RESEARCH REPORT
SWINE HEALTH

Title: Porcine Adenovirus 3 Based Vaccine for Porcine Respiratory and Reproductive Syndrome (PRRS). **NPB #06-126**

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II) Industry Summary

Porcine reproductive and respiratory syndrome (PRRS) virus imposes devastating effects on swine health and productivity. 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, we have constructed recombinant porcine adenovirus 3 expressing vaccine antigen genes (synthesized in the laboratory for increased expression) of PRRS virus. Although use of synthetic (codon optimized) PRRS virus glycoprotein genes helped to increase the level of expression of these glycoproteins in mammalian cells, it was not sufficient to induce a protective immune response in pigs.

III) 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 (PAdV)-3 infects pigs but often does not produce disease, it is a good candidate as a live vaccine. We characterized PAdV-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 PAdV-3 expressing vaccine antigens. We also 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. Here, we demonstrate that use of synthetic (codon optimized) PRRS virus glycoprotein genes helped to increase the level of expression of these glycoproteins in mammalian cells. However, increased expression was not sufficient to induce protective inmmune responses in pigs.

IV) 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 PAdV-3 as a vector for the development of live vectored vaccines for pigs, as a) PAdV-3 is a non-pathogenic porcine virus, which does not cause any serious disease in pigs[1,2], b) it grows to high titers and c) is well characterized[3,4,5].

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[6]. 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 PAdV-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.

V) Stated Objectives from the Original Proposal:

The main objective of the work is to determine if immunization of pigs expressing PRRS virus vaccine antigens induce an immune response and protect the animals from virus challenge.

VI) Materials & Methods:

***A) Cells and Viruses*.** Swine testicular (ST) cells and VIDO R1[3] 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) Animal immunizations. Two to three week old piglets were immunized oronasally twice (four weeks apart) with recombinant PAdV-3 expressing PRRS virus genes. Control animals were inoculated with PAV300 (E3 deleted PAdV-3). Animals were monitored for the induction of mucosal and systemic PRRS virus specific immune responses using standard assays. These animals were challenged intranasally two weeks after second immunization with virulent PRRS virus (ATCC VR-2332). Animals were observed for the development of clinical signs and virus shedding. Finally, animals were euthanized and different tissues examined for lesions.

C) Indirect imunofluorescence assay: MARC- 145 cells were seeded in 96 well plate(s) and grown in DMEM (Dulbeco's minimum essential medium) overnight at 37^0C with 5% CO_2. Next day, the cells were washed with PBS (phosphate buffered saline and incubated with 0.2 ml of media containing 600 $TCID_{50}$/ ml of PRRS virus.

The cells were incubated for 2-3 days, when cells started showing cytopathic effects. The cells were fixed with 100% cold ethanol for 20 min. Finally, the plates were dried at room temperature and washed once with PBS. Test sera were diluted two fold serially ([0 day; 1:2-1:128] [12 day: 1:2-1:1024] and 100μl of each sample was transferred to the infected cell plate. The plates were incubated for 45 min at 37^0C. The plates were washed with PBS and incubated with rabbit anti-swine IgG conjugated to FITC (fluorescein isothiocynate). After incubating for 45 min at 37^0C, the plates were washed with PBS and examined under a fluorescent microscope. Each plate contained a positive control (PRRS antibody positive sera of known titer), a negative control (sera containing PRRS titer ≤ 1:2). In addition, uninfected cells were used as control also. The reciprocal of highest serum dilution showing fluorescence determined the titer of the sera.

D) Virus neutralization test: Initially, 2 fold serial dilutions of heat inactivated sera were made in DMEM in 96 well flat bottom tissue culture plates. About 200 μl of each diluted sera and 200 ul of PRRS virus suspension (containing 200 $TCID_{50}$) were incubated for 1 h at 37 ^{0}C. The incubated mixtures were transferred to MARC-145 cells in triplicates in 96 well plates. The plates were incubated at 37^0C with 5% CO_2. The cells were observed for the appearance of cytopathic effects for 5 days. The reciprocal of the highest dilution of sera which inhibited the development of cytopathic effects produced by PRRS virus in MARC-145 cells defined the neutralization titer of the sera.

