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SUBDIRECCIÓN GENERAL DE
INVESTIGACIÓN Y TECNOLOGÍA
CENTRO DE INVESTIGACIÓN
EN SANIDAD ANIMAL

Preliminary African Swine Fever (ASF) diagnosis and molecular characterization report

REPORT ISSUED BY THE EUROPEAN UNION REFERENCE LABORATORY (EURL-ASF), INIA-CISA

DATE: August 29th, 2019.

ARRIVAL DATE TO CISA: August 19th, 2019

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Num. ARRIVAL REGISTER CISA: REG. 156/2019./ **Num. Output REGISTER:** REG. 413/2019.

TEST REQUESTED: AFRICAN SWINE FEVER (ASF) CONFIRMATORY DIAGNOSIS

Num. SAMPLES RECEIVED: 4 tissues from **4 wild boars** and **9 tissue and 9 blood samples** from **9 domestic pigs (table 1)**:

Table 1 → Identification of the samples TESTED

ID CISA	SAMPLE IDENTIFICATION RECEIVED FROM THE BULGARIA'S NRL*				
	Laboratoy code/Date of testing	*Type of sample*	*Species*	*Type of farm/Owner*	*Region, village*
1.1	715ASF/ 03/07/2019	SPLEEN	DOMESTIC PIG	Backyard; Emil Jelyzakov	Pleven, v. Jernov
1.2	715ASF/ 03/07/2019	BLOOD	DOMESTIC PIG	Backyard; Emil Jelyzakov	Pleven, v. Jernov
2.1	763ASF/ 11/07/2019	SPLEEN	DOMESTIC PIG	Backyard;Krasimir Batilov	Burgas, v. Zidarovo
2.2	763ASF/ 11/07/2019	BLOOD	DOMESTIC PIG	Backyard;Krasimir Batilov	Burgas, v. Zidarovo
3.1	934ASF/ 19/07/2019	SPLEEN	DOMESTIC PIG	Industrial farm "Nikolovo"	Ruse,v Nikolovo
3.2	934ASF/ 19/07/2019	BLOOD	DOMESTIC PIG	Industrial farm "Nikolovo"	Ruse,v Nikolovo
4.1	969ASF/ 23/07/2019	SPLEEN	DOMESTIC PIG	Industrial farm "Brashlen"	Ruse,v Brashlem
4.2	969ASF/ 23/07/2019	BLOOD	DOMESTIC PIG	Industrial farm "Brashlen"	Ruse,v Brashlem
5.1	998ASF/ 25/07/2019	SPLEEN	DOMESTIC PIG	Industrial farm "Golyamo Vranovo"	Ruse,v Golyamo Vranovo

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ID CISA	SAMPLE IDENTIFICATION RECEIVED FROM THE BULGARIA´S NRL*				
	Laboratoy code/Date of testing	*Type of sample*	*Species*	*TYPE OF FARM/oWNER*	*Rgion, village*
5.2	998ASF/ 25/07/2019	BLOOD	DOMESTIC PIG	Industrial farm "Golyamo Vranovo"	Ruse,v Golyamo Vranovo
6.1	1018ASF/ 29/07/2019	SPLEEN	DOMESTIC PIG	Backyard; Natasha Petrova	Vidin,t. Vidin
6.2	1018ASF/ 29/07/2019	BLOOD	DOMESTIC PIG	Backyard; Natasha Petrova	Vidin,t. Vidin
7.1	1030ASF/ 29/07/2019	SPLEEN	DOMESTIC PIG	Industrial farm "Ekoproduct"	Silistra,v Popina
7.2	1030ASF/ 29/07/2019	BLOOD	DOMESTIC PIG	Industrial farm "Ekoproduct"	Silistra,v Popina
8.1	1053ASF/ 30/07/2019	SPLEEN	DOMESTIC PIG	Industrial farm "Bilyana"	Veliko Tarnovo,v B. Slivovo
8.2	1053ASF/ 30/07/2019	BLOOD	DOMESTIC PIG	Industrial farm "Bilyana"	Veliko Tarnovo,v B. Slivovo
9	1084ASF/ 31/07/2019	SPLEEN	WILD BOAR	Game farm; "Renta Grup Ekohotl Zdravetz"	Plovdiv,t Plovdiv
10.1	1112ASF/ 31/07/2019	SPLEEN	DOMESTIC PIG	Industrial farm "Ekoproduct"	Silistra,v. Vetren
10.2	1112ASF/ 31/07/2019	BLOOD	DOMESTIC PIG	Industrial farm "Ekoproduct"	Silistra,v. Vetren
11	1186ASF/ 05/08/2019	SPLEEN	WILD BOAR	State Hunting Farm "Iskar"	Sofia,t. Samokov
12	1249ASF/ 08/08/2019	SPLEEN	WILD BOAR	State Hunting Farm "Varbitsa"	Shumen,v Varbitsa
13	1266ASF /08/08/2019	SPLEEN	WILD BOAR	State Forest Farm "Voden Iri Hisar"	Razgrad,v. Voden

