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Differential antibody responses in sows and finishing pigs naturally infected with African swine fever virus under field conditions

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ABSTRACT

Antibody profile of pigs naturally infected with a virulent African swine fever virus (ASFV) strain under field conditions was studied. Twenty-three serum samples were collected from pigs surviving a natural ASFV infection: 17 samples from finishing pigs (~7 months old) and 6 samples from sows (between 12 and 36 months old). Additionally, 24 serum samples were collected from ASFV-naïve pigs to serve as negative controls. All sera from ASFV-surviving pigs tested positive while all sera from control pigs tested negative by two different commercial ELISA kits. Antibody reactivity of each serum sample was simultaneously measured against six selected ASFV antigens including p12, p32, p54, pp62, C-type lectin and CD2v. All ASFV-surviving pigs had antibody against p32, p54 and pp62 while 91.3% surviving pigs had antibody against p12. Only small portions of ASFV-surviving pigs exhibited antibodies against C-type lectin (34.8%) and CD2v (26.1%). While antibodies against p12, p32, p54 and pp62 were similarly detected in both finishing pigs and sows, antibodies against C-type lectin and CD2v were mainly detected in sows but not in finishing pigs. These results suggest a differential humoral immune response to ASFV infection in sows and finishing pigs. Further studies are needed to better understand the nature of immune responses to ASFV infection in different pig populations.

African swine fever (ASF) is a devastating viral disease of swine with mortality rates that can approach 100%. The disease has spread to many swine producing countries in Europe and Asia, causing large economic losses and affecting global food security. The causative agent of ASF, African swine fever virus (ASFV), is a large double-stranded DNA virus of the genus *Asfivirus,* within the family *Asfarviridae*. The ASFV genome varies between 170 and 193 kbp and contains between 150 and 170 open reading frames (Dixon et al., 2013). The ASFV virion has a very complex structure and contains at least 68 virus-encoded proteins, half of which have no known functions (Alejo et al., 2018).

Most pigs experimentally infected with a virulent ASFV strain die before they mount a detectable level of antibody responses (Guinat et al., 2014). However, pigs surviving an ASFV infection develop virus-specific antibodies that are detectable as early as 7–14 days post-infection (Pan et al., 1982; Reis et al., 2007). Importantly, pigs recovered from a natural ASFV infection or vaccinated with an experimental live-attenuated ASF vaccine acquire a protective immunity that protects them from being reinfected with a homologous strain (Carlson et al., 2016; Mulumba-Mfumu et al., 2016; O'Donnell et al., 2017). Passive transfer of ASFV hyperimmune antisera to naïve pigs resulted in protection against a lethal challenge infection with a virulent ASFV strain, clearly demonstrating that antibody is an important component of the protective immunity against ASFV (Onisk et al., 1994). However, antibody-mediated effector functions associated with the protection remain poorly understood. Virus-neutralizing antibodies are detected in convalescent antisera collected from ASFV-infected pigs, but these antibodies often do not completely inhibit virus infectivity in an *in vitro* virus-neutralization assay (Escribano et al., 2013). Therefore, other antibody effector functions such as complement-mediated cell lysis, antibody-dependent cell mediated cytotoxicity and cell-to-cell transmission inhibition are important for antibody-mediated protection (Arias et al., 2017; Wardley et al., 1985).

In this present study, we analyzed antibody responses in pigs naturally infected with ASFV under field conditions against six ASFV structural proteins that are associated with the induction of putative protective antibody responses. These included p12 (O61R), p32

Abbreviations: ASFV, African swine fever virus; LIPS, luciferase-immunoprecipitation system; ELISA, enzyme-linked Immunosorbent assay; Nluc, nanoluciferase.

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(CP204L), p54 (E183L), pp62 (CP530R), C-type lectin (EP153R) and CD2v (EP402R). Viral membrane protein p12 mediates the attachment of ASFV virions to cellular receptors and blocking the p12-mediated attachment abrogates viral infection (Angulo et al., 1993). Phosphoprotein p32 (also known as p30) is expressed early after viral infection and localized predominantly in the cytoplasm of infected cells (Prados et al., 1993). The transmembrane protein p54 plays an important role in ASFV morphogenesis (Rodriguez et al., 2004). Both p32 and p54 play important roles in viral entry into susceptible cells. Particularly, p54 is involved in viral attachment while p32 is involved in virus internalization (Gomez-Puertas et al., 1998). In addition, p32 and p54 are highly immunogenic and antibodies specific to these two proteins are capable of neutralizing virus infection (Prados et al., 1993). pp62 is a polyprotein essential for viral core development (Alejo et al., 2018; Suarez et al., 2010). C-type lectin and CD2v are two viral glycoproteins that mediate hemadsorption to viral infected cells (Galindo et al., 2000; Rodriguez et al., 1993). Pigs infected with ASFV developed antibodies that inhibit hemadsorption. Hemadsorption- inhibition assay can be used to classify ASFV into serogroups and ASFV strains within a serogroup provide immune protection against one another (Burmakina et al., 2016; Malogolovkin et al., 2015). Thus, antibodies specific to C-lectin and CD2v might be important for immune protection against ASFV.

