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# Adenovirus-vectored African Swine Fever Virus antigen cocktails are immunogenic but not protective against intranasal challenge with Georgia 2007/1 isolate

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ABSTRACT

African Swine Fever Virus (ASFV) causes a hemorrhagic disease in swine and wild boars with a fatality rate close to 100%. Less virulent strains cause subchronic or chronic forms of the disease. The virus is endemic in sub-Saharan Africa and an outbreak in Georgia in 2007 spread to Armenia, Russia, Ukraine, Belarus, Poland, Lithuania, and Latvia. In August 2018, there was an outbreak in China and in April 2019, ASFV was reported in Vietnam and Cambodia. Since no vaccine or treatment exists, a vaccine is needed to safeguard the swine industry. Previously, we evaluated immunogenicity of two adenovirus-vectored cocktails containing ASFV antigens and demonstrated induction of unprecedented robust antibody and T cell responses, including cytotoxic T lymphocytes. In the present study, we evaluated protective efficacy of both cocktails by intranasal challenge of pigs with ASFV-Georgia 2007/1. A nine antigen cocktail-(I) formulated in BioMize adjuvant induced strong IgG responses, but when challenged, the vaccinees had more severe reaction relative to the controls. A seven antigen cocktail-(II) was evaluated using two adjuvants: BioMize and ZTS-01. The BioMize formulation induced stronger antibody responses, but 8/10 vaccinees and 4/5 controls succumbed to the disease or reached experimental endpoint at 17 days post-challenge. In contrast, the ZTS-01 formulation induced weaker antibody responses, but 4/9 pigs succumbed to the disease while the 5 survivors exhibited low clinical scores and no viremia at 17 days post-challenge, whereas 4/5 controls succumbed to the disease or reached experimental endpoint. Overall, none of the immunogens conferred statistically significant protection.

## 1. Introduction

African Swine Fever Virus (ASFV) is an enveloped double-stranded DNA virus which causes a hemorrhagic disease in domestic pigs and wild boars with a mortality rate approaching 100% (Scott, 1957). There is no vaccine or drug available, and control methods include preventive biosecurity and mass slaughter in case of an outbreak (Brown and Bevins, 2018). The virus is present in sub-Saharan Africa, Sardinia, Caucasus region and Russia (Brown and Bevins, 2018; OIE). Since August 2018, there have been multiple outbreaks in China, Vietnam, Cambodia, and North Korea (OIE).(An outbreak in ASFV-free large scale pork producing countries, such as U.S.A, Germany, and Brazil, will pose a serious threat to the swine industry (Brown and Bevins, 2018).

Development of a vaccine is hindered by virus complexity, existence of multiple genotypes, and limited knowledge about protective immunity (Arias et al., 2017; Dixon et al., 2013). However, development of a vaccine is feasible since immunity can be induced with low-virulence isolates or mutants, but protective antigens and correlates of protection are poorly defined (Gallardo et al., 2018; Lacasta et al., 2015). Antibodies probably inhibit virus attachment, but T-cells could be the major protective effectors, especially cytotoxic T lymphocytes (CTLs) (Dixon et al., 2004; Oura et al., 2005; Reis et al., 2007). Depletion of $CD8^+$ T cells decreases protection by attenuated ASFV and following immunization, the frequency of ASFV-specific IFN-$\gamma^+$ T-cells correlate to the degree of protection upon challenge (Oura et al., 2005; Takamatsu et al., 2013). However, this outcome was not reproduced in

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recent studies, perhaps due to the differences in the challenge models (Carlson et al., 2016; O'Donnell et al., 2017).

Attenuated ASFV can confer 100% protection and on-going efforts are geared towards development of safe mutants (O'Donnell et al., 2017; Sánchez-Cordón et al., 2018). It is also rational to develop subunit vaccines given the advantage of safety. Promising outcomes such as delayed onset of viremia, delayed mortality, and partial protection have been observed using either one or a combination of a few antigens (Argilaguet et al., 2013; Neilan et al., 2004). A recent study showed that, although protection was not achieved, a pool of antigens delivered using DNA plasmids and the *Vaccinia* virus vector was able to significantly reduce viral genome loads in blood and lymphatic tissues (Jancovich et al., 2018). Thus, development of a subunit vaccine is possible, but it will likely require incorporation of multiple protective antigens. The main challenges are identification of protective antigens and a delivery system capable of eliciting protective immunity.

We have evaluated the immunogenicity and safety of two live-vectored ASFV multi-antigen cocktails in two independent studies (Lokhandwala et al., 2016, 2017). The first study showed that immunization of pigs with a cocktail of adenoviruses expressing structural antigens (p32, p54, pp62, and p72), induced robust IgGs, IFN-$\gamma^+$ T cells, and CTL responses (Lokhandwala et al., 2016). The second study showed similar outcomes in pigs immunized with a cocktail of adenoviruses expressing novel antigens, namely A151R, B119 L, B602 L, EP402RΔPRR, B438 L, K205R, and A104R (Lokhandwala et al., 2017).

The current study evaluated protective efficacy of the two cocktails against an ASFV Georgia 2007/1 intranasal challenge. We included an additional antigen, pp220 polyprotein, which is processed to generate p150 (the major structural protein), p37, p34, and p14 proteins that are localized within the viral core (Andres et al., 1997). Immunogenicity studies showed that pp220 induced strong antibody responses, robust IFN-$\gamma^+$ T cell, and CTL responses. In addition, predicted strong *SLA*-I binding pp220 peptides induced strong recall IFN-$\gamma^+$ T cells in splenocytes from immunized pigs (manuscript in-preparation). Additionally, pp220 was one of the antigens that was shown to consistently induce strong IFN-$\gamma^+$ responses (Jancovich et al., 2018). Given that the polyproteins, pp220 and pp62, account for 30% of the virion protein mass, we hypothesized that they contribute a significant amount of peptides in the cytoplasm of infected cells that are loaded onto *SLA*-I for presentation to CD8$^+$ T-cells (Andres et al., 2002).

The majority of previous ASFV vaccine studies have used the intramuscular route to challenge pigs (Argilaguet et al., 2013; Burmakina et al., 2016; Gallardo et al., 2018; Jancovich et al., 2018). However, the needle challenge does not directly mimic the natural route of infection. Transmission of ASFV in domestic pigs, especially at present in the Caucasus, Eastern Europe, Baltic countries, and China, in which tick involvement has not been demonstrated, most likely occurs via the mucosal route (Guinat et al., 2016). Thus, to simulate a scenario closer to natural infection, we opted for an intranasal ASFV challenge. Prior to conducting the efficacy studies, we empirically determined an effective intranasal challenge dose of the ASFV Georgia 2007/1 isolate by infecting pigs intranasally with 5 different doses of ASFV. Once the challenge dose to be used was ascertained, we tested whether the adenovirus-vectored antigen cocktails could confer protection against challenge.

## 2. Materials and methods

### 2.1. Generation of recombinant adenoviruses expressing ASFV antigens

The rationale for the selection of ASFV vaccine candidate antigens and the generation of replication-deficient recombinant adenoviruses, designated AdA151R, AdB119 L, AdB602 L, AdEP402RΔPRR, AdB438L, AdK205R-A104R, Adp32, Adp54, Adpp62, and Adp72, as well as the generation of the negative control immunogen Ad-Luciferase (Ad-Luc), has previously been reported (Lokhandwala et al., 2016, 2017). In the current study, we generated new adenoviruses expressing the ASFV p220 polyprotein, an antigen that has been reported to consistently induce strong IFN-$\gamma^+$ responses in pigs (Jancovich et al., 2018). To generate recombinant adenoviruses expressing the p220 polyprotein components (p37, p34, p14, and p150), the amino acid sequence of the pp220 polypeptide (Georgia 2007/1 isolate; GenBank Accession FR682468) was split into three parts designated i) p37-34-14 (contains the p37, p34, and p14 components); ii) p150-I (first half of the p150 protein); and iii) p150-II (second half of the p150 protein). These amino acid sequences were modified to add in-frame, a FLAG- and HA-tag at the N- and C-termini, respectively, and the resultant sequences were used to design codon-optimized synthetic genes. The genes were synthesized (GenScript) and used to generate recombinant replication-incompetent adenoviruses (designated Adp37-34-14, Adp150-I, and Adp150-II, respectively) using the ViraPower™ Adenoviral Gateway™ Expression Kit (Thermo Fisher Scientific K493000).

