ESTONIAN UNIVERSITY OF LIFE SCIENCES
Institute of Veterinary Medicine and Animal Sciences

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COLLECTION OF ORAL FLUID SAMPLES FROM WILD BOAR IN THE FIELD CONDITIONS TO DETECT AFRICAN SWINE FEVER VIRUS (ASFV)

SUUÕÕNE VEDELIKU PROOVIDE KOGUMINE METSSIGADELT VÄLITINGIMUSTES SIGADE AAFRIKA KATKU VIIRUSE MÄÄRAMISEKS

Final Thesis in Veterinary Medicine
Curriculum in Veterinary Medicine

Supervisor: Professor Arvo Viltrop

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LIST OF ABBREVIATIONS

ASF – African swine fever

ASFV – African swine fever virus

CSF – classical swine fever

CSFV – classical swine fever virus

Ct – threshold cycle

DIF – direct immunofluorescence test

DNA – deoxyribonucleic acid

dpi – days post inoculation

ELISA – enzyme-linked immunosorbent assay

et al. – *et alii* (and others)

FAO – the Food and Agriculture Organization of the United Nations

FMD – foot-and-mouth disease

FMDV – foot-and-mouth disease virus

IB – immunoblotting test

IFI – immunofluorescence test

IgA – Immunoglobulin A

IgE – Immunoglobulin E

IgM – Immunoglobulin M

IPT – immunoperoxidase test

mtCyB – mitochondrial DNA cytochrome b gene

No. – *numero* (number)

PCR – polymerase chain reaction

PRRS - porcine reproductive and respiratory syndrome

PRRSV - porcine reproductive and respiratory syndrome virus

pSWAB – pathogen sampling wild animals with baits

ref. – referenced

RNA – ribonucleic acid

RT-PCR – real-time polymerase chain reaction

Taq – Thermus aquaticus

VFL – Estonian Veterinary and Food Laboratory

ABSTRACT

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African swine fever (ASF) is a contagious viral disease that causes a lethal, hemorrhagic fever in domestic pigs and wild boar. The virus has been circulating in Eastern European Union countries since 2014. The aims of the study were to investigate the practical feasibility of non-invasive rope-in-a-bait (pSWAB) oral fluid sampling method for collection of oral fluid samples from wild boar in field conditions, and its suitability for detection of African swine fever virus (ASFV) infection in wild boar population in infected area. The study was conducted in five different feeding grounds in County of Tartu, Estonia. Oral fluid samples were collected with two different pSWABs, loose and fixed baits. Samples were examined in Estonian Veterinary and Food Laboratory for wild boar specific deoxyribonucleic acid (DNA) and ASFV DNA by real-time polymerase chain reaction (RT-PCR). 24 loose bait samples (13%) were obtained out of 183 exposed baits. With fixed baits, 17 samples were obtained with 11 baits. All the fixed bait samples and 71% of the loose bait samples were positive to wild boar DNA. In addition, all the samples were negative to ASFV DNA. Oral fluid sampling with pSWABs is possible, but it is laboriuous and therefore not very practical. In the present study, no ASFV was detected in wild boar, although the virus was circulating in the wild boar population in the immediate vicinity during the period when the study was conducted. More studies are needed to investigate if pSWABs could be used in field to detect other infectious diseases.			
Keywords: pSWAB, rope-in-a-bait, non-invasive sampling			

LÜHIKOKKUVÕTE

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Autor: Hanna Eveliina Männistö		Õppekava: Veterinaarmeditsiin	
Pealkiri: Suuõõne vedeliku proovide kogumine metssigadelt välitingimustes sigade Aafrika katku viiruse määramiseks			
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Sigade Aafrika katk (SAK) on kontagioosne viirushaigus, mis tekitab letaalset hemorraagilist palavikku kodusigadel ja metssigadel. Viirus on tsirkuleerinud Euroopa Liidu idaosa riikides alates aastast 2014. Selle uuringu eesmärkiks oli hinnata metssigadelt suuõõne vedeliku proovide kogumise võimalikkust ja praktilisust mitte-invasiivsel meetodil köispeiutiste (ingl. k. *rope-in-a-bait* – pSWAB) abil välitingimustes, ning selle meetodi sobivust SAK viirusinfektsiooni tuvastamiseks metssea populatsioonides nakatunud aladel.