ASF outbreaks were first reported in Vietnam in early 2019 (Nga et al., 2020). The ASFV strain affecting Vietnamese swine herds belongs to genotype II and is closely similar to the ASFV Georgia 2007 (Truong et al., 2021). Under experimental conditions, all pigs experimentally infected with the Vietnamese ASFV isolate died within 8 days post infection (Lee et al., 2021). Under field conditions, we observed that a small portion of pigs infected with ASFV in Vietnam survived the infection. We were able to collect 23 serum samples from surviving pigs from three farms that were confirmed by to be affected by ASFV. These included 17 samples collected from finishing pigs approximately 5 months after their litter mates experienced clinical signs of ASF and 6 samples from sows between 10 and 20 months after they were confirmed to be infected with ASFV (Table 1). Additionally, 24 serum sample were collected from farms that had never experienced ASF in Vietnam. Serological status of these antisera was determined by using two different commercially available ELISA kits: ASFV Ingezim PPA COMPAC ELISA (Ingenasa, Madrid, Spain), which is a blocking ELISA detecting antibodies against viral protein p72 and ID Screen® African Swine Fever Indirect (IDVet, Grabels, France), which is an indirect ELISA detecting antibodies against three viral proteins p32, pp62 and p72. All 23 serum samples collected from ASFV-surviving pigs tested positive while all 24 samples collected from uninfected farms tested negative by both ELISA kits (Fig. 1).

Genes encoding the six selected proteins derived from the genotype II ASFV strain Georgia 2007 (GenBank accession number FR682468) were synthesized by GenScript (Piscataway, NJ, USA) and cloned in-frame to the 5′ terminus of the *nanoluciferase* gene (Nluc) in the pCI-Nluc plasmid (Luong et al., 2020). The resulting plasmids were transfected to HEK-293T cells to generate Nluc-tagged ASFV antigens which were used to measure antibody reactivity following the luciferase-immunoprecipitation system (LIPS) assay.

Table 1.
Serum samples collected from pigs surviving a natural ASFV-infection.

Farms	Pig type	Number of samples	Times from disease appearance to sample collection (months)
Farm 1	Finishers	17	5
Farm 1	Sows	2	20
Farm 2	Sows	2	16
Farm 3	Sows	2	10

LIPS was done as previously described (Burbelo et al., 2010; Luong et al., 2020). Briefly, test serum samples were diluted 1:40 in buffer A (50 mm Tris, pH 7.5, 100 mm NaCl, 5 mm MgCl2, 1% Triton X-100), and passed through a 0.45 μm filter to eliminate large aggregates. Fifty μL of each diluted sera were transferred to a well of a 96-well plate and mixed with 50 μL of the Nluc-tagged antigen extract containing approximately 10^7 relative light units (RLU). The plate was incubated for 1 h at RT on a rocking platform. Ten μL of protein A Sepharose 4B (Invitrogen, Camarillo, CA, USA) pre-washed and diluted in 50 uL of buffer A was added to each well of the plate. After another 1 h incubation, the entire content from the 96-well plate was transferred to a 96-well filter HTS plates (EMD Millipore, Billerica, MA, USA) for washing on a vacuum manifold. Each well was washed eight times with 200 μL of buffer A, followed by two times with 200 μL of PBS. After the final wash, 50 μL of distilled water followed by 50 μL of Nano-Glo® Luciferase substrate (Promega, Madison, WI, USA) was added to each well. Luminescence signal was measured by using a SpectraMax L reader (Molecular Devices, San Jose, CA, USA). Each serum sample was tested in triplicate. Fetal bovine serum (FBS) was used as negative control. Data were expressed as the ratios between the RLU of test serum sample to the RLU of FBS (S/N ratio).

The results show that sera collected from ASFV-naïve pigs exhibited low S/N ratios against the six tested antigens, ranging from 0.6 to 5.9 (Fig. 2, left panel). On the other hand, sera collected from surviving pigs exhibited a wide range of reactivity against the six ASFV antigens tested (Fig. 2, right panel). Particularly, sera from surviving pigs exhibited the highest antibody levels against p54 (S/N ratio of 140.13 ± 28.85) and the lowest levels against C-type lectin and CD2v (S/N ratios of 4.7 ± 0.97 and 4.78 ± 1.53, respectively). These antisera displayed intermediate antibody levels against p12 (37.52 ± 6.36), p32 (17.15 ± 3.41) and pp62 (21.05± 1.78).