**Table 1**
Adenovirus-ASFV cocktail-I immunization protocol.

| Treatments | No. of pigs | Immunogen | Dose/pig |
|---|---|---|---|
| Cocktail-I-BioMize | 12 | Ad-ASFV cocktail-I[b] | Prime: $8 \times 10^{10}$ IFU<br>Boost: $8 \times 10^{11}$ IFU |
| Luc-BioMize | 12[a] | Ad-Luc | Prime: $8 \times 10^{10}$ IFU<br>Boost: $8 \times 10^{11}$ IFU |

[a] 1 animal died on day 20 post-boost due to an unrelated health issue.
[b] Ad-ASFV cocktail-I = 1 x $10^{10}$ IFU (prime) or 1 x $10^{11}$ IFU (boost) each of AdA151R, AdB119L, AdB602L, AdEP402RΔPRR, AdB438L, AdK205R-A104R, Adpp62 and AdB646L formulated in BioMize 0226 adjuvant.

All the above mentioned recombinant adenoviruses encoding the ASFV vaccine candidate antigens and the Ad-Luc construct, were scaled up in HEK-293A cells (Thermo Fisher Scientific R70507) to generate bulk virus for immunization as previously described (Lokhandwala et al., 2016, 2017). Protein expression by the scaled up recombinant adenoviruses was confirmed by immunocytometric analysis of adenovirus-infected HEK-293A cells using either anti-FLAG or anti-HA monoclonal antibodies [mAbs] and the ASFV-specific convalescent serum as previously described (Lokhandwala et al., 2016, 2017). The convalescent serum, a kind gift from E. J. Kramer, Plum Island Animal Disease Center, was produced from a donor pig that was sequentially infected with a series of tissue culture adapted and wild-type viruses from p72 genotypes I (DR11, Haiti 81, Lisbon 60, Malawi 83, and UG-61), genotype VIII and genotype X. The scaled-up viruses were titrated following the QuickTiter Adenovirus Titer Immunoassay kit (Cell Biolabs, Inc.) protocol with a slight modification as previously described (Lokhandwala et al., 2017). The infected HEK-293A cells were first probed with rabbit anti-adenovirus polyclonal IgGs followed by an alkaline phosphatase-conjugated anti-rabbit IgG (1:1000) (Jackson Immuno-Research 711-055-152). FastRed TR/Naphthol AS-MX was then used as the substrate (F4523; Sigma). Stained cells were counted using a microscope and titer calculated as infectious units (IFU)/ml.

### 2.2. Generation of recombinant ASFV antigens

All recombinant ASFV antigens, except pp62, were expressed using the Bac-to-Bac™ HBM TOPO™ Secreted Expression System (ThermoFisher Scientific A11339) using manufacturer's protocols and as previously described (Lokhandwala et al., 2016, 2017). Briefly, the synthetic genes coding for the FLAG-tagged ASFV antigens were used to prepare recombinant baculoviruses. Infection of High-Five cells (ThermoFisher Scientific) with these recombinant baculoviruses yielded FLAG-tagged ASFV antigens which were then affinity-purified from the cell supernatants using the anti-FLAG M2 affinity gel (Sigma, A2220). Recombinant pp62 was expressed using the Freestyle 293 expression system (ThermoFisher Scientific K900001) as previously described (Lokhandwala et al., 2016). Briefly, the synthetic gene encoding for the

**Table 2**
Adenovirus-ASFV cocktail-II immunization protocol.

| Treatments | No. of pigs | Immunogen | Adjuvant | Dose/pig |
|---|---|---|---|---|
| Cocktail-II-ZTS | 10[a] | Ad-ASFV cocktail-II[b] | ZTS-01 (Zoetis) | Prime : $7 \times 10^{10}$ IFU<br>Boosts : $7 \times 10^{11}$ IFU |
| Luc-ZTS | 5 | Ad-Luc | ZTS-01 | Prime : $7 \times 10^{10}$ IFU<br>Boosts : $7 \times 10^{11}$ IFU |
| Cocktail-II-BioMize | 10 | Ad-ASFV cocktail-II[b] | BioMize (VaxLiant) | Prime : $7 \times 10^{10}$ IFU<br>Boosts : $7 \times 10^{11}$ IFU |
| Luc-BioMize | 5 | Ad-Luc | BioMize | Prime : $7 \times 10^{10}$ IFU<br>Boosts : $7 \times 10^{11}$ IFU |

[a] One pig died on day 17 post-priming due to an unrelated health issue.
[b] Ad-ASFV cocktail-II = 1 x $10^{10}$ IFU (prime) or 1 x $10^{11}$ IFU (boosts) each of Adp32, Adp54, Adpp62, Adp72, Adp37, Adp150-I, and Adp150-II.

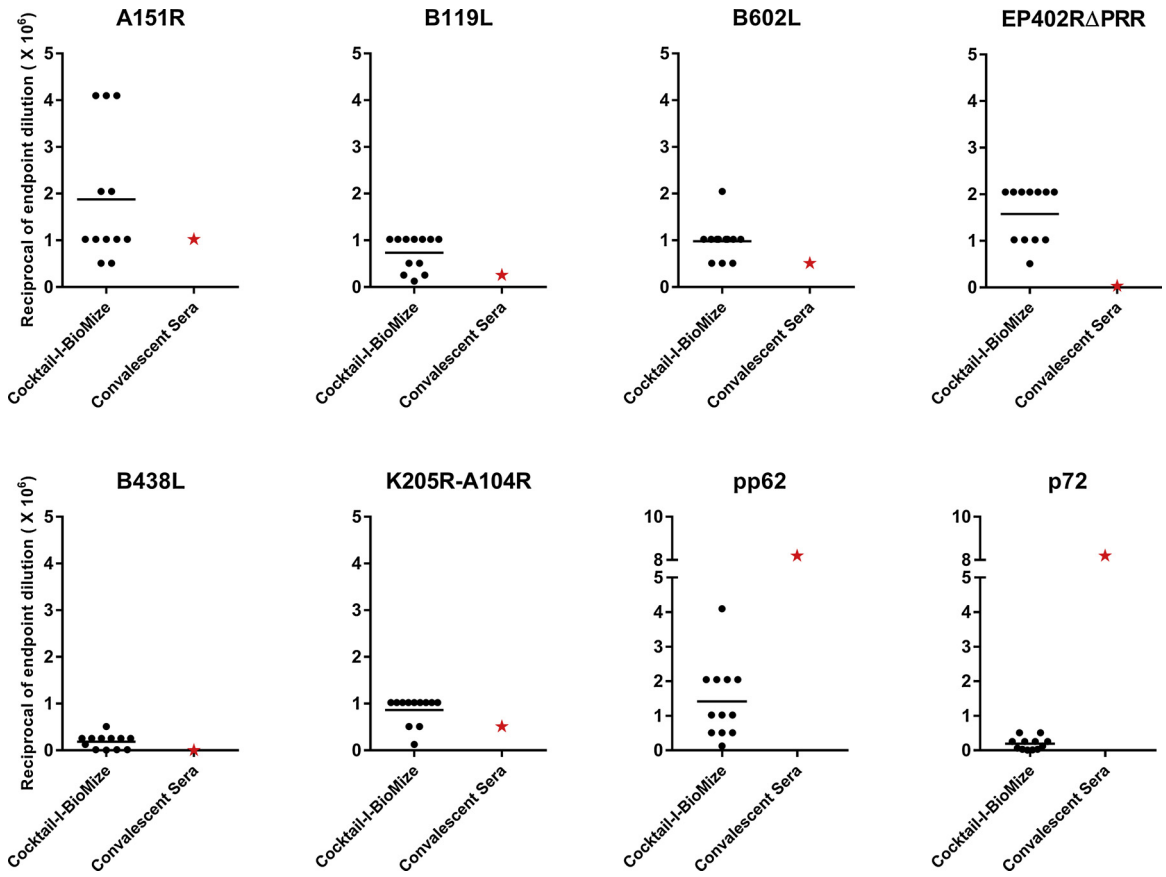


**Fig. 1.** ASFV antigen-specific end-point antibody titers.
Antigen-specific end-point antibody titers, at 2 weeks post-boost, in pigs immunized with cocktail-I-BioMize were determined by ELISA. The endpoint dilution was determined to be the dilution at which the sample OD was higher than the OD of cognate pre-bleed + 3 standard deviations. Data is presented as the reciprocal of the endpoint sera dilution x $10^6$. The highest titers were 1: $4 \times 10^6$ against A151R and pp62. All the titers are reported in Table S1. The ASFV-specific convalescent serum similarly titrated is depicted by the red star symbol. Sera from the negative control pigs showed no reactivity to any of the antigens.

