PORCINE RESEARCH

Clinical and post-mortem investigations of genotype II induced African swine fever

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Abstract. The aim of this study was to analyze specialties of main clinical, biochemical and post-mortem characteristics during the ASF which is induced by intramuscular injection of ASF virus-genotype II (ASFV). We observed routine clinical, laboratorial and post-mortem characteristics of experimentally induced acute ASF. When ASFV is intramuscularly injected, the severity of the disease is likely to increase. Rapid development of clinical symptoms and neutropenia were observed. Also our data shown that onset of thrombocytopenia precedes or coincides with the appearance of fever and the start of viremia. The risk of emerging complications in kidneys, heart and lungs increases. The total bilirubin, direct bilirubin and indirect bilirubin all increased gradually, and the final measured values exceeded a 2.5-fold rise from the norm. The severity of this model of ASF also produced some unusual clinical and pathoanatomical characteristics, like observed for the first time haemarthrosis (most commonly affected joints are hip joint and knee), testicular haemorrhages (usually unilateral) and subdural hematomas.
Key Words: Acute ASF, experimental, bilirubinemia, haemarthrosis, hyperaemia, intramural haemorrhage, neutropenia.

Introduction. African swine fever (ASF) (Pestis africana suum, Montgomery's disease) is a highly contagious and fatal viral disease of animals in the pig family. ASF virus (ASFV) is an icosahedral double-stranded DNA virus that is presently the sole member of the family of Asfarviridae.

ASF affects only members of the pig family (Suidae). Species that can be infected include domesticated swine, European wild boar, warthog (*Phacochoerus africanus*), bush pigs (*Potamochoerus porcus*), giant forest hogs (*Hylochoerus meinertzhageni*) and peccaries (*Tayassu* spp.) (Mebus 1988; Sánchez Botija 1982). Usually some last species show no clinical signs of disease when infected with virulent isolates of ASFV that kill domestic pigs within 5-7 days of infection.

Clinical signs associated with ASFV infection are highly variable, depending on the virulence of the virus isolate and the breed of pig. The lesions observed in the acute ASF described as extensive haemorrhages in lymph nodes (mandibular, renal, and gastrohepatic), spleen, kidney, and sometimes in the heart. Other lesions are pethechiae in the mucous membrane of the urinary bladder, larynx, and pleura. Congestion of the liver, edematous lungs and fluid in the abdominal and thoracic cavities are frequently reported (Rodriguez et al 1996).

The entrance of ASFV into pigs is usually by oronasal routes. Other routes, such as cutaneous scarification, intramuscular, subcutaneous, intraperitoneal, or intravenously injections and tick bites have also been described (Colgrove et al 1968; Karalyan et al

2012). The incubation period varies widely (more often 3-19 days), depending on the ASFV isolate and the route of exposure.

The occurrence of ASF in new areas over the past five years has placed further emphasis on the permanent threat this disease represents to the world pig industry. Due to this increase the actuality of further laboratory studies of the ASFV. Although clinical and post-mortem characteristics of laboratorial and natural infections are slightly different. In our article we analyzed specialties of clinical, biochemical and post-mortem characteristics during the ASF which is induced by intramuscular injection of ASFV (genotype II) distributed in the Republic of Armenia (Rowlands et al 2008).

Material and Method

Virus. Infection was carried out using ASF virus (genotype II, Arm2007) which is distributed in the Republic of Armenia and the Republic of Georgia since 2007 (Rowlands et al 2008). The titre of ASFV for each intramuscular injection was 10^4 50% hemadsorbing doses (HAD50)/mL. Virus quantification was done by titration as described previously and expressed as log10 HAD50/mL for non-adapted cells (Enjuanes et al 1976).

