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Potential use of oral fluid samples for serological diagnosis of African swine fever

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ABSTRACT

African swine fever (ASF) is a complex, highly lethal, notifiable disease of swine. ASF is wide-spread in sub-Saharan Africa and East European countries and there is presently a great risk of spread to neighboring countries. Since there is no vaccine for ASF virus (ASFV), control is based on rapid and early detection of the disease via surveillance. This approach requires collecting blood samples from large number of animals. Laborious and expensive of itself, this process also presents an additional risk because ASFV is present at high concentrations in the blood. The objective of this study was to initiate studies into the potential use of oral fluid as an alternative to serum for ASF diagnosis, for latter studying its possible use in surveillance and control programs. To this end, oral fluid samples collected at different times post infection from eight pigs experimentally inoculated with an attenuated ASFV were assayed using modified protocols of the two validated serological techniques, the enzyme-immune-liked assay (ELISA) and immunoperoxidase technique (IPT). Antibodies against ASFV were detected in oral fluid samples of all animals from early post infection through the end of the experiment by ELISA and IPT. These results confirmed the presence of ASFV antibodies in swine oral fluids samples, the possibility of an oral fluid-based approach in ASF diagnosis and, potentially in ASF surveillance.

1. Introduction

African swine fever (ASF) is one of the most complex infectious diseases of swine species. It is caused by a large doubled-stranded DNA virus, ASF virus (ASFV) belonging to the *Asfaviridae* family (Dixon et al., 2005). The epidemiology of ASF is complex, involving domestic and wild suids of different ages and breeds, as well as ticks from *Ornithodoros* genus. In affected countries, e.g., most of the sub-Saharan African countries, ASFV imposes a severe economic and social burden due to high mortality rates and international trade restrictions. In addition to Africa, ASFV is endemic in the island of Sardinia (Italy) and, since 2007, in a number of Eastern European countries, including the Russian Federation and the Trans Caucasian countries. In the recently infected areas of East Europe, ASFV's rapid expansion into north-western Russia demonstrated its capacity for rapid and far-flung spread, with numerous outbreaks occurring in such geographically distant regions as St Petersburg and the Baltic Sea, i.e., very close to European Union (EU) countries. The current situation in this region, in combination with its increased presence in African continent, highlights ASFV's potential for devastation and the risk it poses to the global pig industry (Sanchez-Vizcaino et al., 2012).

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There are no effective vaccines against ASFV. Therefore control is based on the implementation of strict prevention measures and the early detection of the disease, primarily through rapid laboratory diagnosis. At present, good antibody assays are available for the diagnosis of ASFV infection. These assays are suitable for use both in well-equipped international and national reference laboratories, as well as in basic regional and local laboratories. More recently, rapid assays have been developed for on-site ("point-of-care") use. Antibodies appear early in ASFV infection and persist for a long time, thereby serving as good markers of infection and providing for the detection of carrier animals. Antibody assays are economical, compatible with automation, and suitable for high-throughput screening. These factors make antibody testing the best option for ASFV surveillance (large scale screening) and eradication programmes (Arias and Sanchez-Vizcaino, 2002; 2012).

The limitation of this approach is the expense of collecting and testing blood samples. In particular, the need to handle and bleed animals presents a significant risk for further spread of ASFV as a consequence of the high levels of ASFV present in blood (McVicar, 1984). Oral fluid is an alternative diagnostic specimen that could potentially address these problems. Oral fluid has been proven to be a good diagnostic specimen for the detection of a number of pathogens of swine, including porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2), by both nucleic acid, and antibody detection (Prickett and Zimmerman, 2010; Prickett et al., 2011; Ramirez et al., 2012).

Given the current risk for the spread of ASFV, more cost-effective and rapid methods for ASFV surveillance are needed. Therefore, the objective of this study was to determine if antibodies against ASFV could be detected in oral fluid samples from experimentally infected animals as the first step in evaluating its potential use in ASF surveillance and eradication programmes.

