Puławy, 25.03.2014 r.

Potwierdzenie II przypadku ASF w Polsce przez EURL

W dniu 25.03. br. Europejskie Laboratorium Referencyjne (EURL) ds. ASF w Valdeolmos w Hiszpanii potwierdziło II wynik dodatni uzyskany przez Krajowe Laboratorium Referencyjne ds. ASF w PIWet-PIB w Puławach.

Z próbki śledziony dzika znalezionego pomiędzy miejscowościami Kruszyniany a Ozierany Wielkie w trzecim pasażu wyizolowano wirusa ASF należącego do genotypu II - certyfikat w załączeniu.

MINISTERIO
DE ECONOMÍA Y
COMPETITIVIDAD

INIA
Instituto Nacional de Investigación
y Tecnología Agraria y Alimentaria
SUBDIRECCIÓN GENERAL DE
INVESTIGACIÓN Y TECNOLOGÍA
CENTRO DE INVESTIGACIÓN
EN SANIDAD ANIMAL

African Swine Fever (ASF) diagnosis and molecular characterization.

Report issued by CISA

DATE: 25h March 2014.

ARRIVAL DATE TO CISA: February 18th, 2014 and 3rd March 2014.

RESPONSIBLE FOR SUBMISSION:

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Num. ARRIVAL REGISTER CISA: REG. 33/2014 and REG. 49/2014

TEST REQUESTED: AFRICAN SWINE FEVER (ASF) CONFIRMATORY DIAGNOSIS AND MOLECULAR CHARACTERIZATION.

Num. SAMPLES RECEIVED: samples collected from two positive Polish wild boar identified as;

Table 1 → Identification of the samples received from the NVRI

ARRIVAL DATE TO CISA	Sample No	Sample ID	COLLECTION PLACE
February 18th	1	BONE MARROW	Szudzialowo, Sokolka county, Podlaskie
	2	DNA NVRI BONE MARROW	
March 3rd	1	DNA NVRI from pooled tissues	Kruszyniany, Sokolka county, Podlaskie
	2	DNA NVRI from blood	
	3	Homogeneised tissues (lung and spleen)	
	4	Lung	
	5	Spleen	

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ASF DIAGNOSTIC TESTS PERFORMED:

1. ***ASF virological diagnosis:***

   I. ***ASF genome detection.*** A 10% (w/v) clarified homogenized spleen and lung suspensions were prepared in phosphate-buffered saline [PNT/CISA/PPA/MUESTRAS/1]. The DNA was extracted directly from the undiluted and diluted (1:10) bone marrow and homogenates spleen and lung received for the two separate wild boar suspected ASF cases using the High Pure PCR Template Preparation Kit [Ref. 11796828001 (ROCHE)] following the manufactures procedures [PNT/CISA/PPA/EXTRACCIÓN ADN/1]. For amplification of the ASFV genomic DNA, the OIE conventional [PNT/CISA/PPA/PCR/1], the OIE-real time [PNT/CISA/PPA/PCR/2] PCRs (OIE 2012) and the UPL real time PCR (Fernandez *et* al., 2013) were achieved in the DNAs extracted at CISA-INIA per each sample and in the DNAs received from the NVRI specified in the table 1.

   II. ***ASF virus isolation and haemadsorption (HAD) assay*** has been performed on porcine blood monocytes (PBM) according is described in the OIE Manual (OIE 2012). The PBM has been inoculated at a multiplicity of infection (moi) 1:10 with the bone marrow sample (8 wells/per sample; 10 µl inoculum per well) and with the homogenized spleen and lung suspensions. After inoculation, a preparation of 1% homologous red 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 plates are examined for haemadsorption over a period of seven days. The bone marrow sample and the homogenized spleen and lung suspensions received **have been blind passaged three times.**

2. ***ASF serological diagnosis.*** For ASF antibody detection **the spleen and lung exudates** received for the second wild boar were tested using the indirect immunoperoxidase technique (IPT) [PNT/CISA/PPA/IPT/1].

3. ***ASFV molecular characterization.*** Genetic characterization of ASF virus from the samples received has been achieved by PCR and further sequencing analysing three independent regions of ASFV genome internationally validated for the genotyping of ASF isolates. This comprises, i) the C- terminal end of VP72 coding protein gene, which differentiates up to 22 distinct genotypes (Boshoff *et* al., 2007), ii) the full genome sequence of the *p54*-gene (Gallardo *et* al., 2009) and iii) the central variable region (CVR) within the *B602L*-gene (Gallardo *et* al., 2011). **Twenty three East Europe genotype II-ASFV isolates obtained from both wild and domestic pigs since 2007 up to 2014 including the Lithuanian viruses were included in the study** (table 2)

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Table 2 → ASFV Eastern European isolates selected for genotyping purposes obtained from the outbreaks occurred in East Europe since 2007 until 2014, including the Polish ASFV isolates from the cases in wild boar in 2014 characterized in this study.

