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Next Generation Sequencing of Classical Swine Fever Virus

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“Workshop of the African and Classical Swine Fever National Reference Laboratories (ASF and CSF)”

June 9-10 2015

**National Institute for Agricultural and Food Research and
Technology, (INIA). Ctra de la Coruña km 7,400
28040 Madrid, Spain**

organised by:

EU Reference Laboratory for ASF
Centro de Investigación en Sanidad Animal (INIA-CISA)
Valdeolmos, Spain

and

EU Reference Laboratory for CSF
University of Veterinary Medicine Hannover
Institute for Virology
Hannover, Germany

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1. WORKSHOP PROGRAM

CSF SESSION. Tuesday, 9.06.2015

8.30-9.00 Registration of participants

09.00-9.15 Opening and welcome

Víctor Briones (Director of CISA-INIA).

Francesco Berlingieri (SANCO, EC).

Marisa Arias (EURL/ASF)

Paul Becher (EURL/ CSF)

CSF Session I: Report of the EURL activities

Chairman: Marisa Arias (EURL for ASF)

09.15-10.00 EURL for CSF: Results of the Interlaboratory Comparison Test 2014.
Sophia Austermann-Busch (CSF EURL, DE).

10.00-10.25 EURL activities during 2014-2015. Paul Becher & Alexander Postel (CSF
EURL, DE)

10.25-10.55 *Coffee break*

10.55-11.10 European data base on ASF/CSF in wild boar. Christoph Staubach (DE)

CSF Session II: CSF Country Reports

Chairman: Francesco Berlingieri (SANCO, EC).

11.10-11.20 CSF situation in Bulgaria, 2013-2015. Emiliya Ivanova (BG).

11.20-11.30 Classical swine fever in Croatia- the current situation. Dragan Brnic
(HR).

11.30-11.40 CSF monitoring program in Romania. Daniel Donescu (RO).

11.40-11.50 CSF situation in Latvia. Ieva Rodze (LV).

CSF Session III: CSF Diagnosis and Control

Chairman: Andrzej Lipowski

11.50-12.10 Classical swine fever virus marker vaccine strain CP7_E2alf: shedding
and dissemination studies in boars. Anja Petrov (DE).

12.10-12.40 Evaluation of a newly developed Classical Swine Fever Erns ELISA
Denise Meyer (EURL for CSF, DE).

- 12.40-13.00 Inactivation of CSFV by reagents commonly used in research and diagnosis and the effect of inactivation treatments on ELISA detection of antigen and antibody. Helen Crooke (UK).
- 13.00-14.00 *Lunch*
- 14.00-14.20 Next Generation Sequencing of Classical Swine Fever Virus. Thomas Bruun Rasmussen (DK).
- 14.20-14.40 Characteristics of early infection with three attenuated strains of classical swine fever virus. Katarzyna Podgorska (PL).
- 14.40-15.10 Validation and long term use of a CSF antibody ELISA. Willie Loeffen (NL).
- 15.10-16.00 Final discussion; conclusions and recommendations.
- 16.15 Bus to Madrid. (Moncloa)

ASF SESSION. Wednesday, 10.06.2015

- 08.45-9.00 Registration of participants
- 09.00-9.10 Opening and welcome
 Víctor Briones (Director of CISA-INIA).
 Francesco Berlingieri (SANCO, EC).
 Marisa Arias (EURL/ASF)

ASF Session I: ASF Country Reports

Chairman: Francesco Berlingieri
 (SANCO, EC).

- 09.10-09.30 ASF in Sardinia: a new deal in the management of eradication strategy. Francesco Feliziani (IT).
- 09.30-09.50 ASF current situation in Lithuania. Mindaugas Morkunas (LT).
- 09.50-10.10 ASF situation in Latvia. Ieva Rodze (LV).
- 10.10-10.30 ASF current situation in Poland. Andrzej Kowalczyk (PL)
- 10.30-10.50 ASF current situation in Estonia. Imbi Nurmoja (EE)
- 10.50-11.20 *Coffee break.*

ASF Session II: Report of the EURL activities

Chairman: Sophia Austermann

- 11.20-11.50 ASF diagnosis in affected countries, the EURL perspective. Carmina Gallardo (ASF EURL, ES).
- 11.50-12.30 EURL: Results of the Interlaboratory Comparison Test 2014-2015. Virginia Pelayo (ASF EURL, ES).
- 12.30-13.00 EURL activities during 2014-2015. Marisa Arias (ASF EURL, ES).
- 13.00-14.00 *Lunch*

ASF Session III: Diagnosis.

Chairman: Marie-Frédérique Le Potier

- 14.00-14.15 Comparative validation of commercial and published real-time PCR methods for detection of ASF. Adolf Steinrig, (AT).
- 14.15-14.30 Monitoring of African swine fever antibodies by Ingenasa ELISA in wild boar population in non-infected area, 2014 in Slovenia. Ivan Toplak (SI).
- 14.30-15.45 Round table: Laboratory representatives from affected EU countries and EURL Laboratory coordinator to share experience with questions from participants
- 15.45-16.30 Final Discussion, Conclusions and Recommendations.
- 16.30 Bus to Madrid. (Moncloa)

2. LECTURES: Abstracts

a. CSF SESSION

**EU Reference Laboratories
Classical Swine Fever and African Swine Fever
Wild Boar Surveillance Database**

Christoph Staubach^a, Mathias Merboth^a, Stefan Kowalczyk^a, Thorsten Schrapps^a,
Sophia Austermann-Busch^b, Franz-Josef Conraths^a, Marisa Arias Neira^c, Paul Becher^b

^aFriedrich-Loeffler-Institut, Institute of Epidemiology, Wusterhausen, Germany

^bEURL CSF, Institute of Virology, University of Veterinary Medicine Hannover, Germany

^cEURL ASF, Center for Animal Health Research, National Institute for Agricultural and Food Research and Technology, Spain

The occurrence of CSF cases in wild boar within the EU and the successful implementation of the database in recent years have clearly demonstrated the need for continuation and extension of the project.