To determine the frequency of ASFV-surviving pigs that exhibited antibodies against these six antigens, an arbitrary cutoff equal to mean plus five standard deviations of the S/N ratios of naïve antisera measured against all six antigens was calculated. Based on this cutoff, sera from ASFV-negative pigs did not exhibit antibodies against the six ASFV antigens, except one pig that displayed an S/N ratio for p54 antigen slightly above the cutoff (Fig. 2). We believe this was a false positive since this sample tested negative by both commercial ELISA kits. On the other hand, all sera collected from ASFV-surviving pigs had antibodies specific to p32, p54 and pp62 while 91.3% of these pigs had antibody specific to p12. The frequency of ASFV-surviving pigs exhibited antibodies against C-type lectin and CD2v were 34.8% and 26.1%, respectively (Fig. 2).

In this study, ASFV convalescent sera were collected from two different cohorts of pigs: finishing pigs ($n = 17$) and sows ($n = 6$). Thus, the antibody levels were plotted by pig cohorts to see if there were any differences in the antibody profiles of these two pig populations. As shown in Fig. 3, finishing pigs and sows exhibited similar levels of antibodies against p12, p32, p54 and pp62. However, sera from sows exhibited significantly higher levels of antibodies against C-type lectin and CD2v than sera from finishing pigs. Similarly, frequency of sows that had antibodies against C-type lectin and CD2v was higher than that of finishing pigs (Fig. 3).

The ASFV strain currently affecting Asian and Eastern European countries belongs to genotype II. Under experimental conditions, all pigs experimentally infected with this ASFV strain die before they mount detectable levels of antibody responses (Guinat et al., 2014; Lee et al., 2021). It is therefore difficult to study the host immune response to virulent ASFV-genotype II. In this study, we utilized serum samples collected from pigs surviving a natural ASFV infection under field conditions to study antibody responses. The main limitation this approach is that we could not control the time, dosage, and routes of infection. In addition, the pigs might be co-infected wither other pathogens which might influence their immune responses to ASFV infection. However,

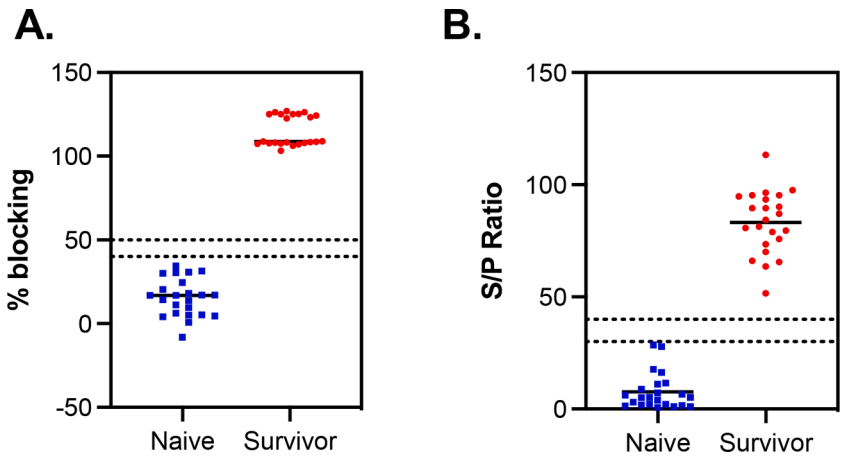

Fig. 1.. *Serological statuses of the field sera samples.* Antisera collected from ASFV negative farms (Naïve, $n = 24$) and from farms broke with ASFV (Survivors, $n = 23$) were tested using two different commercial ELISA kits: (A) ASFV Ingezim PPA COMPAC ELISA (Ingenasa, Madrid, Spain) and (B) ID Screen® African Swine Fever Indirect (IDVet, Grabels, France). Two dotted lines represent the cutoffs of the tests. Samples with values equal or above the upper dotted line are positive; equal or below the lower line are negative and between the two lines are suspicious.

