Short Term Scientific Mission at the Estonian University of Life Sciences in Tartu, Estonia

Scientific report

Title: Further validation of alternative sampling methods for the passive African swine fever (ASF) surveillance in wild boar under field conditions

Background:

Introduction of ASF into a free area is generally accompanied by an increased occurrence of fallen animals and thus, hunted wild boar and carcasses are the main target for passive surveillance. However, the encouragement of hunters to report and sample these animals is rather difficult. One part of the problem is the labor- and contact-intensive sampling of decomposed carcasses. Here, pragmatic sampling approaches such as swabs could facilitate sampling actions.

Recently, we were able to show that a blood-soaked forensic livestock swab is suitable for both molecular swine fever diagnosis (Petrov et al., 2014a) and ASFV antibody detection (Blome et al., 2014) under laboratory conditions.

The aim of the short term scientific mission at the Estonian University of Life Sciences was the assessment and validation of this approach under controlled field conditions (proof-of-concept in an affected country). The exercise was done in collaboration with the Estonian Veterinary and Food Laboratory in Tartu, which acts as Estonian National Reference Laboratory (NRL) for ASF and CSF. In the latter, field-collected swab samples were processed and tested by molecular (polymerase chain reaction, PCR) and serological (enzyme-linked immunosorbent assays, ELISA) techniques.

Mission plan

It was foreseen to stay in Tartu for two weeks. During this time, as many swabs as possible should be collected from the field. Should this proof impossible, alternatively archived blood samples at the NRL in Tartu would be the subject of investigations. In the laboratory, nucleic acids should be extracted from the swab followed by routine real-time PCR. In addition, a piece cut from the forensic livestock swab should be used for the ASFV antibody ELISA using the established protocol for filter paper punches.

Outcome:

During the two weeks of the Short Term Scientific Mission only two wild boar carcasses were reported to the Estonian University of Life Sciences (EMU) in Tartu and could thus be directly sampled with the above mentioned livestock swabs. In addition to these samples 42 forensic livestock swabs were soaked in archived samples at the NRL in Tartu.

Material and Methods:

Processing of the swabs

The livestock swabs (Prionics, Zurich, Switzerland) were soaked in different kinds of samples (see Fig. 1).

sample No.	county	sample type	sample No.	county	sample type	sample No.	county	sample type
1	Tartu	organsuspension	15	Saaremaa	blood	29	Lääne	blood
2	Põlva	organs	16	Rapla	blood	30	Pärnu	blood
3	Põlva	organs	17	Saaremaa	organs	31	Saaremaa	blood
4	Saaremaa	organs	18	Saaremaa	blood	32	Pärnu	blood
5	Saaremaa	organs	19	Tartu	blood	33	Saaremaa	bone marrow
6	Jõgeva	organsuspension	20	Pärnu	blood	34	Lääne	blood
7	Lääne	organs	21	Saaremaa	bone marrow	35	Lääne	blood
8	Saaremaa	bone marrow	22	Saaremaa	bone marrow	36	Lääne	blood
9	Harju	blood	23	Saaremaa	blood	37	Lääne	blood
10	Saaremaa	blood	24	Lääne-Viru	bone marrow	38	Lääne	blood
11	Saaremaa	blood	25	Rapla	blood	39	Rapla	blood
12	Saaremaa	blood	26	Rapla	blood	40	Saaremaa	blood
13	Saaremaa	blood	27	Pärnu	blood	41	Saaremaa	blood
14	Saaremaa	blood	28	Pärnu	blood	42	Lääne-Viru	blood

Fig. 1: List of the archived samples used to challenge the livestock swabs with different qualities and types of sample material.

After soaking, the livestock swabs were stored at room temperature for 72 hours to imitate sample transport. Then three pieces were cut from each swab: Two pinhead-sized pieces for the nucleic acid extraction (both manual and automated) and one larger piece (around 5 x 5 mm) for the ELISA.