**INIA-CISA is not responsible for the identification/information of the samples provided by the client.*

EXECUTION DATE: From 19th August to 28th August 2019.

ASF DIAGNOSTIC TESTS PERFORMED:

1. ***ASFV genome detection.*** 10% (w/v) clarified homogenized tissue suspensions have been prepared in phosphate-buffered saline [PNT/CISA/PPA/MUESTRAS/1]. The DNA has been extracted from the tissue homogenates and blood samples using the High Pure PCR Template Preparation Kit [Ref. 11796828001 (ROCHE)] following the standardised procedure [PNT/CISA/PPA/EXTRACCIÓN ADN/1]. For amplification of the ASFV genomic DNA the UPL real-time PCR (Fernández-Pinero *et al.*, 2013) [PNT/CISA/PPA/PCR/3] has been carried out in the undiluted and diluted 1/10 extracted DNA.

2. ***ASF virus isolation and haemadsorption (HAD) assay*** [PNT/CISA/PPA/VI/1] has been done on porcine blood monocytes (PBM) according is described in the OIE Manual (OIE 2019). The PBM has been inoculated at a multiplicity of infection (moi) 1:10 with the 13 PCR positive tissues (8 wells/per sample; 10 µl inoculum per well). Samples have been filtered and treated with antibiotic (gentamicin sulphate) [PNT/CISA/PPA/MUESTRAS/1]. After inoculation, a preparation of 1% homologous red

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blood cells in phosphate-buffered saline has been added to each well and incubated at 95% relative humidity with 5% CO_2, at 37°C. The plate has been examined for haemadsorption over a period of seven days. This technique is ongoing.

3. ***ASF serological diagnosis*** → For ASF antibody detection the blood samples and the tissue exudates have been tested using the indirect immunoperoxidase technique (IPT) [PNT/CISA/PPA/IPT/1] in two fold dilutions starting from 1/20 in blood and 1/5 in tissue exudates samples.

4. ***ASFV molecular characterization***→ Genetic characterization of ASFV has been performed from the 13 spleen samples by PCR throughout the analysis of three variable regions of the ASFV genome which comprises: i) the intergenic region located between the *I73R* and *I329L* genes and characterized by the presence of tandem repeat sequences (TRS) (Gallardo *et* al., 2014), ii) the central variable region (CVR) within the *B602L*-gene (Gallardo *et al.*, 2011) and iii) the intergenic region between the multigene family (MGF) 505 9R and the 10R genes of ASFV genome (Elsukova, A., et al., 2016). Sequence analysis is ongoing.

   For easy differentiation and on the basis on the PCR-size variation, ASFV genotype II European isolates representatives of the different CVR, IGR, and MGF circulating variants have been included as controls (Gallardo et al., 2018).

RESULTS

WILD BOAR

1. **ASF virus detection** → a positive result has been obtained by the UPL real-time PCR in the 4 wild boar samples received. After seven days (first passage) the ASFV has been isolated, showing the haemadsorbing pattern, in 3 out of the four PCR positive samples received. Further passages on the remaining negative sample are ongoing. The results obtained in virus detection are summarized in the **table 2**.

