RESEARCH REPORT
SWINE HEALTH

Title: Development of recombinant vaccinia virus vectored African swine fever vaccines - #15-126

Investigator: Wenjun Ma

Institution: Kansas State University

Date Submitted: 8/15/2017

Industry Summary: African swine fever (ASF) is an infectious and economically important disease of domestic pigs that poses a significant threat to worldwide swine industry including the US. Currently, there is no vaccine for ASF. In this proposal, 3 recombinant vaccinia virus vectored African swine fever vaccine candidates have been developed. Two different ASFV antigens were expressed in each recombinant virus. The immunogenicity of recombinant vaccine candidates was evaluated in pigs that were co-infected with 3 recombinant vaccine candidates. The results showed that an ELISA titer against ASFV antigen was detected in immunized pigs, indicating that the vaccine candidates are immunogenic in pigs. Nevertheless, ASF vaccine candidates have been developed and their efficacy need to be evaluated in pigs that will be challenged with a virulent ASFV when additional funding is available. If they are demonstrated to be able to protect pigs against the virulent ASFV challenge, they can be used as potential vaccines to protect the swine industry.

Dr. Wenjun Ma (Email: wjma@ksu.edu; phone 785-532-4337).

Keywords: African swine fever virus, vaccinia, vectored vaccine, antigen, immunogenicity

Scientific Abstract: African swine fever (ASF) is an infectious and economically important disease of domestic pigs that poses a significant threat to worldwide swine industry including the US. Currently, there is no vaccine for ASF. Herein, we used the vaccinia virus as the vector to develop three recombinant vaccinia vectored African swine fever vaccine candidates. Each recombinant virus expresses two important ASFV antigens such as p72+p15, p54+pp62 and p30+CD2-like, and has been shown stably to express each inserted gene. The immunogenicity of recombinant vaccine candidates was evaluated in pigs that were co-infected with 3 recombinant vaccine candidates. The results showed that the ELISA titers against ASFV p15 and p54 were detected in immunized pigs, indicating that the vaccine candidates are immunogenic in pigs. The efficacy of the developed vaccinia vectored ASF vaccine candidates need to be evaluated in pigs that will be challenged with a virulent ASFV. Nevertheless, ASF vaccine candidates have been developed and they can be used as potential vaccines if their efficacy can be demonstrated in pigs.

Introduction: African swine fever (ASF) is a highly contagious and economically important disease of domestic pigs caused by African swine fever virus (ASFV) that belongs to the family of Asfarviridae. Infection with ASFV causes a lethal hemorrhagic disease in domestic pigs and Eurasian wild boar with up to 100% mortality if they are infected with a virulent strain while African wild pigs (warthogs and bush pigs) typically show no signs of disease. ASF is enzootic in many African countries and was confirmed for the first time in Georgia in 2007. ASFV has spread rapidly into Russia, Ukraine and Belarus with the potential for international spread, a major threat to the swine industry worldwide (Alonso, 2013).

There is no vaccine for ASF, and all attempts to develop a vaccine have so far been unsuccessful (Dixon et al., 2013). Traditional inactivated ASF vaccines have been shown not to induce protection in pigs. The major obstacle of development of an ASF vaccine is the complexity of the virus whose genome contains more than 160 genes, approximately 50% of which have unknown functions. Although the mechanisms of the protective immune responses are not well understood, it has been shown that both humoral and cell mediated responses contribute to the protection from ASFV and passively transferred antibodies specific for ASFV are sufficient to protect pigs from lethal infection (Onisk et al., 1994). Immunization with the Baculovirus expressed ASFV proteins p30 and p54 together provide partial protection to infection, with 50% of immunized pigs surviving a virulent ASFV challenge (Barderas et al., 2001). A monoclonal antibody against ASFV protein p72 has high levels of neutralizing activity against various virulent ASFV isolates. To date, published studies show that the use of individual recombinant proteins as immunogens provides only partial protection, indicating that complete protection may require responses to multiple viral proteins and might involve cell-mediated immunity. Previous studies demonstrated that pigs immunized with attenuated ASFV isolates can be partially and completely protected from a homologous challenge (King et al., 2011; Oura et al., 2005), and that CD8+ T cells are necessary for inducing protection. This protection correlates with the frequency of ASFV specific IFN-gamma producing memory T cells (Oura et al., 2005). Although ASF attenuated live vaccines are promising, challenges in the use of them as vaccines include safety concerns and potential for reversion to virulence. Extensive research for producing a safe and efficacious ASF live attenuated vaccine is still required before these strains can be used as vaccines in the field.