FLAG-tagged pp62 protein was cloned into the pcDNA3.3 TOPO vector (Invitrogen). The resultant plasmid was used to transfect FreeStyle 293-F cells (ThermoFisher Scientific R79007) using 293fectin transfection reagent and the protein was then affinity-purified from the cell supernatant as described above.

### 2.3. Challenge inoculum

Spleen from a pig previously infected with ASFV Georgia 2007/1 was used to prepare the challenge inoculum. The homogenate from spleen tissue was back-titrated on primary cultures of porcine alveolar macrophages (PAMs). Quadruplicate 10-fold dilutions in RPMI complete media supplemented with 10% FBS, Pen/Strep (80 units/ml) and Fungizone (3 ug/ml) were added to washed confluent monolayers of PAMs in 96-well plates, incubated for 1 h at 37 °C and media replaced. After 3 days, the infected cells were visualized by IFA using monoclonal antibody against ASFV p30 protein and the $TCID_{50}$/ml was calculated (Reed and Muench, 1938).

### 2.4. Swine infections and immunizations

#### 2.4.1. Determination of intranasal ASFV Georgia 2007/1 challenge dose

Infection studies were performed at the biosafety level 3 (BSL3-Ag) biocontainment facilities at the Kansas State University Biosecurity Research Institute (BRI). Five-week-old Large White x Landrace pigs (n = 20) were housed in separate pens (n = 4/pen) and acclimated for four days prior to infection. Pigs were infected with different doses ($10^1$, $10^2$, $10^3$, $10^4$, or $10^5$ $TCID_{50}$) of ASFV Georgia 2007/1 via

**Table 3**
Viremia in pigs infected intranasally with graded amounts of ASFV.

| ASFV Georgia/07 dose | Day post-infection | | | |
|---|---|---|---|---|
| | Day 0 | Day 3 | Day 5 | Day 7 |
| G1: 1 $\log_{10}$ $TCID_{50}$ | | | | |
| 1 | – | – | – | – |
| 2 | – | – | – | – |
| 3 | – | – | – | – |
| 4 | – | – | – | 35.6 (1) |
| G2: 2 $\log_{10}$ $TCID_{50}$ | | | | |
| 5 | – | – | – | 32.9 (1.7) |
| 6 | – | – | 32.2 (1.6) | |
| 7 | – | – | 37.8 (0.9) | |
| 8 | – | – | 32.4 (1.7) | |
| G3: 3 $\log_{10}$ $TCID_{50}$ | | | | |
| 9 | – | – | 20.0 (5.4) | |
| 10 | – | – | 28.2 (3) | |
| 11 | – | – | 31.1 (2.2) | |
| 12 | – | 32.9 (1.7) | | |
| G4: 4 $\log_{10}$ $TCID_{50}$ | | | | |
| 13 | – | 27.5 (3.2) | | |
| 14 | – | – | 20.0 (5.3) | |
| 15 | – | – | 18.3 (5.8) | |
| 16 | – | – | 25.8 (3.7) | |
| G5: 5 $\log_{10}$ $TCID_{50}$ | | | | |
| 17 | – | – | 18 (5.9) | |
| 18 | – | – | 28.4 (2.9) | |
| 19 | – | – | 20.9 (5.1) | |
| 20 | – | 30.1 (2.5) | | |

Results are shown as Ct value following ASFV PCR. The number in parentheses shows the estimated concentration of virus (logTCID50/ml).

intranasal inoculation. Clinical signs and rectal temperatures were monitored daily. Blood samples were collected at 0, 3, 5, and 7 days post-infection (DPI).

*2.4.2. Immunization and challenge of pigs*

*2.4.2.1. Immunizations*. Weaned piglets were acquired and housed under BSL-2 conditions in the Large Animal Research Center (LARC) at Kansas State University. The piglets were randomly allocated into immunization and control groups (n = 12) and allowed to acclimatize for a week prior to immunizations. In the first study, the immunization group designated cocktail-I-BioMize, received the Ad-ASFV cocktail-I (AdA151R, AdB119 L, AdB602 L, AdEP402RΔPRR, AdB438 L, AdK205R-A104R, Adpp62, and Adp72) formulated in BioMize 0226 adjuvant (VaxLiant), whereas the control group, designated Luc-BioMize, received a similarly formulated equivalent amount of Ad-Luc virus (Table 1). The inoculum was administered intramuscularly in the neck area behind the ears. All the pigs were boosted four weeks post-priming (Table 1). The ASFV proteins (A151R, B119 L, B602 L, B438 L, K205R and A104R) included in cocktail-I are novel immunogenic antigens whose protective efficacy has previously not been tested. In addition, we included a modified CD2v antigen (EP402RΔPRR), in which the immunomodulatory cytoplasmic proline-rich repeats were deleted (Lokhandwala et al., 2017). The rationale for including pp62 and p72 was to enable comparison of immune responses across experiments since these two antigens are highly immunogenic and are strongly recognized by ASFV convalescent serum (Lokhandwala et al., 2017).

In a separate study, a total of 30 piglets were randomly distributed into two immunization groups of 10 pigs each and two cognate control groups of 5 pigs each. The immunization groups, designated cocktail-II-ZTS and cocktail-II-BioMize, respectively, received the Ad-ASFV cocktail-II (Adp32, Adp54, Adpp62, Adp72, Adp37-34-14, Adp150-I, and Adp150-II) formulated in either ZTS-01 (Zoetis) or BioMize 0226 (VaxLiant) adjuvant, respectively (Table 2). These ASFV proteins were included in cocktail-II because: i) they are immunogenic structural antigens which together account for about 50% of the virion protein mass (Andres et al., 2002, 1997; Jancovich et al., 2018; Lokhandwala et al., 2016); and ii) protective efficacy of an immunogen composed of adenovirus expressing these antigens has not been evaluated in pigs. The two negative control groups, designated Luc-ZTS and Luc-BioMize, respectively, received equivalent amounts of the Ad-Luc virus similarly formulated in the two different adjuvants. The adjuvants constituted 80% of final inoculum (v/v). All the animals were boosted twice at three week intervals with a dose one log higher than the priming dose. The animals were monitored twice daily after immunizations to record any adverse effects associated with the immunizations. Blood was collected weekly via jugular venipuncture to monitor serum antibody responses.

*2.4.2.2. Challenge*. All animals were moved to the BSL3-Ag biocontainment facility at the Kansas State University BRI prior to challenge with ASFV. The immunization and control group pigs were housed in separate pens and acclimated for four days prior to infection. The cocktail-I-BioMize vaccinees and the Luc-BioMize controls were challenged intranasally with 1 ml of $10^4$ $TCID_{50}$/ml of ASFV Georgia 2007/1. The cocktail-II-ZTS and the cocktail-II-BioMize vaccinees, and the cognate Luc controls were similarly challenged, but with $10^3$ $TCID_{50}$/ml. Each challenge inoculum was back-titrated on PAMs to ascertain the correctness of the administered dose. Blood samples for sera were collected at 0, 3, 7, 10, 14, and 17 days post-challenge or when an animal was euthanized, via jugular venipuncture. During the course of challenge, the pigs were monitored daily for appearance of clinical signs associated with ASF and scored as described in Table S1. The basis for experimental end point was a fever of > 40 °C for 2 consecutive days and/or if a pig showed a rapid deterioration of health, it was humanely euthanized. Death was also an acceptable end point. The study was terminated on day 17 post-challenge and all surviving animals were euthanized.

