RESEARCH REPORT
SWINE HEALTH

Title: Porcine adenovirus 3 based vaccine for porcine respiratory and reproductive syndrome (PRRS) - **NPB# 04-110**

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I) *Abstract*

Infectious diseases remain the major cause of death and economic losses in animals. One way to reduce this is by vaccination. The use of safe and effective vaccines against diseases is crucial not only to improve the health of the animals but also to reduce the widespread use of antibacterial drugs, which can end up as contaminants in meat products. Although immunization has a great impact on the economics of livestock production and on animal suffering, today's vaccines produced by conventional means are still imperfect in many respects including excessive virulence and less-than-optimal efficacy. Through the use of genetic engineering, we are now able to generate live vaccines that are safer and possibly more effective than conventional vaccines. By introducing multiple gene deletion mutations in a directed way in the genome of a virus, one can virtually eliminate the agent's ability to cause disease, and the chance of reversion, as well as make room for the insertion of genes encoding vaccine antigens.

As porcine adenovirus (PAV)-3 infects pigs but often does not produce disease, it is a good candidate as a live vaccine. We characterized PAV-3 at the molecular level by determining the complete genome sequence and transcriptional map. We also have demonstrated the feasibility of manipulating and constructing recombinant PAV-3 expressing vaccine antigens. Now, we have demonstrated the feasibility of a) constructing synthetic genes encoding vaccine antigens of the porcine respiratory and reproductive (PRRS) virus and b) constructing recombinant PAV-3s expressing these vaccine antigens of the PRRS virus.

II) *Introduction*

Vaccination of animals is one way to reduce losses from infectious diseases. One of the impediments to developing better vaccines is ***Delivery***. Subunit vaccines require strong adjuvants (a few are licenced); they often do not induce the breadth of immunity required (cell-mediated, mucosal immunity, etc.); and are not economical to produce for use in ***Veterinary Medicine***. As compared to sub-unit vaccines, live vaccines are the better inducers of mucosal immunity when administered orally or intranasally. In addition, production of live vaccines is cost-

effective. We have choosen porcine adenovirus-3 (PAV-3) as a vector for the development of live vectored vaccines for pigs, as a) PAV-3 is a non-pathogenic porcine virus, b) it grows to high titers and c) it does not cause any serious disease in pigs.

We have choosen the porcine respiratory and reproductive (PRRS) disease of pigs as a model since it causes significant economic losses due to pnemonia in young pigs, reproductive failures in sows and an increase in preweaning mortality. Although the successful control of the disease depends on vaccination, the ***efficacy of currently available vaccines has been a major issue***. Some of the recent outbreaks of PRRS have been linked to the use of a live attenuated vaccine. Moreover, current vaccines do not allow differentiation between vaccinated and infected animals. Use of a PAV-3 based PRRS vaccine will a) eliminate the problems associated with the use of live attenuated PRRS vaccines; and b) allow differentiation between vaccinated and infected pigs.

III) ***Stated Objectives from the Original Proposal:***

i) Construction of synthetic PRRS virus genes
ii) Construction of recombinant PAV-3 expressing PRRS virus genes

IV) ***Materials & Methods:***

A) Cells and Viruses:

Swine testicular (ST) cells and VIDO R1 (Reddy et al., 1999) cells were grown and maintained in Eagle's Minimum Essential Medium (MEM) in the presence of 10% fetal bovine serum (FBS). The wild-type and recombinant PAdV-3 were cultivated in ST cells in MEM with 2% FBS.