The alternative approach is the development of ASF vaccines that can induce both humoral and cellular immunity for complete protection against challenge with virulent ASFV isolates. Vectored vaccines such as poxviruses could be the alternative strategy since they have been used as expression vectors for human and veterinary vaccine development (Draper & Heeney, 2009; Kieny et al., 1984; Paoletti, 1996; Smith et al., 1983a; Smith et al., 1983b). There are many attractive advantages to using poxviruses as vaccine vectors, including their ability to infect multiple species, including pigs, their induction of strong immune responses, their well-established construction technology, their sufficient safety information, their ability to accommodate large fragment of foreign DNA and high expression levels. There are many veterinary vaccines utilizing poxvirus vectors such as wildlife rabies vaccine and equine flu etc (Brochier et al., 1995; Rupprecht et al., 1986; Wiktor et al., 1984; 1992). There are also many poxvirus-based vaccines in human clinical trial (Moss, 2013).

The vaccinia virus strain Copenhagen (VACV-COP) had been used as a vaccine strain in humans during the global campaign to eradicate smallpox and is considered safe in humans. There are other appealing advantages to using the VACV-COP as an animal vaccine vector. First, many of the gene functions have been studied and this knowledge can be used to enhance the vector by further reducing its virulence and improving its immunogenicity. Second, it is a replicating vector that can dramatically reduce the immune dosage and the cost of vaccine production. Third, it has been used in veterinary vaccine development with demonstrated efficacy and safety (Papin et al., 2011). In this proposal, we will use the vaccinia virus VACV-COP to construct African Swine Fever Virus (ASFV) vaccine candidates by expressing ASFV proteins p15, p72, p30 and p54. The recombinant VACV-COP viruses will be characterized and used as vaccine candidates in pigs to test their immunogenicity.

Objectives: The proposed project is to develop efficacious vaccinia virus vectored African swine fever vaccine candidates and includes 2 objectives:
Objective 1: Construct and characterize recombinant vaccinia VACV-COP viruses expressing both ASFV proteins p30 and p54, and both ASFV proteins p15 and p72
Objective 2: Evaluate immunogenicity of recombinant vaccinia VACV-COP viruses in pigs including specific humoral and cellular immune responses

Materials & Methods:

Construct recombinant VACV viruses expressing ASFV antigens. Large foreign DNA fragments can be inserted into the vaccinia genome for expression. The synthesized p15, p30, p54, and p72 genes based on the Georgia/2007 virus sequence encode membrane, capsids or other structural proteins of ASFV, and their epitopes have been shown to elicit neutralizing antibodies to ASFV (Argilaguet et al., 2012; Cubillos et al., 2012). These genes were used for VACV-COP-based ASFV vaccine development. We constructed two recombinant viruses: one expressing ASFV p15 and p72 and another expressing ASFV p30 and p54. In both cases, the ASFV genes were placed under the control of a strong VACV early/late promoter. The expression of ASFV antigens at both early and late time of vaccinia replication is critical to elicit the host immune response as the early gene expression has been shown to be important for CD8+ T cell response, while late gene expression is important for B cells and CD4+ T cells responses (Yang et al., 2011). In this attempt, we first cloned the ASFV genes into a vector together with flanking vaccinia sequence into a plasmid using In-Fusion cloning system according to the manufacturer's instruction (Clontech). The ASFV genes in the plasmid are designed to replace the GFP gene in VACV-COP-GFP, so that the virus without GFP expression after recombination can be selected for further analysis. Briefly, BS-C-1 cells were infected with VACV-COP-GFP at a MOI of 1 followed by transfection of DNA containing ASFV p15 and p72 (or p30 and p54) under the control of a strong VACV early/late promoter and flanking VACV DNA sequences which are able to recombine to target region of viral genome. The cells were harvested 48 h later and lysed by three freeze-thaw cycles. The suspension was diluted and plated onto BS-C-1 monolayers. Recombinant viruses not exhibiting green fluorescence were purified by three or four rounds of plaque isolation as described (Earl et al., 2001). The inserted genes and location of the inserted DNA were verified by PCR and sequencing. The confirmed recombinant viruses VACV-COP-p30+p54 and VACV-COP-p15+p72 were selected for further characterization.