E) Western blot. Monolayers of swine testicular (ST) cells ($1X10^5$ per well) in 6 well tissue culture dishes were infected with recombinant PAdV-3 at a multiplicity of infection (MOI) of 1 and harvested at 24 h post infection. The cells were lysed in 100 μl of radioimmunoprecipitation buffer (RIPA) buffer (0.15M NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP40, 1% deoxycholate, 0.1% SDS). Proteins from the lysates of PAdV-3 infected ST cells were separated on 10% sodium –dodecyl sulphate (SDS)-polyacryl -amide gel electrophoresis (PAGE) under reducing conditions, transferred to nitro- cellulose membrane and blocked in tris-buffered saline (TBS) containing 3% skim milk. The membrane was probed with anti-PRRSV sera. The membrane was washed three times, exposed to goat anti swine IgG (H+L) (Jackson ImmnoResearch labs) conjugated to alkaline phosphatase, and developed using BCIP/NBT substrate (Sigma) according to manufacturer's directions.

F) Virus isolation. Pieces of lung tissue of individual pig were collected and placed into tubes containing 2.4 mm Zicornia beads and 1 ml of media. Tubes were vibrated on mini-bead beater to prepare lung homogenates. Homogenates from samples collected from the same pig were pooled together. A 10 fold serial dilution of each sample was made in media. Hundred μl of each sample dilution (in triplicate) was added to MARC-145 cells seeded in 96 well plates. The plates were incubated at 37^0C in CO_2 for at least 5 days. The cells were observed for the development of cytopathic effects. Appropriate positive and negative controls were included in each plate. The highest dilution showing cytopathic effects determined the titer of the virus.

G) RT-nPCR for detection of viral RNA. RNA was isolated from serum as described earlier [10,11] using guanidine thiocyanate method. The presence of viral RNA in the serum was determined by amplifying Orf7 specific sequences of VR-2332 using reverse transcriptase nested polymerase chain reaction[10,11].The outer forward and reverse primers were 5'-TCGTGTTGGTGGCAGAAAAGC-3' AND 5'-GCCATTCACCACAC ATTC TTCC-3' which amplified a product of 484bp. The nested primers were 5'-CCAGATGC TGGGTAAGATCATC-3' and 5'-CAGTGTAACTTATCCTCCCTGA-3',which amplified a product of 236bp.

VII) Results & VIII) Discussion

The recombinant viruses a) PAV300 (E3 deleted PAdV-3; control), b) PAV310 (PAV300 containing wild-type Orf3 inserted in E3 region), c) PAV310a (PAV300 containing synthetic Orf3 in E3 region), d) PAV312 (PAV300 containing wild-type Orf 5 inserted in E3 region), and e) PAV312a (PAV300 containing

synthetic Orf 5 inserted in E3 region) were made (Fig. 1) using standard procedures[7,8]. They were passaged and grown in VIDO R1 cells to make viral stocks.

In order to determine the immune response, 2-3 week old crossbred piglets were randomly allocated in different groups. All pigs were seronegative for PRRS antibodies as tested by ELISA (Herdcheck PRRS 2XR kit). However, all pigs had low level of anti-PAV-3 titers in the serum. The piglets were vaccinated twice, three weeks apart with recombinant PAV310 (expressing wild-type Orf3), PAV312 (expressing wild- type Orf5) PAV310a (synthetic Orf3), PAV312a (synthetic Orf5), or PAV300 (E3 deleted; control) oronasally ($5X10^7$ IU/ml). At day 32, all pigs were challenged intranasally with 10^6 $TCID_{50}$/ml of PRRS virus (ATCC VR-2332 grown in pig lung). Animals were observed for the development of any clinical sign(s) and temperature. Blood for Serum was collected at days 0, 32, and 44, and analysed for humoral immune responses and virus isolation. In addition, blood was also collected in on day 32 for isolation of PBMCs.