*2.5. ELISA*

Antigen-specific IgG end-point titers in serum from blood collected at 2 weeks post-boost were determined using an ELISA as previously described (Lokhandwala et al., 2016). Briefly, 96-well microplates coated overnight with 1 μg/ml (100 μl/well) of recombinant affinity-purified ASFV antigens and then blocked with 10% non-fat dry milk in PBST (0.1% Tween 20), were incubated (in triplicates) with 2 fold serum dilutions starting from 1:4000 to 1:8 × $10^6$. Pre-immunization sera similarly diluted served as negative control, whereas convalescent serum from a pig that was inoculated with multiple live ASFV genotypes served as the positive control (Lokhandwala et al., 2016). Peroxidase-conjugated anti-swine IgG (Jackson ImmunoResearch, Cat No. 114-035-003) was the secondary antibody and Sure Blue Reserve TMB (KPL, Cat No. 53-00-02) was the substrate. The absorbance at 450 nm was read using a microplate spectrophotometer (BioTek Epoch). The end-point titers were calculated as described in the figure legend (Fig. 1). In the cocktail-II vaccine study, antigen-specific IgG antibody responses were similarly evaluated, but using the serum at a dilution of 1:1000.

*2.6. DNA extraction and PCR*

Following challenge, total DNA was extracted from 50 μl of serum using MagMAX-96 viral isolation kit (Applied Biosystems) following the manufacturer's instructions. The samples were analyzed for the presence of ASFV genomic DNA by quantitative PCR (qPCR), as previously described (King et al., 2003). All PCR reactions were carried out on a CFX96 Touch Real-Time PCR Detection System (BIO-RAD) using a 96 wells plate format. A standard curve was prepared by making serial dilutions containing 6 points ranging from $10^6$ to $10^1$ logs of template viral DNA from the spleen virus stock. The standard curve was used to estimate ASFV viremia. The PCR results were reported as $\log_{10}$ ASFV

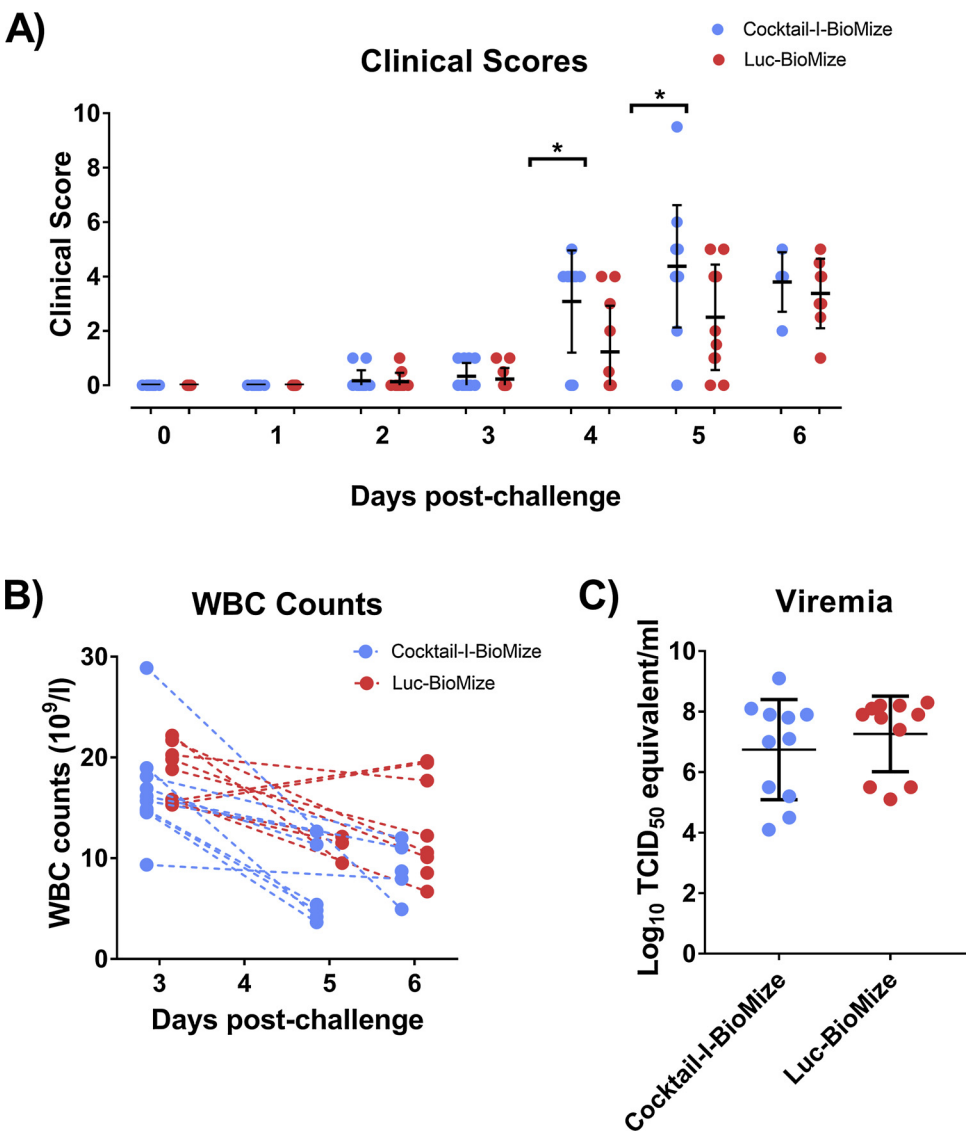


**Fig. 2.** Clinical manifestations and viremia post-challenge.
A) Individual and mean clinical scores of the cocktail-I-BioMize and Luc-BioMize groups, observed on days 0–6 post-challenge. Asterisk denotes statistically significant differences between the immunization and the control groups (*$p < 0.05$); B) WBC counts in the immunization and control groups on day 3 post-challenge and on the day of termination (day 5 or day 6 post-challenge). The WBC decline in the immunization and the control groups was not statistically different; C) Viremia (detected by qPCR) in blood samples from the immunization and control pigs on the day of termination (day 5 or day 6 post-challenge).

DNA starting quantity (copy number) per PCR reaction.

### 2.7. White blood cell count

Whole blood samples were collected from the cocktail-I-BioMize vaccinees and controls at day 3, and on either day 5, or 6 post-challenge when an animal was euthanized on reaching experimental end-point. Blood samples were collected from the cocktail-II-ZTS and cocktail-II-BioMize vaccinees as well as the cognate controls at day 0, 7, 10, and 14 post-challenge or at day 17 when the study was terminated. White blood cells (WBC) were enumerated in EDTA-stabilized samples by using a semi-automated animal blood cell counter (VetScan HM5 Hematology system; Abaxis, USA). To ensure instrument quality control, a normal control sample purchased from the supplier was used to calibrate the whole blood analyzer before every use. All samples were analyzed on the day of acquisition.

### 2.8. Statistical analysis

All analyses were performed with GraphPad Prism 7.02 and a $P < 0.05$ was considered significant. The differences in the clinical scores and viremia between the treatment and control groups in the first study were compared using unpaired t-tests. The differences in the mean antigen-specific antibody responses between the two treatment groups and between the treatment and control groups in the second study were analyzed using one-way ANOVA, followed by Sidak's multiple-comparison test.

### 2.9. Ethics statement

Experiments involving animals and virus were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University Institutional Animal Care and Use Committees (IACUC registration # 3871) and Institutional Biosafety Committee (IBC registration # 1200). Animals were humanely euthanized using pentobarbital following the American Veterinary Medical Association (AVMA) guidelines for the euthanasia of animals, and all efforts were made to minimize suffering.