Animals. In our study, eight healthy pigs of the same age (3-month-old) and weight (30 kg) were used for infection and five for control. Eight pigs were infected by intramuscular injection and five pigs (intramuscular injection of physiological solution – 1.0 mL) were used as the uninfected control. Animal care and euthanasia were done according to the AVMA Guidelines on Euthanasia, and local guideline for animal care and use (Institutional Review Board/ Independent Ethics Committee of the Institute of Molecular Biology of NAS, IRB00004079). Carbon dioxide inhalation (75-80% carbon dioxide for 20 min) was used for euthanasia of infected and control animals after 7 days post infection (dpi).

Blood smears, Giemsa staining. Peripheral blood was obtained from the ophthalmic venous sinus of either infected or uninfected pigs as described previously (Stier & Leucht 1980). For white blood cells analysis blood smears were used, slides were fixed in pure methanol and stained by Giemsa modified solution (azure B/azure II, eosin and methylene blue) according to the manufacturer's protocol (Sigma-Aldrich). White blood cells were examined under the light microscope at 1,250× magnification in a random sequence. At least 200 white blood cells in each sample were classified. The evaluation of cells based on morphologic characteristics was described previously (Karalyan et al 2012).

Biochemical studies. For clinical chemistry, whole blood without additives was allowed to clot at room temperature for several hours and centrifuged for 5 min at 1,500 rpm. Serum was transferred to clean tubes and analyzed promptly on a chemistry analyzer, according to the manufacturer's instructions. The following biochemical parameters were determined: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), total bilirubin (TB), indirect bilirubin (IB), direct bilirubin (DB).

Clinical and post-mortem observations. Clinical signs of infection were recorded every day. Pathological and anatomical characteristics were observed during routine post-mortem examinations.

Statistical analysis. Statistical analyses were performed using the Student's t-test. SPSS version 17.0 software package (SPSS Inc., Chicago, Ilinois) was used for statistical analyses.

Results and Discussion

Experimental infection. ASF experimental infection had been described earlier (Karalyan et al 2012). It was characterized by early viraemia from 1-2 dpi. Viraemia

peaked on 5 dpi (virus titers were 5.0-5.25 log10 HAD50/mL). The high titres of ASFV were determined in all pigs up to 7 dpi.

Clinical manifestations. Incubation period of acute ASF aroused by intramuscular injection of the virus is from 18 to 24 h. The first clinical signs were observed after 20-36 h post infection (HPI) when all infected pigs demonstrated loss of appetite and slight diarrhea. In some swine there is dyspnoea, vomiting, incoordination, and prostration.

There were (beginning 2-3 dpi) extensive petechial haemorrhages of the skin (size from 1-2 mm to 10 mm), especially over the ears, flanks, back, ventral areas of the thorax and abdomen (Figure 1). On skin single hemorrhages shows tendency to fuse, indicated on 3-5 dpi. Hemorrhages colour varies from dark pink to dark violet. The main clinical signs were observed after 24-48 h post infection.

Figure 1. Petechial hemorrhages of the skin over the ears at 4 dpi.

From 3 to 4 dpi, infected animals displayed hyperthermia simultaneously, decreased activity in behavior; difficulties in breathing and reddening of the skin were detected. As follow from Figure 2, body temperature (skin measured) usually ranged between 39°C and 41.1°C, in some animal increased up to 40-42°C (but generally about 39.1-39.7°C). Also in clinical signs were presented by anorexia, shallow and rapid respiration and depression. Vomiting (in rare cases), bloody diarrhea and ataxia were seen at 5-6 dpi, and therefore all infected animals were sacrificed according to guidelines at 7 dpi at terminal stage of disease. 2 pigs (25%) of 8 died earlier. Death of this pigs occurred within 5-6 days after intramuscular injection (within 3-4 days of onset of clinical signs).

