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African Swine Fever Virus Genome Detection Using Real Time Q PCR Polymerase Chain Reaction Method- Comparison of two Sample Specimen (Blood and Organs)

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Abstract. *The study aims to show the result of CT (threshold cycle) data collected over a three-year study conducted in the molecular biology laboratory of D.S.V.SA Constanta using the Real Time Polymerase Chain Reaction method to demonstrate the pathogenetic effect of African swine fever virus during the replication and vascular changes present in all affected organs.*

Keywords: Polymerase Chain Reaction (PCR), African Swine Fever virus

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1. Introduction

African Swine Fever (ASF) is a contagious viral disease of domestic pigs and wild boars. It is a notifiable disease to OIE (World Organization for Animal Health). Since Eustace Montgomery had first studied the virus in 1921 the reports had shown a pattern of significant increase of outbreaks around the globe. The virus is highly pathogenic with 100% mortality in pigs. There is no effective vaccine or treatment for this disease. In Europe many countries have reported the disease as a first occurrence to OIE through WAHIS (World Animal Health Informational System WAHIS) from 2016-2022: Moldova notified the disease in September 2016, Check Republic in June 2017, followed by Romania 2018, Hungary in April 2018, Bulgaria in August 2018, Slovakia in July 2019, and most recently Serbia in January 2020 and Greece in February 2020. Since January 2020, 8 countries have reported ASF as a first occurrence in the country, while 12 countries reported its spread to new zones (Hungary, Italy, Latvia, Moldova, and Romania). This highlights a continuous spread of the disease into new countries, and new zones in countries already affected. In Asia and the Pacific, China notified the presence of the disease for the first time in August 2018, Philippines in July 2019, Korea in September 2019 Indonesia in November 2019 (OIE World Organization for Animal Health[1]

African Swine Fever is a large icosahedral DNA virus which replicates predominantly in the cytoplasm of infected cells. The ASF double - stranded DNA genome varies in length from abought 170 to 193 kbp depending on the isolate and contains between 150-167 open readings frames (ORF)[2] .These are closely spaced and

read from both DNA strands. The virus genome termini are covalently closet by imperfectly based-paired hairpin loops that are present in tow forms that are complimentary and inverted with respect to each other. Head to head concatemeric genome replication intermediates have been described. A similar mechanism of replication to Poxviruses has been proposed for ASF. Virus genome transcription occurs independently of the host RNA polymerase II and virus particles contain all the enzyme and factors required for early transcription. DNA replication begins in perinuclear factory areas about 6h post- infection although an earlier stage of nuclear DNA synthesis has been reported. The virus genome encodes enzymes required for transcription and replication of the virus genome and virion structural proteins. Enzymes that are involved in a base excision repair pathway may be an adaptation to enable virus replication in the oxidative environment of the macrophage cytoplasm. Variation between the genomes of different ASF isolates is most commonly due to gain or loss of members of multigene families, MGFs 100,110,300,360,505/530 and family p22. These are located in the left terminal 40kbp and right terminal 20kbp[3].

2. Genome structure

The ASF genome is a linear double – stranded DNA molecule. The gene families encoded are named according to the average number of amino acids in in the proteins encoded by each family, the direction they are read in and position in that family from left genome end. In addition, related genes encoding an early membrane protein, p22, which is close to the left genome terminus are present in some isolates in 1 or 2 copies close to the right genome end. The gene encoded by the genome are closely spaced and encoded on both DNA strands with no clear bias for coding of genes on either strand across the complete genome [1].

3. Entry mechanism of African Swine Fever

Is a complex and dynamic interaction with the host cell's that determine the virus tropism and pathogenesis. The clinical evolution of the disease can by acute form characterized by the development of ASF over 7- day period compared with 10-20 days for subacute from of the disease. Chronic ASF causes clinical lesions that are not specific and at this stage the infection is detected by serological screening for ASF- virus antibodies [4].

In the affected pig's monocytes and macrophages appear to be the main ASF virus target cell for replication shown using immunohistochemical techniques revealing the nucleic changes and ASF replication sites and later are evident as rounded organelle free area containing elongated membranous structures and both mature and immature viral particles. Mature particles are seen as icosahedral structures (175-190nm in diameter). Mature viral particles are associated with ribosomes in the viral replication site. Virus replicates mainly in mononuclear phagocytic cell in tonsils and mandibular and other lymph nodes spreading through lymph and blood to secondary organs replication (in macrophages) where it may be detected within 2-3 dpi, but in the middle and final phase of acute ASF (5-7 dpi) evidence of virus replication is found in non-monocytes-macrophage cell, epithelial and/or mesenchymal cell. Viral particles, but not virus replication, have been observed in lymphocytes and platelets [5].These elements act as

passive vehicles for spared of the virus through the body similar to the function of the erythrocytes.