## 3. Results

### 3.1. The minimum intranasal dose that infected 100% of pigs was $10^2$ $TCID_{50}$ ASFV

To determine the optimal dose of ASFV (Georgia 2007/1) for an

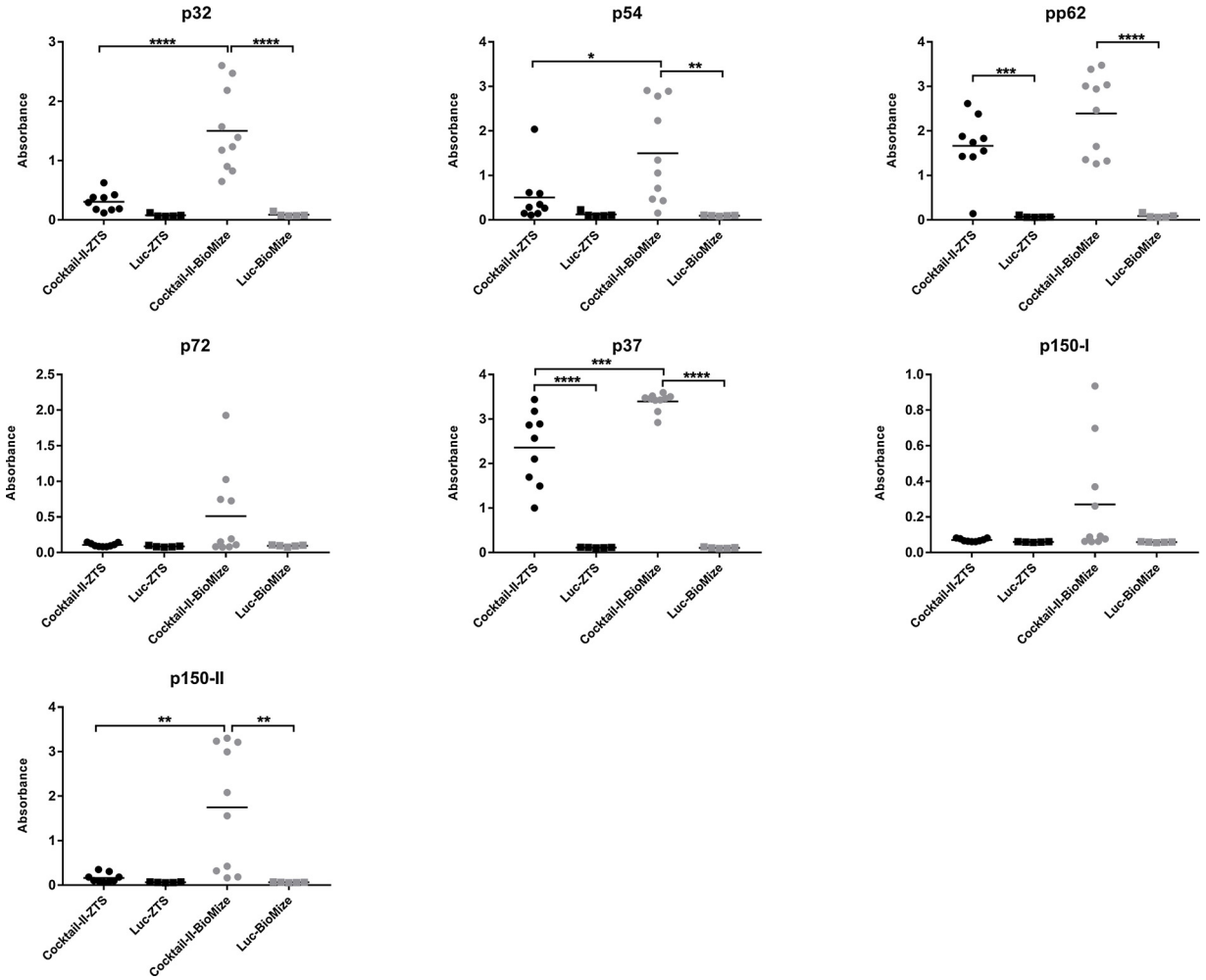


**Fig. 3.** Antigen-specific IgG responses post-boost.
Antigen-specific IgG responses by pigs inoculated with Cocktail-II-ZTS; Luc-ZTS; Cocktail-II-BioMize or Luc-BioMize at 1 week post-boost were evaluated by ELISA. The serum dilution was 1:1000. The asterisks denote a significant difference between the groups indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

intranasal challenge, five groups of four pigs each, were infected with an increasing dose (log increments) of the ASFV Georgia 2007/1 (Table 3). Successful infection of individual pigs was determined by the clinical signs of ASFV infection such as fever, depression, and loss of appetite. A change in behavior from bright, alert and responsive to depressed was observed as early as day 3 post-infection (DPI) in pigs belonging to groups 3–5 (high dose groups). One pig from each of the groups 4 and 5 had to be euthanized on day 4 post-infection, while the remaining pigs in both groups were euthanized on day 5. The study was terminated on day 7 post-infection on account of surviving pigs from groups 2 and 3 becoming pyrexic, anorexic, dehydrated and non-responsive to NSAID treatment. Evaluation of viremia in blood by qPCR showed that at day 3 post-infection, one pig from each of the groups 3, 4, and 5 were viremic and by day 5 post-infection, all the pigs had detectable levels of viremia (Table 3). Three pigs from the group infected with $10^2$ $TCID_{50}$ were viremic at day 5 and by day 7, the remaining pig was positive, whereas only one pig from the group that received $10^1$ $TCID_{50}$ was viremic at day 7 post-infection. Therefore, a dose of $10^2$ $TCID_{50}$ contained the lowest amount of virus sufficient to result in a 100% infection (all pigs positive by PCR) within one week following intranasal inoculation of the ASFV Georgia 2007/1 strain.

### *3.2. Immunization of pigs with Ad-ASFV cocktail-I induced immune responses that were counter protective upon intranasal challenge*

Our previous studies had shown that immunization of pigs with Ad-vectored ASFV antigen cocktails induced immune responses to all the vaccine candidate antigens (Lokhandwala et al., 2016, 2017). To determine whether these immune responses are protective, pigs were immunized with cocktail-I-BioMize, whereas controls received Luc-BioMize and then all the pigs were challenged (Table 1). Evaluation of antigen-specific end-point antibody titers in sera from blood collected at two weeks post-boost showed that a majority of the pigs in the immunization group had antibody titers $\geq 1{:}1 \times 10^6$ against antigens A151R, B119 L, B602 L, EP402RΔPRR, K205R-A104R, and pp62 (Fig. 1). The highest titer was $1{:}4 \times 10^6$ against A151R in three of the vaccinees and against pp62 in one vaccinee. A comparison of the antigen-specific antibody titers in sera from the vaccinees with the antibody titer of the ASFV-specific convalescent serum revealed that the cocktail-I-BioMize induced titers higher or equivalent to the convalescent serum in all vaccinees for antigens B602 L, EP402RΔPRR, and B438 L. In the case of antigens A151R, B119 L, and K205R-A104R chimera, 83%, 91%, and 92% of the vaccinees, respectively, had antigen-specific antibody titers higher or equivalent to the convalescent serum. Antibody titers for p72 antigen in the vaccinees were much lower than in the convalescent serum, which was consistent with our previous outcome (Lokhandwala et al., 2016). The data are in

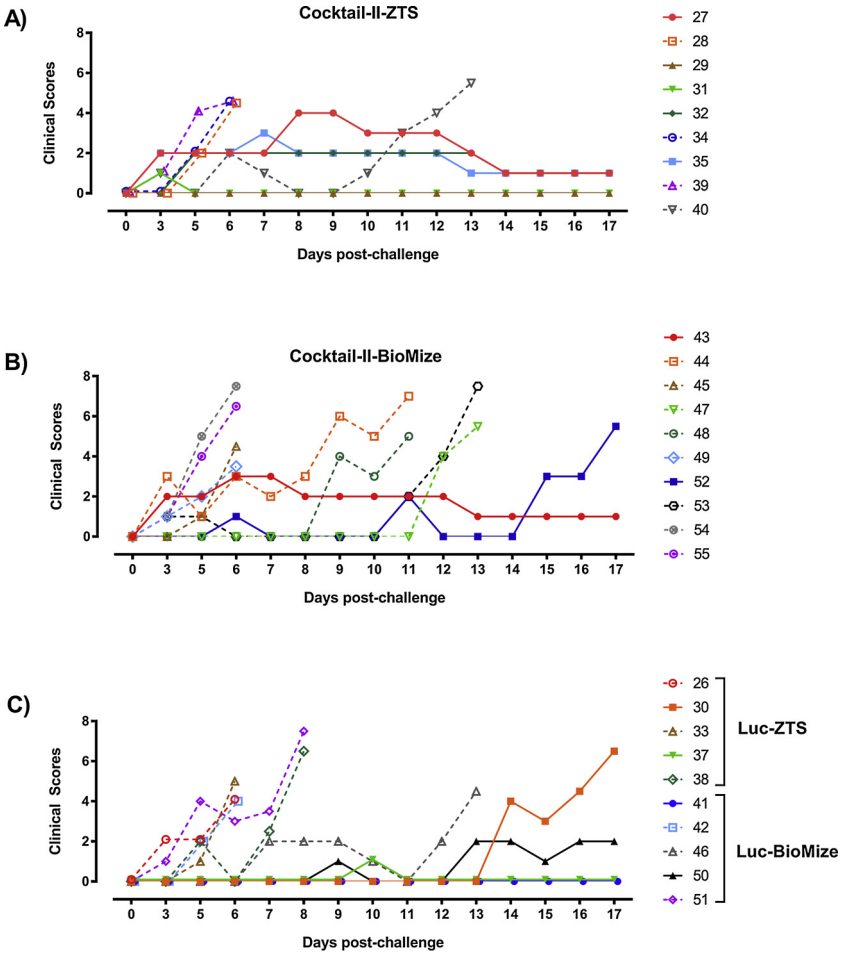


**Fig. 4.** Clinical scores post-challenge.
Individual clinical scores of pigs post-challenge from: A) Cocktail-II-ZTS; B) Cocktail-II-BioMize; and C) Luc-ZTS and Luc-BioMize groups. The pigs that were alive when the study was terminated (day 17 post-challenge) have solid markers and the pigs that were euthanized have hollow markers.

agreement with the outcomes from our previous immunogenicity study and confirms that, except for the p72 antigen, two doses of the cocktail-I-BioMize induced higher antigen-specific titers than the convalescent serum from an animal that received multiple inoculations of live ASFV (Lokhandwala et al., 2016, 2017).