Post-mortem examination. A post-mortem examination was performed on all eight pigs, which died or were euthanasied during the course of the infection. Well-known, poor-known and almost unknown post-mortem characteristics were observed at acute ASF aroused by intramuscular injection of the virus.
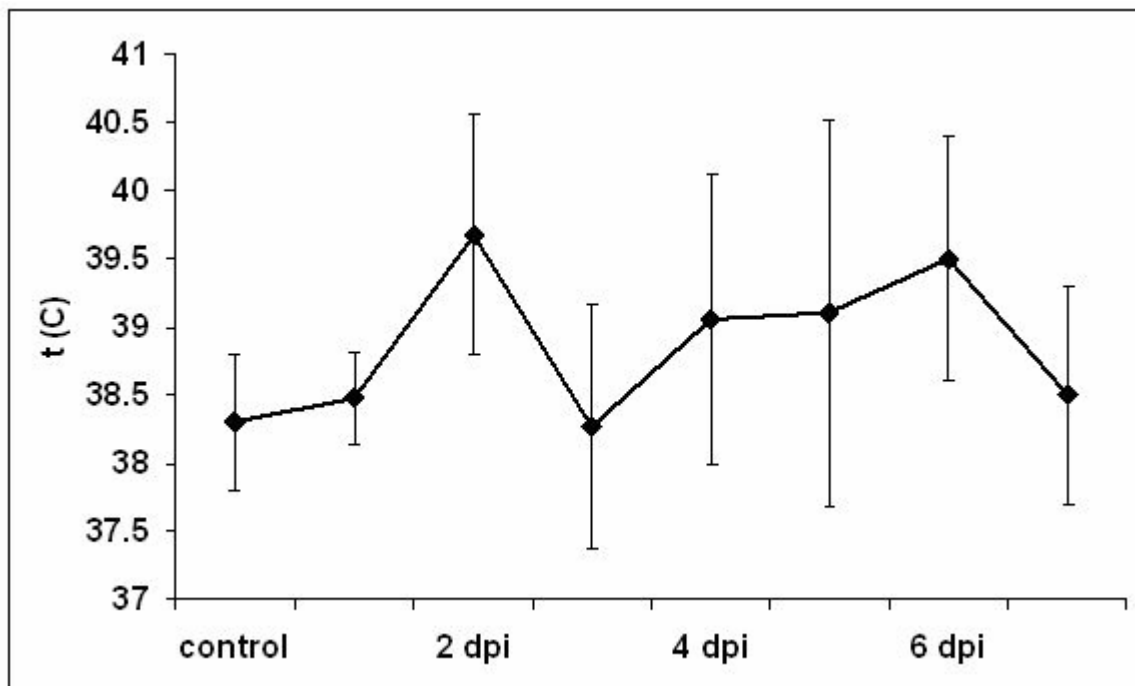

Figure 2. Temperature curve of acute African swine fever. The ordinate axis shows external temperature of body (°C). The abscissa axis shows control and days post infection.

Necropsy of the dead animals showed numerous hemorrhages on the pleural and peritoneal membranes and in organs of peritoneal and thoracic cavities. Post-mortem study also showed numerous hemorrhages on the peritoneum and mesentery (Figure 3A). Several lymph nodes are enlarged and friable. Histologically, these nodes show an advanced or final stage of the hemorrhagic infiltration.

The spleen of animals was plethoric, friable, dark-colored and remarkably enlarged (sometimes 2 to 3 times), splenic pulp flabby, soften, dark-red colored. At necropsy pigs had slightly enlarged plethoric livers. Lung was swollen and contained excess fluid and blood, with hemorrhages, sometimes observed oedema. Pathologic lesions in lung also included well-demarcated regions of hemorrhagic pulmonary infarction. Pulmonary infarctions more often observed in marginal lobs (Figure 3B).

The pleural cavities contain slightly turbid red or dark-red colored fluid usually measured at 100-150 mL (Figure 3C). Abdominal cavities often contain dark-red colored fluid usually measured at more high volumes.

Post-mortem examinations in numerous animals (50%) show hemorrhages in skeletal muscles. Intramuscular hemorrhages were found in the musculature of the extremities and in the cervical and laryngeal muscles also in the musculature of the neck and back (Figure 3D).