During the period 01.10.2002 – 31.05.2015, the currently 13 participating member states entered 742,363 data records into the CSF database. A total of 164 defined restriction zones and vaccination areas are recorded in the region/zone database. Furthermore, additional data for German scientific work has been entered into the database for the period 01.01.1999 – 30.09.2002 (a total of 41,352 data records).

Recently Austria and Latvia joined the database and are presently in a testing phase. Further EU Member States (e.g. Estonia, Lithuania and Poland) are interested to join the database and Serbia, as accession candidate, has also indicated its intention in participation.

The maps and display of ecological information plays an important role during the evaluation process of the CSF surveillance data. Therefore, a map viewer displaying several base maps from different sources (e.g. topographic map, satellite map, forest map, administrative and statistical units map etc.) using modern technology was developed on the demand of the member states. In the pipeline for future development of the database is the change to a regular update procedure of the spatial data using the official data of the member states (e.g. EuroBoundaryMap) provided through EuroGeographics, a specialized agency of the EC.

In view of the current ASF situation in the eastern member states as well as in bordering third countries and the already available information in the laboratories concerning ASF in wild boar derived from differential diagnostic testing for CSF the database is now able to integrate information also on ASF. The extension of the CSF database will allow the input of ASF data analogous to the CSF data. The current ASF situation can be analysed and illustrated with the same tools as implemented in the CSF module. Due to the integration of ASF, the database name will change to “CSF/ASF wild boar surveillance database”. The new version the database is still under active development, but can be accessed using the test site: <https://asf-wildboar.eu>.

The EURL CSF-DB can be accessed via a secure internet connection following the linked website <https://csf-wildboar.eu> and any further information is available via the public part of the website <http://public.csf-wildboar.eu>

CLASSICAL SWINE FEVER SITUATION 2013 – 2015 IN BULGARIA

Emiliya Ivanova

National Diagnostic and Research Veterinary Medical Institute – Sofia

In 2006 – 2011 after the ceasing of prophylactic vaccinations of domestic and East-Balkan pig populations in Bulgaria there were investigated a lot of samples from internal organs and sera samples of swine. There were found some outbreaks of CSF. It was detected a new CSF virus – 2.3* Bulgaria. For the last six years /2009 – 2015/ there were not found outbreaks of CSF in wild, domestic and East-Balkan pig populations in Bulgaria. The last CSF case in wild boars was registered in 2009.

Bulgarian domestic pig population: a total of 26 904 farms with a total of 517 741 pigs; 52 industrial pig farms with 424 139 pigs; 118 family farms type “A” with 37 518 pigs; 810 family farms type “B” with 8 468 pigs; 25 856 backyard pig holdings with 41 843 pigs and 68 herds with 5 773 East Balkan pigs.

For the 2013 there were investigated 11 354 sera samples: 9 221 from domestic pigs and 2 133 – from East-Balkan pigs. For the 2014 there were investigated 7 227 sera samples: 6 626 from domestic pigs and 601 only – from East-Balkan pigs. Seroreagents were not found.

Bulgarian wild boars population: 79 281 animals. There were investigated in 2013 a total of 5 905 sera samples from wild boars with 484 positive samples from vaccination zones and 3 811 samples of internal organs from shot wild boars. The positive samples were additionally investigated in VNT, resulted with high titers of antibodies detected. 12, 85 % of investigated wild boars age 0 – 1 year were found positive for antibodies; 41,76 % were positive from age group 1-2 years and 53,54 % - from age group > 2 years.

For 2014 – 3829 sera samples with 375 positive and 6 506 samples of internal organs were investigated.

Classical swine fever in Croatia- the current situation

Dragan Brnić, Lorena Jemeršić, Tomislav Keros, Jelena Prpić

Classical swine fever (CSF) is a highly contagious viral disease that infects domestic pigs and wild boars worldwide. A prompt laboratory diagnosis is of crucial importance in confining the viral spread. From 2005, the CSF control policy accompanied by diagnostic procedures in the Republic of Croatia is completely in compliance with EU regulations. Croatia has banned vaccination of domestic pigs against CSF since the beginning of 2005, which was followed by CSF outbreaks in domestic pigs and wild boars in the following years (2006-2008) with the last outbreak being detected in March 2008. Since then, strict measures regulated by two Ordinances and two National surveillance programs, have resulted in the absence of the virus. Until today, the number of CSF antibody positive domestic pigs and wild boars has intensively decreased, with only the recognition of few positive wild boars from the recent years. Even so, all positives were restricted to several Counties neighbouring Bosnia and Herzegovina and Serbia. The presentation will give an emphasis on the activities of the National Reference Laboratory for CSF at the Croatian Veterinary Institute in the implementation of two National surveillance programs during the past three years.