3.1. Macrophage activation

Research suggests that despite virus replication in macrophages and their subsequent necrosis during ASF there is an increase in macrophage number in all organs in which virus replication take's place. This increase is associated with enhance macrophage secretory activity and release of cytokines including (TNF-α; IL-1α; IL-1b;IL-6;IL-8) that has a pro-inflammatory, pro-coagulant and pro- apoptotic profile that leads to pulmonary oedema, hemorrhage more common in organs not containing a fixed vascular macrophage population, particularly gastro-hepatic and renal lympho-nodes, but they may also occur in other organs including the intestine and the heart(although these contain fixed macrophages.

3.2. Endothelial damage

As cause of hemorrhages, was initially attributed to direct action of the virus on endothelial cell. However, ultrastructural examination has shown that when bleeding is first observed in the lymph nodes and kidney of pigs infected with virulent ASF strains, there is no evidence of infection or virus replication in the endothelial cell of these organ. However, coincides with another phenomenon which may account for the endothelial lesions, phagocytic activation of capillary endothelial cells, accompanied by lysosome proliferation and the presence of phagocytosed cell debris, give raise to endothelial hypertrophy which may lead to complete occlusion of some capillary lumina, increasing intravascular pressure and this leads to endothelial disruption, cell debris and erythrocytes appearing in the interstitium give raise to hemorrhages. This phenomenon coupled with the necrosis of splenic cord macrophages prompts activation of the coagulation system [4].

4. Experimental

My research study began with the collection and preparing of the specimen received in the laboratory based on the national legislation Ordin Nr 35 on 30th March 2016, published by National Veterinary and Food Safety Authority[6]

The extraction phase performed as follows:

Organ specimens are prepared using MagNa Lyser equipment and after preparing 10%organ suspension in PBS pH 7.2- 7.4 (by transferring 0.1g of sample to a special tube Green Beads containing ceramic pearls in 900µl PBS. After starting the device, the rotation speed is set at 30 seconds per 6000 rpm. The lysis process with the help of the MagNA Lyser is based on the collision of the ceramic beads with the sample, followed by centrifugation to express the supernatant.

Blood samples on anticoagulant (EDTA) is processed as follows: shake gently the vacutainers in which the blood samples were collected and transfer about 1ml of the sample into Eppendorf tubes (avoid any clots formed).

The samples thus obtained will be kept at -20ºC for a short time (until entering the work) and at -37 ºC for longer periods of time (6 months or more).

Positive controls, represented by internal reference material (viral strain), strain of the thermally inactivated ASF virus Ba71VR, standard received from the National Reference

Laboratory African Swine Fever at Diagnostic Institute of Animal Health (IDSA). They are stored in a saucerised high temperature freezer - 80ºC.

4.1. The extraction and purification phases performed as follows:

The 200µl samples are transfer to 2ml Eppendorf tube and lysed under highly denaturing condition at room temperature (15°C - 25°C) in the presence of Proteinase K and Buffer VXL, which together ensure the inactivation of nucleases. Adding Buffer ACB adjusts the biding condition for the copurification of DNA. The lysate is transferred to an IndiSpin Column. During centrifugation, nucleic acids is absorbed onto the silica membranes while contaminants pass through. Tow efficient wash step remove the remaining contaminants and enzyme inhibitors. Finally nucleic acids is eluted in Buffer AVE.

4.2. The preparation of Master Mix and DNA mixing:

The amplification kit allows the realization of a fast, specific, and sensitive PCR test by using Tag Man enzyme. Reagents necessary for the preparation of the denaturing mix (Mater Mix SSO Advance Bio Rad and specific ASF virus Primary and Probe) stored in the freezer. All reagents removed from the freezer and stored on ice until use. Pipetted into PCR plaques 15µl reaction mixture and 5µl DNA. The pipetting takes place on the ice.

4.3. Ensuring the validity of the results

In each PCR experiment, four controls will be included: two positive (positive extraction control and positive coupling control) and two negatives (negative extraction control and no templet control). The positive extraction control is represented by the internal reference material (viral strain) strain characterized by the National Reference Laboratory for African Swine Fever within the IDSA, and the positive control of coupling is represented by a previously extracted positive DNA. The negative extraction and no templet controls are represented by water, the sample that does not contain biological material.

It is considered validated the test in which the positive test/samples and the positive controls are positive, and the negative test/s and the negative controls are negative.