2. Material and methods

2.1. Cells and viruses

A Spanish strain of ASFV isolated in 1970 (E70) and adapted to grow in a monkey stable (MS) kidney cell line (ECACC, 91070510) was used for OIE-antigen production following the method described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012). For the experimental in vivo studies, the attenuated and non haemadsorbing Portugal ASFV isolate NH/P68 (NHV) belonging to p72 genotype I and the virulent Armenia ASFV Arm07 isolate (genotype II) were used. Finally, isolate BA71V, recovered in Spain in 1971 and adapted to Vero cells (ATCC CCL 81) was employed for the production of the fixed indirect immunoperoxidase plates. The ASFV isolates were propagated and titrated according with Carrascosa et al. (2011). All ASFV isolates were obtained from the collections of the European Union Reference laboratory for ASF (CISA-INIA).

2.2. Experimental design and sampling collection

The ability to detect antibodies against ASF in oral fluid was evaluated using samples collected over time in two independent studies involving experimentally-infected pigs: (i) four Landrace × Large White pigs intramuscularly inoculated with 10^5 TCID50/ml of the ASFV isolate NH/P68 (NHV) and challenged at day 30 post infection (dpi) with 10 HAD50/ml of the heterologous virulent Armenia ASFV Arm07 isolate; and (ii) four Landrace × Large White pigs intramuscularly inoculated with 10^3 TCID50/ml of the attenuated and non haemadsorbing Portugal ASFV isolate NH/P68 (NHV). Paired oral fluid and serum samples were obtained at 0, 11, 14, 18, 21, 30, 37, 44, 52, 58 and 65 dpi(s). Both animal experiments were conducted at the BSL3 animal facilities at CISA-INIA and performed in accordance with the EC Directive 86/609/EEC, following the recommendation 2007/526/EC for the accommodation and care of animals used for experimental and other scientific purposes.

Oral fluid samples were collected from individual pigs using cotton rope 12 mm diameter and 25 cm length. Pigs were allowed to chew the rope for 10 min, i.e., until the rope was sufficiently wet. The wet end of the rope was cut, placed in a syringe (50 ml), and compressed to recover the oral fluid. A volume of ~5 ml was obtained from each animal on each collection day. Serum samples were collected using conventional methods.

2.3. ASF antibody detection in serum and oral fluid samples

ASFV antibody in serum samples was measured using the OIE indirect ELISA (OIE, 2012) and the Indirect Immunoperoxidase test (IPT). The IPT was performed using fixed VERO cells infected with isolate Ba71V ASFV following the same procedure described in COS1-cells by Gallardo et al., 2012.

The ASF ELISA and IPT serological tests were adapted to anti-ASFV antibody detection in oral fluid samples by adjusting incubation time, incubation temperatures, blocking buffers, concentrations of the antigen and the oral fluid samples, as well as the type and concentration of the conjugate (secondary anti-pig and/or protein A horseradish peroxidase [HRP] conjugated).

3. Results

3.1. Modification of OIE ASFV indirect ELISA and IPT protocols for oral fluid specimens

Optimization of the OIE ELISA and IPT tests for the detection of ASFV antibody in oral fluid specimens was carried out by comparing the responses of varying concentrations of antigen and conjugates using a checkerboard titration procedure. The optimum response for the indirect ELISA was achieved when microtitration plates were coated with 1 μg of ASFV cytoplasmic antigen per well, which correlated to a 1:800 working dilution. In addition, the OIE ASFV ELISA protocol was modified to enhance the detection of antibody in oral fluid, e.g., oral fluid samples were assayed without

dilution, the incubation time was increased to 16 h (overnight) at 4 °C, and protein A-HRPO conjugate was used at a 1:1000 dilution. Under these conditions, the optical density of the positive oral fluid samples collected late in the infection was approximately 10 times greater than the negative oral fluid samples collected prior to inoculation.

The conditions of the IPT test that optimized the positive/negative (P/N) ratio and produced the lowest background used oral fluid diluted 1:2 and protein A-HRPO diluted 1:1000. The IPT test was employed as confirmatory technique.