Kidneys enlarged, the sub-capsular surface of the kidney and the mucosa of the urinary bladder were petechiated (Figure 3E). Sometimes were observed confluent hemorrhages in kidneys. Kidney with confluent hemorrhages became bigger and dark-red colored (Figure 3F).

Heart often enlarged, in the myocardium, sometimes observed intramural hemorrhage and hyperaemia. The pericardial cavities often contain fluid yellowish (3 g), red or dark-red, it volume sometimes exceeds 50-100 mL. Hydropericardium and epi- to endocardial hemorrhages were described in majority of cases (Figure 3H).
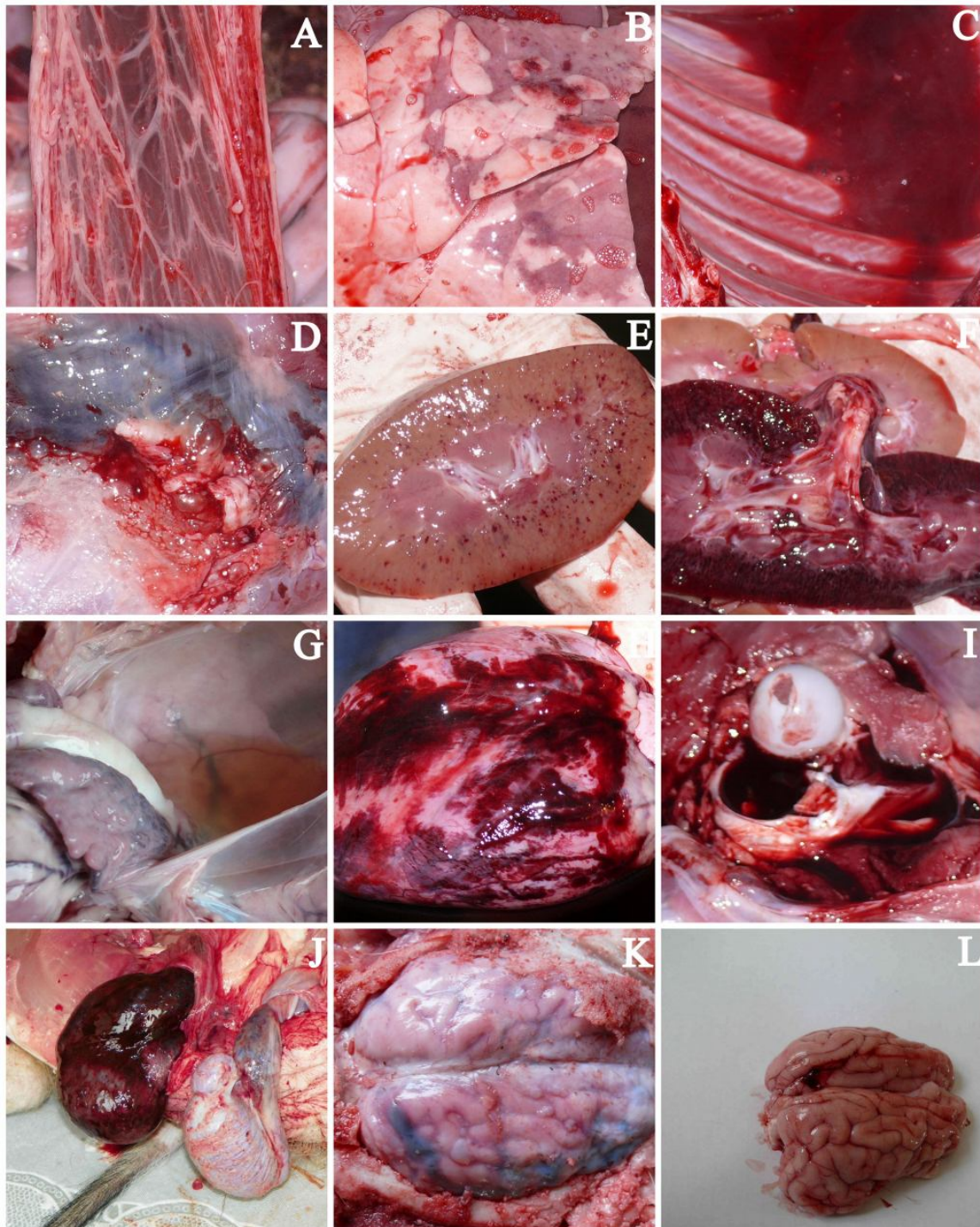
Figure 3. Post-mortem examination in acute ASF (gross autopsy study). A - Multiple hemorrhages in mesentery; B - Pulmonary infarction; C - Blood-stained pleural effusion; D - intramuscular hemorrhages; E - Petechiae in the renal pelvis and papillae; F – Confluent hemorrhage of a pig kidney; G - Pericardial effusion; H - hemorrhage in the pericardium; I – Haemarthrosis of hip joint; J - Unilateral testicular hemorrhage; K - Brain in the cranial case. Blood is visible through the intact dura mater; L - The dura mater has been removed from the brain.