B) Construction of Synthetic genes

Overlapping 100 base pair oligonucleotides were synthesized for construction of a 621 base pair Orf5 synthetic sequence *in vitro*. Eight oligonucleotides were designed in pairs with a 30 base pair overlap. Oligonucleotide pairs were annealed at 70^{o}C for 30 min and the DNA sequence was extended using DNA polymerase (Klenow Fragment). The resulting 170 base pair double-stranded DNA fragments were analyzed by polyacrylamide gel electrophoresis (PAGE), isolated and then blunt-end ligated into the pBluescriptKSII^{+} vector at the *SmaI* site. The *E.coli* cells were transformed by plasmid DNA. In total, four Orf5 fragments (Fragment 5A, 5B, 5C and 5D) have been constructed and the cloning products have been analyzed by restriction digestion with Orf5 specific sites and DNA sequencing. Each fragment is flanked by corresponding restriction enzyme sites for ligation of the fragments to form a full-length Orf5 sequence. Fragment 5A, which represents the Orf5 N-terminus, is flanked with a *BamHI* site at its 5' end and a *KpnI* site at its 3' end. Fragment 5B has a corresponding *KpnI* site at its 5' end for ligation with Fragment 5A, and a *PstI* site at its 3' end for ligation with fragment 5C. Ligation of fragment 5D (Orf5 C-terminus) to 5C utilized a *StuI* restriction site present at the corresponding ends of each fragment. Construction of the full-length clone was accomplished by sequential ligation of each fragment into the pBluescriptKSII^{+} containing fragment 5D (Orf5 C-terminus) followed by transformation of *E.coli* cells (Fig 1A). Initially, 10 clones were selected (based on restriction enzyme analysis) and sequenced to confirm the DNA sequence of the synthetic gene. Two of the clones contained the desired DNA sequence.

A 543 base pair synthetic Orf M was constructed in the same manner using four pairs of oligonucleotides for production of four double-stranded DNA fragments (fragments M-A, M-B, M-C and M-D). Annealing of oligonucleotides, extension and analysis of the double-stranded DNA fragments were performed as described above. Fragment M-A (OrfM N-terminus) was ligated into the pUC18 vector at the restriction sites *EcoRI* and *XbaI*. Ligation of fragment M-B with M-A has been performed at the *XbaI* site. The C-terminus of OrfM, fragment M-D, was ligated into pUC18 at the *BamHI* and *HindIII* restriction sites. Ligation of fragment M-C with M-D at the *BamHI* site has been performed. Finally, the M-C/M-D fragment and the M-A/M-B fragment were digested at an internal site with *MunI* followed by ligation and transformation of *E.coli* cells (Fig.1B). Initially, 10 clones were selected (based on restriction enzyme analysis) and sequenced to confirm the DNA sequence of the synthetic gene. Three of the clones contained the desired DNA sequence.

The full-length synthetic Orf3 DNA sequence is 783 base pairs in length and was constructed in the same manner using five pairs of synthetic oligonucleotides. The oligonucleotides have been annealed and extended in double-stranded DNA fragments as described above. The Orf3 fragments, designated 3-A, 3-B, 3-C, 3-D and 3-E were analyzed and isolated by PAGE. All fragments were subcloned into the pTZ18A vector by blunt-end ligation followed by transformation of *E.coli* cells. The ligation of the N-terminus of Orf3 (fragment 3-A) with fragment 3-B at a *SacI* site has been accomplished. This was followed by ligation of fragment 3-C onto fragment 2-B at a *BlpI* site. The C-terminus of Orf3, fragment 3-E, was subcloned into pTZ18A at the *BglII* and *HindIII* sites followed by ligation with fragment 3-D at a *BglII* restriction site. The full-length Orf3 synthetic sequence was constructed by ligation of fragment 3-A/3-B/3-C with 3-D/3-E at an internal *EaeI* site (Fig.1C). Initially, 10 clones were selected (based on restriction enzyme analysis) and sequenced to confirm the DNA sequence of the synthetic gene. One of the clones contained the desired DNA sequence.

For simultaneous expression of ORF M and ORF 5, a bicistronic expression cassette was constructed first by cloning of the ORF M sequence at the *MscI* and *EcoRV* sites downstream of the internal ribosome entry site (IRES) sequence in the pCITE-1 vector (Novagen). Then, the IRES-ORF M sequence was cloned downstream of the ORF 5 sequence in pGEM7zf- by ligation at the *EcoRI* and *SphI* sites (Figure 2B).