Uuring teostati viiel erineval söögiplatsil Tartu maakonnas. Suuõõne vedeliku proovid võeti kasutades kahte erinevat tüüpi köispeibutisi, lahtiseid ja fikseerituid. Proovid uuriti Veterinaar- ja Toidulaboratooriumis metssea spetsiifilise desoksüribonukleiinhape (DNA) ja SAK viiruse DNA suhtes RT-PCR meetodil.

Kokku 183-st söötmisplatsidele paigutatud lahtisest peibutisest leiti üles 24 (13%) peibutist. 11 fikseeritud peibutisest võeti vaatlusperioodil vältel 17 proovi. Kõik fikseeritud peibutistest võetud proovid ja 71% lahtiste peibutiste proovidest olid positiivsed metssea DNA suhtes. Kõik proovid olid negatiivsed SAK viiruse DNA suhtes.

Suuõõnevedeliku kogumine köispeibutiste abil on võimalik, kuid on töömahukas ning seetõttu mitte väga praktiline. Käesoleva uuringu käigus SAK viirust metssigadel tuvastada ei õnnestunud, ehkki viirus ringles lähipiirkonna metsseapopulatsioonis uuringu läbiviimise perioodil.

Kõispeibutiste kasutamise sobivus teiste infektsioonhaiguste tuvastamiseks vajab edasist uurimist.

Märksõnad: pSWAB, köispeibutis, mitte-invasiivne proovivõtmine

INTRODUCTION

African swine fever (ASF) is a contagious viral disease of domestic pigs and wild boar, that has been continuing its spread in Eastern European Union since 2014. It has caused major economic losses in affected countries, set limits to pig production and threatened food security (Costard *et al*. 2013). There is no vaccine against the disease and therefore the control of its spread is limited. Among other things, infected wild boar are found to have a significant role in the spread of the disease (EFSA AHAW Panel 2014).

Diagnosis of the disease is approached mainly by clinical signs and post mortem lesions combined with laboratory confirmation (Beltrán-Alcrudo *et al*. 2017). In the laboratory, it is possible to detect ASF virus or antibodies from excretions, secretions, and blood and tissue samples of swine (EURL-ASF 2018). In wild boar, most of the sampling procedures are linked to hunting activities. Therefore, it would be valuable to find a non-invasive animal sampling method for detecting infected populations.

In the present study, two different non-invasive rope-in-a-bait (pSWAB) oral fluid sampling methods were used to evaluate the practical feasibility of oral fluid sample collection from wild boar to detect ASFV in field conditions. Loose and fixed baits were used in experiments that were held in wild boar feeding grounds in County of Tartu, Estonia. The sampling methods were based on wild boar natural curiosity and feeding behavior, such as rooting and foraging on the ground.

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1. LITERATURE REVIEW

1.1. Collection of oral fluid samples from pigs and wild boar for detection of viruses

Oral fluid sampling has been used for several years as a means to diagnose infectious diseases of humans and domestic animals. Oral fluid consists of a mixture of saliva, oral mucosal transudate and gingival crevicular fluid, which present a similar composition to serum. In response to an infection, pathogen-specific IgA, IgM and IgG antibodies and a variety of infectious agents, such as viruses, are shed in oral fluid. Oral fluid sampling is a non-invasive and 'animal friendly' method to collect diagnostic specimens. (Prickett, Zimmerman 2010.)