After two immunizations with PAV312, PAV312a, or PAV300, very low levels of antibody titers were detectable by indirect immunofluorescence (IIF) assay (Fig.2) at 0 day post challenge. There was no significant difference between vaccinated animals and control animals ($P>0.05$). However, 12 days post challenge, pigs immunized with either PAV312 or PAV312a developed higher antibody titers compared to pigs immunized with PAV300 as detected by IIF (Fig. 2). However, the difference was significant only in pigs immunized with PAV312a compared to pigs immunized with PAV300 ($P<0.001$) or PAV312 (($P<0.001$). Although low levels of PRRS virus neutralization (VN) antibodies (Fig. 3) were detected in pigs immunized twice with either PAV312 or PAV312a at 0 day post challenge, there was significant difference between pigs immunized with PAV300 to pigs immunized with PAV312a ($P<0.001$). However 12 days after challenge, pigs immunized with PAV312a developed significantly higher VN titer than pigs immunized with either PAV312 ($P<0.001$), or PAV300 ($P<0.05$) (Fig. 3).

Similarly, very low level of antibody titers were detected in pigs immunized with PAV310a or PAV300 as detected by IIF (Fig. 2) at day 0 post challenge. The level of antibodies increased in pigs immunized with PAV310a compared to PAV310 on day 12 post challenge as detected by IIF (Fig. 2). However, the difference was significant in pigs immunized with PAV310a than pigs immunized with PAV300 ($P<0.05$) . Low levels of PRRS VN antibodies (Fig. 3) were detected in pigs immunized twice with PAV310 and PAV310a at day 0. However 12 days after challenge pigs immunized with PAV310a developed significantly higher VN titers than pigs immunized with either PAV310 ($P<0.001$) or PAV300 ($P<0.05$).

To assess PRRSV-specific cellular immune responses, PBMCs were isolated from blood samples on day 32. PBMCs were seeded into 96 well plates (and stimulated with inactivated PRRSV virus (10μg\ml) or ConA (5μg\ml). At 48 h post stimulation, cell culture media were harvested and assayed using BIOSOURCE swine INF-γ. Although INF-γ could be detected in ConA stimulated cells, none of the samples stimulated with inactivated PRRSV was positive for INF-γ.

To determine the virus load, 10 fold serial dilution of homogenates of pieces of lung from each pig were added to the monolayer of MARC-145 cells. The cells were observed for the development of cytopathic effects. There was really no significant difference in the PRRS virus load between animals immunized with PAV300, PAV310, PAV310a and PAV312 (titers ranged from 10^4-10^5/pig) at 12 days post challenge (Fig.4). However, pigs immunized with PAV312a contained significantly less virus than pigs immunized with PAV300 ($P <0.001$) or PAV310 ($P <0.001$).

To determine the presence of virus in serum, RT.nPCR was performed. Viral RNA could be detected in animals immunized with PAV300 (10 out of 10 animals), PAV310 (10 out of 10 animals), PAV310a (10 out of10 animals), PAV312 (10 out of 10 animals) and PAV312a (8 out of 10 animals). Our results suggests that immunization with recombinant PAdV-3 expressing vaccine antigens of PRRS virus did not prevent the

development of viremia. Moreover, it appears that there is no correlation between the viremia and the virus neutralization titers. Similar results have been reported earlier[9,10].

No significant difference in clinical signs including rectal temperature could be observed amongst the groups. Moreover, no significant gross lesion(s) could be detected in the lungs resulting from the virus challenge in any of the groups.

Conclusions

One of the major impediments to effective vaccines is delivery. We have choosen to develop PAdV-3 as a live viral vector for delivery of vaccine antigens.
Earlier, we showed that recombinant PAdV-3 expressed low levels of PRRS glycoprotein (wild-type) vaccine antigens. Moreover, immunization of pigs with recombinant PAdV-3 expressing PRRS wild-type glycoproteins produced did not induce protection in the animals from PRRS challenge. Here, we demonstrated that use of synthetic (codon optimized) PRRS virus glycoprotein genes helped to increase the level of expression of these glycoproteins. However, the level of vaccine antigen expression was not sufficient to induce protection in challenged pigs.