3.2. Comparison of oral fluid samples versus sera for anti-ASFV antibody detection

A comparison of the results of serum samples tested using the OIE-indirect ELISA, with the IPT used as a confirmatory test, versus oral fluid samples tested using the modified protocols showed that the levels of antibodies in serum and oral fluid samples followed a similar pattern, albeit oral fluid contained lower concentrations of antibodies. In the eight animals (C1 to C8) inoculated with the attenuated Portuguese isolate NHV/P68 (with or without further challenge), ASFV antibody was first detected in serum samples at 8 to 11 dpi(s) and, thereafter, through the end of the experiment. In oral fluid samples, ASFV antibody was first detected at 11 to 30 dpi(s) (Fig. 1A) and was detectable through the end of the study. Statistically significant differences were found in the level of antibodies of oral fluid samples by IPT analysis among the inoculated animals. The differences detected among the eight individuals were highly correlated with the serum antibody titers. In general, serum antibody titers $\leq$1:2500 were negative for ASFV antibodies in oral fluid.

4. Discussion

Cumulatively, the absences of vaccines, the early appearance and long-term persistence of ASFV antibodies, and the lower cost of antibody assays relative to PCR-based methods, justifies the crucial role of antibody detection to the control of the disease (Arias and Sánchez-Vizcaíno, 2012). In the present situation, there is a pressing need for more intensive and cost-effective ASFV surveillance, if further spread is to be avoided and control ultimately achieved. However, such efforts must be based on diagnostically effective, yet less costly, sampling procedures.

Previous studies have described the use of oral fluid samples as an economical alternative to serum for the surveillance of both human and swine diseases using either antibody and virus detection (Prickett and Zimmerman, 2010). Based on these reports, the objective of this study was to evaluate the feasibility of using oral fluid samples to determine the onset, level, and duration of antibodies against ASF from experimentally inoculated pigs. To this end, oral fluids from eight pigs inoculated with an attenuated ASFV isolate were tested using the optimized OIE-indirect ELISA and IPT tests. The results were compared to those obtained in standard serum samples analyzed by the validated OIE-indirect ELISA and IPT test.

The results revealed that antibodies against ASFV were detected in oral fluid samples from 11 to 30 dpi(s), depending on the animal, and maintained through the end of the experiment. The antibody response in oral fluid correlated with antibody titers in serum although a small delayed (median of 6 days) was shown in oral fluid samples of most of the infected animals. This fact could be explained by the lower quantity of antibodies present in

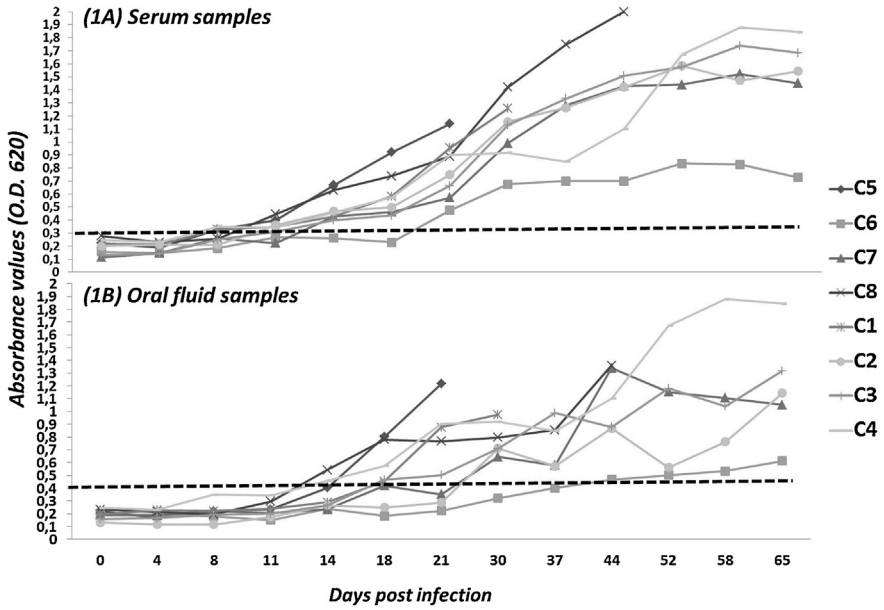

Fig. 1. Detection of ASFV-specific antibodies in serum (A) and oral fluid (B) samples using the OIE-indirect ELISA from eight pigs experimentally infected with the attenuated virulent ASFV Portuguese isolate (NHV). Cut off optical density value is represented by dots.

oral fluid compared with serum samples (Cameron and Carman, 2005). This lower concentration is responsible of the required adaptation of the diagnostic techniques, and nevertheless, results in a lower sensitivity of the test. The key issue for the future application of this sampling method is if it possible to assume this decrease of sensitivity, mainly referred to a delayed detection at early stages of infection. Future research should try to solve this problem, assaying other conditions for the assays, in order to achieve a similar sensitivity than with serum samples.