In swine, oral fluid samples have been successfully used for detecting different pathogens such as foot-and-mouth disease virus (FMDV) (Mouchantat *et al*. 2014b; Vosloo *et al*. 2015), porcine reproductive and respiratory syndrome virus (PRRSV) (Grau *et al*. 2015; Langenhorst *et al*. 2012; Prickett *et al*. 2008), classical swine fever virus (CSFV) (Dietze *et al*. 2017; Grau *et al*. 2015; Mouchantat *et al*. 2014a) and African swine fever virus (ASFV) (Grau *et al*. 2015). For detection of ASFV genome, PCR-based assays are used by the recommendation of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals by World Organization for Animal Health (OIE 2012). Additionally, the possibility to detect ASFV antibodies from oral fluid samples by using enzyme-linked immunosorbent assay (ELISA) and immunoperoxidase technique (IPT) has been demonstrated (Mur *et al*. 2013).

In domestic pigs, oral fluid can be collected by using a pure cotton rope that is suspended over a pen of pigs (Figure 1) (Seddon *et al*. 2012). Cotton-based materials are found to be the most effective material for collecting oral fluid samples for PCR-based assays (Olsen *et al*. 2013). The cotton rope absorbs the oral fluid as pigs chew on the rope, and the samples can be collected by squeezing the fluid into the collection tube. (Seddon *et al*. 2012). In the study presented by Grau *et al*. (2015), pigs infected with ASFV were interested in chewing the ropes until the presentation of severe clinical signs or sudden death, which occurred 5–6 days post inoculation (dpi).

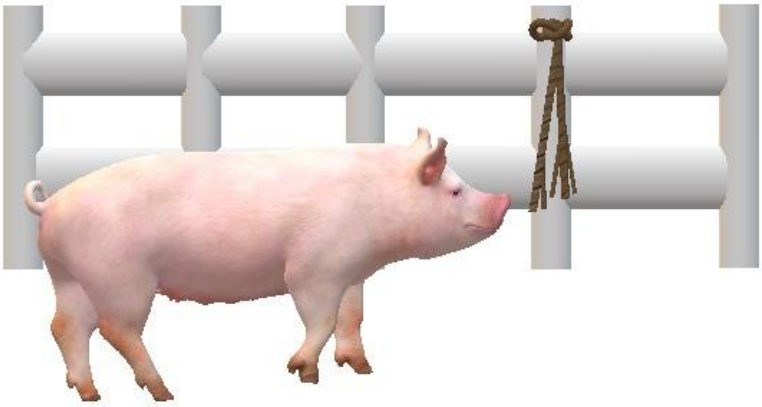


Figure 1. Diagram of oral fluid sampling method used in domestic pigs.

In 2014, Mouchantat *et al*. introduced a non-invasive rope-in-a-bait sampling method called pSWAB (pathogen sampling wild animals with baits) for collecting oral fluid samples which are used to detect the classical swine fever (CSF) viral genome in wild boar. Several months later, the same method was presented for detection of FMDV (Mouchantat *et al*. 2014b). The method used in the aforementioned studies was based on the use of a 10 cm long and 0.8 cm in diameter cotton rope embedded in a cereal-based bait matrix. The pSWABs were placed on the floor of the pen and chewed ropes were recollected and examined for viral genome or antibody detection. In the experimental study conducted with CSFV, thorough evaluation of the sampling method could not be obtained due to an unexpected mild course of the infection during the study (Mouchantat *et al*. 2014a). In the Mouchantat's *et al*. (2014b) study conducted with foot-and-mouth disease (FMD), viral ribonucleic acid (RNA) was detected from the samples as soon as 24 hours after infection. During the study, wild boar were additionally sampled with conventional saliva swabs – the sensitivity of pSWABs proved almost comparable with them. Therefore, the authors suggest pSWABs to be a sensitive, cheap and feasible method for a non-invasive sampling of wild boar, although the practical use of pSWABs in the field requires further evaluation.