C) Construction of plasmids

i) *Construction of pPAV300CI.* The transfer plasmid pPAV300CI was constructed by cloning a chimeric intron sequence into the *SnaBI* site of the E3 region in pPAV300 (Reddy et al., 1999). This intron was amplified from the pCI-Neo vector (Promega) by PCR using primers (5′ GTAGTTAACGTAAGTATCAAGGTTAC AAGAC 3′) and (5′ GTATACGTACTG TGGAGAGAAAGGCAAAG 3′). A 137 bp amplified product was isolated which has a *HincII* site at its 5′ end and a *SnaBI* half site at its 3′ end for reconstruction of the *SnaBI* site after blunt-end ligation in the appropriate orientation. The chimeric intron sequence consists of a human *beta-globulin* gene 5′-donor site taken from the first intron, together with the branch and 3′-acceptor site taken from the intron of an *immunoglobulin* gene heavy chain variable region (Senapathy et al., 1990).

ii) *Construction of recombinant PAV-3 plasmids containing PRRS ORF 5, 3 and M.*
A 609 bp ORF 5 sequence was isolated by digestion with *BamHI* and *HindIII* from pGEM7zf (Fig. 2A). Orf 3 (831 bp) was isolated by digestion with *EcoRI* and

HindIII (Fig. 2A). Digestion with *SphI* and *SacI* of pGEM7zf- containing ORF5-IRES-ORFM (Fig. 2B) resulted in an 1836 bp fragment. These sequences were blunt-end repaired by Klenow polymerase and ligated into pPAV300CI at the *SnaBI* restriction site, downstream of the chimeric intron to avoid the occurrence of aberrant mRNA splicing products from cryptic 5′-donor splice sites within the PRRS sequences. The *SphI-SpeI* fragments of the pPAV300CI containing the individual PRRS transgene insertions in the E3 region were isolated and individually recombined with *SnaBI* linearized pFPAV300 plasmid in BJ 5183 *E.coli* cells (Reddy et al., 1999). Full-length clones were rescued for E3 insertion of synthetic PRRS genes ORF3 (pFPAV310a), ORF5 (pFPAV312a) and ORF5-IRES-ORFM (pFPAV317a) (Figure 2).

D) Transfection

About 80% confluent monolayers of ST or VIDO R1 (Reddy et al., 1999) cells grown in 60 mm dishes were transfected with 5.0 or 7.5 μg of PacI-digested individual recombinant full-length plasmid DNA (pFPAV310a, pFPAV321a or pFPAV317a) using lipofection (invitrogen). Following transfection, cells were maintained in MEM containing 3% FBS at 37°C for 2-3 weeks until cytopathic effects appeared. Cells showing cytopathic effects were harvested, freeze-thawed five times and the recombinant viruses propagated in ST cells

V&VI ***Results & Discussion***

A. Construction of synthetic PRRS genes.

1) <u>*Sequence analysis of the PRRS genes Orf5, Orf3 and OrfM:*</u> The DNA sequences of three PRRS virus genes, Orf5, Orf3 and OrfM were retrieved from the 15 kb PRRS genome using the GenBank DNA database. The DNA sequences of Orf5, Orf3 and OrfM were adjusted to encode the same amino acid sequence using codons that are optimally used in pigs to ensure high levels of expression. Also, the DNA sequence was analyzed for the presence of cryptic RNA splice sites using the Splice Site Prediction software by Neural Network (Berkley Drosophila Genome Project). The RNA splice sites were removed from the sequences, while maintaining optimal codon usage and the amino acid sequence. Within the Orf5, Orf3 and OrfM sequences, restriction enzyme sites were engineered into the DNA sequence without altering the amino acid sequence.

ii) <u>Construction of Synthetic genes:</u> In order to obtain optimal expression of vaccine antigen (Orf3, Orf5 and Orf M), PRRS virus genes (Fig. 1 A,B,C) were chemically synthesized *in vitro* by adjusting the codon bias (based on usage of codons in abundantly expressed proteins of pig) and by removing potential splice site sequences (Splice Site Prediction software by Neural Network [Berkley Drosophila Genome Project]) without affecting the amino acid sequence of vaccine antigens. This was achieved by synthesizing and ligating oligonucleotides of desired DNA sequences using appropriate existing or newly created restriction enzyme sites. The identity of the re-synthesized genes was confirmed by determining the DNA sequence.