Future works should consider this option, as well as the use of a higher number of animals in the study. Authors are aware of the small size of this experimental study (8 pigs) in terms of statistical inferences and significances. However, our intention in this first approach was to elucidate if it was possible to detect ASF antibodies in oral fluid from infected animals or not; and for that purpose, a small group of animals was considered to be enough. This fact also explains the use of a non haemoabsorbing attenuated ASFV isolate (NHV) in the experimental infection. This ASFV isolate induces chronic clinical forms in infected animals which presents low viraemia in a late phase of infection (after 14 days post infection) combined with a high antibody responses developed at early stages (Leitão et al., 2001). This last point gave us the opportunity to study the antibody response through the time, trying to emulate a situation occurring in endemic areas with the presence of carriers circulating pigs, or in such places where moderate virulence ASF viruses are present, where the detection of antibodies are the recommended tests for the control of the disease. In these situations, oral fluid sampling should be considered as a potential non-invasive sampling method to be used as alternative in the control and eradication programmes in endemic areas.

However, this scenario differs from other situations occurring with virulent ASFV isolates, as those currently circulating in some parts of Africa and Russian Federation. Not because of the performance of the diagnostic techniques with different isolates (Gallardo et al., 2012); but for the acute form of the disease. In those cases, the problematic is the same with serum than oral fluid samples; the death of the animals could occur at very early stage of the disease, before the appearance of antibodies. However, even in those cases, it is important to no forget that the mortality of the virus is not always 100%, so oral fluid could be a good alternative for analysing the farms surrounding the outbreak, in surveillance programmes or checking the animals after the quarantine for antibody detection.

Overall, these data demonstrated that oral fluid could be used as an alternative diagnostic specimen for ASFV antibody detection using the modified ELISA and IPT tests. This study represents the first step of the research needed for the last goal of applying this test in ASF surveillance programmes. The use of oral fluid samples has many potential advantages in surveillance programmes, especially at this juncture in history when the risk for ASF introduction into the EU is high and targeted, enhanced surveillance is severely needed. In this study, oral fluid samples were collected from individual pigs in order to compare in parallel the results obtained in serum and oral fluid samples. However, as has been done elsewhere, future research should focus on pen-based samples, i.e., oral fluids collected from animals within the same pen, in order to study the detection of antibodies against ASFV in groups. Considering that the EU is free from ASFV (with the exception of Sardinia), and even more, that ASF is a mandatory notifiable disease, the detection of a single positive result implies immediate control actions. The use of pen-based oral fluid samples would obviate the need to differentiate individual animal status, thereby expediting the efficient detection of ASFV while saving money and time. A critical question related to this approach is the limit of detection, e.g. what percentage of pigs in a pen need to be ASFV-infected for the pen-based sample to test positive.

In addition, further studies will be required to fully validate this approach for ASF diagnosis in the field and its latter implementation in surveillance and control programmes. Further studies should increase the number of infected pigs, including a representative sample size and use other virulent isolate (e.g. the ASFV circulating in Russia) that develop an acute/subacute form of the disease. These future experiments would certainly clarify the potential application of this technique to the current situation in Europe, potentially including the detection of ASFV genome by PCR techniques. Based on the experience obtained during this experiment authors suggest to perform a daily collection of the samples until the first detection of antibodies, for the detailed analysis of the results and detection limits. It would be also interesting to include individual and group sampling in order to stablish the detection limits of the procedure. Finally, we also recommend to standardize and adapt the collection process to field conditions, fully accommodating it to farm structure, pig behavior, and the needs of specific disease surveillance programmes. It is important to note that the use of oral fluid samples always requires re-optimization of the assay because antibodies are present in oral fluid samples in lower concentrations than serum. Most serum antibody assays can be adapted to the detection of antibody in oral fluid by manipulating sample volume, incubation time and/or temperature, reagents, and cutoff threshold (Cameron and Carman, 2005), as it was done in this work for two serological techniques for ASF detection or in previous works and as was done previously for a commercial PRRSV antibody ELISA (Kittawornrat et al., 2012).