According to the article by Rossi *et al*. (2015), the cereal-based bait matrix has been used for CSF oral vaccination baits in wild boar. It contains corn meal, milk powder, almond aroma, paraffin wax, and hardened coconut oil. The consist is based on several studies about wild boar bait acceptance and taste preferences. In the infield study conducted by Kaden *et al*. (2000) in the 1990's, the bait uptake rate was determined to be between 85 and 100%. Wild boar have been found to start to consume the baits from the age of 3.5 months (Brauer *et al*. 2006).

1.2. African swine fever

1.2.1. Etiology

African swine fever (ASF) is a contagious viral disease that causes a lethal, hemorrhagic fever in domestic pigs and wild boar. It is caused by African swine fever virus (ASFV), which belongs to the genus *Asfivirus* in the family *Asfarviridae* (Dixon *et al*. 2009). ASFV is the only member of its family with 22 different genotypes of it identified (Boshoff *et al*. 2007; Galindo, Alonso 2017). Virulence of the virus isolates vary from highly pathogenic strains that cause almost 100% mortality to low-virulence isolates that can be difficult to diagnose (CFSPH 2015).

ASFV is a large, double-stranded DNA virus by which its virions have a complex multilayer structure (Figure 2). An internal core structure contains the nucleoid. It is 70–100 nm in diameter and is surrounded by a thick 30 nm protein layer, core shell. The core is coated by an internal lipid layer and an icosahedral capsid which is 170–190 nm in diameter. Beyond that, extracellular virions have an external lipid-containing envelope that is obtained when the virus buds out through the plasma membrane. The average diameter of the ASFV virion is 200 nm. The replication of the virus primarily occurs in the cytoplasm of macrophages. (Dixon *et al*. 2009; Galindo, Alonso 2017; Salas, Andrés 2013.)

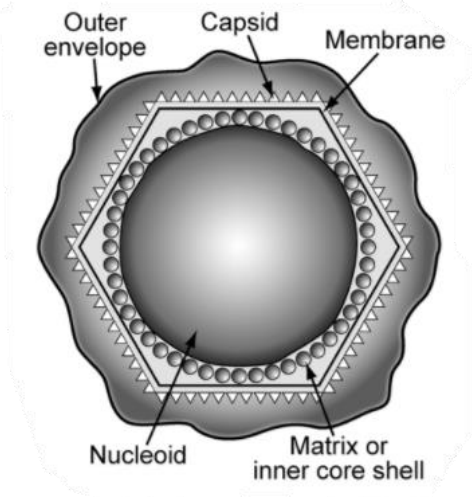


Figure 2. Diagram of extracellular ASFV virions. (Image: Pippa Hawes, Institute for Animal Health, UK)

1.2.2. Species affected

ASFV affects species which belong to the *Suidae* family. The species that can get infected are, among others, domestic pig (*Sus scrofa domesticus*), European wild boar (S*us scrofa ferus*), bush pigs (*Potamochoerus* porcus), warthogs (*Phacochoerus africanus*) and giant forest hogs (*Hylochoerus meinertzhageni*) (Beltrán-Alcrudo *et al*. 2017). Domestic pigs, European wild boar and feral pigs are the hosts that suffer from the disease (OIE 2013). African wild pigs, such as warthogs, bush pigs and giant forest hogs are resistant to the pathogenic effects of the virus and develop no clinical signs of the disease (Beltrán-Alcrudo

et al. 2017). They are, together with soft ticks (*Ornithodoros*) which can transmit the virus through their bites, thought to be wildlife reservoirs for ASFV in Africa (CFSPH 2015, Beltrán-Alcrudo *et al*. 2017). ASFV replicates in soft ticks (Sanchez Vizcaino *et al*. 2015) and can be retained in them for long periods of time (Costard *et al*. 2013). Furthermore, ASFV has been detected to cause mortality in soft ticks (Kleiboeker *et al*. 2007, ref. Costard *et al*. 2013).