Evaluate and characterize the recombinant viruses in cultured cells. The recombinant viruses were evaluated for their foreign protein expression and stability *in vitro* and protein expression was confirmed by Western blotting. To evaluate the stability of the foreign gene, each of the recombinant viruses was passed for 10 generations in cultured Vero cells and the protein expression levels of the recombinant viruses was measured at 1, 3, 6, and 10 generations. To evaluate the growth of the recombinant virus, the growth kinetics of recombinant viruses was compared with that of a parental virus by infecting Vero cells at a MO1 of 0.01 and measuring the viral titers at different time points by the plaque assay. The stable recombinant viruses that efficiently express ASF specific proteins were selected for the pig study in order to evaluate their immunogenicity.

Evaluate immunogenicity of recombinant VACV-COP viruses in pigs

Pig studies using recombinant VACV-COP viruses. The immunogenicity of the selected recombinant VACV-COP viruses was evaluated in piglets that are seronegative for swine influenza virus and porcine reproductive and respiratory syndrome virus. Briefly, a group of 10 piglets (vaccination) and one group of 5 piglets (control) was used in the study. Each pig in vaccinated group was administered with combination of both recombinant viruses (10*6 PFU/pig/per virus). The 5 control piglets were administered a placebo and served as negative controls. Each pig in the vaccination group was boosted with the same dose of mixture of recombinant viruses at 14 days post vaccination. Recombinant virus were administered intramuscularly (IM) in the neck and clinical signs such as fever, swelling and pox-like lesions in vaccination site were observed and monitored throughout the experiment. Piglets were bled for serology by venipuncture on days 0, 14, and 28 post vaccination. Serum samples and PBMC cells

were prepared and stored at -80oC until used. All pigs were euthanized on day 28 post vaccination. Lymph nodes were collected from all pigs at the necropsy day for immunological analysis.

To determine specific humoral immune responses induced by recombinant viruses in pigs, sera was tested for antibodies to recombinant vaccinia viruses expressing ASFV proteins by using an ELISA assay with recombinant ASFV protein as coating antigens.

Results:

Objective 1: In our original design to generate a recombinant vaccinia virus expressing both p30 and p54, we generated 2 recombinant vaccinia viruses expressing both p54 and pp62, or both p30 and pCD2-like instead of one since pp62 and CD2-like proteins are also important immunogenic antigens of ASFV for protection. In order to increase our chance to achieve sufficient immunity induced by the vaccine candidates we made change in original design, described in materials and methods and generated 3 recombinant vaccinia viruses expressing 2 ASFV proteins each: VACV-COP-p72+p15, VACV-COP-p54+pp62 and VACV-COP-p30+CD2-like. These 3 recombinant viruses were stable since the inserted genes could be detected after 10 passages in cell culture. Furthermore, in recombinant viruses VACV-COP-p72+p15, VACV-COP-p54+pp62 expression of p15 and p54 ASFV proteins was confirmed by IFA (Figure 1) and Western Blotting (Figure 2) assays using the anti-p15 monoclonal antibody and the anti-p54 polyclonal antibody respectively. We could not confirm expression of the recombinant proteins in VACV-COP-p30+CD2-like virus by IFA or Western Blotting as no antibodies were available for detection.