Classical Swine Fever monitoring program in Romania

D. Donescu

Institute for Diagnosis and Animal Health, Bucharest, Romania

The CSF was maintained under control between 1974-2001 using the live vaccine in constant and repeated compulsory vaccination of domestic pigs and after 1987 even of some feral pig populations. As an acceding state to the EU, Romania adopted in 2001 the EU non-vaccination policy for CSF. The CSF was first diagnosed in Romania beginning with 2001. After introducing the policy of no vaccination the number of outbreaks increased yearly and disease became endemic in all territory of Romania. From 2001 to 2007 in domestic pigs were detected 2860 outbreaks of CSF in 1202 localities. The peak of outbreaks was achieved in 2005. Most of the outbreaks were in back yards. In 2007 CSF was diagnosed only in 3 commercial farms. The number of pigs affected in backyard outbreaks during 2001 -2007 was 27149.

The control of CSF in Romania was based on emergency vaccination, surveillance, EU legislation and a support legislation to regulate the movement of pigs, especially from back yards. In December 2006 until 2009 Romania implement emergency vaccination. Since 2007 in Romania have not been diagnosed outbreaks of CSF and after 2011 Romania implement a surveillance program to demonstrate CSF free status. In this purpose, was put in place a serological and virological surveillance program in domestic pig and wild boar population.

In case of wild boar, all hunted and found dead wild boar were tested virologically and serologically. In wild boar population in 2014 were tested 14259 serum samples and 10033 set of organs by RT-PCR.

The surveillance program for domestic pigs from nonprofessional holdings was design using the hypothesis that the virus is present and still circulates in pig populations at a prevalence of 5% with 95% confidence (as is mentioned in Decision 106/2002). In 2012 the prevalence of 5% was applied at 2 levels: herd level (proportion of infected herds in a locality) and animal level (proportion of infected animals in an infected holding). In 2013 and 2014 this prevalence was applied at 3 levels: locality level (percent of infected localities in a county), herd level and animal level.

Using this design prevalence in 2012 were tested 49723 holdings and 208475 serum samples. In 2013, applying the 3-th level of prevalence were tested 84864 back yards and 280146 serum sample and in 2014 were tested 94002 back yards and 231580 serum samples. Based on this design, after 3 years of serological surveillance, it was not detected any positive results for CSF and permit to reject the hypothesis that the virus is present and still circulates in pig populations at a prevalent.

Classical swine fever virus marker vaccine strain CP7_E2alf: shedding and dissemination studies in boars

Carolin Dräger¹, **Anja Petrov**¹, Martin Beer¹, Jens P. Teifke², Sandra Blome¹

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²*Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald - Insel Riems, Germany*

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a severe multi-systemic disease of pigs, which can lead to tremendous economic losses. While prophylactic vaccination is prohibited in most countries with industrialized pig production, emergency vaccination is foreseen to reduce the socio-economic and animal welfare impact of CSF outbreaks. However, the lack of suitable marker vaccines has so far prevented implementation of emergency vaccination in domestic pigs.

Over the last decade, the pestivirus chimera “CP7_E2alf” was presented as a promising modified-live marker vaccine in several studies, and recently it has been licensed by the European Medicines Agency. However, some safety issues are still not completely elucidated. The presented study was undertaken to gain further background data with regard to distribution and shedding of the vaccine, especially in urine, faeces, and semen of reproductive boar.

In detail, after a single intramuscular vaccination with a tenfold vaccine dose, the dissemination and shedding pattern of the vaccine strain “CP7_E2alf” was assessed in twelve adult boars. Upon vaccination, neither local nor systemic adverse effects were observed in the experimental animals. Four and seven days post vaccination, six animals were subjected to necropsy and triplicate samples were obtained from reproductive and lymphatic organs as well as urine, faeces, blood, and several additional organs and matrices. The sampling days were chosen based on pre-existing data that indicated the highest probability of virus detection. It was confirmed that primary replication is restricted to the lymphatic tissues and especially the tonsil. While viral genome was detectable in several samples from the lymphatic tissues at four and seven days post vaccination, infectious virus was only demonstrated at four days post vaccination in one tonsil sample and one parotid lymphnode. Sporadic detection at a very low level occurred in some replicates of liver, lung, bone marrow and salivary gland samples. In contrast, viral genome was not detected in any sample from reproductive organs and accessory sex glands, in faeces, urine or bile.

The presented data on the dissemination of the vaccine virus “CP7_E2alf” in adult boars are supplementing existing safety and efficacy studies and indicate that the use of the vaccine is also safe in reproductive boars.

Evaluation of a newly developed Classical Swine Fever Erns ELISA

D. Meyer¹, S. Fritsche², M. Beyerbach³, L. Kreienbrock³, C. Engemann², C. Schroeder², A. Postel¹ and P. Becher¹

¹ Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine, Hannover, Germany

² QIAGEN Leipzig GmbH, Leipzig, Germany

³ Institute for Biometry, Epidemiology and Information Processing, University of Veterinary Medicine, Hannover, Germany

Successful implementation of marker vaccines (e.g. CP7_E2alf) is dependent on a reliable accompanying diagnostic assay that allows differentiation of infected from vaccinated animals (DIVA strategy). As induction of a protective immune response relies on virus neutralizing antibodies against the E2 protein of the CSF virus (CSFV), the most promising DIVA strategy is based on detection of Erns-specific antibodies.