4.4. Analysation of amplification phases

Using the Applied Biosystems 7900HT Fast Real-Time PCR system uses fluorescent-based PCR chemistries to provide quantitative detection of nucleic acid sequences using real-time analysis.

DNA polymerase is an essential component for PCR because of its key role in synthesizing new DNA strands. Because of the sensitive and specific nature of PCR, it is important to choose high-quality enzymes and reagents to produce optimal results. Since thermal cycling is a key feature of the conditions that enable the repetitive chain reaction of amplifying DNA, thermostability of the DNA polymerase to be used is also an important feature. Highly thermostable DNA polymerases are recommended for amplifying GC-rich or long templates that often require prolonged high-temperature reactions.

5. The principle of PCR- Polymerase Chain Reaction

The polymerase chain reaction is carried out in a reaction mixture which comprises the DNA extract (template DNA), Taq polymerase, the primers, and the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution. The tubes containing the mixture reaction are subjected to repetitive temperature cycles 40 of times in the heating block of a thermal cycler Applied Biosystems 7900HT Fast Real-Time PCR system which has an enclosure where the sample tubes are deposited and in which the temperature can vary, very quickly and precisely, from 0 to 100°C by Peltier effect). The apparatus allows the programming of the duration and the succession of the cycles of temperature steps. The process of the PCR is subdivided into three stages as follows:

5.1. Denaturation

It is the separation of the two strands of DNA, obtained by raising the temperature. The first period is carried out at a temperature of 95°C for 3 minutes, called the denaturation temperature. At this temperature, the matrix DNA, which serves as matrix during the replication, is denatured: the hydrogen bonds cannot be maintained at a temperature higher than 80°C and the double-stranded DNA is denatured into single-stranded DNA (single-stranded DNA).

5.2. Aliment of primers

It is carried out at a temperature called primer hybridization temperature. Decreasing the temperature allows the hydrogen bonds to reform and thus the complementary strands to hybridize. The primers, short single-strand sequences complementary to regions that flank the DNA to be amplified, hybridize more easily than long strand matrix DNA. The higher the hybridization temperature, the more selective the hybridization, the more specific it is.

5.2. Elongation

The third period is carried out at a temperature of 60°C, called elongation temperature. It is the synthesis of the complementary strand. Taq polymerase binds to primed single-stranded DNAs and catalyses replication using the deoxyribonucleoside triphosphates present in the reaction mixture. The regions of the template DNA downstream of the primers are thus selectively synthesized. In the next cycle, the fragments synthesized in the previous cycle are in turn matrix and after a few cycles, the predominant species corresponds to the DNA sequence between the regions where the primers hybridize. It takes 40 cycles to synthesize the DNA present.

To address the need for a quantification, Real Time PCR techniques have been developed in which the amount of DNA is quantified after each cycle with the help of fluorochromes and the increasing signal yield fluorescent it is directly proportional to the number of molecules generated of the PCR amplification product called amplicon. The data collected during the exponential phase of the reaction provides information on the quantified efficiency of the beginning amount of the amplified hold. Fluorescence exchanges along the reaction are measured by combining thermal cycles with fluorochrome scanning. Through the graphic representation of the fluorescence relative to the number of cycles and thus the Applied Biosystems 7900HT Fast Real-Time PCR system device generates an amplification graphic that represents the accumulation of products on the duration of a complete Run PCR.

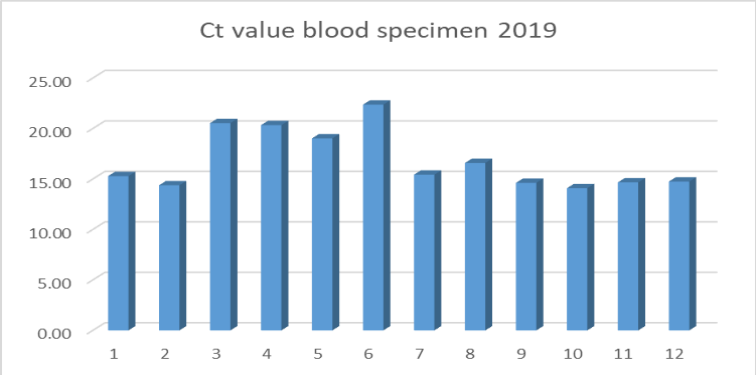
5.4. CT value

It is that cycle in which the florescent signal of the reaction crosses the threshold line The CT value is invertible in relation to the starting amount of the target AND in conclusion the amount and subtracts the CT value will grow.