An ideal vaccine for PRRS should induce both humoral and cellular immune responses against the virus. Although a number of strategies including DNA vaccination[11] and vectored (e.g Bacille Calmette-Guerin vector[12], herpes virus vector[13], vaccinia virus [14]) vaccination are being evaluated for producing / delivering vaccines against PRRS disease in pigs, none of these strategies have so far been successful in inducing protection against PRRS disease in pigs for a number of reasons including choice of vaccine antigen(s) used and non inclusion of vaccine antigen(s) inducing T cell responses As both humoral and cellular immune responses are required for inducing protection against PRRS disease in pigs, the future recombinant vaccines should contain antigens, which induce a potential cellular immune responses. Thus, more studies need to be done (e.g. effect of insertion site, effect of different transcriptional elements, route of immunization) to determine the full potential of this approach for delivering vaccines in porcine and to find a way of developing increased pre challenge PRRS virus neutralizing antibodies.

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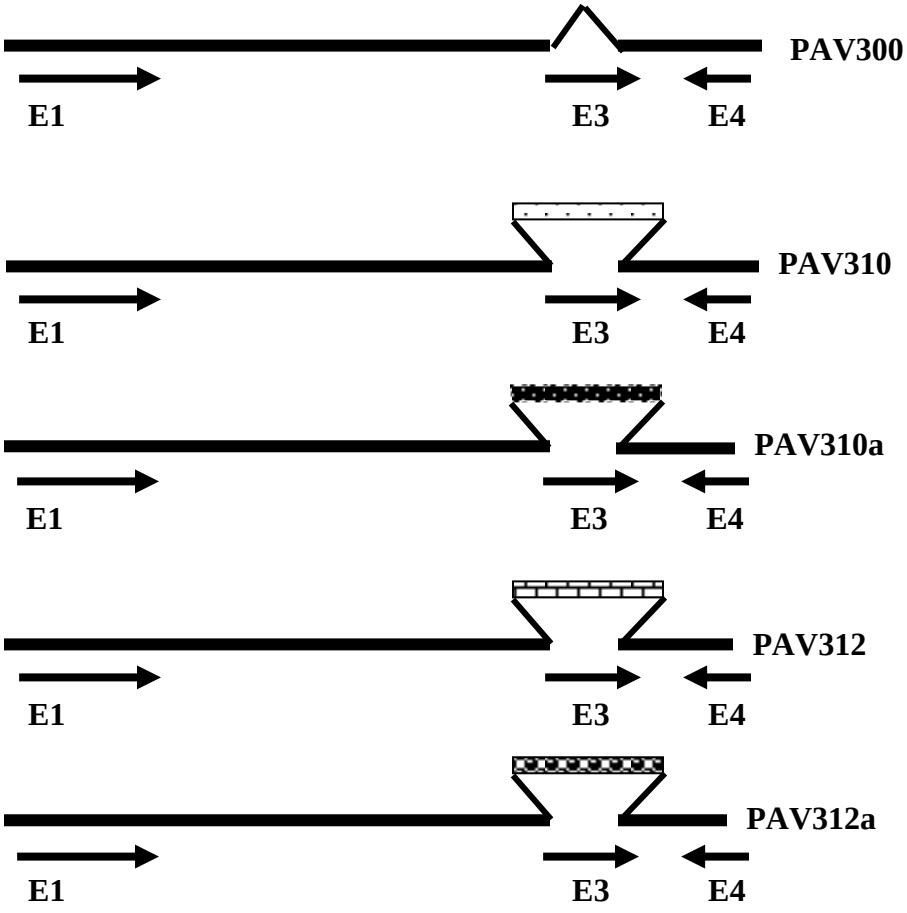

Fig1. Schematic diagram of genomes of recombinant PAdV-3.