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Table 2 → ASF virus detection in wild boar samples

Sample ID CISA	Sample laboratory code at the Bulgaria´s NRL			UPL-real time PCR [a]				VI-HAD [b] (1 passage)
	ID tube	ORIGIN	Type of sample	1st PCR CT value	2nd PCR CT value	2nd PCR CT value	Result	
9	1084ASF	Plovdiv,t Plovdiv	SPLEEN	17.53	NT	NT	POSITIVE	POSITIVE
11	1186ASF	Sofia,t. Samokov	SPLEEN	16.61	NT	NT	POSITIVE	NEGATIVE
12	1249ASF	Shumen,v Varbitsa	SPLEEN	16.86	NT	NT	POSITIVE	POSITIVE
13	1266ASF	Razgrad,v. Voden	SPLEEN	15.44	NT	NT	POSITIVE	POSITIVE

NT = not tested

*(a) **UPL-real time PCR** → Real time PCR test described by Fernández-Pinero et al.2013 based on the Universal Probe Library (UPL).*

PNT/CISA/PPA/PCR/3]

*(b) **VI-HAD** → Virus isolation and haemadsorption test on PBM cells as is described in the OIE Manual of diagnosis for ASF (Chapter 2.8.1. OIE seventh edition 2019).*

2. **ASF antibody detection by IPT**→ **the presence of antibodies has been confirmed by IPT in 3 out of the 4 WB samples.** The results obtained in ASF antibody detection are summarized in **table 3.**

Table 3 → ASFV antibody detection by IPT in wild boar samples

Sample ID CISA	Sample laboratory code at the Bulgaria´s NRL			EURL-IPT [a]		CONCLUSION
	ID tube	ORIGIN	Type of sample	Titer	Result	
9	1084ASF	Plovdiv,t Plovdiv	SPLEEN	1/10	WEAK	WEAK
11	1186ASF	Sofia,t. Samokov	SPLEEN	1/40	WEAK	WEAK
12	1249ASF	Shumen,v Varbitsa	SPLEEN	NEG	NEG	NEGATIVE
13	1266ASF	Razgrad,v. Voden	SPLEEN	1/20	WEAK	WEAK

WEAK= IPT Titre <1/80;

*A) **URL-IPT→ Indirect immunoperoxidase technique** on E70-MS infected cells using the protocol standardized and validated by the European Union Reference Laboratory (EURL). [PNT/CISA/PPA/IPT/1]*

3. **ASFV molecular characterization**→ Genetic characterization of the ASFV has been initially done by PCR from **the 4 PCR positive wild boar samples** analysing three independent regions of the ASFV genome. **On the basis of different-sized PCR products**, the CVR within the *B602L* gene, the intergenic region between the *I73R* and *I329L* genes (IGR $_{I73R\text{ - }I329L}$) and the intergenic region between the MGF 505 9R and the 10R genes (MGF) have been amplified. All samples yielded an amplicon of ≈400 bp for the CVR, ≈367-bp for the IGR $_{I73R\text{ - }I329L}$ and ≈489-bp for the MGF which corresponds in size to the European genotype II ASFV isolates belonging to the CVR-1 variant, IGR-2 variant and MGF-1 variant. **Sequence analysis is ongoing to confirm these results.**

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DOMESTIC PIGS

1. **ASF virus detection** → a positive result has been obtained by the UPL real-time PCR in **all domestic pig samples received.** After seven days (first passage) the **ASFV has been isolated,** showing the haemadsorbing pattern, in **7 out of the 9 spleen samples tested.** Further passages on the remaining negative spleen samples are ongoing. The results obtained in virus detection are summarized in the **table 4.**

Table 4 → ASFV virus detection in domestic pig samples

Sample ID CISA	Sample laboratory code at the Bulgaria's NRL			UPL-real time PCR [a]				VI-HAD[b] (1 passage)
	ID tube	ORIGIN	Type of sample	1st PCR CT value	2nd PCR CT value	2nd PCR CT value	Result	
1.1	715ASF	Pleven, v. Jernov	SPLEEN	19,25	NT	NT	POSITIVE	POSITIVE
1.2	715ASF	Pleven, v. Jernov	BLOOD	18,57	NT	NT	POSITIVE	NT
2.1	763ASF	Burgas, v. Zidarovo	SPLEEN	19,95	NT	NT	POSITIVE	POSITIVE
2.2	763ASF	Burgas, v. Zidarovo	BLOOD	17,75	NT	NT	POSITIVE	NT
3.1	934ASF	Ruse,v Nikolovo	SPLEEN	16,66	NT	NT	POSITIVE	NEGATIVE
3.2	934ASF	Ruse,v Nikolovo	BLOOD	20,17	NT	NT	POSITIVE	NT
4.1	969ASF	Ruse,v Brashlem	SPLEEN	18,42	NT	NT	POSITIVE	POSITIVE
4.2	969ASF	Ruse,v Brashlem	BLOOD	18,19	NT	NT	POSITIVE	NT
5.1	998ASF	Ruse,v Golyamo Vranovo	SPLEEN	16,61	NT	NT	POSITIVE	POSITIVE
5.2	998ASF	Ruse,v Golyamo Vranovo	BLOOD	19,23	NT	NT	POSITIVE	NT
6.1	1018ASF	Vidin,t. Vidin	SPLEEN	18,8	NT	NT	POSITIVE	POSITIVE
6.2	1018ASF	Vidin,t. Vidin	BLOOD	16,56	NT	NT	POSITIVE	NT
7.1	1030ASF	Silistra,v Popina	SPLEEN	16,98	NT	NT	POSITIVE	NEGATIVE
7.2	1030ASF	Silistra,v Popina	BLOOD	16,65	NT	NT	POSITIVE	NT
8.1	1053ASF	Veliko Tarnovo,v B. Slivovo	SPLEEN	16,29	NT	NT	POSITIVE	POSITIVE
8.2	1053ASF	Veliko Tarnovo,v B. Slivovo	BLOOD	18	NT	NT	POSITIVE	NT
10.1	1112ASF	Silistra,v. Vetren	SPLEEN	18,84	NT	NT	POSITIVE	POSITIVE
10.2	1112ASF	Silistra,v. Vetren	BLOOD	23,59	NT	NT	POSITIVE	NT