We also detected haemarthrosis, especially in big joints. Blood could be readily identified in the joint cavity at about the 4-5th dpi. The blood when found in the joint was always fluid and only occasionally several small clots could be detected. The most commonly

affected joints are hip joint, knee, others (like elbow, and ankle) very rare (Figure 3I). Testicular infarction is an uncommon finding in veterinary practice, but it was described in acute ASF (Figure 3J). There was no change in the scrotal skin color. After necropsy was observed that the epididymis appeared to be incorporated in the mass.

Also necropsy examination in 25% cases showed lateral subdural hematomas. In the first photo, the dura mater is in place (Figure 3K). In the second photo, the dura mater removed (Figure 3L).

Changes in peripheral blood cells. Changes in white blood cells were observed previously by us (Karalyan et al 2012). Shortly, the peripheral white blood of uninfected pigs mainly consisted of lymphocytes, band and segmented neutrophils as well as monocytes (Karalyan et al 2012), nucleated red blood cells absent. Band-to-segmented neutrophils ratio (B:S ratio) was about 0.65 at 1 dpi (compared with 0.3 in control), B:S ratio became higher (3.5) at 4 dpi, which shows an increased percentage of band neutrophils in population. However, marked lymphopenia and neutropenia (mature forms), with 2.5-fold reduction of lymphocytes and neutrophils at 7 dpi, were detected from 2 to 3 dpi (Figure 4).
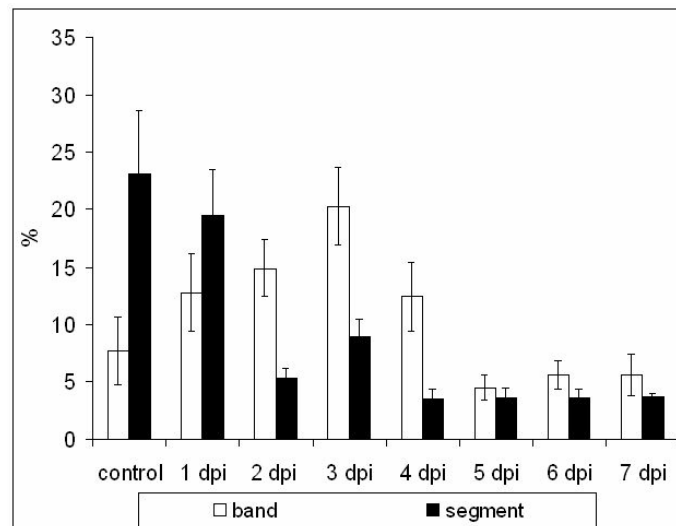