Thus, the aim of the present study was to develop an Erns-specific ELISA which may be used as an accompanying discriminatory test for the marker vaccine CP7_E2alf and other future marker vaccines based on the same principle. For the detection of CSFV-specific antibodies the concept of a double antigen ELISA was applied and tested. Therefore, a representative number of serum samples (> 1000 samples) including CSFV antibody positive sera (\leq 21 days post infection and > 21 days post infection), CSFV antibody negative sera, and sera taken from animals vaccinated with the marker vaccine CP7_E2alf were analyzed.

The concept of a double antigen ELISA is shown to be a reliable strategy for development of a serological CSFV assay. Serum antibodies against CSFV isolates of different genotypes were detectable and, when compared to an E2 antibody ELISA, an increased sensitivity was observed for sera taken at early time points after infection. Moreover, differences in reactivity between serum samples taken from infected and CP7_E2alf vaccinated pigs were observed when the novel double antigen ELISA was applied. With respect to its possible application as a DIVA ELISA, test performance in terms of sensitivity and specificity will be presented and compared to a licensed Erns ELISA.

Inactivation of classical swine fever virus by reagents commonly used in research and diagnosis and the effect of inactivation treatments on ELISA detection of antigen and antibody.

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^b*Department of Comparative Pathology, Veterinary Faculty, University of Córdoba-Agrifood Excellence International Campus (ceiA3), Edificio Sanidad Animal, Campus de Rabanales, 14014, Córdoba, Spain*

Objective: The impact of classical swine fever virus on the swine industry obliges laboratories handling the virus to use appropriate measures to prevent its release into the environment. Various reagents and treatment are used to inactivate viruses prior to handling of samples outside of high containment facilities. However, data demonstrating efficacy of CSFV inactivation under the particular conditions used is often not available. The objective of this study was to demonstrate how efficacious certain laboratory reagents and treatments are in inactivation of classical swine fever virus (CSFV). The effect of heat and chemical inactivation treatments on the subsequent detection of CSFV antigen and antibody was also assessed.

Methods: Reagents commonly used for RNA extraction, cell fixation prior to flow cytometry or immunoperoxidase staining, chemical inactivation or disinfection were incubated with CSFV or sera from CSFV infected animals for times and temperatures relevant to the treatment in question. The amount of viable virus remaining was determined by titration on PK15 cells followed by immunoperoxidase staining and the viral reduction factor determined by comparison to untreated control virus. For assessment of toxic cell fixative treatments, PK15 cells infected with CSFV were treated and then washed 3 times in PBS prior to titration of virus on PK15 cells. To assess the effect of heat or chemical inactivation treatments on subsequent detection of CSFV antibody or antigen, sera from pigs vaccinated with C-strain and inoculated with CSFV UK2000/7.1 were either heated to 56°C for 90 minutes or treated with β -propiolactone (0.1% v/v) for 2 hours at 37°C. Detection of antigen and antibody, compared to untreated sera, was assessed by ELISA.

Results: No viable CSFV was detected after treatment of samples with the commercial RNA extraction lysis buffers; Buffer AVL, Buffer RLT (Qiagen) or Tri Reagent (Ambion). However, the toxicity of these reagents, and some cell fixatives used for flow cytometry and immunoperoxidase staining, limited the ability to demonstrate high viral reduction factors. An alternative strategy of treating CSFV-infected cells and washing prior to titration was therefore applied for those agents that did not lyse cells. Reduction factors of $> 6 \log_{10}$ were demonstrated for BD FACS™ Lysing Solution, BD CellFIX™ solution, BD CytoFix/Cytoperm™, 4% paraformaldehyde and 100% and 80% acetone treatments. Notably, incubation in 20% acetone at room temperature for 10 minutes, which is included as a fixative method for OIE prescribed serum neutralising tests for CSFV, resulted in little or no reduction in the amount of viable CSFV. Whilst a 1:1 TMB ELISA substrate/ 1M sulphuric acid stop solution mixture reduced virus by $>3.3 \log_{10}$, viable virus was occasionally detected after treatment at a level below the quantifiable limit of the assay. FAM30, an approved disinfectant for CSFV when used at a contact time of 30 minutes, resulted in $>3 \log_{10}$ reduction in virus even after short contact times of 1 to 5 minutes.

In contrast to heat treatment of sera at 56°C, which is not a reliable method of inactivating CSFV, β -propiolactone treatment resulted in $>4 \log_{10}$ viral reduction factor. Both heat treatment and β -propiolactone had minimal effect on antibody detection but both treatments reduced OD values obtained in CSFV antigen ELISA compared to untreated sera.

Conclusion: The results of this study provide useful information for laboratories handling CSFV for research and diagnosis and will facilitate improved protocols that minimise the risk of accidental release of virus. For example, use of 20% acetone as a cell fixative must not be considered as a method that inactivates CSFV. The demonstration of efficacy of disinfectants after short contact times will facilitate the rapid transfer of samples between high containment animal and laboratory facilities. Effective inactivation protocols will allow handling of samples in lower biosafety level facilities that will provide substantial cost savings and facilitate analyses, such as flow cytometry, not available or possible within existing containment facilities.

