RESEARCH REPORT
SWINE HEALTH

TITLE: Development of African swine fever diagnostic assays for oral fluids – NPB #13-048

Investigator: Jeffrey Zimmerman (jjzimm@iastate.edu)

Co-investigators: Dr. Luis G Gimenez-Lirola, Dr. DL Hank Harris

Institution: Iowa State University

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INDUSTRY SUMMARY

African swine fever virus (ASFV) is of concern to pork producers because of its high mortality rate, its severe economic impact, and its rapid geographic expansion in recent years. Prevention and control of ASFV is complicated by the absence of effective vaccines. Therefore, control is based on identification of infected animals and herds.

Detection of antibodies is useful for ASFV diagnosis and surveillance because antibodies are a definitive and reliable indication of infection (Sanchez-Vizcaíno et al., 2012). An earlier study showed that ASFV antibodies could be detected in oral fluids and suggested that oral fluids could serve as a suitable specimen for ASFV surveillance (Mur et al., 2013). The objective of NPB #13-048 was to develop a commercially-viable dual-matrix (serum or oral fluid) ASFV p30 antibody ELISA. The following steps were carried out:

1. The best recombinant antigen to use in the ELISA (p30) was selected by evaluating the serum antibody response of ASFV-infected pigs against 3 His-tagged fusion recombinant polypeptides (rp30, rp54, rp72) using a multiplex fluorescent microbead-based immunoassay (FMIA; Luminex® Corporation).
2. Serum and oral fluid antibody-positive samples were generated by experimental inoculation of 9 pigs with an attenuated ASFV isolate (NHV) that produces chronic infection. Paired oral fluid and serum samples were sequentially collected from individual pigs (DPI 0, 6, 12, 15, 19, 26, 33, 40, 47, 54, and 61). The specificity of the ELISA was evaluated using 200 serum and oral fluid samples submitted to the Iowa State University Veterinary Diagnostic Laboratory from swine herds in the U.S., i.e., a known ASFV-negative population.
3. The results of testing showed that IgG antibodies were detected by DPI 12 in both serum and oral fluid specimens. Evaluation of ASFV negative field samples showed ELISA specificities of 99.5% and 100% for serum and oral fluid samples, respectively.

4. Thus, the test detected ASFV antibodies in either oral fluid or serum samples early in the infection, yet was highly specific (few false positives) for both specimen types. Given the increased surveillance efficiency provided by oral fluid sampling and the ability to confirm results using serum samples, the ASFV rp30 antibody ELISA will be highly useful under conditions that require ASFV surveillance.

KEYWORDS - Foreign Animal Disease, African swine fever, surveillance, ELISA, oral fluids, serum.

SCIENTIFIC ABSTRACT

Several ASFV structural and non-structural proteins have being identified as candidate antigens for serological tests (Gallardo et al., 2009; Cubillos et al., 2013). Among these, structural proteins p30, p54, and p72 have been identified as the principal serological immunodeterminants of ASFV (Kollnberger et al., 2002, Cubillos et al., 2013) and are the antigens most widely used in commercial ASFV serum antibody ELISAs.

We evaluated the serum antibody response against the three major ASFV proteins (p30, p72 and p54) using a multiplex fluorescent microbead-based immunoassay (FMIA). As shown in Figure 1, p30 provided the earliest response and best discrimination between known negative and known positive samples. Therefore, subsequent ELISA development was based on ASFV p30.

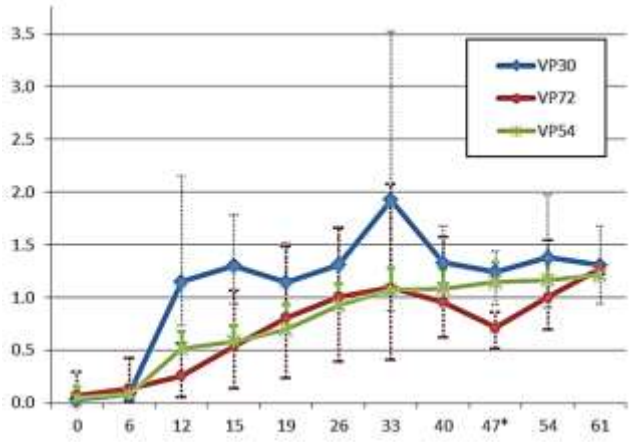

Fig 1. ASFV p30, p54, & p72 serum antibody responses (S/P ratio) over time as detected using a multiplex fluorescent microbead-based immunoassay (FMIA)

To create recombinant p30 for coating ELISA plates, a plasmid containing the p30 gene was over-expressed in *E. coli* and the recombinant His-tagged fusion p30 protein was purified from extracts of *E. coli* using nickel-affinity chromatography. ELISA conditions, e.g., rp30 coating/blocking conditions, sample/conjugate dilutions, buffers, and incubation times were optimized for antibody detection in serum and oral fluid specimens.