Viral genome detection

One pinhead-sized piece of each genotube swab was directly cut into a 1.5 ml tube containing 560 µl AVL lysis buffer and carrier RNA (included in the QIAamp Viral RNA Mini Kit, QIAGEN) and then vortexed thoroughly. The tube was incubated for 10 minutes at room temperature and the nucleic acid extraction was performed according the manufacturer's instruction.

To prepare the second pinhead-sized genotube piece for the automated extraction 200 µl RNAse free water were added and the tubes were vortexed thoroughly. Then 140 µl of the liquid were transferred to a new tube to get rid of the genotube piece and subsequently automated extraction was performed using the QIAcube (QIAGEN) automated extraction platform.

After the extraction qPCR was performed on all samples according to the protocol published by Tignon et al. (2011) using swine β-actin as an endogenous control.

Detection of antibodies by commercial ELISA

For the detection of antibodies a commercial ELISA kit (Ingezim PPA COMPAC, Ingenasa) was carried out following the manufacturer's instructions with slight modifications in the first step: The above mentioned piece of the genotube swab (around 5 x 5 mm) was soaked in 100 µl of the sample diluent-buffer of the ELISA kit and vortexed thoroughly. Then 100µl of the "swab-dilution" was added directly to the ELISA plate, adding the 50µl of the diluent buffer only to the wells for the control sera. The next steps were performed following the manufacturers instructions. The Ingezim PPA ELISA detects antibodies directed against the ASFV specific protein p72 in a competitive format.

Penside tests for antibody and antigen detection

25 blood samples were also tested with Lateral Flow Devices (LFD) INGEZIM PPA CROM (INGENASA) based on the technique of direct immunochromatography which detects antibodies against VP72 of ASFV. To challenge the LFDs, samples of bad quality were chosen such as hemolysed or fatty blood and compared to samples of normal quality. Tests were performed according the manufacturer's instructions.

In addition, eight blood samples were tested in LFDs INGEZIM PPA CROM (INGENASA) *Prototype* based on the technique of direct immunochromatography which uses a Monoclonal Antibody (MAb) specific for VP72 of ASFV to detect antigen. Samples of different ct-values (16-36) were chosen to evaluate the cut-off. Tests were performed according the manufacturer's instructions.

Sampling the wild boar carcasses

The two wild boar carcasses were reported from northern Estonia and after bone marrow samples were taken for routine diagnostic they were additionally sampled directly in the forest with genotube swabs. Since the carcasses were already severely decomposed or eaten by other animals (see Fig. 2 + 3) and the outdoor temperature was around -10°C it was challenging to take samples.

Fig. 2: carcass no. 1

Fig. 3: carcass no. 2

To sample carcass no. 1 around 2 ml of lukewarm RNA/DNAse free water was poured on a spot of the carcass where some tissue material was left. Thus, it was possible to take a swab sample from the deeply frozen decomposed tissue. Since the second carcass was less decomposed it was possible to soak the genotube swab in some thawed body liquids (see Fig. 4)

Fig. 4 body liquid of carcass no. 2

The two taken genotube swabs were extracted manually and tested in qPCR analogue to the archived samples (see detailed description above).

Results:

Viral genome detection

Comparing the qualitative outcome of the qPCR results of the genotube pieces with the original sample the results are equal in all but two cases regarding the manual extraction. The automatically extracted samples failed in six cases to reproduce the original qualitative

qPCR result (see Fig. 5). Regarding the ct-values the manually extracted genotubes showed higher genome loads in 25 samples compared to the original automatically extracted sample. Compared to the original ct-value the automated extraction of the genotube piece yielded higher genome loads in 8 samples (blood and organs) and equal results in 3 samples. In all other cases the original sample showed higher genome loads compared to the automatically extracted samples.