Next Generation Sequencing of Classical Swine Fever Virus

Thomas Bruun Rasmussen

DTU Vet, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark

Next Generation Sequencing (NGS) has rapidly become the preferred technology in nucleotide sequencing, and can be applied to unravel molecular adaptation of RNA viruses such as classical swine fever virus (CSFV). In this talk, NGS data from an immunisation/challenge experiment will be presented. Groups of pigs were immunised with CSFV vaccine candidates, i.e. vR26 and vR26_E2gif, and subsequently challenged with the highly virulent CSFV strain “Koslov”. NGS data of Koslov RNA derived from serum of vaccinated pigs and mock-vaccinated pigs was obtained and analysed. The variation analysis revealed significant differences in single-nucleotide polymorphisms (SNPs) between the CSFV sequence data from the vaccinated and the mock-vaccinated groups. The viral sequences obtained from the mock-vaccinated pigs had a similar SNP distribution as the challenge virus, which was not the case for the sequence data from the vaccinated group where a complete change of the SNP distribution was observed. Additionally, new detectable non-synonymous SNPs were found in the vaccinated pigs indicating selection pressure onto the challenge virus, which was not observed in the mock-vaccinated group.

Characteristics of early infection with three attenuated strains of classical swine fever virus (CSFV)

Podgórska K., Kus K., Szczotka-Bochniarz A., Stępniewska K., Jabłoński A., Małek B., Szymanek K., Pejsak Z

National Veterinary Research Institute, Swine Diseases Department, Pulawy, Poland

Vaccines based on attenuated strains of CSFV do not comply with DIVA requirements. However, their efficacy is a point of reference for evaluation of new generation vaccines. Despite different or obscure origin of attenuated strains their biological features and immunogenic properties are commonly perceived as comparable.

The objective(s) of the present study was/were to (1) characterize an early infection with selected attenuated CSFV strains of three most genetically divergent attenuated strains and selection of best candidate strain for comparative analysis of new generation vaccines efficacy, development of new marker vaccines and emergency vaccination.

Two of the strains selected for the study, Chinese strain (C-strain) and Norden, belonged to two distinct clusters within subgenotype 1.1, while third strain KPS87 localized within lineage 1.2, together with Rovac and TVM-1 strains. The sequence identity within E2 region between C-strain and KPS87 strain was estimated for 91.7% and between C-strain and Norden reached 95.7%.

Three groups of twelve 6 wks old pigs were inoculated with 10^5 TCID₅₀ of selected strains. Three pigs received placebo. Body temperature and clinical scores were monitored daily and blood samples were collected twice a week. Three pigs from each group were euthanized weekly (at 7, 14, 21 and 28 days post inoculation (dpi)). Sera and tissue samples were tested for the presence of infectious virus and viral RNA and the development of humoral immunity was followed by ELISA and NPLA.

The results of the study indicated that the course of early infection, distribution of the virus in tissues, dynamics of seroconversion and production of neutralizing antibodies as well as the shedding status varied between analysed strains.

In all groups virus persisted in tonsils until the end of the study (28 dpi). However, in case of C-strain group the concentrations of viral RNA were lower compared to other strains. In KPS87 and Norden group viral RNA was detected in serum between 3-8 dpi, while no viremia was observed in C-strain inoculated pigs. Moreover, the replication and distribution in tissues had higher level in case of KPS87 and Norden compared to C-strain. Analysis of oral fluid samples collected daily during the study indicated that in Norden-inoculated group the virus was shed to the environment. On the other hand, the earliest and the most robust development of neutralizing antibodies was observed in C-strain group.

Abovementioned results indicate that C-strain characterized with the highest level of attenuation and induced stronger immunity compared to other strains used in the study. Therefore, it should be a strain of choice for emergency vaccination as well as comparative efficiency studies of new generation vaccines.

Validation and long term use of a CSF antibody ELISA

Willie Loeffen.

CVI Lelystad, The Netherlands

In the framework of ISO17025 accreditation, CVI carries out in-house validations of all diagnostic tests, including those purchased as commercial kits. Validations carried out by manufacturers are in general not considered representative for the conditions under which the test is carried out by CVI. Neither are samples used in manufacturers' validations considered representative for the target population. For classical swine fever antibody ELISA's for instance, it is important to consider the situation with respect to BVD and BD in the target population when properly validating the test. Important parameters for validation are analytical and diagnostic sensitivity, analytical and diagnostic specificity, reproducibility, repeatability and robustness. Through a proper validation the behaviour and limitations of the test are better understood and the reliability of the results will be increased. Once validated, it is crucial that the performance of a diagnostic test remains constant in time within acceptable limits. Changes in the test that affect validated parameters require additional validation. It is our experience that manufacturers tend to modify their tests with limited or no communication about potential changes in relevant outcome parameters. Furthermore, batch to batch variability can be high, even without intentional changes to a test. Commercial test kits therefore require ongoing monitoring of their performance. Each batch needs to be tested with an extensive batch control panel for its suitability to be used in the diagnostic laboratory. Furthermore, two in-house prepared Internal Control samples are used on each test plate to monitor performance of test plates within one batch in time.

During the EURL meeting the validation and ongoing monitoring (through batch controls and additional IC's) of a CSF antibody ELISA will be presented. An additional tool for ongoing monitoring of ELISA's may be universal standard serum samples, which would allow for harmonized evaluation, but also calibration of antibody ELISA's. The need for, and the use of, such standard serum samples could be discussed during the EURL meeting.

b. ASF SESSION

African swine fever in Sardinia: a new deal in the management of eradication strategy **Francesco Feliziani**

Istituto Zooprofilattico Sperimentale Umbria e Marche – Perugia (Italy)

African swine fever (ASF) was introduced in Sardinia in late 1978 and currently remains endemic. During this long period, different plans were implemented to eradicate the infection; however, unfortunately, the outcomes were poor. In this context, the only good result obtained is that the ASF virus did not “escape” Italy.