NT = not tested

*(a) **UPL-real time PCR** → Real time PCR test described by Fernández-Pinero et al.2013 based on the Universal Probe Library (UPL).*

PNT/CISA/PPA/PCR/31

*(b) **VI-HAD** → Virus isolation and haemadsorption test on PBM cells as is described in the OIE Manual of diagnosis for ASF (Chapter 2.8.1. OIE seventh edition 2019).*

2. **ASF antibody detection by IPT**→ the presence of antibodies has been confirmed by IPT in 12 out of the 18 domestic pig samples tested (a WEAK positive result have been obtained in ID CISA samples 4.1, 4.2, 5.1, 6.1, 7.1, 8.1 and 10.1). The results obtained in ASF antibody detection are summarized in **table 5.**

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Table 5 → ASFV antibody detection by IPT in domestic pig samples						
Sample ID CISA	Sample laboratory code at the Bulgaria´s NRL			EURL-IPT [a]		CONCLUSION
	ID tube	ORIGIN	Type of sample	*Titer*	*Result*	
1.1	715ASF	Pleven, v. Jernov	SPLEEN	1/640	POS	POSTIVE
1.2	715ASF	Pleven, v. Jernov	BLOOD	NEG	NEG	NEGATIVE
2.1	763ASF	Burgas, v. Zidarovo	SPLEEN	NEG	NEG	NEGATIVE
2.2	763ASF	Burgas, v. Zidarovo	BLOOD	NEG	NEG	NEGATIVE
3.1	934ASF	Ruse,v Nikolovo	SPLEEN	1/80	POS	POSTIVE
3.2	934ASF	Ruse,v Nikolovo	BLOOD	NEG	NEG	NEGATIVE
4.1	969ASF	Ruse,v Brashlem	SPLEEN	1/10	WEAK	WEAK
4.2	969ASF	Ruse,v Brashlem	BLOOD	1/20	WEAK	WEAK
5.1	998ASF	Ruse,v Golyamo Vranovo	SPLEEN	1/20	WEAK	WEAK
5.2	998ASF	Ruse,v Golyamo Vranovo	BLOOD	1/1280	POS	POSTIVE
6.1	1018ASF	Vidin,t. Vidin	SPLEEN	1/40	WEAK	WEAK
6.2	1018ASF	Vidin,t. Vidin	BLOOD	1/10240	POS	POSTIVE
7.1	1030ASF	Silistra,v Popina	SPLEEN	1/40	WEAK	WEAK
7.2	1030ASF	Silistra,v Popina	BLOOD	1/20480	POS	POSTIVE
8.1	1053ASF	Veliko Tarnovo,v B. Slivovo	SPLEEN	1/10	WEAK	WEAK
8.2	1053ASF	Veliko Tarnovo,v B. Slivovo	BLOOD	NEG	NEG	NEGATIVE
10.1	1112ASF	Silistra,v. Vetren	SPLEEN	1/20	WEAK	WEAK
10.2	1112ASF	Silistra,v. Vetren	BLOOD	NEG	NEG	NEGATIVE

WEAK= IPT Titre <1/80;