To evaluate the performance of the ASFV p30 ELISA, serum and oral fluid samples were collected from 9 pigs following inoculation with an attenuated ASFV isolate (NHV) that produces chronic infection. Oral fluid and serum samples were collected over days post inoculation (DPI 0, 6, 12, 15, 19, 26, 33, 40, 47, 54, and 61). ELISA specificity was evaluated using samples (200 oral fluid and 200 serum samples submitted to the Iowa

State University Veterinary Diagnostic Laboratory for routine diagnostic testing from swine herds in the U.S., i.e., a known ASFV-negative pig population.

The mean ASFV rp30 antibody ELISA ODs for serum and oral fluid are shown in Figure 2. IgG antibody was detected by DPI 12 in both serum and oral fluid specimens. The evaluation of known ASFV negative field samples showed specificities of 99.5% and 100% for serum and oral fluid samples, respectively.

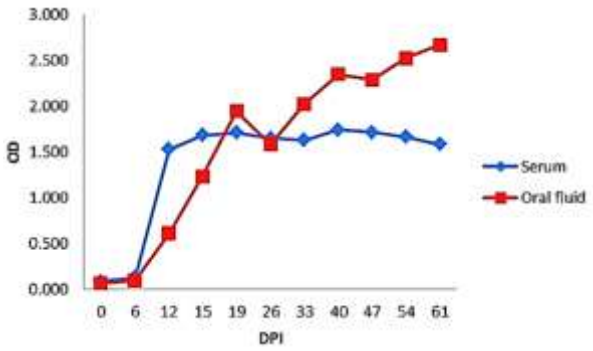

Fig 2. Mean optical density for serum (♦) and oral fluid (■) specimens collected over time from pigs inoculated with ASFV isolate NHV

INTRODUCTION

African swine fever virus (ASFV) is of major concern because of its high mortality rate, its severe economic impact, and its recent rapid geographic expansion. Prevention and control of ASFV is complicated by the absence of effective vaccines and because of a complex transmission cycle involving domestic pigs and feral/wild boars.

In the absence of an efficacious vaccine, antibodies are a definitive indication of infection and are detectable for a prolonged period of time (Sanchéz-Vizcaino and Arias, 2012). Antibody detection has been widely used along with viral antigen detection in ASFV surveillance and control programs in the Iberian Peninsula and Sardinia as a tool for the detection of ASFV carrier animals. It follows that a major limitation of the current surveillance system is the logistical complexity and cost of collecting and testing statistically appropriate numbers of samples. Previous studies have validated the use of oral fluids for the detection of swine pathogens including PRRSV (Kittawornrat et al., 2012; Prickett et al., 2008), PCV2 (Prickett et al., 2008), influenza A virus (Detmer et al., 2010; Panyasing et al., 2012; Ramirez et al., 2012), and others (Prickett and Zimmerman, 2010). Initial research showed that ASFV antibodies could be detected in oral fluids (Mur et al., 2013) and suggested that oral fluids could serve as a suitable specimen for ASFV diagnostic and surveillance. Compared to serum, collecting oral fluid sampling requires little labor, is stress-free ("welfare-friendly") for both animals and humans, and is more sensitive than individual samples for detecting infections in populations (Olsen et al., 2012).

OBJECTIVES

The goal of the present study was to develop a commercial ASFV antibody ELISA capable of detecting antibody in serum and oral fluid using non-infectious specimens as internal controls and recombinant proteins as target antigen.