5. Conclusions

The results of this pilot study demonstrated the presence of antibodies against ASFV in oral fluid samples collected from infected animals at early stages of the infection. These data strongly suggest that oral fluid is a viable sample for the detection of antibodies against ASFV, potentially providing in the future a safe, efficient, cost-effective, and welfare-friendly alternative to serum for ASFV surveillance, control, and eradication programs. Such an approach would allow for fulfilling our responsibilities to animal health and commitment to animal welfare simultaneously.

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References

Arias, M., Sanchez-Vizcaino, J.M., 2002. African swine fever eradication: the Spanish model. In: Morilla, A., Jin, K., Zimmerman, J. (Eds.), Trends in Emerging Viral Infections of Swine. Iowa State University Press, Iowa, United States of America, pp. 133–139.

Arias, M., Sánchez-Vizcaíno, J.M., 2012. African swine fever. In: Zimmerman, J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine. 10th ed. John Wiley and Sons, United States of America, pp. 396–404.

Cameron, S.O., Carman, W.F., 2005. The use of the OraSure collection device for hepatitis virus testing in health care settings. J. Clin. Virol. 34, 22–28.

Carrascosa, A.L., Bustos, M.J., de Leon, P., 2011. Methods for growing and titrating African swine fever virus: field and laboratory samples. Curr. Protoc. Cell Biol. 53, 26.14.1–26.14.25.

Dixon, L.K., Escribano, J.M., Martins, C., Rock, D.L., Salas, M.L., Wilkinson, P.J., 2005. Asfarviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London, pp. 135–143.

Gallardo, C., Soler, A., Nieto, R., Carrascosa, A.L., De Mia, G.M., Bishop, R.P., Martins, C., Fasina, F.O., Couacy-Hymman, E., Heath, L., Pelayo, V., Martín, E., Simón, A., Martín, R., Okurut, A.R., Lekolol, I., Okoth, E., Arias, M., 2012. Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests. Vet. Microbiol. http://dx.doi.org/10.1016/j.vetmic.2012.08.011.

Kittawornrat, A., Prickett, J., Wang, C., Panyasing, Y., Ballagi, A., Rice, A., Main, R., Johnson, J., Rademacher, C., Hoogland, M., Rowland, R., Zimmerman, J., 2012. Detection of porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody ELISA. J. Vet. Diagn. Invest. 24, 262–269.

Leitão, A., Cartaxeiro, C., Coelho, R., Cruz, B., Parkhouse, R.M., Portugal, F., Vigário, J.D., Martins, C.L., 2001. The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. J. Gen. Virol. 82, 513–523.

McVicar, J.W., 1984. Quantitative aspects of the transmission of African swine fever. Am. J. Vet. Res. 45, 1535–1541.

OIE, 2012. African swine fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012, Office International des Epizooties, Paris, France (Chapter 2.8.1).

Prickett, J.R., Zimmerman, J.J., 2010. The development of oral fluid-based diagnostics and applications in veterinary medicine. Anim. Health Res. Rev. 11, 207–216.

Prickett, J.R., Johnson, J., Murtaugh, M.P., Puvanendiran, S., Wang, C., Zimmerman, J.J., Opriessnig, T., 2011. Prolonged detection of PCV2 and anti-PCV2 antibody in oral fluids following experimental inoculation. Transbound. Emerg. Dis. 58, 121–127.

Ramirez, A., Wang, C., Prickett, J.R., Pogranichniy, R., Yoon, K.-J., Main, R., Rademacher, C., Hoogland, M., Hoffmann, P., Johnson, J.K., Kurtz, A., Kurtz, E., Zimmerman, J., 2012. Efficient surveillance of pig populations using oral fluids. Prev. Vet. Med. 104, 292–300.

Sanchez-Vizcaino, J.M., Mur, L., Martinez-Lopez, B., 2012. African swine fever: an epidemiological update. Transbound. Emerg. Dis. 59 (Suppl. 1), 17–35.