1.2.3. Transmission

According to Fernández and White (2010), ASFV is transmitted in three different ways; direct contact, indirect contact through contaminated objects, and with tick vector. Direct transmission involves contact between infected and susceptible animals. The virus is shed in blood, tissues, secretions and excretions of sick and dead animals. Therefore, pigs may become infected via an oro-nasal route as a consequence of contact with excretions from infected pigs. As Olesen *et al*. (2017) and Donaldson *et al*. (1977, ref. Olesen *et al*. 2017) proved in their studies, infectious ASFV can also be transmitted via air over short distances. Excretions and secretions may become aerosolized by means of sneezing and coughing for example, or when excretions or secretions containing dust become aerosolized (de Carvalho Ferreira *et al*. 2013). De Carvalho Ferreira *et al*. (2013) found in their study that ASFV excretion to the air is more likely to occur during an acute disease. In several experimental studies, inoculated pigs or wild boar have been able to transmit the disease when in contact with naïve pigs (Gabriel *et al*. 2011; Gallardo *et al*. 2017; Guinat *et al*. 2014; Olesen *et al*. 2017).

Indirect transmission can follow an infection route via infected meat, fomites such as contaminated premises, vehicles, machineries and clothes, and soft ticks (*Ornithodoros*), which act as biological vectors (Fernández, White 2010). Soft ticks do not only transmit the virus through their bites but they also act as a reservoir host with the ability to transmit the virus transstadially, transovarially, and venerally (Beltrán-Alcrudo *et al*. 2017).

ASFV can survive for long time periods in meat and can be isolated in chilled meat for up to 155 days (Kovalenko *et al*. 1972, ref. EFSA AHAW Panel 2014) and in frozen meat for up to 1 000 days (Adkin *et al*. 2004, ref. EFSA AHAW Panel 2014). Therefore, chilled and frozen meat from infected animals have been ranked to be at high to very high risk of containing and retaining the infectious ASFV (EFSA AHAW Panel 2014). According to the findings of Davies *et al*. (2017), excretions containing ASFV may serve as an important

route of ASFV transmission. In their study utilizing the ASFV Georgia 2007/1 isolate, temperature was found to have an effect on the survival of the virus in urine and feces over time. ASFV DNA was detected in feces from 35 days (at 21°C and 37°C) up to at least 98 days (at 4°C and 12°C) and in urine for at least 126 days (from 4 to 37°C). In oral fluid, ASFV DNA was detected from 14 days (at 12°C and 21°C) to 35 days (at 4°C). At temperature of 37°C, ASFV DNA was not detectable in oral fluid. Infectious ASFV was estimated to survive in feces from 3.7 days (at 37°C) to 8.5 days (at 4°C) and in urine from 2.9 days (at 37°C) to 15.3 days (at 4°C).

1.2.4. Occurrence

African swine fever is considered to be endemic in sub-Saharan Africa, Sardinia, parts of the Caucasus, and Eastern Europe (Beltrán-Alcrudo *et al*. 2017). It was first identified in Kenya in the 1920s (Montgomery 1921, ref. Galindo, Alonso 2017). According to the review article by Penrith (2009), the distribution of the disease limited to Africa until it appeared in Portugal in 1957, where it proceeded to continue its spread to several countries in Europe. It later spread to South America in 1971. The disease was effectively eradicated from Europe in the 1990s, with the exception of Sardinia (Galindo, Alonso 2017). In 2007, ASFV spread from eastern Africa to Georgia, and from there to neighboring countries (Gallardo *et al*. 2014).

The first cases of infected wild boar in the European Union countries were detected in Lithuania in January 2014 (Gallardo *et al*. 2014). Later in the same year, the disease was reported in Poland and Latvia (OIE WAHID 2018). In Estonia, ASF was reported for the first time in September 2014 (Ministry of Rural Affairs of the Republic of Estonia 2018). Genotype circulating in Eastern Europe, Russia and Caucasus region is highly virulent p72 genotype II (Gallardo *et al*. 2014).