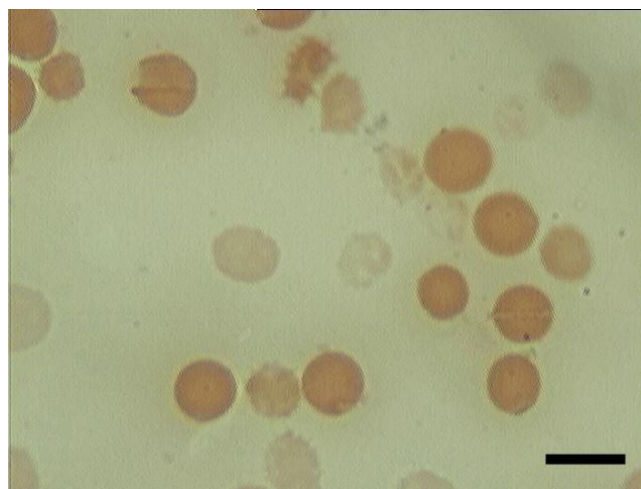Figure 4. Dynamics of band-to-segmented neutrophils ratio in pigs with African swine fever.

Figure 5. Erythrocyte ghosts, in erythrocytes from a pig at 7 dpi Wright-Giemsa stain cells were examined under the light microscope at 1,250× magnification. Scale bar: 10 μm.

Blood analysis at 6-7 dpi has been shown arising of 'ghost' erythrocytes (Figure 5).

Biochemical results. We measured the liver enzymes, bilirubin to determine liver injury. After intramuscular injection of ASFV, the values of aspartate aminotransferase began to increase significantly and reached a peak at 5-7 dpi with over 8-fold increases from the baseline (Figure 6C). Low creatine kinase activities in pigs with ASF have been observed beginning 2-3 dpi. There were no any significant changes in activity of purine nucleoside phosphorylase (Figure 6A).
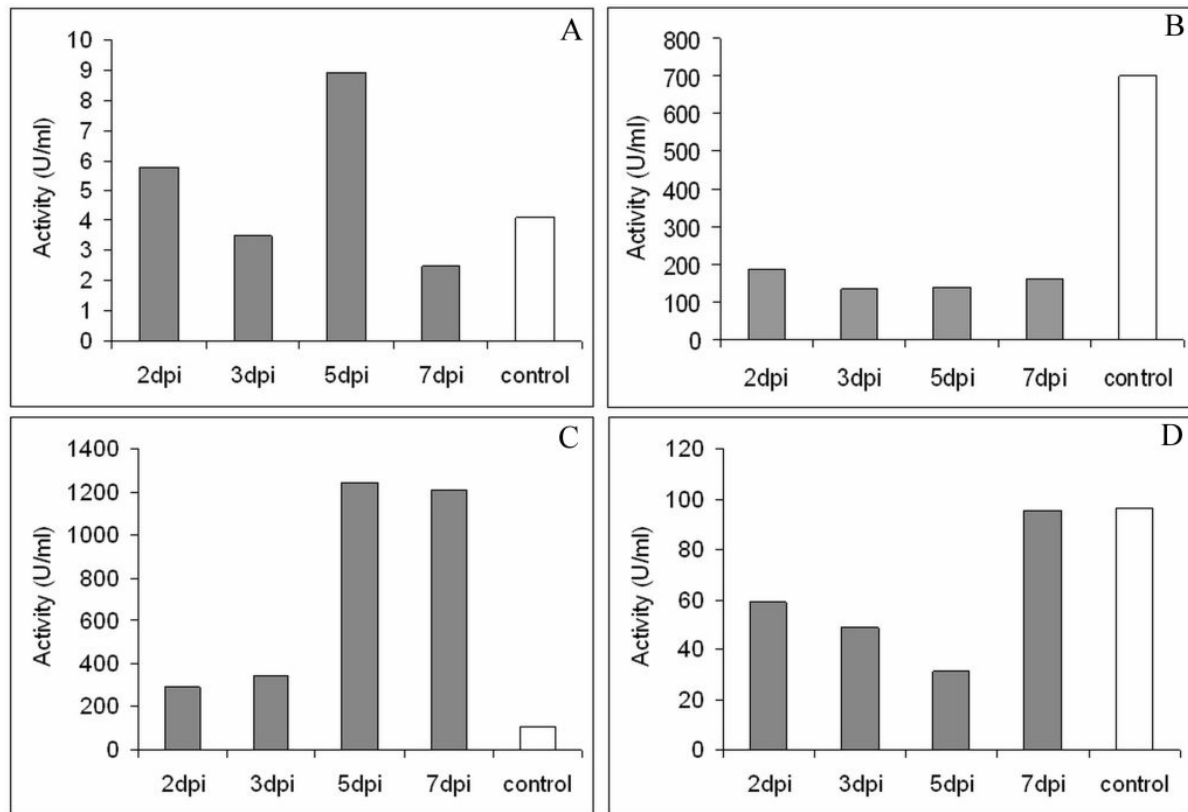