ASFV isolate	Country	Source (District)	Host	Date of onset of outbreak
Abk07	Georgia	Abkhazia Republic Gulripish	Domestic Pig	04/07/2007
Arm07	Armenia	Dilijan town	Domestic Pig	07/08/2007
Che07	Russia	Chechnya Republic Shatoysky	European Wild boar	04/12/2007
Az08D	Azerbaijan	Qebele district	Domestic Pig	22/01/2008
Az08B	Azerbaijan		Domestic Pig	22/01/2008
Ing08	Russia	Ingushetia Republic Sunzhensky	European Wild boar	21/07/2008
Oren08	Russia	Orenburg Chernorechye	Domestic Pig	10/07/2008
NO08/Av	Russia	Republic North Osetia Vladikawkaz	Domestic Pig	18/07/2008
NO08/Ap	Russia	Republic North Osetia Prigorodni	Domestic Pig	21/07/2008
Dagestan09	Russia	Tarumovsky, Respublika Dagestan	European Wild boar	11/09/2009
StPet09	Russia	Kirovsky, Leningradskaya Oblast	Domestic Pig	01/10/2009
Kalmykia09	Russia	Yashaltinsky, Kalmykiya-Khal'mg Tangch	Domestic Pig	10/10/2009
Rostov09	Russia	Krasnosulinsky, Rostovskaya Oblast	Domestic Pig	20/10/2009
Tver0511/Torjo	Russia	Torjo, Tver region	Domestic pig	31/05/2011
Tver0312/Novo	Russia	Novozavidovskii, Tver region	Domestic pig	14/03/2012
Tver0312/Torjo	Russia	Torjo, Tver region	European Wild boar	28/03/2012
Tver0712/Les	Russia	Lesnoi, Tver region	Domestic pig	16/07/2012
Ukr12/Zapo	Ukraine	Zaporozhye region	Domestic Pig	30/07/2012
Tver0812/Bolo	Russia	Bologovskii, Tver region	European Wild boar	15/08/2012
Tver1112/Zavi	Russia	Zavidovo, Tver region	European Wild boar	20/11/2012
Bel13/Grodno	Belarus	Lelyukinskiy, Ivye, Grodno	Domestic Pig	19/06/2013
LT14/1490	Lithuania	Salcininkai, Vilnius	European Wild boar	21/01/2014
LT14/1482	Lithuania	Alytus-Varena	European Wild boar	21/01/2014
Pol14/Sz	**Poland**	**Szudzialowo, Sokolka county, Podlaskie**	**European Wild boar**	**14/02/2014**
Pol14/Krus	**Poland**	**Kruszyniany, Sokolka county, Podlaskie**	**European Wild boar**	**17/02/2014**

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RESULTS

1. ***ASF virological diagnosis*** → **Positive ASF result** has been obtained by the conventional and real time PCRs **in the bone marrow, lung and spleen samples and the NVRI DNAs received from the two cases in wild boar tested previously as positive at the NVRI.** The detailed results are summarized in the **table 3**.

Table 3 → ASF virological results obtained on samples received from Poland.

ARRIVAL DATE TO CISA	Sample No	OIE-PCR (a)		OIE-real time PCR (b)		UPL- real time PCR (c)		VI-HAD (d)	
		Result	Ct value	Result	Ct value	Result	Nº passage	Result	
February 18th	1- BONE MARROW	POS	25.82	POS	25.19	POS	3	NEG	
	2- DNA NVRI BONE MARROW	POS	26.22	POS	27.26	POS	-	-	
	1- DNA NVRI from pooled tissues	POS	20.03	POS	19.77	POS	-	-	
	2- DNA NVRI from blood	POS	28.58	POS	28.74	POS	-	-	
March 3rd	3- Homogeneised tissues	-	-	-	-	-	3	NEG	
	4- Lung	POS	28.03	POS	25.74	POS	3	NEG	
	5- Spleen	POS	22.89	POS	22.85	POS	3	POS	

(a) ***OIE-PCR*** → *Conventional PCR (Aguero et al., 2003) technique as is described in the OIE Manual of diagnosis for ASF (Chapter 2.8.1. OIE seventh edition 2012).* **[PNT/CISA/PPA/PCR/1]**

(b) ***OIE- real time PCR*** → *real time PCR (King et al., 2003) technique as is described in the OIE Manual of diagnosis for ASF (Chapter 2.8.1. OIE seventh edition 2012).* **[PNT/CISA/PPA/PCR/2]**

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

(d) ***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 2012).*

After three passages in PBM cells **the ASF virus has been isolated from the spleen sample** received from the second wild boar case **showing the specific haemadsorbing pattern of ASF**.

2. ***ASF antibody detection*** → The tissue exudates analysed from the second wild boar gave **negative result for antibodies against ASFV.**

3. ***ASFV molecular characterization*** → The expected 478 bp and 558 bp of the C-terminal *p72*-gene and the full length *p54*-gene, respectively, were obtained in the bone marrow, spleen and lung samples received from the two wild boar cases. The PCR amplicons were directly sequencing and compared with the homologous sequences from the 23 East European ASFV isolates specified in Table 1. The p72 and p54 virus genome segments amplified by PCR from the Polish samples **[named Pol14/Sz and Pol14/Krus]** were identical at nucleotide level to all East European ASF viruses, including the Lithuanian and Belarus isolates, across the 478 bp C-terminal p72-gene and the 558 bp full length *p54*-gene.