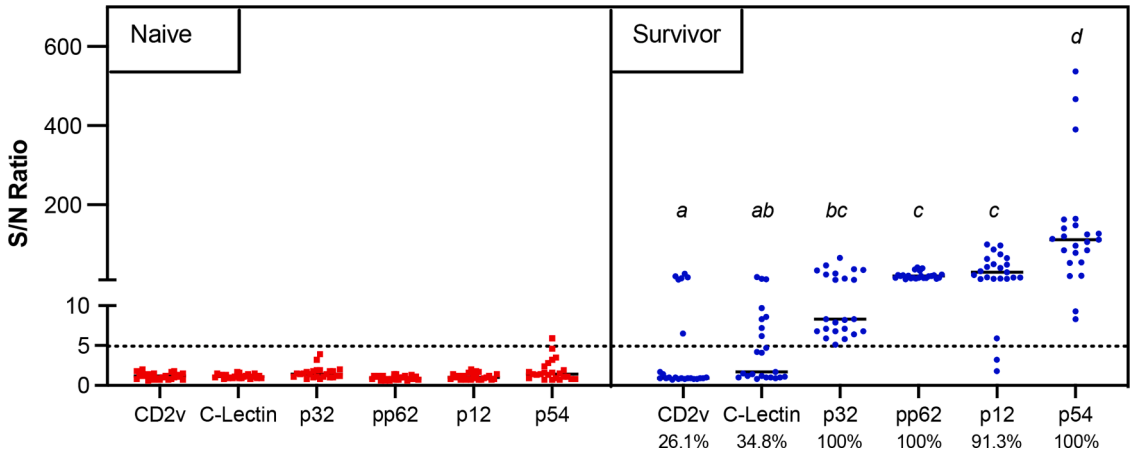

Fig. 2.. *Antibody reactivity against six ASFV antigens.* Antibody levels of each serum sample were simultaneously measured against six selected ASFV structural proteins following the LIPS assay. Fetal bovine serum (FBS) was incorporated to each run to serve as a negative control. Data are expressed as sample to negative (S/N) ratios. The dotted line at S/N ratio of 4.9 indicates the cutoff of the assay. Numbers below the antigen names indicate the proportion of survivor's serum samples tested positive for the respective antigens. Friedman's test, a non-parametric test, was used for finding statistical differences in S/N ratios of the survivor antisera measured against the six tested antigens. Subsequently, Dunn's multiple comparisons test was used for pair-wise comparison of the S/N ratios among the six antigens. The superscripts at the top of the graph denote the statistical comparison of the S/N ratios of the survivor antisera. Antigens with different superscripts are statistically different from each other ($p < 0.05$).

the advantage of this approach is that the results truly reflect the host immune responses to a highly virulent ASFV strain. Additionally, the results will be useful for serological diagnostic test development since the tests will be eventually used to detect ASFV in the field.

All 23 sera collected from ASFV-surviving pigs had antibodies against p32, p54 and pp62 antigens. These three proteins are targets of commercially available ELISA tests for serodiagnosis of ASF. Thus, our results demonstrate that LIPS is a reliable assay for studying swine humoral immune response to ASFV infection. We observed that 91.3% sera collected from ASFV-surviving pigs reacted to p12. Moreover, the S/N ratio for p12 was compatible to that for p32 and pp62. Therefore, p12 appears to be a high immunogenic protein which might be a potential target for serodiagnosis of ASF.

C-type lectin and CD2v mediate hemadsorption to viral infected cells (Galindo et al., 2000; Rodriguez et al., 1993). There exists a notion that antibodies specific to C-type lectin and CD2v might be important for immune protection against ASFV (Burmakina et al., 2016; Malogolovkin et al., 2015). We observed in this study that antibodies against C-type lectin and CD2v were only detected in ASFV-infected sows, but not in finishing pigs. At this moment, we do not know the possible mechanisms for the differential antibody responses against C-type lectin and CD2v in these two cohorts of pigs. However, it should be noted that differential antibody responses against different viral proteins have been observed in children and adult people infected with SARS-CoV-2 (Weisberg et al., 2021). Particularly, children infected with SARS-CoV-2 develop IgG antibody predominantly against the viral spike (S) protein but not the nucleocapsid (N) protein. On the other hand, adults infected with SARS-CoV-2 had IgG antibodies against both S and N proteins. In this study, finishing pigs were exposed to ASFV when they were approximately 2 months old, and samples were collected approximately 5 months later. Sows were also infected with ASFV when they were approximately 2 months old, but the samples were collected between 10 and 20 months later. Thus, the main difference between these two serum sample sets is that samples from sows were collected at significantly later time than those from finishing pigs. ASF is endemic in Vietnam. The farms where samples were collected for this study did not implement any measures to eliminate the virus. We therefore speculate that these sows were exposed to ASFV multiple times. It is possible that repeated exposure to ASFV might drive the development of antibodies against C-type lectin and CD2v in sows.