A) ***URL-IPT→ Indirect immunoperoxidase technique** on E70-MS infected cells using the protocol standardized and validated by the European Union Reference Laboratory (EURL). [PNT/CISA/PPA/IPT/1]*

3. <u>ASFV molecular characterization</u>→ Genetic characterization of the ASFV has been initially done by PCR from **the 9 spleen samples of the 9 domestic pigs** analysing three independent regions of the ASFV genome. **On the basis of different-sized PCR products**, the CVR within the *B602L* gene, the intergenic region between the *I73R* and *I329L* genes (IGR $_{I73R - I329L}$) and the intergenic region between the MGF 505 9R and the 10R genes (MGF) have been amplified. All samples yielded an amplicon of ≈400 bp for the CVR, ≈367-bp for the IGR $_{I73R - I329L}$ and ≈489-bp for the MGF which corresponds in size to the European genotype II ASFV isolates belonging to the CVR-1 variant, IGR-2 variant and MGF-1 variant. Sequence analysis is ongoing to confirm these results.

CONCLUSION

1. **The presence of ASF has been confirmed throughout ASFV genome and/or antibody detection in all samples received belonging to four wild boar cases and nine domestic pigs from outbreaks in Bulgary.**

2. **The ASF virus has been isolated from samples belonging to three out of the four wild boar and seven out the nine domestic pigs showing the characteristic haemadsorbing pattern.**

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3. **The preliminary genetic results suggest that, on the basis of the different-sized PCR products of the two intergenic regions ($IGR_{I73R-I329L}$ and MGF) and the CVR within the ASFV genome, the ASF viruses from the wild boar and domestic pig outbreaks occurred in Bulgaria belong to the p72 genotype II variants CVR-1, IGR-2 and MGF1.**

Virus isolation and sequence analysis to confirm the preliminary molecular characterization results are ongoing.

REFERENCES

1. Carmina Gallardo, Jovita Fernández-Pinero, Virginia Pelayo, Ismail Gazaev, Iwona Markowska-Daniel, Gediminas Pridotkas, Raquel Nieto, Paloma Fernández-Pacheco, Svetlana Bokhan, OlegNevolko, ZhannaDrozhzhe, Covadonga Pérez, Alejandro Soler, Denis Kolvasov, and Marisa Arias (2014). Genetic Variation among African Swine Fever Genotype II Viruses, Eastern and CeNTral Europe. *Emerg. Infect. Dis.* Volume 20, Number 9—September2014.
2. Elsukova, Aleksandra; Shevchenko, Ivan; Varentsova, Alisa; Zinyakov, Nikolay; Igolkin, Aleksey; Vlasova, Natalia. *African swine fever (ASF), intergenic region, 9R/10R, NGS, tandem repeat sequences in the intergenic region MGF 505 9R/10R is a new marker of the genetic variability among ASF Genotype II viruses*"", EPIZONE, 10th Annual Meeting, 27-29 September 2016, MADRID, SPAIN
3. Fernández-Pinero J, Gallardo C, Elizalde M, Robles A, Gómez C, Bishop R, Heath L, Couacy-Hymann E, Fasina FO, Pelayo V, Soler A, Arias M. Molecular diagnosis of African swine fever by a new real-time PCR using universal probe library (2013). *Transbound Emerg Dis* 60 (2013) 48–58. doi: 10.1111/j.1865-1682.2012.01317.x. Epub 2012 Mar 7.
4. Gallardo C, Anchuelo R, Pelayo V, Poudevigne F, Leon T, Nzoussi J, Bishop R, Pérez C, Soler A, Nieto R, Martín H, Arias M. (2011). African swine fever virus p72 genotype IX in domestic pigs, Congo. *Emerg. Infect. Dis.* Aug: 17(8):1556-8.
5. Goller KV, Malogolovkin AS, Katorkin S, Kolbasov D, Titov I, Höper D, et al. Tandem repeat insertion in African swine fever virus, Russia, 2012 [letter]. Emerg Infect Dis. 2015 Apr [date cited]. http://dx.doi.org/10.3201/eid2104.141792
6. OIE. May 2019, posting date. Chapter 3.8.1. African swine fever (Infection with African swine fever virus). In *Manual of diagnostic tests and vaccines for terrestrial animals*2012. World Organization for Animal Health, Paris, France https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.08.01_ASF.pdf

In Valdeolmos, Madrid (Spain) 29th August 2019

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