Wild boar density (estimated number/ km^2) and quality of the habitat available for them have been considered to be risk factors for the spread of ASFV among the wild boar population (Depner *et al*. 2017). According to Estonian Environment Agency (2017), there were estimated to be 20 600 wild boar in Estonia in 2015. In 2016, the number was 12 220 and in 2017 it was only 5620. In Estonia, the prevalence of ASF antibody-positive and ASFV genome positive wild boar have been calculated among hunted animals by Nurmoja *et al*. (2017b). Antibody-positive wild boar prevalence was presented to be from 1.5 to 7.4%

whereas ASFV genome positive wild boar prevalence was from 2 to 13.7% depending on region.

1.2.5. Pathogenesis and clinical manifestation

ASFV penetrates into an organism usually via the tonsils or dorsal pharyngeal mucosa and transports to the mandibular or retropharyngeal lymph nodes. Occasionally, the first target organs are bronchial, mesenteric or gastrohepatic lymph nodes. The replication of the virus occurs primarily in monocytes and macrophages. From the affected lymphoid organs, the virus spreads through the blood and the secondary viral growth occurs in spleen, body lymph nodes, liver and lungs. Eventually, all tissues contain the virus. (Sánchez-Vizcaíno *et al*. 2009.)

Clinical signs of the disease depend, among the others, on the virus virulence, route of exposure, infectious dose, and endemicity status in the area (Beltrán-Alcrudo *et al*. 2017). According to Sánchez-Vizcaíno *et al*. (2009), the disease appears in four forms – peracute, acute, subacute and chronic. Highly virulent isolates may cause a peracute or an acute disease with clinical signs such as high fever, anorexia, depression, gastrointestinal signs, or a sudden death.

In the Food and Agriculture Organization of the United Nations (FAO) ASF Manual for Veterinarians by Beltrán-Alcrudo *et al*. (2017) the incubation period of the disease has presented to vary from 4 to 19 days depending on the virus, host and route of infection. Excretion of the virus may begin up to 2 days prior to the occurrence of clinical signs. Wild boar that were experimentally inoculated either orally or intramuscularly with highly virulent ASFV Caucasus isolates became viremic from 2 to 7 days post inoculation (dpi) (Blome *et al*. 2012; Gabriel *et al*. 2011). In the study conducted by Gabriel *et al*. (2011), clinical signs developed after an incubation period from 3 to 4 days. The virus was excreted into oropharyngeal fluid mainly from 6 to 7 dpi until the occurrence of death. Mortality of the disease in experimental infections caused with Caucasus isolates reached 100% in less than 10 days (Blome *et al*. 2012, 2013; Gabriel *et al*. 2011).

The virus strains circulating in the European Union have been investigated by Guinat *et al*. (2014), Gallardo *et al*. (2017), Nurmoja *et al*. (2017a) and Olesen *et al*. (2017). Pigs infected intramuscularly with ASFV strains from Georgia and Lithuania became viremic approximately 3 dpi (Gallardo *et al*. 2017; Guinat *et al*. 2014). With the Georgia 2007/1

strain, the virus was isolated from oral fluid swabs generally two days after it was detected from blood (Guinat *et al*. 2014). Clinical signs occurred in both cases 4–5 dpi (Gallardo *et al*. 2017; Guinat *et al*. 2014). Following intranasal inoculation with the ASFV isolate from Poland, the incubation period was approximately 6 days (Olesen *et al*. 2017). In the study presented by Nurmoja *et al*. (2017a), the ASFV strain circulating in north-eastern Estonia was re-isolated and wild boar were inoculated intramuscularly with this isolate. Most of the animals became viremic 4 dpi and started to show clinical signs from 4 to 6 dpi.