B) Construction of Recombinant PAdV-3 expressing PRRS virus Genes

To increase the expression of PRRS virus genes, we initially inserted a chimeric intron sequence into the E3 region of plasmid pPAV300 creating plasmid pPAV300CI.The full-length synthetic ORF3 or ORF5 were individually inserted into the E3 region of pPAV300CI and finally recombined into the E3 region of the

pFPAV300 genome in the same transcriptional orientation as E3, using the homologous recombination machinery of *E. coli* BJ5183 (Degryse, 1996). The PacI-digested pFPAV310a or pFPAV312a plasmid DNA was transfected into ST / VIDO RI cells. The cells showing 50% cytopathic effects were collected, freeze-thawed three times and recombinant viruses were propagated in ST cells. The recombinant viruses were named pAV310a (pFPAV310a) and PAV312a (pPFPAV312a). The viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with restriction enzyme . Expected bands were observed in PAV300 and recombinant viruses PAV310a and PAV312a. Presently, the recombinant viruses are being passaged in order to evaluate the stability of the recombinant genomes. Once we produce stable recombinant viruses (after 10 passages), we will check the expression of recombinant protein by Western blot or by immunoprecipitation using protein-specific antibodies.

In addition to the proposed objectives, we also constructed the full-length plasmid pFPAV317a containing ORF5-IRES-ORFM inserted in the E3 region of PAV300. The PacI-digested plasmid pFPAV317a DNA was transfected into ST cells or VIDO RI cells. Presently, the transfected cells are being observed for cytopathic effects.

References

Degryse, E (1996). In vivo intermolecular recombination in Escherichia coli. Application to plasmid construction. Gene 170, 45-70.

Reddy, P. S., Idamakanti, N., Babiuk, L. A., Mehtali, M., Tikoo, S. K. (1999). Porcine adenovirus-3 as a helper-dependent expression vector. J. Gen. Virol.80, 2909-2916.

Senapathy, P., Shapiro, M. B., Harris, N. L., (1990). Splice junctions, branch point sites and exons: sequence statistics, identification, and applications to genome project. Meth. Enzymol. 183, 252-278.

VII ***Lay interpretation***

Porcine reproductive and respiratory syndrome (PRRS) virus imposes devastating effects on swine health and productivity. In the U.S., PRRS virus causes approximately $560 million in losses each year. Vaccination of animals is one way to reduce losses from infectious diseases including PRRS. Current modified live and killed vaccines have some success, although differentiation between vaccinated and infected animals has been a problem. In addition, live attenuated vaccines have resulted in appearance of PRRS like symptoms in sow herds and altering semen quality of boars. As such, new approaches are being utilized to develop better PRRS vaccine. Using harmless porcine adenovirus 3 (which infects respiratory tract of pigs), we have constructed recombinant porcine adenovirus 3 expressing vaccine antigens genes (synthesized in the laboratory for increased expression) of PRRS virus. These recombinant PAV-3s will be used to evaluate their ability to induce a protective immune responses in pigs against PRRS virus challenge. The advantages of our approach of using harmless PAV-3 to deliver PRRS vaccine antigens to pigs is that such vaccines are ***safe, cost-effective, and highly efficacious.***