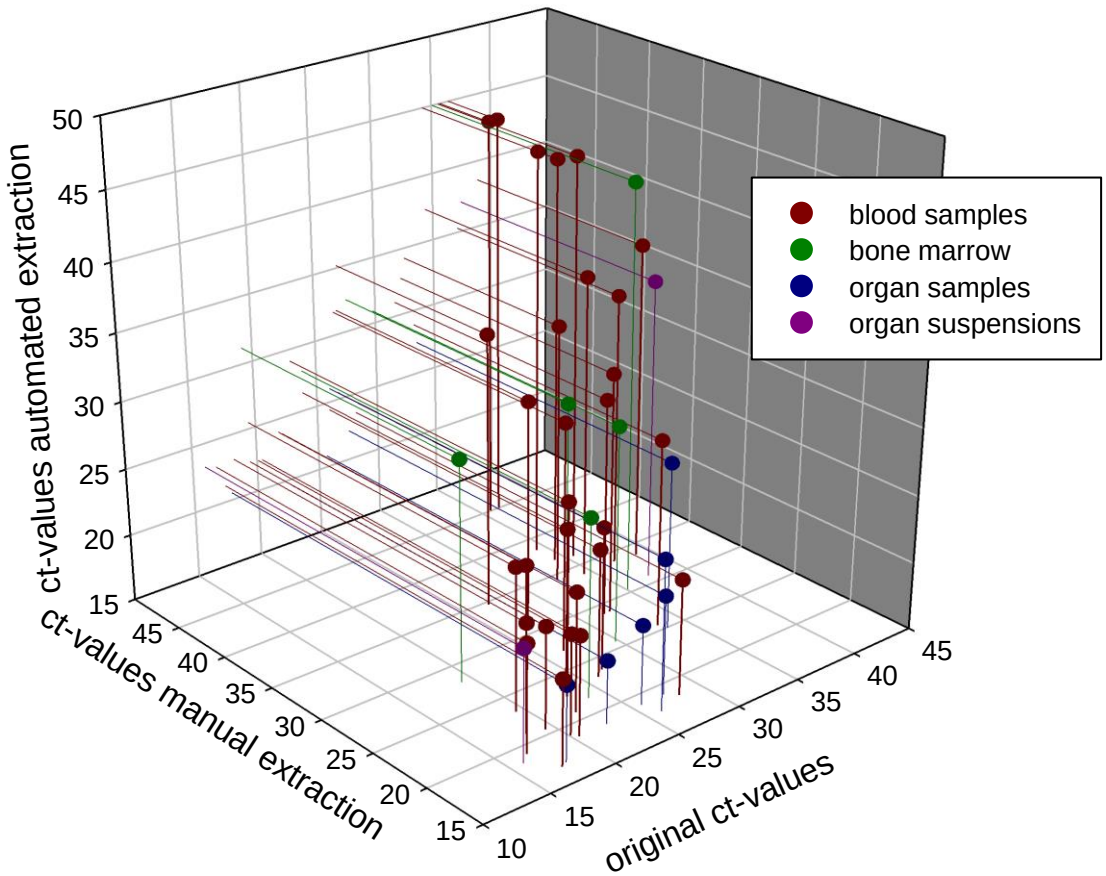

Fig. 5: qPCR results of the genotube pieces (manual versus automated extraction) compared to results of the original sample

The two genotube swab samples from the directly sampled carcasses yielded positive results in the qPCR with ct-values of 37,48 (carcass no.1) and 37,36 (carcass 2). In both cases the genotube swabs showed significantly lower genome loads compared to the corresponding bone marrow samples yielding ct values around 28.

Antibody detection

The comparison of the ELISA results of the original blood sample with the genotube piece revealed a specificity of 100 % without any false positive results. With three false negative results in the genotube samples (25 samples in total) the sensitivity is 80 % (excluding the

doubtful results) and 85,7 % (doubtful results included). Three genotube samples showed a doubtful result in the genotube sample while they were positive in the original one.

Penside tests for antibody and antigen detection

The lateral flow devices for the penside antibody detection showed a specificity and sensitivity of 100 % without any false negative or false positive results.