Four weeks post-boost, all the pigs in the cocktail-I-BioMize and the Luc-BioMize groups were challenged intranasally with $10^4$ $TCID_{50}$ of the ASFV Georgia 2007/1 isolate. By day 4 post-challenge, 9/12 of the pigs in the cocktail-I-BioMize group, compared to 4/11 of the pigs from the Luc-BioMize group, had high fever (body temp > 41 °C) and appeared listless. Other clinical signs of acute ASF such as hemorrhaging in the ears and cutaneous erythema were observed in 5/12 of the pigs from the cocktail-I-Biomize group on day 5, but not in the Luc-BioMize control pigs. The immunization group had higher mean clinical scores than the control group on days 4, 5, and 6 post-challenge (Fig. 2A) with the difference being significant ($P < 0.05$) on days 4 and 5 post-challenge (Fig. 2A). In addition, the animals in the cocktail-I-Biomize group exhibited a sharper decline in WBC counts compared to the controls (Fig. 2B). Leukopenia is a characteristic indicator of ASFV infection and the sharper drop in the number of cells in the cocktail-I-Biomize animals, compared to the controls, is consistent with increased severity of disease. On day 5 post-challenge, 6/12 of the pigs in the cocktail-I-Biomize group and 3/11 of pigs in the control group were euthanized for animal welfare reasons. All remaining animals had to be euthanized by day 6 post-challenge. No significant difference was observed in viremia in blood collected on the day of termination between the immunization and control groups (Fig. 2C).

### 3.3. Survival of pigs immunized with Ad-ASFV cocktail-II was influenced by adjuvant

To evaluate protective efficacy of the Ad-ASFV cocktail-II, two groups of pigs were immunized with the cocktail formulated in either ZTS-01 (cocktail-II-ZTS) or BioMize (cocktail-II-BioMize) adjuvant (Table 2). The cognate control groups, designated Luc-ZTS and Luc-BioMize, received the Ad-Luc formulated in either adjuvant, respectively (Table 2). Analysis of humoral responses post-priming revealed lower antibody response as compared to the previous immunogenicity study (Lokhandwala et al., 2016) and thus, the animals were boosted twice. Thus, all animals received one prime and two boosts, at three week intervals. Evaluation of antigen-specific IgG responses in sera from all animals at one week following the second boost showed that the mean IgG response of the cocktail-II-BioMize vaccinees was higher than the response of the cocktail-II-ZTS vaccinees for all 7 antigens in the cocktail (Fig. 3). This difference between the two groups was significant for antigens p32 ($P < 0.0001$), p54 ($P < 0.05$), p37 ($P < 0.001$), and p150-II ($P < 0.01$). The data indicated that the BioMize adjuvant induced a higher ASFV antigen-specific antibody response as compared to the ZTS-01 adjuvant.

Following intranasal challenge with $10^3$ $TCID_{50}$ of ASFV Georgia 2007/1, some pigs in all the four groups were pyrexic (> 41 °C) by day 5 post-challenge. Notably, one animal from the cocktail-II-BioMize group (pig 53), two animals (pigs 26 and 38) from the Luc-ZTS group, and one animal (pig 51) from the Luc-BioMize group exhibited mild joint swelling, a clinical sign more typically associated with chronic

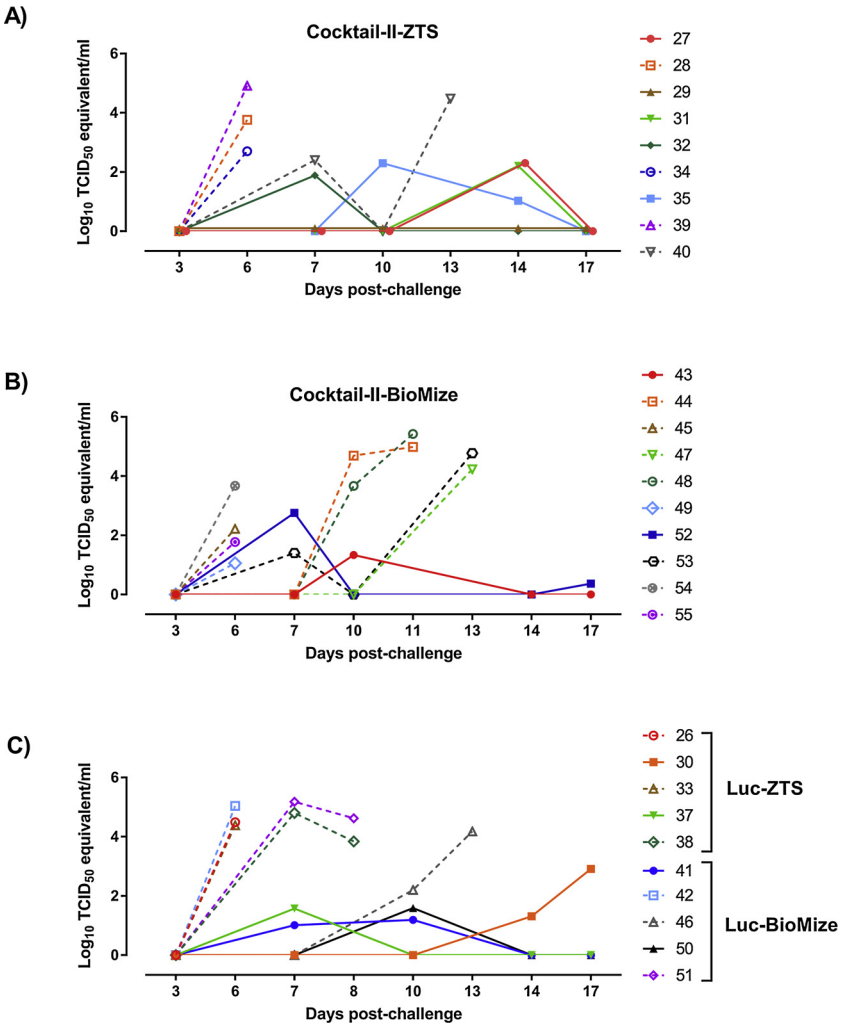


**Fig. 5.** Viremia post-challenge.
Post-challenge viremia in blood samples from: A) Cocktail-II-ZTS; B) Cocktail-II-BioMize; and C) Luc-ZTS and Luc-BioMize groups. Viremia was measured by qPCR for all animals on day 3, on the day of euthanasia for animals with severe clinical disease and on days 7, 10, 14, and 17 for surviving animals. The pigs that were alive when the study was terminated (day 17 post-challenge) have solid markers and the pigs that were euthanized have hollow markers. The color scheme used for the individual animals is same as Fig. 4.