MATERIALS AND METHODS

Recombinant antigens. Three ASFV antigens (p30, p54, and p72), were evaluated for their use in the ELISA. ASFV (isolate BA71V) genes p30, p54 and p72 were commercially produced (GenScript, Pistacaway, NJ, USA) and cloned into a pHUE plasmid. The constructs were analyzed by restriction enzyme digestion and DNA sequencing, and then transformed into *E. coli* BL21(DE3)pLysS (Rosetta cells) (Invitrogen™, Carlsbad, CA, USA) for expression. Recombinant his-tagged fusion proteins p30 and p54, and his-tagged p72 over-expressed in inclusion bodies, were purified from clarified extracts of Rosetta cells under denaturing conditions (urea 8M) using a nickel-nitrilotriacetic acid (Ni-NTA) chelate affinity chromatography kit (PrepEase® His-tagged protein purification kit, USB Corporation Cleveland, OH, USA) performed according

to manufacturer's instructions. The purified antigens were analyzed and their apparent molecular mass was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Multiplex fluorescent microbead-based immunoassay (FMIA). The serum antibody response against p30, p54, and p72 was evaluated using a multiplex fluorescent microbead-based immunoassay (FMIA; Luminex Corp., Austin, TX, USA) and serum samples from animals of known ASFV infection status using procedures previously described (Giménez-Lirola et al., 2013).

rp30 antibody ELISA. ELISA conditions, e.g., rp30 coating and blocking conditions, sample and conjugate dilutions, buffers, and incubation times were optimized for antibody detection in serum and oral fluid specimens simultaneously. The reagents used in the assay, i.e., sample and conjugate diluents, substrate, stop solution, and wash solution, were provided by a commercial entity (IDEXX Laboratories, Inc., Westbrook, ME USA).

***Preparation of ELISA plates*.** 96-well microtitration plates (Nunc, Thermo Fisher Scientific, Agawam, MA, USA) were manually coated with 100 µl of the rp30 polypeptide per well at a concentration of 5 µg per ml in phosphate-buffered saline (PBS) pH 7.4 (Gibco®, Life Technologies) and were then incubated at 4°C overnight. The concentration of the antigen was optimized for simultaneous antibody detection in serum and oral fluid specimens and to provide the best discrimination between positive and negative samples. After incubation, plates were washed 5 times and blocked with 300 µl per well of a blocking solution containing 1% bovine serum albumin (Jackson ImmunoResearch Inc.) and incubated at 25°C for 2 h. Plates were then dried at 37°C for 4 h and stored at 4°C in a sealed bag with desiccant packs. Plate lots with a coefficient of variation $\geq$10% were rejected.

Source of ELISA negative and positive controls. To safely establish a bank of serum and oral fluid samples containing antibodies specific for ASFV p30, p54, and p72, pigs were vaccinated with RP vaccines expressing ASFV antigens p30, p54, and/or p72 (Harrisvaccines Inc., Ames, IA, USA). ELISA negative and positive controls consisted of pooled sera from p30 replicon particle-vaccinated and non-vaccinated control animals, respectively. Replicon particles (RP) are single-cycle RNA vectors capable of expressing foreign antigens *in vivo*.

ELISA procedure. Assessment of the detection of ASFV antibodies in oral fluid and serum specimens was performed using samples collected over time from ASFV-inoculated pigs and field samples submitted to the Iowa State University Veterinary Diagnostic Laboratory. Serum samples were diluted 1:100 and oral fluid samples were diluted 1:2, after which plates were loaded with 100 µl of diluted sample per well. Antibody-positive and negative controls were run in duplicate on each ELISA plate. Plates were incubated at 25°C for 1 h and then washed 5 times. Thereafter, 100 µl of peroxidase conjugated goat anti-swine IgG (H+L) antibody (Jackson ImmunoResearch, Inc.) diluted 1:60,000 for serum or 1:25,000 for oral fluid was added to each well and then plates were incubated at 25°C for 30 min. After a washing step, the peroxidase reaction was visualized by adding 100 µl tetramethylbenzidine-hydrogen peroxide (TMB) substrate solution per well. Plates were incubated at room temperature for 10 min, 50 µl of stop solution were added to stop the reaction, and plates were read immediately thereafter. Reactions were measured as optical density (OD) at 450 nm using an ELISA plate reader (Biotek® Instruments Inc., Winooski, VT) operated with commercial software (GEN5TM, Biotek® Instruments Inc.).

S/P ratio calculation. Serum and oral fluid antibody response were represented as sample-to-positive (S/P) ratios calculated as:

$$\text{S/P ratio} = \frac{\text{(sample OD – negative control mean OD)}}{\text{(positive control mean OD – negative control mean OD)}}$$

Statistical analysis. The performance of rp30 antibody ELISA was evaluated by receiver operating characteristic (ROC) curve analysis using statistical software (MedCalc Statistical Software verions 13.0, Ostend, Belgium).