Nucleotide and aminoacid sequences obtained were then compared with homologous sequences available in GenBank from at least 1 virus representative of at each of 22 (I-XXII) p72 described genotypes (Boshoff *et al*., 2007). The phylogeny inferred placed the ASF **Polish viruses** within **genotype II** together with the **Lithuanian viruses** **[named LT14/1482 and LT14/1490]** and all the ASFV isolates from **Georgia (2007), Armenia (2007), Azerbaijan (2008), Russia (2008-2012), Ukraine (2012) and Belarus 2013** *(Figure 1).*

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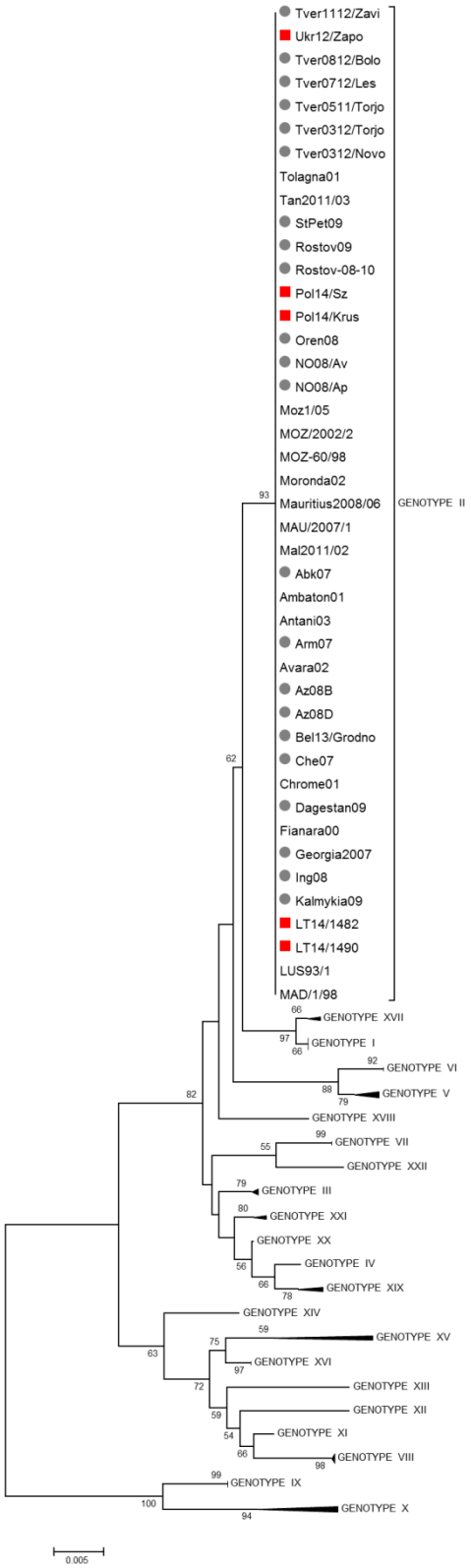

Figure 1. Minimum Evolution phylogenetic tree of ASF Polish and Lithuanian viruses (LT14/1482 and LT14/1490) based on C-terminal end of the p72 protein relative to the 22 p72 genotypes (labelled I-XXII). ASF Polish and Lithuanian 2014 viruses genotyped are marked in red in red (■) whereas genotyped Caucasus ASFV isolates since 2007 to 2013 are marked in grey (●)

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In addition, and for easy genotyping on the basis of different-sized PCR products, the CVR within the *B602L* gene was amplified. The bone marrow, spleen and lung samples yielded an amplicon of ≈200 bp, which **corresponded in size and sequence to the other East Europe ASFV genotype II isolates**. Further sequence analysis revealed ten copies of aminoacid tetramer repeats (type BNDBNDBNAA) unique to the group of viruses circulating in the Eastern European regions since 2007 (Rowlands *et al*., 2008; FAO Empress 2009; Malogolovkin *et al*., 2012). **The nucleotide sequences were 100% identical to the homologous sequence of the ASFV Lithuanian viruses and to the Belarus isolate obtained in 2013 in the Grodno region.**

CONCLUSION

1. **The presence of ASF has been confirmed by PCR tests in the samples received from the two European wild boar collected in Szudzialowo (Sokolka county, Podlaskie province) and in a forest area (Kruszyniany area) in Eastern Poland.**
2. **The ASF virus has been isolated from the spleen sample** received from the second wild boar (Kruszyniany area) **showing the specific haemadsorbing pattern of ASF.**
3. **The p72 genotyping of Polish viruses clustered the virus responsible of the two cases in wild boar within genotype II circulating in the Eastern European countries since the first introduction in Georgia in 2007.**
4. **The subtyping using the CVR of ASFV genome showed than the ASF Polish viruses were 100% homologous to the Lithuanian and Belarus ASFV isolates.**

In Valdeolmos, Madrid (Spain), March 25th, 2014.

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