Figure 6. Blood biochemical parameters of the enzymatic activities in pigs infected with ASFV. The abscissa and ordinate represent measured time and activity, respectively. A - Purine nucleoside phosphorylase; B - Creatine kinase; C - Aspartate aminotransferase; D - Alanine aminotransferase. Gray histograms – infected pigs. White histograms – control.

The total bilirubin, direct bilirubin and indirect bilirubin all increased gradually, and the final measured values exceeded a 2.5-fold rise from the corresponding baseline values (Figure 7).

Clinical signs associated with ASFV infection are highly variable, depending on the virulence of the virus isolate and the breed of pig. Anorexia, cyanosis, and incoordination may be present one or two days before death (Karalyan et al 2012). In our experiments first symptom was anorexia at 2-3 dpi (detected earlier compared with literature data). Usually the incubation period is from 3 to 15 days, and clinical signs vary depending on the virulence of the strain (Colgrove et al 1969). The incubation period of acute ASF aroused by intramuscular injection of the virus is from 20 h to 34 h. Shortness of incubation period may be resulted by intramuscular injection of the virus.

The majority of above post mortem observations are in agreement generally with the clinical and pathological changes earlier described for ASF (Colgrove et al 1969; McVicar 1984; Murphy et al 1999). However, the haemarthroses, subdural hematomas and testicular haemorrhages described in the report haven't been observed earlier in ASF. Lesions in lymph nodes are well known and mainly can be distinguished as swelling

and hyperaemia, haemorrhagic infiltration, and dense infiltration with red blood corpuscles, indicated by the dark red colour of the lymph node parenchyma (Mebus 1988).
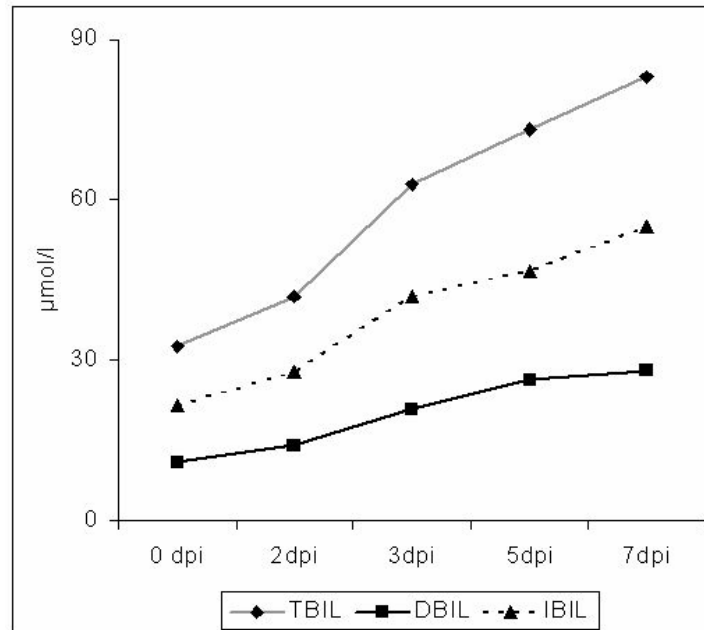

Figure 7. Blood biochemical parameters of the bilirubin levels (μmol/L) in pigs infected with ASFV. The abscissa and ordinate represent measured time and value, respectively.