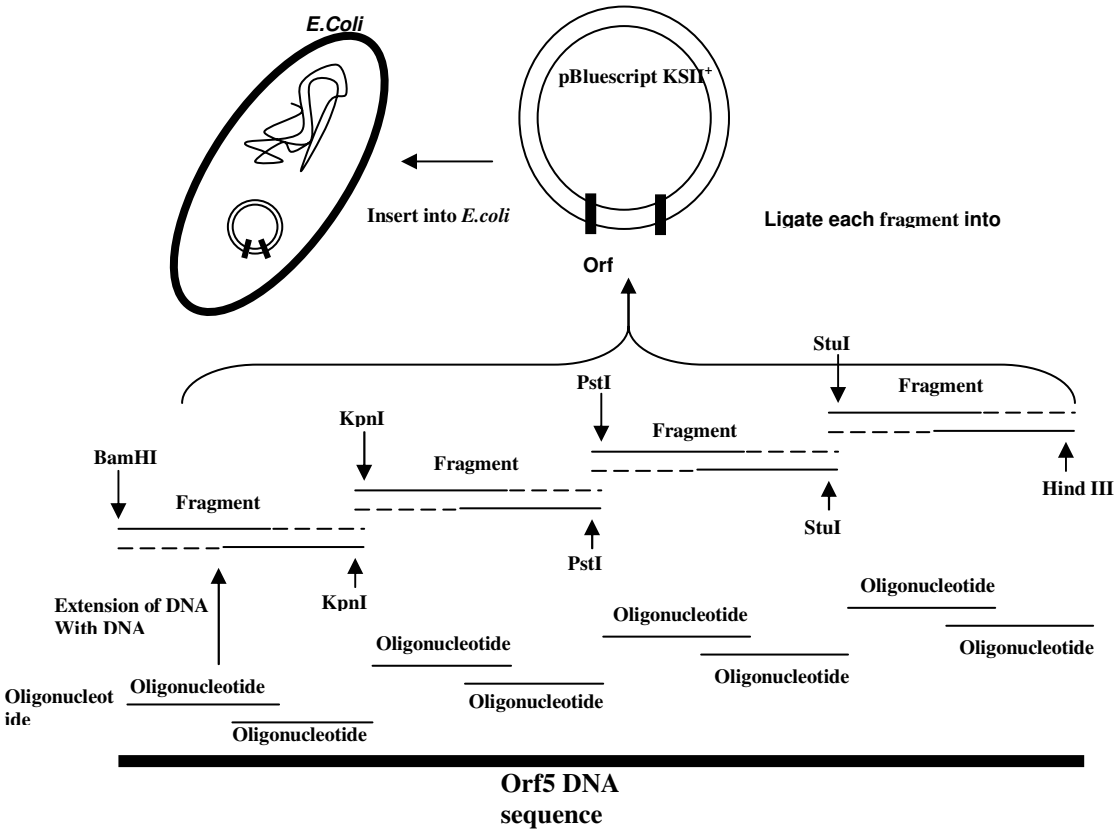

Fig. 1A. Schematic diagram showing the construction of synthetic Orf 5

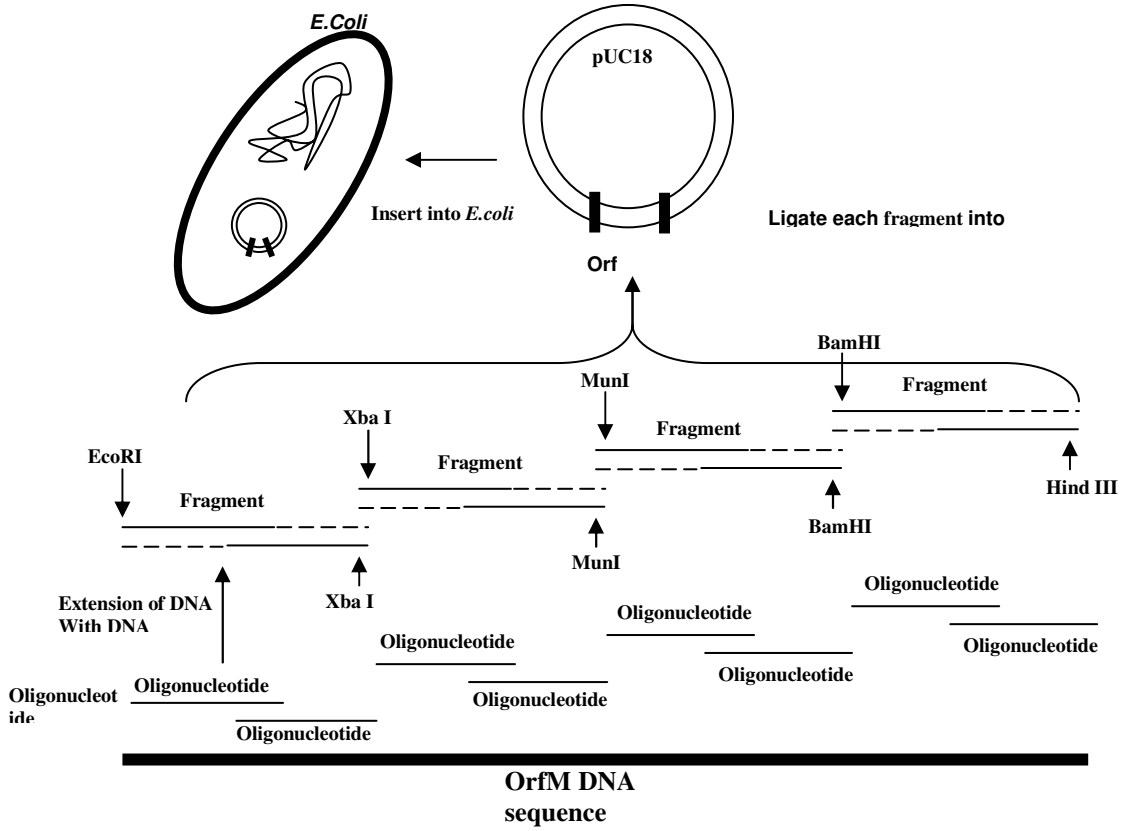

Fig. 1B. Schematic diagram showing