The lateral flow devices for the detection of ASFV-antigen showed a sensitivity of 37,5 % with 3 true positive results and 5 false negative results out of 8 samples. Four of five false negative samples were tested positive for ASFV-specific antibodies. Since all tested samples were positive regarding the qPCR results no statement for the specificity could be made.

Discussion

Targeted surveillance and early warning actions are crucial to detect ASFV either in free countries but also in already infected areas. As former studies (Petrov et al. (2014b), Blome et al. (2014)) showed, the genotube swabs can be used to detect both African and classical swine fever virus and the corresponding antibodies. Since this has been proved already for blood and serum samples of good quality the present study completes the proof towards the testing of “real-life”-samples (poor quality) and organ material. Genotube livestock swabs are easy to handle and can be stored without cooling which minimizes the effort for the involved hunters. This could improve the compliance of the hunters which is urgently needed for surveillance strategies. Also the risk of contamination is lower using the genotube swabs compared to the so far practiced sampling technique where the blood tube is dipped directly into the blood of the wild boar. Regarding the processing of the sample in the diagnostic labs the workload is slightly higher with the genotubes compared to the common blood samples because a cutting step is needed. Concerning tissue samples the work load is lower with the genotube swabs because no shredding of the organs is needed. The detection of viral genome failed only in two of the genotube samples after the manual extraction. This can be explained with the low viral genome loads of the original blood samples (ct-values around 36). On the other hand, higher genome loads were detected in most of the samples (25 out of 42) after the manual extraction due to the already known higher sensitivity of the manual extraction process compared to the automated extraction of the original sample. Since the detection of the viral genome failed in 6 cases in which the genotube swab was extracted automatically the processing needs to be improved by eventually cutting a larger piece. The same modification might help to solve the false negative results in the antibody detection in the genotube swabs. Regarding the LFDs for the detection of antibodies, they can be recommended as reliable penside tests without restrictions even for samples of very poor quality while the LFDs for antigen detection definitely need improvement before they are ready to use. Also the role of inhibiting antibodies has to be considered.

Acknowledgements

I would like to thank Prof. Dr. Arvo Viltrop, University of Life Sciences in Tartu for hosting me in his faculty and sharing his great knowledge about African swine fever. Further I would like to thank Imbi Nurmoja, Estonian Veterinary and Food Laboratory in Tartu for the opportunity to work in her lab, sharing her experience on AFSV diagnostic and providing the field samples. Moreover, I acknowledge the whole staff from the department of Molecular Analysis and the Virology-Serology department for their constant help and support with the lab work.
Many thanks also to INGENASA for providing some *Prototype* lateral flow devices (INGEZIM PPA CROM) for the detection of ASFV antigen.
Furthermore, I would like to thank the European Cooperation in Science and Technology (COST) for partially funding the Short Term Scientific Mission (Reference Code: COST-STSM-ECOST-STSM-CA15116-290117-082147) within the COST Action Understanding and Combating African Swine Fever in Europe (ASF-STOP).

References

Blome, S., Goller, K.V., Petrov, A., Drager, C., Pietschmann, J., Beer, M., 2014. Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar--extension towards African swine fever virus antibody detection. Veterinary microbiology 174, 607-608.

Petrov, A., Schotte, U., Pietschmann, J., Drager, C., Beer, M., Anheyer-Behmenburg, H., Goller, K.V., Blome, S., 2014a. Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar. Veterinary microbiology.

Petrov, A., Schotte, U., Pietschmann, J., Drager, C., Beer, M., Anheyer-Behmenburg, H., Goller, K.V., Blome, S., 2014b. Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar. Veterinary microbiology 173, 360-365.

Tignon, M., Gallardo, C., Iscaro, C., Hutet, E., Van der Stede, Y., Kolbasov, D., De Mia, G.M., Le Potier, M.F., Bishop, R.P., Arias, M., Koenen, F., 2011. Development and inter-laboratory validation study of an improved new real-time PCR assay with internal control for detection and laboratory diagnosis of African swine fever virus. J Virol Methods 178, 161-170.