Considering the CT values obtained while analysing the positive samples bellow

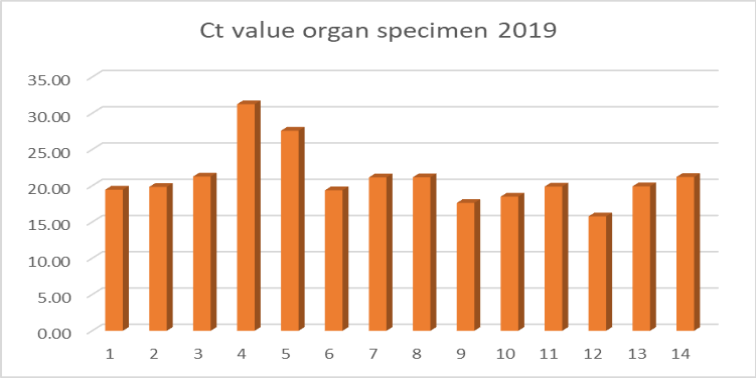
2019						
Swine	**Total probs analyzed**	**Blood specimen**	**Positive blood specimen**	**Organ specimen**	**Positive organ specimen**	**Total positive result**
Domestic	**1337**	**351**	**12**	**986**	**8**	**20**
Wild boar	**224**	**6**	**0**	**218**	**6**	**6**

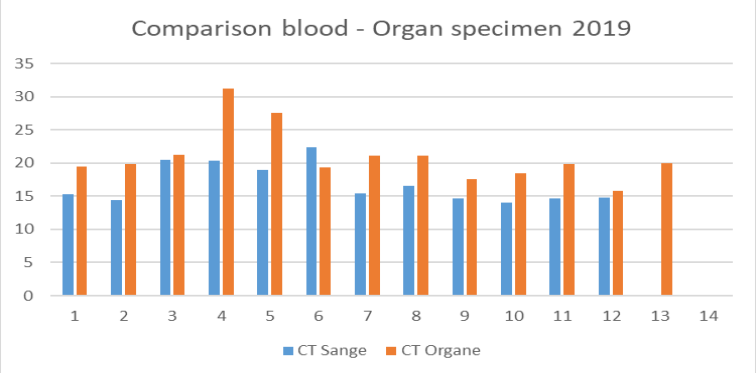
2019		
Specimen analysed	**Number of samples**	**CT values**
Blood	**1**	**15.29**
Blood	**2**	**14.38**
Blood	**3**	**20.54**
Blood	**4**	**20.34**
Blood	**5**	**19.03**
Blood	**6**	**22.37**
Blood	**7**	**15.43**
Blood	**8**	**16.59**
Blood	**9**	**14.62**
Blood	**10**	**14.09**
Blood	**11**	**14.67**
Blood	**12**	**14.58**



2019		
Specimen analysed	**Number of samples**	**CT values**
Organ	**1**	**19.47**
Organ	**2**	**19.85**
Organ	**3**	**21.28**
Organ	**4**	**19.52**
Organ	**5**	**31.26**

Organ	**6**	**27.60**
Organ	**7**	**19.39**
Organ	**8**	**21.16**
Organ	**9**	**21.18**
Organ	**10**	**17.64**
Organ	**11**	**18.51**
Organ	**12**	**19.90**
Organ	**13**	**15.79**
Organ	**14**	**19.93**



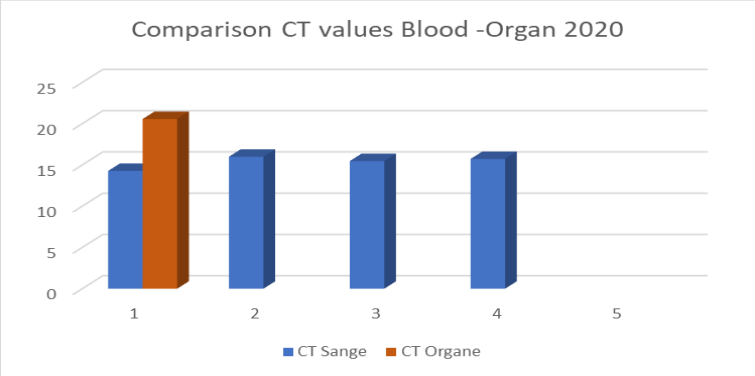


2020						
Swine	**Total probs analyzed**	**Blood specimen**	**Positive blood specimen**	**Organ specimen**	**Positive organ specimen**	**Total positive result**
Domestic	**1012**	**485**	**4**	**527**	**0**	**4**
Wild boar	**156**	**0**	**0**	**156**	**1**	**1**
2020						

African Swine fever Virus Genome detection using real time q PCR Polymeras chain Reaction Method-comparison of two sample Specimen (Blood and Organs)