the construction of synthetic Orf M

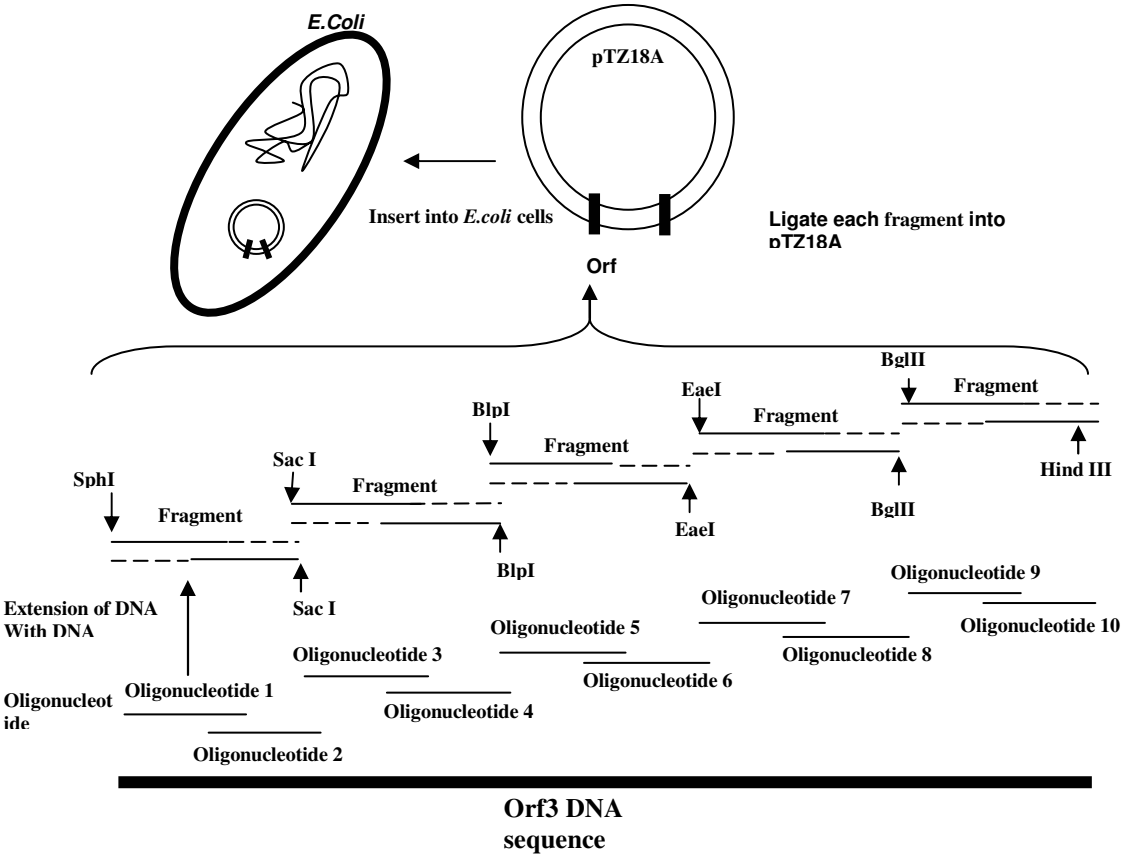

Fig. 1C. Schematic diagram showing the construction of synthetic Orf 3

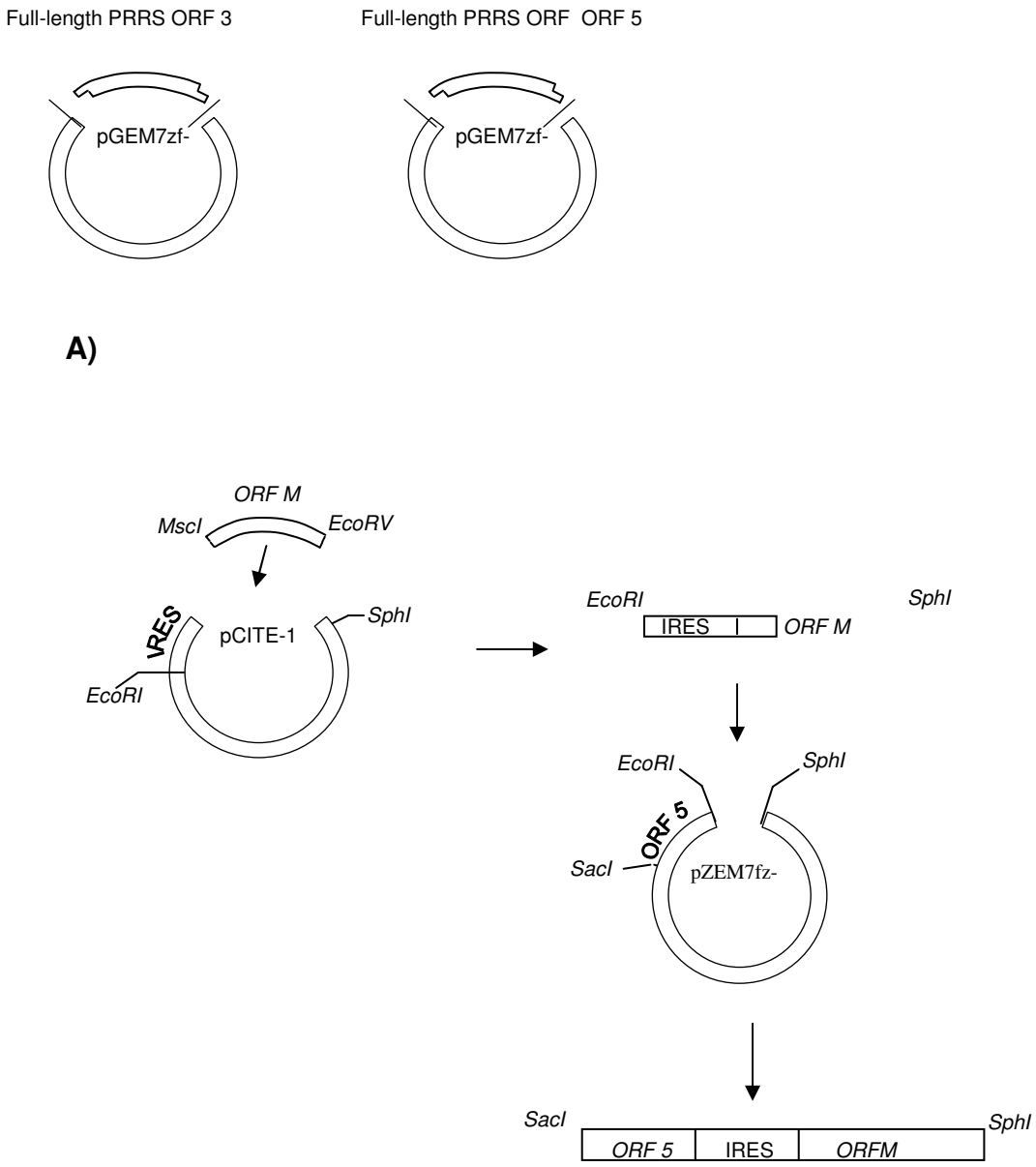
Full-length PRRS ORF 3
Full-length PRRS ORF ORF 5
pGEM7zf-
pGEM7zf-
A)
ORF M
MscI
EcoRV
IRES
pCITE-1
SphI
EcoRI
EcoRI
SphI
IRES
ORF M
EcoRI
SphI
ORF 5
SacI
pZEM7fz-
SacI
SphI
ORF 5
IRES
ORFM
B)