In fact, the presence of ASF in Sardinia was not considered a priority threat to the surrounding European countries at least until 2007 when the infection spread to Eastern Europe, even involving European Union countries (Lithuania and Poland). The alert status was changed, and the European Commission was urgently requested to intervene, also because, at the same time, the epidemiological situation in the Mediterranean Island had worsened.

In Sardinia, beginning in 2011, pork exports were banned and an extremely rigorous monitoring was imposed, primarily comprising the efficient registration of swineherds, increased biosecurity at swine farms, and comprehensive (clinical, serological, and virological) surveillance of the swine population including wild boars. Furthermore, the European Commission proposed taking strict action to eliminate the illegal breeding of feral pigs, which are considered the ASF reservoir, particularly in east-central Sardinia.

Despite adopting this onerous eradication plan, the ASF incidence has not significantly decreased, and questions about the long-term sustainability of these measures have arisen. In this context, considering the history and the recent epidemiologic dynamic of ASF infection, a new approach was proposed by the regional government, primarily taking into account several social and economic aspects that negatively influence the eradication process.

A specific task force was implemented by regional authorities, the board of which included international experts and representatives of national and local institutions, with the goal to carry out a new comprehensive strategy to eradicate ASF infection in at least 2–3 years. On this basis, a new plan was presented and approved by the European Commission that is particularly committed to fight the illegal practice of free-ranging breeding of swine.

Although the progress is slow and sometimes difficult, the regional machinery is now beginning to work and great expectations are placed on this new plan that seems to be capable of achieving the eradication of infection in Sardinia.

African Swine Fever situation in Lithuania

Mindaugas Morkunas

National Food and Veterinary Risk Assessment Institute, LT-08409, Vilnius, Lithuania

The purpose of this presentation is to demonstrate the epidemiological situation of African Swine Fever (ASF) in Lithuania and the activities of National Food and Veterinary Risk Assessment Institute of Lithuania (NFVRAI).

First cases of ASF in Lithuania were detected on 24 of January 2014 – a hunted wild boar, and a wild boar found dead were tested positive for ASF at NFVRAI in Vilnius. Intensive wild and domestic animal monitoring programme was started. No new cases of ASF were detected for 6 month. During this period ~24000 animals were tested for ASF (>10000 domestic pigs, >13000 wild boar). Disease reappeared on 23rd of July in Ignalina district in a in large (~20000 pigs were kept) commercial pig holding with the highest biosecurity. 290 pigs were sampled from infected farm, and 102 were found positive. 19 217 pigs were killed and destroyed by burying on the territory of the farm. Despite all efforts no clear conclusions could be made about the source of infection or possible ways of virus introduction. During 2014 ASF outbreaks continued to appear, the very next one occurred in same county in a backyard farm two weeks later. During August a total of 5 ASF outbreaks in domestic pigs have been detected in 3 district municipalities of Lithuania, bordering each other (9 domestic pigs were tested ASF positive). During 2014 a total of ~29000 domestic pigs were tested for ASF.

After first wild boar ASF cases detected in January 2014 next wild boar cases were found only in August in Ignalina district again. During 2014 a total of 45 locations where ASF positive wild boars were found dead (25 locations, 54 animals) or hunted (20 locations, 22 animals) were determined. During 2014 a total of ~16500 wild boars were tested for ASF.

During 2015 no ASF cases in domestic pigs were detected (~10500 animals were tested). So far only dead (28 animals in 16 locations) or hunted (13 animals in 13 locations) wild boars were found to be ASF positive (~3000 animals were tested).

During 2014-2015 the Institute has received 32 food samples (meat products) confiscated on the border with Belarus, and found 4 of these to be positive for ASFV.

At NFVRAI ASF samples are analysed by Ab ELISA, IPT (since 2015 January), PCR, and pathological examination. Lithuanian ASF team is in close contact with ASF EURL – methodology and staff qualification is constantly updated via trainings and discussions.

ASF surveillance in Poland: update on current situation.

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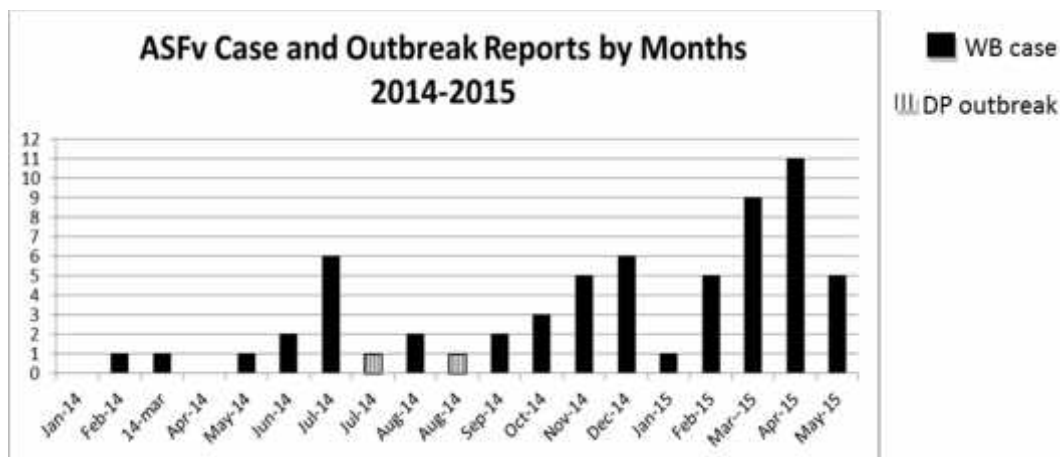
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The presence of African Swine Fever (ASF) in the Caucasus region and Russian Federation had increased concerns that wild boars introduced the ASF virus into the European Union (EU). Since the 1st of January 2014 till 20th of May 2015, 63 cases of ASF in wild boar (WB) and 3 outbreaks of ASF in domestic pigs (DP) have been detected in Poland. These events occurred in the same area parts of 3 districts in Podlaskie region, within the Area Under Restrictions listed in Part III of Annex to decision Decision 2014/178/EU.