ASF infection. On day 6 post-challenge, 3/9 pigs in the cocktail-II-ZTS group; 4/10 pigs in cocktail-II-BioMize group; 2/5 pigs in Luc-ZTS group; and 1/5 pig in Luc-BioMize group were euthanized due to high fever of 40.5–42 °C for 2 days, combined with dullness. The experiment was terminated and all animals were euthanized on day 17 post-challenge. Five of the nine remaining pigs from the cocktail-II-ZTS group, representing 55.5% of animals survived until day 17 post-challenge and were clinically healthy when euthanized (Fig. 4A). Virus was detected in blood samples of pigs 27 and 31 from this group on day 14, but not on day 17 post-challenge (Fig. 5A; Table S2). Pig 32 from the cocktail-II-ZTS group was PCR positive only on day 7 but not thereafter and pig 35 from the same group was PCR positive on days 10 and 14, but not on day 17 post-challenge (Fig. 5A). Pig 29 from this group was the only animal that was negative for viremia at all time-points tested (Fig. 5A). Presence of antibodies to ASFV antigen p22 (not included in the cocktail) at 17 days post-challenge confirmed that pig 29 had been infected although it did not develop clinical signs or viremia (Fig. S1). In contrast, only 2 of 10 pigs from the cocktail-II-BioMize group survived until day 17 post-challenge (Fig. 6). However, one animal (pig 43) from this group had an elevated clinical score (due to watery diarrhea) throughout, but never developed fever. Pig 52 from the same group had a low fever on days 6 (40.2 °C) and 11 (40.7 °C) and developed pyrexia (> 41 °C) from day 15 onwards along with cutaneous erythema and listlessness by the time of euthanasia on day 17 post-challenge (Fig. 4B). Virus was detected in blood of both pigs 43 and 52 at earlier time points, on day 10 and day 7, respectively, but not at day 14 and day 10, respectively. Consistent with the re-appearance of clinical signs, pig 52 was PCR positive for virus again on day 17 (Fig. 5B). Two control pigs (pig 30 and 37) from the Luc-ZTS group and two pigs (pig 41 and 50) from the Luc-BioMize group survived up to day 17. However, one animal from Luc-ZTS group (pig 30) exhibited severe clinical symptoms by day 17 that were sufficient to justify euthanasia (Fig. 4C). In agreement with the clinical symptoms, pig 30 was also positive for virus on day 14 and day 17 (Fig. 5C). One animal (pig 50) from Luc-BioMize group was viremic on day 10 and negative thereafter, however it was lethargic and had mildly swollen joints from day 14 onwards (Figs. 4 and 5C). Although, no significant difference was observed in mean terminal viremia across the groups, the mean viremia of the cocktail-II-ZTS group was lower than cocktail-II-BioMize group and both the control groups, mainly due to the 5 vaccinees in the cocktail-II-ZTS group that were clinically healthy (Fig. 6). Evaluation of WBC counts post-challenge (Fig. S2) showed that higher clinical scores and increased viremia coincided with characteristic ASFV-induced leukopenia while the survivors from all groups had fairly stable WBC counts at the time of euthanasia. Overall, 5/9 pigs from the cocktail-II-ZTS group were healthy at study termination compared to one pig from the

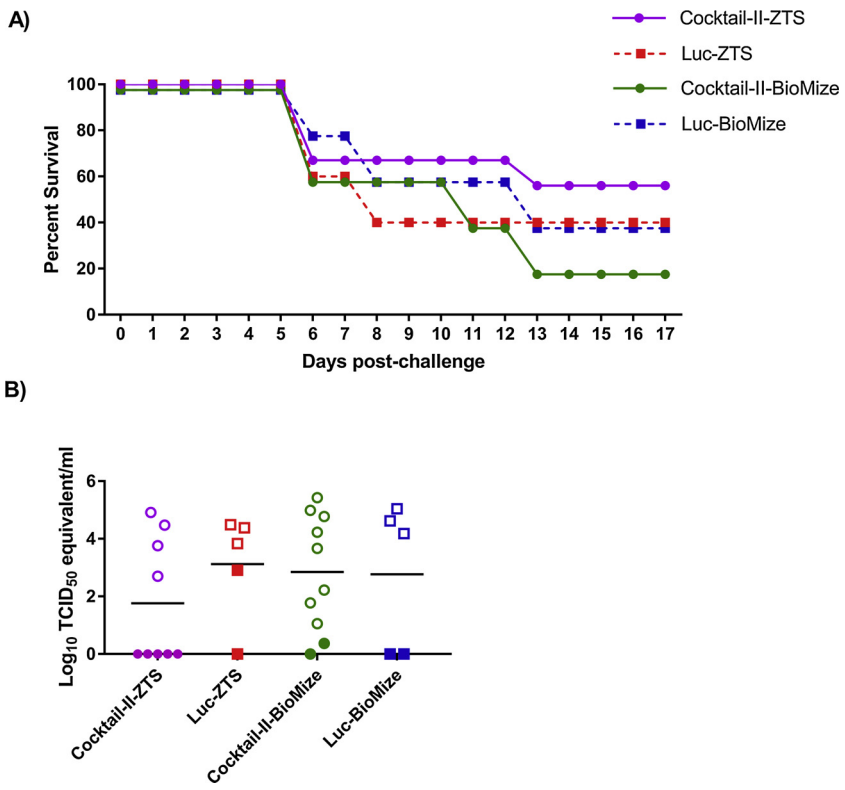


**Fig. 6.** Pig survival and terminal viremia post-challenge. (A) Survival of the pigs in Cocktail-II-ZTS; Luc-ZTS; Cocktail-II-BioMize; and Luc-BioMize groups. Pigs were euthanized based on disease severity as judged by the attending veterinarian. The pigs that were alive when the study was terminated (day 17 post-challenge) have solid markers and the pigs that had to be euthanized have hollow markers. (B) Terminal viremia was measured for all the pigs by qPCR on the day of their euthanasia. The difference in survival and mean terminal viremia between the groups was not statistically significant.

cocktail-II-BioMize group (Fig. 6). One pig from each of the control groups exhibited clinical signs similar to the five survivors of cocktail-II-ZTS group.

## 4. Discussion

The protective efficacy of two adenovirus-vectored ASFV antigen cocktails was evaluated to extend previous work in which safety, tolerability, and immunogenicity of these immunogens had been evaluated (Lokhandwala et al., 2016, 2017). The animals were challenged intranasally to simulate the typical infection route observed in ASFV outbreaks as supported by a previous study in which comparison of intraoropharyngeal, intranasopharyngeal, intramuscular, and direct contact infection methods had revealed that intranasal route may be better for ASFV pathogenesis and vaccine studies (Howey et al., 2013). We demonstrated that intranasal challenge with a minimum dose of $10^2$ $TCID_{50}$ ASFV Georgia 2007/1 was sufficient to infect 100% of pigs within one week. Induction of immune responses and protective efficacy of two antigen cocktails, Ad-ASFV-I and Ad-ASFV-II, containing nine and seven antigen components, respectively, was evaluated in separate studies, the second of which also involved a comparison of the Ad-ASFV-II cocktail formulated in two different adjuvants. The Ad-ASFV-I cocktail in BioMize adjuvant, tested in the first of these experiments, contained structural and non-structural ASFV proteins (A151R, B119 L, B602 L, EP402RΔPRR, AdB438 L, K205R and A104R, pp62, and p72), whereas the Ad-ASFV-II cocktail that was used for the adjuvant comparison study contained structural proteins (p32, p54, pp62, p72, and pp220 polyprotein components) which together constitute about 50% of the virion protein mass (Andres et al., 1997). The pp62 and p72 antigens were included in both cocktails to enable comparison of immune response to the same antigens across experiments.

All the pigs from the cocktail-I-BioMize and the Luc-Biomize groups developed severe disease and the study was terminated at day 6 post-challenge. Surprisingly, the cocktail-I-BioMize vaccinees exhibited significantly ($p < 0.05$) higher mean clinical scores on days 4 and 5 post-challenge compared to the Luc-BioMize controls. This outcome suggested an immune-response dependent enhancement of disease in the vaccinees. Enhanced susceptibility to certain virus infection due to pre-existing immunity can occur through mechanisms involving antibodies, activated macrophages, $CD4^+$ T-cells, and dendritic cells (Chapman, 2016; Huisman et al., 2009). One such mechanism, antibody-dependent enhancement [ADE] of infectivity is well documented for viral pathogens such as dengue virus and HIV (Beck et al., 2008; Halstead, 2014). Our observation is consistent with two previous reports where immune-mediated disease enhancement for ASFV was reported, but the underlying mechanisms responsible have not yet been investigated (Blome et al., 2014; Jancovich et al., 2018).