Intramuscular hemorrhages are described rarer. There was no correlation between severity of clinical manifestation, length of disease before death and intensity or localization of the intramuscular hemorrhages.

It is important to mention that increase of lymphocytes starts from 1-2 dpi, but credible lymphopenia starts from 3 dpi. This coincides with some literature data. According to (Gómez-Villamandos et al 1997), early lymphopenia began as a result of apoptosis of lymphocytes and suppression of their proliferation.

Also we showed decrease of neutrophils, which is contrary to some literature dates. For example indicated neutropenia have never been shown previously (Gómez-Villamandos et al 1997). Authors reported neutrophilia as a result of rejuvenation of the neutrophil population. We also indicated increase of immature forms of neutrophils (band). This change in the population of neutrophiles is similar to the data of Wardley & Wilkinson (1977). Consequently we observed significant shift to left in neutrophil population, with arising of high level of metamielocytes at 3-5 dpi.

It is important to mention that in our experiments, the increase of neutrophil proliferation does not compensate their simultaneous lost. This occurred as result of uncompensated lost of segments. An ability of compensated replacement of neutrophiles takes place until 3-4 dpi (during successful compensatory process some increase of total level of neutrophils observed). Than begun decompensation, and neutropenia in porcine peripheral blood.

African swine fever (ASF) is a viral disease characterized by hemorrhages possibly associated with early intense thrombocytopenia. Our data shown that onset of thrombocytopenia precedes or coincides with the appearance of fever and the start of viremia. Over the years, a number of studies (Villeda et al 1993; Gómez-Villamandos et al 1998) have set out to identify the pathogenic mechanism giving rise to this thrombocytopenia; various hypotheses have been advanced, some of them clearly contradictory.

Subnormal activity of creatine kinase in serum has been observed in a variety of clinical conditions. Creatine kinase (CK), the central regulatory enzyme of energy

metabolism and its subnormal activity may be found as a consequence of diminished efflux of the muscle enzyme into serum from reduced physical activity caused by illness or advanced age or may result from reduced muscle mass accompanying muscle wasting or cachectic states. Low serum CK values also reported in acute viral hepatitis (Rosalki 1998). But more likely that low serum CK activity should be interpreted in our experiments with liver disorders and multiple organ failure developed in pigs at terminal stages of ASF (Rosalki 1998).

Serum liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), are commonly elevated at liver diseases and thus may reflect the status of liver injury. In cases of acute viral hepatitis, aminotransferase levels usually peak before jaundice appears and have a more gradual decrease thereafter, and there is a greater increase in serum bilirubin levels (Giannini et al 2005). This interpretation corresponded with our data in serum bilirubin measurement.

Increased levels of bilirubin can be associated with increased intravascular erythrocyte destruction. When intravascular haemolysis occurs, possible observation of erythrocyte ghosts (Harvey 2001). During circulation blood is filtered continuously through thin-walled splenic cords into the splenic sinusoids and normal erythrocyte can deform itself and pass through the openings in the splenic cords. Erythrocytes with structural alterations are phagocytized and destroyed by macrophages (Dhaliwal et al 2004). Involvement of spleen pathology in intravascular red blood cell destruction was described about 50 years ago. Subhiyah & Al-Hindawi (1967) assert from their own studies that "splenic destruction of red cells is an important, if not the main, factor in the haemolytic process".

Conclusions. When ASFV genotype II is intramuscularly injected, the severity of the disease is likely to increase. We observed rapid development of clinical symptoms and neutropenia also increases the risk of emerging complications in kidney, heart and lung. Severity of this model of ASF also produced some unusual clinical and pathoanatomical characteristics.

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