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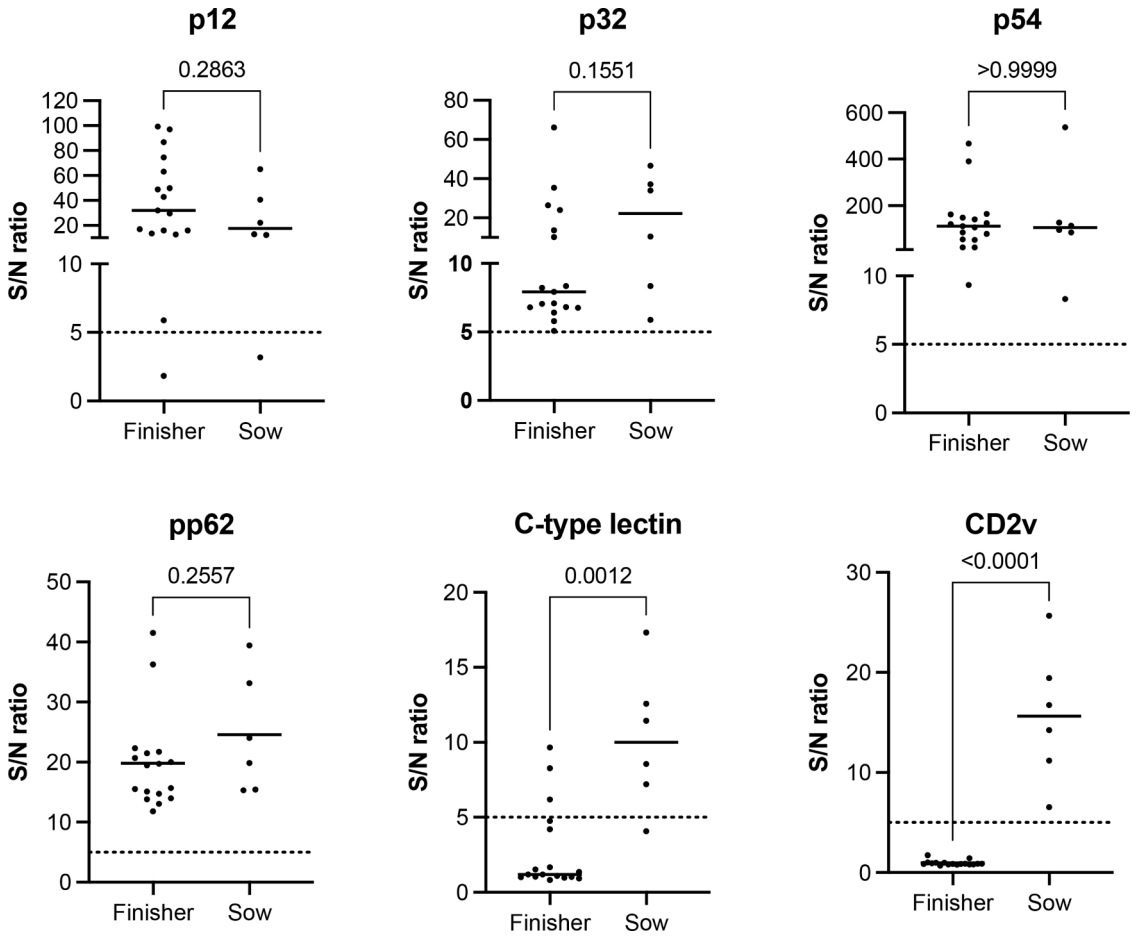

Fig. 3.. *Sows exhibit a different antibody profile compared to finishing pigs.* Antibody levels against each of the six tested antigens are plotted by the cohorts of pigs: finishing pigs ($n = 17$) and sows ($n = 6$). The dotted line at S/N ratio of 4.9 indicates the cutoff of the assay. A two-tailed Mann-Whitney test was used for statistical comparison of antibody levels. Numbers on top of the graphs are the p values of the statistical test.

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Ethical approval

This study was approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee under the protocol number 1792. This protocol was also reviewed and approved by the Committee for Animal Care and Use of Vietnam National University of Agriculture under the protocol number VNUA-20,201.

CRediT authorship contribution statement

Hung Q. Luong: Investigation, Methodology, Data curation, Visualization, Writing – original draft. **Huong TL. Lai:** Supervision, Resources, Project administration, Writing – review & editing. **Luc D. Do:** Resources. **Bo X. Ha:** Resources. **Giap V. Nguyen:** Resources. **Hiep LX. Vu:** Conceptualization, Methodology, Project administration, Funding acquisition, Resources, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The author(s) declare that there are no conflicts of interest

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