The organised sampling has been undertaken. The epidemiological surveillance of African Swine Fever in WB and DP populations requires controlled collection of numerous samples of biological materials for molecular and serological testing. Serological survey was conducted using commercial ELISA (PPA Ingezim Compact 11.PPA.K3, Ingenasa). Positive or doubtful samples were retested using Immunoperoxidase test (IPT) and/or Immunoblotting (IB). Since 01.01.2014 till 20.05.2015 out of 8548 WB tested sera, 460 were confirmed by ELISA as positive or doubtful for ASFV antibodies. All those samples were retested using IPT (and/or IB) and 450 (97.8%) samples gave negative signal and were confirmed as false positive in the ELISA test. Summary of presented data including only confirmed positive results of serological examination and confirmed PCR (Fernandez-Pineiro et al. 2013) results is shown in Tab. 1.

Survey	Domestic Pig			Wild Boar		
	samples	results		samples	results	
		+	-		+	-
PCR	31353	11	31342	20269	102	20167
serological	2410	0	2410	8548	10	8538
Total number of examined animals	31361	11	31350	20269	104	20165

All cases and outbreaks were found at a distance not exceeding 25 km from the border with Belarus. Distance between the extreme outer two points is approx. 82km. Numbers of ASF cases and outbreaks by months are shown in Fig. 1.



In the region of Poland, where the ASFV prevalence in WB was confirmed, there is only 4.9% of entire Polish pork production. In this region non-professional holdings are dominant and it is comprised of small producers (94,7% of pig holdings have <15 pigs) and apply little to no biosecurity measures. This region was determined by permanent grasslands, set-aside zones and idle lands (appx 42% area). The fact that WB population in the Area Under Restrictions is one of the highest in Eastern Poland (3.5 WB/km²) is cause for particular concern. However, it is worth stressing the fact that this density is limited only to the Area Under Restrictions and this zone is surrounded by low WB density regions (0.5 WB/km²).

Conclusions. Based on 17 months experience, of Poland and other affected by ASF EU countries we can draw presented below conclusions:

1. The annual prevalence estimated in the affected region was approximately 2%.
2. Infected wild boar are, so far, the main vector of transmission of ASFV.
3. Dynamics of spreading of ASF is related to the density of wild boar population; epidemics are self-limiting.
4. Control of wild boar population and closing of all backyards farms, which did not apply necessary biosecurity rules, and are located in “restriction” or “protection” areas are the most important steps towards to stop spreading of ASFV to other regions.

African swine fever current situation in Estonia

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The population of domestic pigs kept in Estonia is about 380 thousand on 920 farms, mainly in Central and Southern Estonia. Pig farms in Estonia are very concentrated: 98.5% of pigs are raised in farms with more than 100 pigs (in total 374 400 pigs kept there). While the number of small scale and backyard farms are small: around 73% of pig farms have 10 pigs or fewer (in total 0.6% of pigs are kept on such farms). The number of farms keeping wild boar is 20, with 440 animals. The hunters' estimation of the population of wild boar is around 22,400.

The first case of ASF in Estonia was diagnosed in September 2014, near to the Latvian border. In total, in 2014, 73 ASFV-positive wild boars were found in seven different infected areas. Five of these areas are located in the southern part of the country, and the other two are in north-eastern Estonia. In 2015, so far (15th of May), 125 ASFV positive wild boar have been found. During 2015, two new infected areas in the southern part of Estonia, has been additionally recorded, not far from previously reported locations. It can be viewed as the normal, natural spread of the disease. Up to now, no positive cases among domestic pigs have been found.

The ASFV strain currently circulating in Eastern Europe, including Estonia, belongs to the p72 genotype II, which has been circulating in Eastern European countries since the introduction of the virus into Georgia in 2007. The subtyping of virus by the analysis of the intergenic regions between *I73R* and *I73L* genes of ASFV genome has been showed that the ASFV from Estonia is 100% homologous with the virus isolated from wild boar and domestic pigs in Latvia, Lithuania and Poland in 2014 and 2015, and with the ASF virus isolated in Belarus in 2013 (Bel13/Grodno).

ASF diagnosis in affected countries, the EURL perspective.

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ASF diagnosis requires the identification of animals that are or have previously been infected with ASFV. Thus, an appropriate diagnosis involves the detection and identification of ASFV-specific antigens or DNA and antibodies. The OIE-recommended tests for virus detection include virus isolation, fluorescent antibody tests (FAT) and both real-time and conventional PCR assays. These PCRs are the most widely used at National Reference Laboratory (NRL) level within the EU. New real-time PCRs, such as the UPL real time PCR (Fernández *et al.*, 2013), developed in recent years have shown to provide greater sensitivity for detecting animals that have survived infection. Other assays such as antigen detection ELISA, which allows for large-scale testing of samples, are also available at NRL level but have been reported as having lower analytical sensitivity than PCR tests.