RESULTS

As shown in Figure 2 (above) an assessment of the ASFV rp30 antibody ELISA showed that the test is both diagnostically sensitive and specific for the detection of ASFV antibodies in either oral fluid or serum samples. Unique to this ELISA, serum and oral fluid samples from the same pig(s) can be run on the same plate using the same reagents. This will provide for greater testing flexibility and efficiency.

DISCUSSION

The current epidemiological situation of ASFV in the Caucasus and the Russia Federation, including the incursion of the disease in Belarus, Ukraine, Lithuania, and Poland indicates that a further geographic spread of ASF is likely to occur. Given this scenario, technologies related to ASFV diagnosis and surveillance are critical components in preparedness.

ASFV surveillance is vital, but a robust, cost-effective approach must be developed based on (1) efficient, low-cost specimen collection and (2) accurate diagnostic testing. Recovered ASFV carrier pigs and persistently infected feral/wild pigs constitute the biggest problems in controlling the disease because of their potential for spreading the disease. However, these pigs also develop a specific and long-term humoral (antibody) response against ASFV. Thus, in the absence of effective vaccine, antibodies are a definitive indication of infection (Arias and Sánchez-Vizcaíno, 2012).

For antibody detection, it has become widely recognized that oral fluid specimens are a viable option to serum, are easily collected, and provide diagnostic information equivalent or superior to serum samples (Kittawornrat et al., 2012; Olsen et al., 2012). Mur et al. (2012) reported the detection of anti-ASFV antibodies in oral fluids from pigs infected with ASFV under experimental conditions. The aim of this study was to pursue this line of research and develop an ASFV dual matrix serum/oral fluid antibody ELISA and in this endeavor we were successful: the results showed that the ASFV rp30 indirect ELISA detects ASFV antibodies in either oral fluid or serum samples. Given the increased surveillance efficiency provided by oral fluid sampling (Olsen et al., 2013) and the ability to corroborate results using serum samples, the ASFV rp30 antibody will be highly useful for ASFV surveillance.

PUBLICATIONS: PROCEEDINGS AND ABSTRACTS

Giménez-Lirola LG, Mur L, Rivera B, Wang C, Goodell C, Rowland RB, Harris DL, Gallardo C, Arias M, Sánchez-Vizcaíno J, Zimmerman JJ. Development of dual-matrix serum/oral fluid ASFV p30 antibody ELISA test. 7th International Symposium on Emerging and Re-emerging Pig Diseases, Kyoto Japan (*submitted for poster presentation*)

Giménez-Lirola LG, Mur L, Rivera B, Wang C, Goodell C, Rowland RB, Harris DL, Gallardo C, Arias M, Sánchez-Vizcaíno J, Zimmerman JJ. December 2014. African swine fever virus (ASFV) rp30 ELISA detects antibody in serum and/or oral fluid specimens. Proc 95th Ann Meet Conference of Research Workers in Animal Diseases. Chicago, Illinois. Abstr #172.

Giménez-Lirola LG, Mur L, Rivera B, Lizano S, Goodell C, Rowland RR, Mogler M, Harris DL, Gallardo C, Arias M, Sanchez-Vizcaino JM, Zimmerman J. October 2014. Simultaneous detection of African swine fever antibodies in serum and oral fluid using a recombinant p30 antibody ELISA. Proceedings 57th Annual Conference, American Association of Veterinary Laboratory Diagnosticians. Kansas City, Missouri, p. 135.

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serum and/or oral fluid specimens. Proceedings, 23nd International Pig Veterinary Society Congress. Cancún, México, Abstract O.155.

ORAL PRESENTATIONS

Giménez-Lirola LG, Mur L, Rivera B, Wang C, Goodell C, Rowland RB, Harris DL, Gallardo C, Arias M, Sánchez-Vizcaíno J, Zimmerman JJ. December 9, 2014. African swine fever virus (ASFV) rp30 ELISA detects antibody in serum and/or oral fluid specimens. 95th Ann Meet Conference of Research Workers in Animal Diseases. Chicago, Illinois.

Giménez-Lirola LG, Mur L, Rivera B, Lizano S, Goodell C, Rowland RR, Mogler M, Harris DL, Gallardo C, Arias M, Sanchez-Vizcaino JM, Zimmerman J. October 19, 2014. Simultaneous detection of African swine fever antibodies in serum and oral fluid using a recombinant p30 antibody ELISA. American Association of Veterinary Laboratory Diagnosticians. Kansas City, Missouri.

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