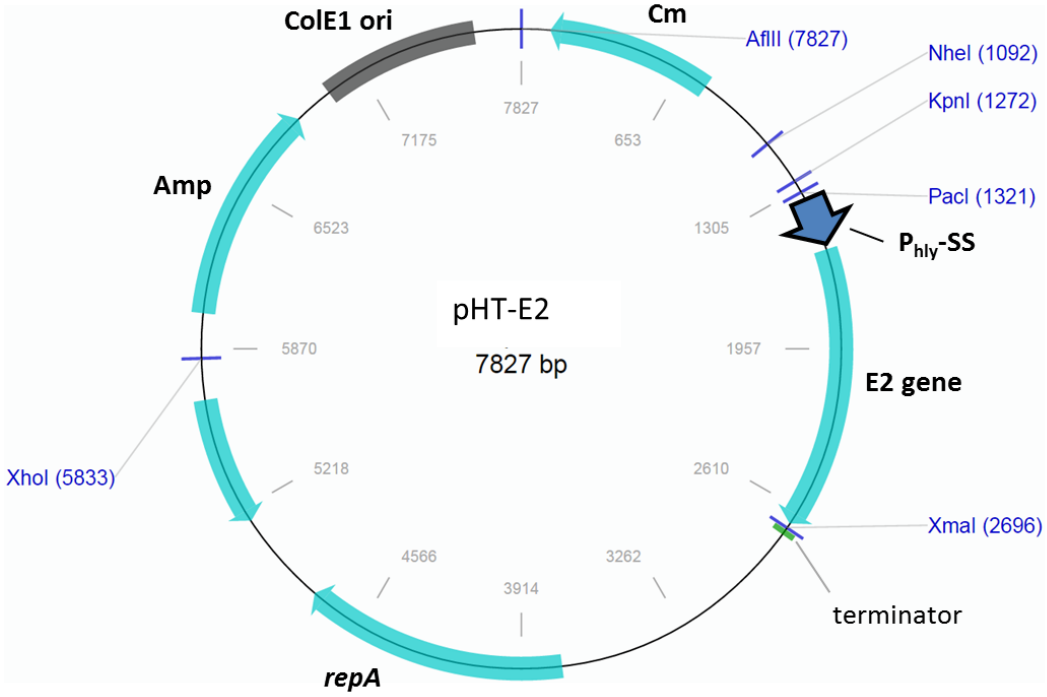


Figure 2.1 Construction of the *pHT-E2* shuttle vector and organization of *L.m* vgc locus. (A) *L.m* VGC locus consists of three operons, the *plcA-prfA* operon, the *hly* operon and the lecithinase operon (modified from Portnoy, D.A., 1992) [56]. (B) Synthesized multiple cloning site (MCS) with *hly* promoter (P_{hly}) and signal sequence (*hly* SS) in it. (C) Map of pHT-E2 vector. ColE1, *E. coli* replication origin; repA, major replication element in *Bacillus* plasmids pBS72; E2, ectodomain of CSFV E2 gene; P_{hly}-SS, promoter and signal sequences of *L.m* hly gene. Map was developed using Addgene (www.addgene.org).

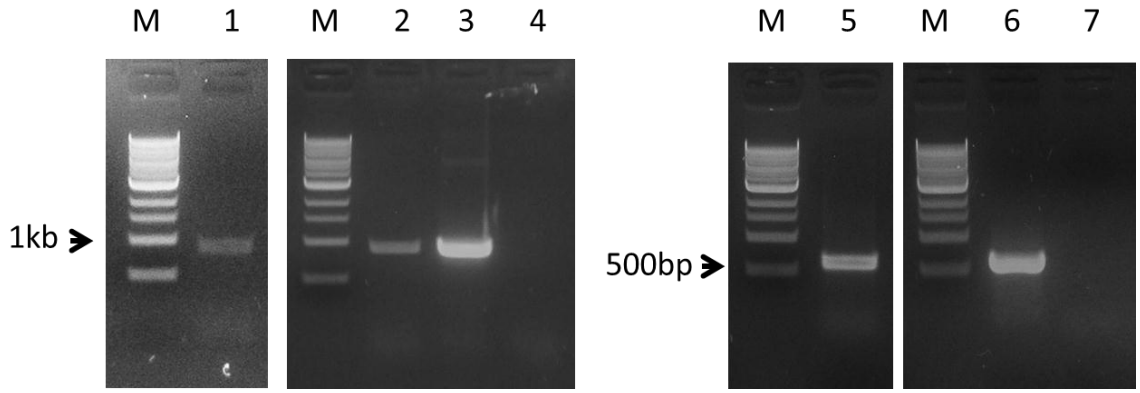


Figure 2.2 PCR confirmation of recombinant *L.inn* strains, *L.inn::e2 and L.inn::vgc1+e2* . M = Quick-Load 1 kb Extend DNA Ladder (NEB); 1-4 = amplification of the E2 gene (target band: 906bp) from *L.inn::e2*, *L.inn::vgc1+e2*, plasmid pHT01-Part1a-E2 (positive control), *L.inn* transformed with pHT01 vector (negative control), respectively. 5-7 = amplification of VGCpart1a (target band: 605bp) from *L.inn::vgc1+e2*, plasmid pHT01-Part1a-E2 (positive control), *L.inn* transformed with pHT01 vector (negative control), respectively.

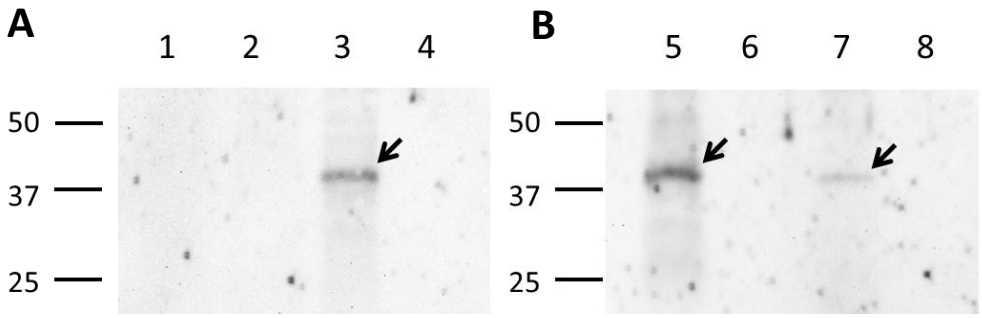


Figure 2.3 Western blot for the recombinant *L.inn* strains. Proteins in both supernatants and total cell lysates of recombinant *L.inn* cultures were extracted and subjected to immunoblotting test. Proteins were probed with a monoclonal anti-E2 antibody (WH211). (A) 1, 3 = cell lysates of *L.inn* transformed with pHT01 vector (negative control) and *L.inn::vgc1+e2*; 2,4 = supernatant from *L.inn* transformed with pHT01 vector (negative control) and *L.inn::vgc1+e2*, respectively; (B) 5, 7 = cell lysates of *L.inn::vgc1+e2* and *L.inn::e2*; 6,8 = supernatant from *L.inn::vgc1+e2* and *L.inn::e2*, respectively.

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