For the detection of ASF antibodies the OIE-prescribed assays involve the use of an ELISA test for antibody screening, backed up by Immunoblotting (IB) or Indirect Immunofluorescence (IIF) as confirmatory tests. The indirect immunoperoxidase test (IPT), validated by the European Union Reference Laboratory (EURL) for ASF, has effective analytical and diagnostic sensitivity and can be used as an alternative confirmatory test for the detection ASF in porcine sera. In addition, it can be applied with ease to a large number of samples and does not require expensive fluorescence microscope equipment. Currently, three commercial ELISA kits are available for the detection of ASF antibodies (INGENASA, IDVET and SVANOVIR), of which the INGEZIM PPA COMPAC, K3 from INGENASA is the most widely used at EU level.

The techniques currently in use for ASF diagnosis give a confident diagnosis of the disease in any epidemiological situation. However, ASF diagnosis is complex and not always easy due to the wide range of clinical forms and the similarity of its symptoms to those of other viral infections such as Classical Swine fever (CSF). The current epidemic situation of ASF in the EU has created a need to review the sensitivity and specificity of current diagnostic tests and their ability to diagnose ASF in both domestic and wild *Suidae* in affected areas. To this end, the EURL has performed, in collaboration with the NRLs of the four affected EU countries, a comparative study using all the ASF diagnostic tests that are currently being used across the EU to analyse experimental and field samples collected from both domestic and wild pigs during the epidemic outbreaks occurred in 2014 and 2015. In conclusion, the UPL-PCR in combination with the IPT has been the most trustworthy methods for detecting ASF during the epidemic outbreaks affecting EU countries. The use of the most appropriate diagnostic tools is critical when implementing effective control programs.

COMPARATIVE VALIDATION OF COMMERCIAL AND PUBLISHED REAL-TIME PCR METHODS FOR DETECTION OF AFRICAN SWINE FEVER

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1. Introduction

The first detection of African swine fever (ASF) in Poland and the Baltic states in 2014 has rendered ASF a recent threat also for Central and Western European countries. Apart from raising awareness and country-specific surveillance strategies, for earliest possible detection of ASFV introduction it is necessary to provide well characterized diagnostic tools to deal with suspicious cases as well as for routine screening. Nowadays, direct ASFV detection is usually based on real-time PCR. Since comparative studies on ASFV real-time PCR performance are sparse, we performed a validation of several published and commercial ASFV real-time PCR methods.

2. Material and methods

The diagnostic performance of two commercial ASFV real-time PCR kits und three published real-time PCR methods (1-3) was comparatively assessed based on a panel of 176 samples with pre-defined ASFV-status. This panel consisted of proficiency test samples obtained over several years from the EU Reference Laboratory for African swine fever (Spain), samples received from the Friedrich-Loeffler-Institute (Germany) and samples from our own clinical routine. We considered both the analytical and diagnostic sensitivity and specificity of the different methods, as well as their ability to detect sample inhibition by implementation of respective control assays. Furthermore, we looked at whether the tests were capable of simultaneously detecting *Classical swine fever virus* (CSFV).

3. Results and discussion

In direct comparison, both commercial ASFV real-time PCR kits did not perform better than the published methods. On the contrary, one commercial kit even resulted in an increased rate of false-positives. We found that in our hands a multiplex real-time PCR method for simultaneous detection of ASFV and CSFV (3) performed best. Since ASF and CSF are indistinguishable by clinical symptoms alone, laboratory diagnostic testing of suspicious cases for both diseases is warranted. Thus, such a combined ASFV/CSFV test also helps to streamline and cheapen laboratory testing. However, in order to guarantee sensitive detection of both DNA- und RNA-viruses in the same nucleic acid extract, it is necessary to also validate nucleic acid extraction procedures for all potential sample matrices.

4. References

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Monitoring of African swine fever virus antibodies by Ingenasa ELISA in wild boar population in non-infected area in Slovenia in 2014

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African swine fever (ASF) is a complex and lethal disease of swine with major negative impact on regional, national and international trade. After introduction of ASF in 2007 in Georgia, virus was spread in several neighbouring countries of Caucasus region and in 2014 the disease was introduced for the first time into four countries in East part of European Union. The wild boar can be important source of virus infection for domestic pig population and indicator of ASF virus persistence in the environment. The early appearance and long term persistence of ASF antibodies make the antibody detection techniques together with virus detection essential in control programmes. Since there is no vaccine available, the laboratory detection of antibodies can be an important tool for diagnosis in infected and non infected areas. In Slovenia ASF has never been detected. In 2014 serum samples from 504 wild boars were collected within national annual decree for ASF and tested by Ingenasa ELISA (INGEZIM PPA Compac 1.1.PPA.K3). According to OIE recommendation all positive and doubtful samples were tested with confirmatory immunoblotting assay (IB). Out of 504 tested samples 7 were interpreted positive and 2 doubtful in ELISA according to the producer instructions, later all 9 samples were found ASF antibody negative in confirmatory IB. The low percent (1,78 %) of false positive results are probably linked to the haemolysis of collected samples, even though all bad preserved and haemolytic samples were excluded prior testing in ELISA. These results showed, that confirmatory methods are important part of diagnosis also in a wild boar surveillance programme in non infected areas as well as they can provide an important information for usefulness of this ELISA in eradication programme in infected areas. Wild boar population in Slovenia was monitored for the first time in 2014 for ASF antibodies and all samples were negative.