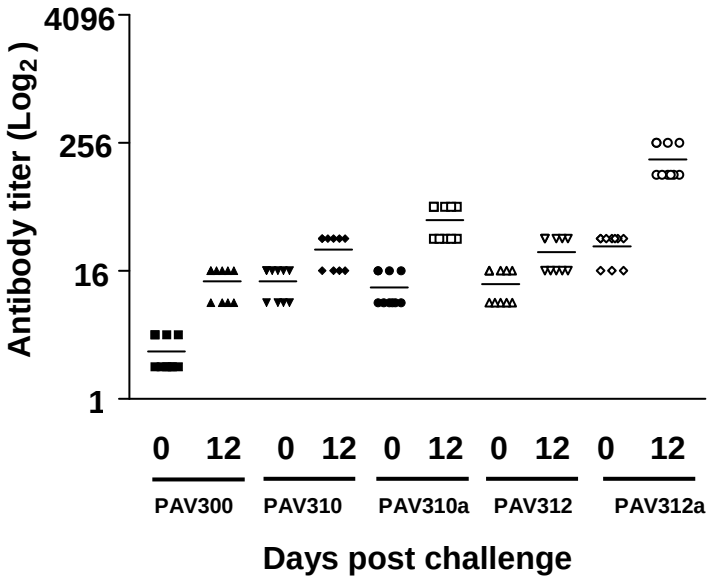

Fig 2. Antibody titers in sera as determined by IIF(Method described in the text).

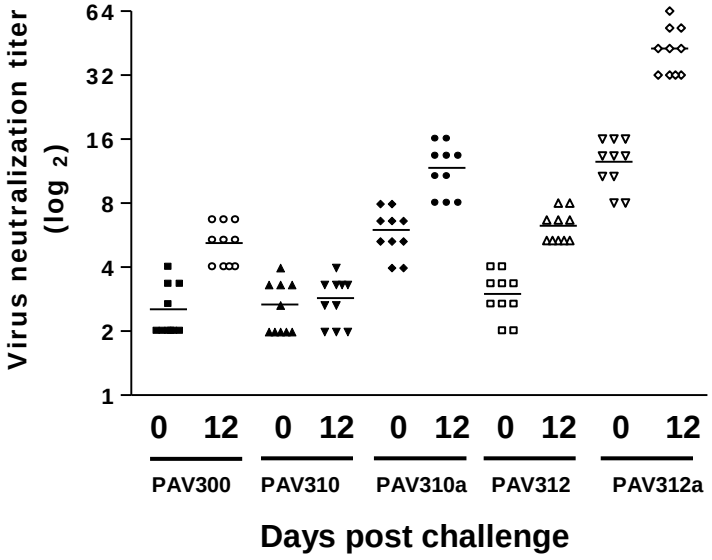

Fig. 3. Virus neutralization titers in sera of pigs. (Method described in the text)

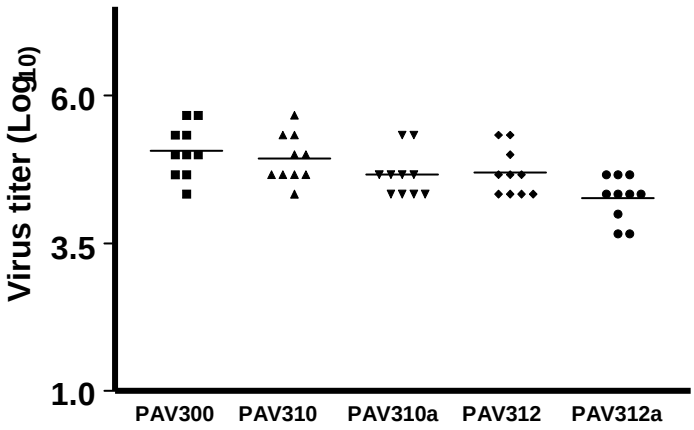

Fig. 4 Virus titers in lungs of infected pigs. (Method described in the text)