In the cocktail-I-BioMize efficacy study, challenge with $10^4$ $TCID_{50}$ rapidly induced fatal disease in all pigs. Therefore, the challenge dose for the cocktail-II and the cognate controls was lowered to $10^3$ $TCID_{50}$ with the rationale of providing a window that would allow identification of protective immune responses that are associated with significant delay to death or resolution of clinical disease and viremia. Antigen-specific antibody responses in the cocktail-II-BioMize group were significantly higher than in the cocktail-II-ZTS group for four out of the seven antigens. Upon challenge, 2/10 cocktail-II-BioMize pigs and 2/5 Luc-BioMize controls were alive at 17 days post-challenge. However, one of the 2/10 survivors and one of the 2/5 controls exhibited clinical symptoms that justified euthanasia. In comparison, all 5/9 survivors from the cocktail-II-ZTS group were healthy, but 2/5 Luc-ZTS controls were also alive at 17 days post-challenge. However, one of the Luc-ZTS control pig was moribund. Thus, only one pig from each of the cocktail-II-BioMize and both the Luc control groups exhibited clinical symptoms similar to the 5/9 survivors from the cocktail-II-ZTS group. Since both the cocktail-II groups contained the same antigens, but different adjuvants, the 5/9 survival in the ZTS group compared to 1/10 survival in BioMize adjuvant group was an interesting outcome that warrants further evaluation using a larger number of animals. The four pigs from both the Luc control groups that were alive at 17 days post-challenge, implies that the $10^3$ $TCID_{50}$ was a sub-lethal dose. However, it should be noted that all pigs, with the exception of pig number 29 in the

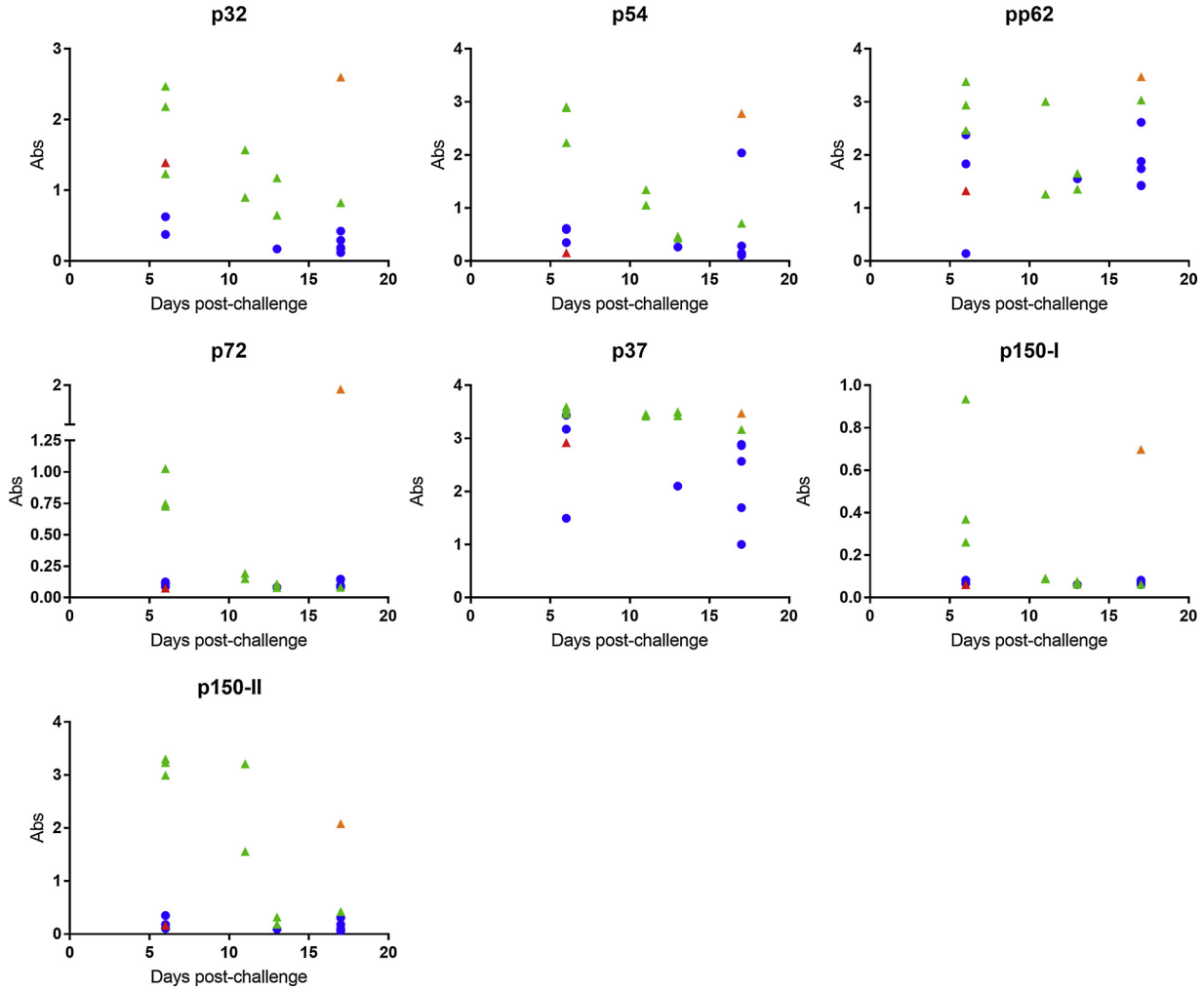


**Fig. 7.** Correlation of antigen-specific antibody response with the survival of pigs.
Antigen-specific antibody response (absorbance values) at 1 week post-boost was correlated with the survival post-challenge (in days). Cocktail-II-ZTS vaccinees: blue circles; and cocktail-II-BioMize vaccinees: green triangles. Pigs 43 and 49 from the cocktail-II-BioMize group are shown as orange and red triangles, respectively. A negative trend between antibody response and survival is observed for 8/10 of the cocktail-II-BioMize vaccinees for antigens p32, p54, p72, p150-I and p150-II with pigs 43 and 49 being the outliers. Upon excluding these outliers, negative correlation is significant for p32 ($p < 0.05$, r -0.7469); p54 ($p < 0.01$, r -0.9007); p72 ($p < 0.01$; r -0.8942) and p150-II ($p < 0.01$; r -0.8465).

cocktail-II-ZTS group, developed viremia upon challenge.

The high levels of antigen-specific antibodies, but poor survival of the pigs in the cocktail-II-BioMize group mirrored the outcome observed in the pigs inoculated with the cocktail-I-BioMize. A similar observation has been made previously in pigs immunized with inactivated ASFV formulated in adjuvants (Blome et al., 2014). The authors reported an increase in clinical progression of the disease in vaccinees with the highest antibody responses. An attempt to inversely correlate the antigen-specific antibody response with the survival of the pigs in this study, failed to yield statistically significant outcomes (Fig. 7). However, a negative trend was observed between the strength of the antigen-specific antibody response and survival of the cocktail-II-BioMize pigs for some antigens (Fig. 7). Interestingly, two animals were outliers that may have affected the statistical outcome given the limited experimental group sizes dictated by the availability of biocontainment space. One animal (pig 43) had a high antibody response for almost all antigens and survived up to day 17, whereas pig 49 had the lowest response to all antigens and had to be euthanized as early as day 6 post-challenge. These observations highlight the complexity of the role of antibodies in protection against ASFV. The antibody responses induced by the adenovirus-vectored antigens in both cocktails were non-protective. However, it is important to note that there is evidence in the literature emphasizing the role of antibodies induced by viral infection in protection against challenge (Escribano et al., 2013). Passively acquired anti-ASFV antibodies have been shown to confer protection and in addition, it has also been demonstrated that homologous protection may be haemadsorption-inhibition serotype-specific (Burmakina et al., 2016; Onisk et al., 1994).

In conclusion, we tested protective efficacy of twelve ASFV antigens in two cocktails. Some evidence of disease enhancement in vaccinees was observed with both Ad-ASFV cocktail-I and cocktail-II when formulated with the BioMize adjuvant, but not with cocktail-II when formulated with the ZTS-01 adjuvant. The cocktail-II-ZTS vaccinees had better survival rate, but clinical disease was not prevented. Further studies will be required to evaluate the efficacy of novel antigens formulated in a suitable adjuvant, such as ZTS-01.

## Author contributions

The study design was conceptualized by SL, SDW, RB, RRR, and WM. The laboratory experiments were performed by SL, VP, NS, AS, LE, and JB. Animal immunization and infection experiments were carried out by SL, VP, LP, NS, CE, AS, MO, LE, MS, and WM. Data analysis and result interpretation was done by SL and WM. Manuscript was written

and reviewed by SL, VP, AS, SDW, RB, RRR, and WM.

## Competing interests

The authors declare no financial or non-financial competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.006.

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