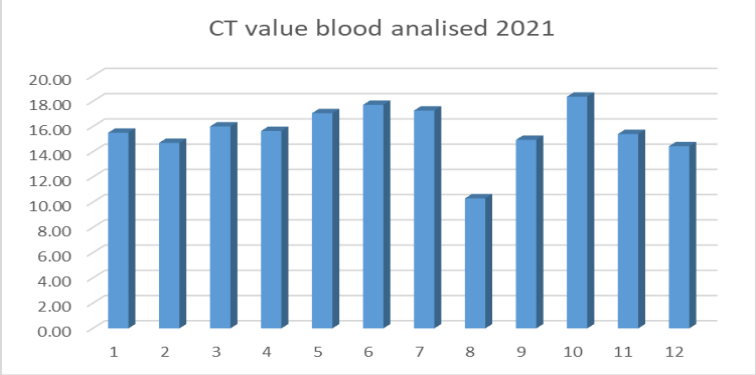
Specimen analysed	Number of samples	CT values
Blood	**1**	**14.28**
Blood	**2**	**16.00**
Blood	**3**	**35.47**
Blood	**4**	**15.73**

2020		
Specimen analysed	**Number of samples**	**CT values**
Organ	**1**	**20.57**

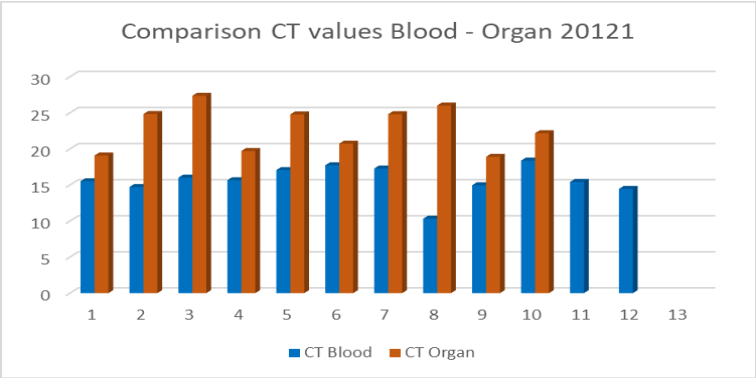


2021						
Swine	**Total probs analyzed**	**Blood specimen**	**Positive blood specimen**	**Organ specimen**	**Positive organ specimen**	**Total positive result**
Domestic	**1031**	**511**	**13**	**520**	**8**	**21**
Wild boar	**96**	**0**	**0**	**96**	**0**	**0**

2021		
Specimen analysed	**Number of samples**	**CT values**
Blood	**1**	**15.51**
Blood	**2**	**14.71**
Blood	**3**	**16.01**
Blood	**4**	**15.65**
Blood	**5**	**17.06**
Blood	**6**	**17.72**
Blood	**7**	**17.27**
Blood	**8**	**10.31**
Blood	**9**	**14.95**
Blood	**10**	**18.37**
Blood	**11**	**15.41**
Blood	**12**	**14.44**
Blood	**13**	**12.35**



2021		
Specimen analysed	**Number of samples**	**CT values**
Organ	**1**	**19.08**
Organ	**2**	**24.83**
Organ	**3**	**27.36**
Organ	**4**	**19.71**
Organ	**5**	**24.78**
Organ	**6**	**20.72**
Organ	**7**	**24.81**
Organ	**8**	**26.01**



6. Conclusions

- The analysis of CT values to conduct a comparison between blood samples on the EDTA and those of organs (spleen, kidneys, lympho-nodes). The study indicated that the moment of amplification of the target genomic sequence occurs differently during the amplification reaction depending on the different matrices used in the experiment. This finding was made by comparing the CT (Threshold Cycle) values obtained through the Real Time PCR amplification program for a period of 3 years (2019 to 2021).

- My package of records includes a population consisting of wild boars and domestic pigs and the CT values obtained for the blood matrix and respective organs allows me to compare the average CT values for blood matrix is 15.72 compared to the average CT values for organ matrix is 24.33. I can conclude that following the comparison made between the two matrices we can say that in the blood the amount of virus is higher than in the organs. Based on scientific literature the viral replication take's place in macrophage and monocytes but also in other endothelial cells producing cellular apoptosis that activates proinflammatory cytoleukins. And in the subacute phase of evolution, haemorrhage is caused mainly due to the increase in vascular permeability. These findings show that the EDTA blood matrix has a higher specificity for detecting the viral genome, using Real Time PCR technic, both in the early stages of the disease and in the late stages of evolution.

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