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Short Communication

Evaluation of hemostaseological status of pigs experimentally infected with African swine fever virus

Hovakim Zakaryan [a], Elena Karalova [a], Henrik Voskanyan [a,b], Zarine Ter-Pogossyan [a], Narek Nersisyan [a], Astghik Hakobyan [a], David Saroyan [a], Zaven Karalyan [a,*]

[a] Laboratory of Cell Biology and Virology, Institute of Molecular Biology of NAS RA, P.O. Box 0014, Yerevan, Armenia
[b] Scientific Center of Stock Breading and Veterinary RA, P.O. Box 0071, Nubarashen Sarahat 2, Yerevan, Armenia

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ABSTRACT

African swine fever is a highly contagious hemorrhagic disease of pigs caused by African swine fever virus (ASFV). Hemorrhages are the most frequently reported lesions in acute and subacute forms of ASF. Hemorrhagic lesions are accompanied by impaired hemostasis, which includes thrombocytopenia and changes in the coagulation system. In the present study, experimental infection was conducted to elucidate whether a highly virulent ASFV genotype II circulating in the Trans-Caucasus and Eastern Europe affects the hemostasis of infected pigs. Platelet count changes and platelet size, as well as coagulation parameters were evaluated upon experimental infection. In contrast to other ASFV strains, ASFV genotype II showed a significant decrease in the number of platelets from 3rd dpi onwards. Furthermore, a decrease in platelet size was observed throughout the entire period of experiment. A significant increase in the number of platelet aggregates was observed from the beginning of infection. Unlike other ASFV strains, ASFV genotype II induced a slight shortening of an activated partial thromboplastin time (aPTT) throughout the experiment. Thrombin time (TT) was prolonged from day 5 onwards, whereas no changes in prothrombin time (PT) were found upon infection. The level of D-dimers was permanently higher than in control with a peak on day 3 post-infection. ASFV induced a significant decrease in the level of fibrinogen from day 5 till the end of experiment. Thus, it can be concluded that ASFV genotype II isolated in Armenia affects the hemostasis of infected pigs and causes changes that differ from that of other ASFV strains described previously.

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1. Introduction

African swine fever virus (ASFV) is the causative agent of African swine fever (ASF), a highly contagious hemorrhagic disease of pigs for which no effective vaccine is available. ASFV is a large, icosahedral DNA virus that is the only member of the *Asfarviridae* virus family (Takamatsu et al., 2011). As it infects argasid ticks of the genus *Ornithodoros*, ASFV is considered to be the only DNA virus transmitted by arthropods (Kleiboeker and Scoles, 2001). In domestic pigs the virus replicates predominantly in tissue macrophages and blood monocytes. Their massive infection plays a major role in the pathogenesis of ASF due to the release of cytokines, complement factors and metabolites (Gómez-Villamandos et al., 2013).

Depending on the virulence of the virus strain and host factors, ASFV infection of domestic pigs results in several disease forms, ranging from highly lethal with up to 100% mortality to subclinical. ASFV genotype II circulating in the Trans-Caucasus and Eastern Europe causes acute disease

* Corresponding author. Tel.: +374 94 202405; fax: +374 10 282061.
E-mail address: z_karalyan@mb.sci.am (Z. Karalyan).

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with high mortality rates (Chapman et al., 2011; Karalyan et al., 2012). Pigs infected with a highly virulent ASFV genotype II exhibit symptoms such as high fever, loss of appetite, severe depression, high respiratory and pulse rates, reddened skin, bloody diarrhea, and hemorrhagic lesions, which are the most frequently reported lesions in acute and subacute forms of ASF (Sánchez-Vizcaíno et al., 2009). Pigs usually die within 10 days of infection.

Hemorrhagic lesions in ASF are accompanied by impaired hemostasis and thrombocytopenia (Blome et al., 2013). These pathological changes have been well described for ASFV strains of different virulence (Edwards et al., 1984, 1985; Villeda et al., 1993; Rodriguez et al., 1996a; Perez et al., 1997). However, there is no study describing the hemostaseological status of pigs infected with highly virulent ASFV genotype II. Thus, in this study, the effect of ASFV genotype II on the hemostasis of infected pigs was investigated. To this means, platelet count changes and platelet size, as well as coagulation parameters were evaluated upon experimental infection.