Fig. 2

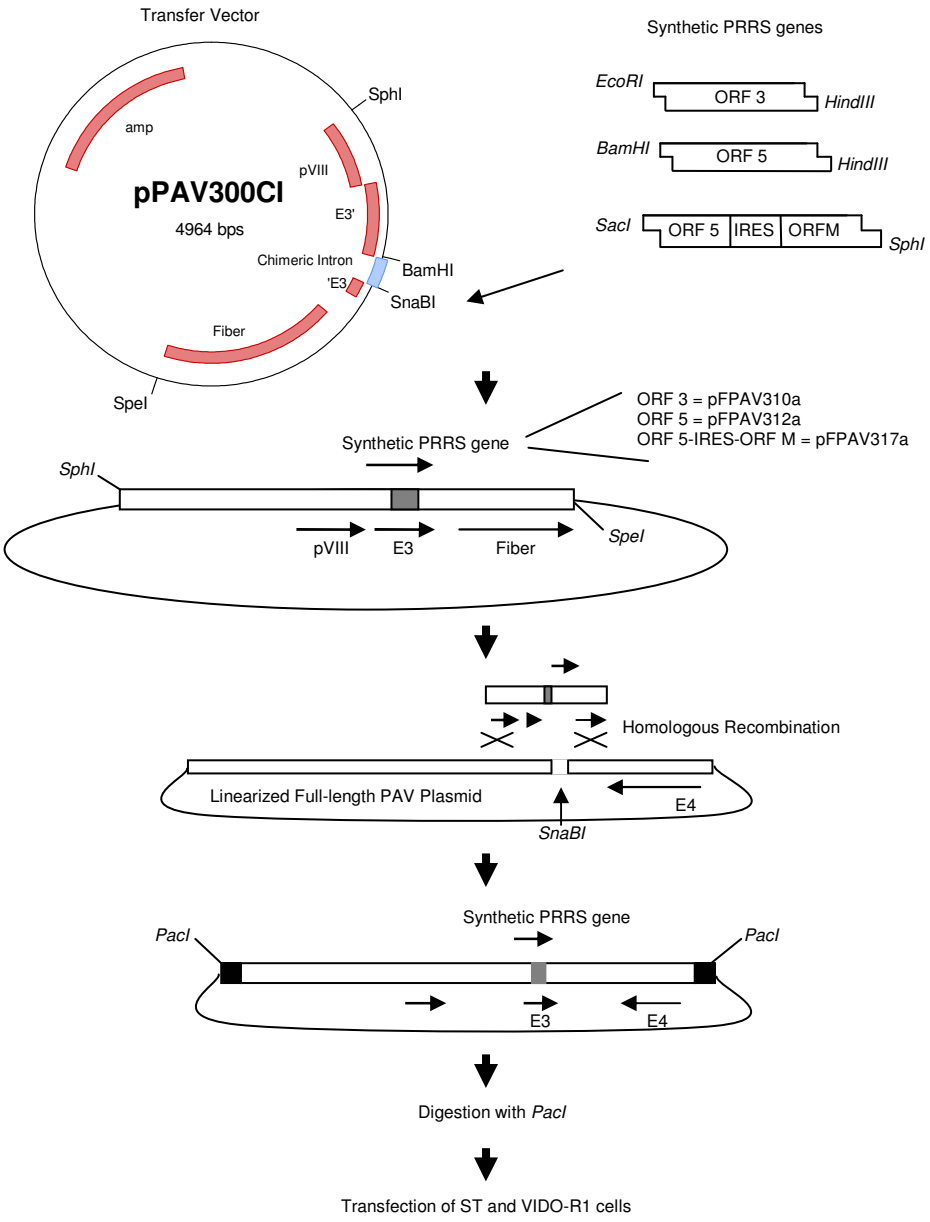
Transfer Vector
SphI
amp
pVIII
pPAV300CI
4964 bps
E3'
Chimeric Intron
'E3
BamHI
SnaBI
Fiber
SpeI
Synthetic PRRS genes
EcoRI
ORF 3
HindIII
BamHI
ORF 5
HindIII
SacI
ORF 5
IRES
ORFM
SphI
ORF 3 = pFPAV310a
ORF 5 = pFPAV312a
ORF 5-IRES-ORF M = pFPAV317a
Synthetic PRRS gene
SphI
pVIII
E3
Fiber
SpeI
Homologous Recombination
Linearized Full-length PAV Plasmid
E4
SnaBI
Synthetic PRRS gene
PacI
PacI
E3
E4
Digestion with PacI
Transfection of ST and VIDO-R1 cells

Fig. 3