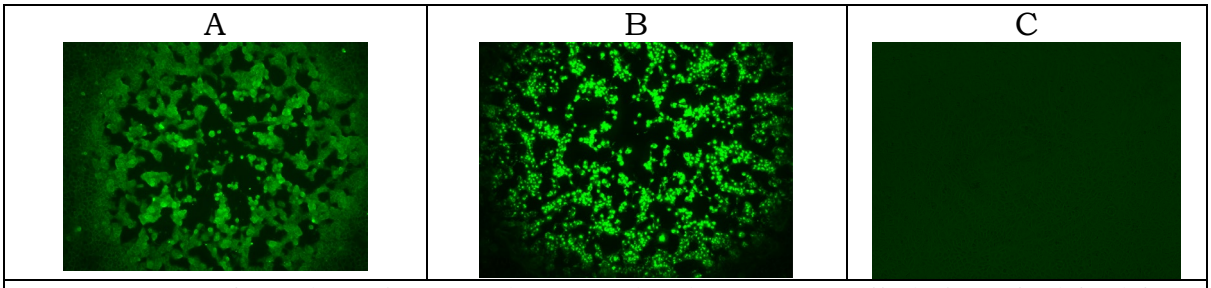

Figure 1: IFA detection of expression proteins in BS-C-1 cells infected with either the recombinant VACV-COP-p72+p15 (A) or VACV-COP-p54+pp62 (B) virus or Medium as the control (C) using the anti-p15 monoclonal antibody or anti-p54 polyclonal antibody respectively.

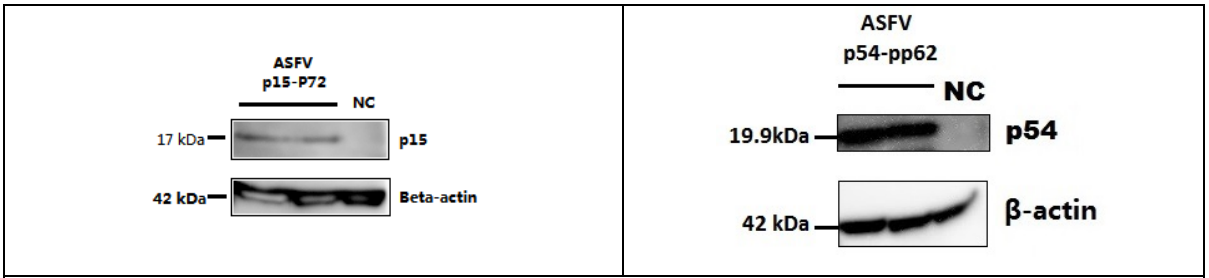

Figure 2: ASFV proteins p15 and p54 were detected in BS-C-1 cells infected with either the recombinant VACV-COP-p72+p15 or the recombinant VACV-COP-p54+pp62 virus by Western Blotting

Objective 2: The immunity induced by a single ASFV antigen has been shown not protect pigs that are exposed to a virulent virus, indicating that synergistic functions of antigens are needed to provide effective protection in pigs. Therefore, we immunized pigs with a mixture of vaccine candidates instead of using a single recombinant vaccinia virus.

We have performed a pig study including 2 groups of pigs to evaluate immunogenicity of developed vaccinate candidates. A group of 10 pigs were immunized with a mixture of VACV-COP-p72+p15, VACV-COP-p54+pp62 and VACV-COP-p30+CD2-like (10*6 PFU of each virus), and each pig was boosted the same amount of mixture after 2 weeks post immunization. A group of 5 pigs were mock-immunized and boosted with PBS. After 14 days post 2nd immunization, serum samples were collected for further analysis.

Serum samples were tested by the specific ELISA assays targeting p15, p30 and p54 as we have a positive serum samples against these proteins as positive controls and this assay can be performed in BSL-2 laboratory. The results showed that 2 out of 10 vaccinated pigs had an ELISA titer against p54 (1:200 and 1:800), and one additional pig had the ELISA titer against both p15 and p54 (1:50), but not p30. No ELISA titers against each protein were detected in all 5 control pigs.

Discussion:

In this project, we have developed 3 recombinant vaccinia viruses that stably express ASFV antigens. The results of the pig study show that the vaccinia based vaccines are immunogenic in pigs although not all pigs had an ELISA titer. It should be noticed that during the study the pigs were infected by the porcine respiratory and reproductive syndrome virus (PRRSV). This could impact the immunogenicity of vaccine candidates because the PRRSV infection normally results in immunosuppression in pigs. This is probably the reason why the vaccine candidates did not induce immune responses in all immunized pigs. In addition, vaccination regime used in this study such as both the administration route and interval period between prime and boost probably is also not ideal, which could affect immunogenicity. The optimized vaccination regime for these vaccine candidates in pigs needs to be determined in future studies. Nevertheless, the vaccinia based ASF vaccine candidates have been developed and their efficacy need to be evaluated in pigs that are challenged with a virulent ASFV in a BSL-3 level facility if funds are available. If their efficacy is demonstrated in pigs, they could be used as potential vaccines to protect swine industry.