2. Materials and methods

2.1. Animal experiment and viral stock

In our study, ten pigs (Landrace breed) of the same age (3-month-old) and weight (30–32 kg) were used for infection. All animals were clinically healthy at the beginning of the experiment. Animals were kept in separate stables, where they had access to a commercial feed twice per day and to clear water at all times. Infections were carried out using ASFV (genotype II) distributed in the Republic of Armenia and the Republic of Georgia (Rowlands et al., 2008). Ten pigs were infected by intramuscular injection. The titer of ASFV for each intramuscular injection was 10^4 50% hemadsorbing doses (HAD_{50})/ml. Virus titration was done as described previously and expressed as log10 HAD_{50}/ml for non-adapted cells (Enjuanes et al., 1976). From 1 to 7 days post-infection (dpi), the blood samples were taken from the ophthalmic venous sinus as described previously (Stier and Leucht, 1980). Pre-inoculation blood samples were collected from all pigs for control values. Animal experiments were approved by the Institutional Review Board/Independent Ethics Committee of the Institute of Molecular Biology of NAS RA (reference number IRB00004079).

2.2. Blood smears and Giemsa staining

Fresh blood was used in preparing the blood smears by a routine wedge method (Houwen, 2002). For platelet visualization, count and size measurements, slides were fixed in pure methanol for 10 min and stained by Giemsa modified solution (azure B/azure II, eosin and methylene blue) according to the manufacturer's protocol (Sigma–Aldrich, Germany).

2.3. Platelet evaluation

Whole blood platelet evaluation was done in Geimsa stained blood smears using a light microscopy. Platelet counting was performed by a method described previously with little modification (Malok et al., 2007). In brief, platelets were counted in 200 oil immersion fields (at 1250× magnification) and the average number of platelets was multiplied by 20,000 to yield a platelet count estimate per mm^3. The blood smears were scanned for platelet aggregates and 200 oil immersion fields (at 1250× magnification) were used for the estimation of an average number of aggregates per field, as well as platelets per aggregate. The image cytometry of platelet aggregates and single platelets were done using image analysis software (ImageJ, National Institutes of Health, MD, USA). For platelet size, at least 4×10^3 platelets were measured in each sample in a random sequence.

2.4. Coagulation tests

All coagulation tests were carried out using plasma obtained from citrated blood (3.8% sodium citrate as an anticoagulant at 9:1 volume ratio) by centrifugation at $1500 \times g$, for 10 min, at room temperature. Plasma isolates were stored in aliquots at −70 °C and thawed immediately before each experiment. Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), D-dimers, Antithrombin III (ATIII Activity) and fibrinogen levels were measured by COBAS INTEGRA 400 analytical system using commercially available diagnostic products and their instructions (CoaChrom Diagnostica, Austria).

2.5. Statistical analysis

Statistical tests were performed using SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA). The normality of the data was analyzed using the Kolmogorov–Smironov test. The results of platelet number and aggregated platelets were evaluated by Student's *t*-test. Mann–Whitney *U*-test was used to compare the results of coagulation tests between control and infected group, as well as to compare changes in platelet sizes. Pierson *r*-test was used to define a correlation between the virus titer and the number of platelets. All results are expressed as mean value ($n = 10$) ± standard deviation. Differences between control and infection were considered significant at the $P < 0.05$ level.

3. Results

3.1. Course of infection

Upon infection with ASFV genotype II, the first clinical signs were observed at 3 dpi, when all infected animals demonstrated loss of appetite and depression. From 3 to 4 dpi, the body temperature of infected animals rose above 41 °C. Simultaneously, difficulties in breathing, decreased activity in behavior and reddening of the skin were exhibited. Diarrhea became bloody only in two pigs. Lethargy was observed at 5–6 dpi, and therefore all infected animals were sacrificed at 7 dpi. Infection was confirmed in all pigs by virus titration. Although the first clinical signs of infection appeared at 3 dpi, viremia was observed after the 1st day of infection. The viral titer

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