According to FAO ASF Manual for Veterinarians by Beltrán-Alcrudo *et al*. (2017), the excretion period of the virus depends on the ASFV strain and its virulence. Swine infected with less virulent virus strains could stay infectious for more than 70 dpi. In the experimental inoculation studies done with ASFV strains circulating in Caucasus, the virus excretion continues throughout the course of the disease until death (Blome *et al*. 2012, 2013; Gabriel *et al*. 2011). In a study performed by Nurmoja *et al*. (2017a) applying the ASFV strain circulating in north-eastern Estonia, one animal was able to recover completely from the disease. The conclusion of the experiment showed that neither the virus nor the viral genomes could be detected in the survivor, however, high antibody levels were present.

1.2.6. Laboratory diagnosis

According to European Union Reference Laboratory for African Swine Fever (EURL-ASF 2018), ASF virus can be detected by virus isolation, detecting ASFV genome by polymerase chain reaction (PCR), or detecting ASF antigen by direct immunofluorescence test (DIF) or antigen ELISA test. ASF antibody detection, which is recommended for subacute and chronic forms and for large-scale testing and ASF eradication programmes, can be done by indirect ELISA test, immunoblotting (IB) test, indirect immunoperoxidase test (IPT), or indirect immunofluorescence test (IFI).

For ASF virus detection from blood, serum and organ samples, PCR is a sensitive, specific and rapid method. It allows detection of the virus from infected animals even before they show clinical signs of the disease or when no infectious virus is detected by virus isolation. (EURL-ASF 2018.)

2. AIMS OF THE STUDY

The aims of the study were to investigate

- the feasibility of the non-invasive rope-in-a-bait (pSWAB) sampling method for collection of oral fluid samples from wild boar in field conditions
- the suitability of pSWAB sampling method for detection of ASFV infection in wild boar population in infected areas

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in the County of Tartu, in the area where ASF outbreaks have been reported. According to Estonian Environment Agency (2017), there were estimated to be 1380 wild boar in the area in 2015. The number reduced to 570 in 2016, and so on to 250 in 2017. The sampling was carried out in five different feeding grounds which were organized by the local hunters and used for hunting wild boar. The geographical distribution of the sampling sites is shown in Figure 3. Altogether seven observation periods were carried out. The study period lasted approximately 1.5 years. ASF infections detected in wild boar during the study is shown in Figure 4.

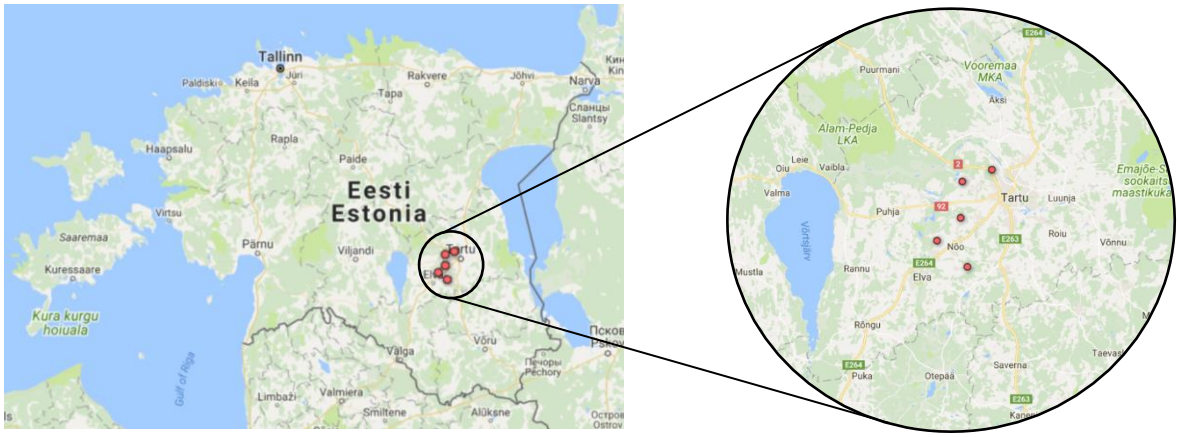
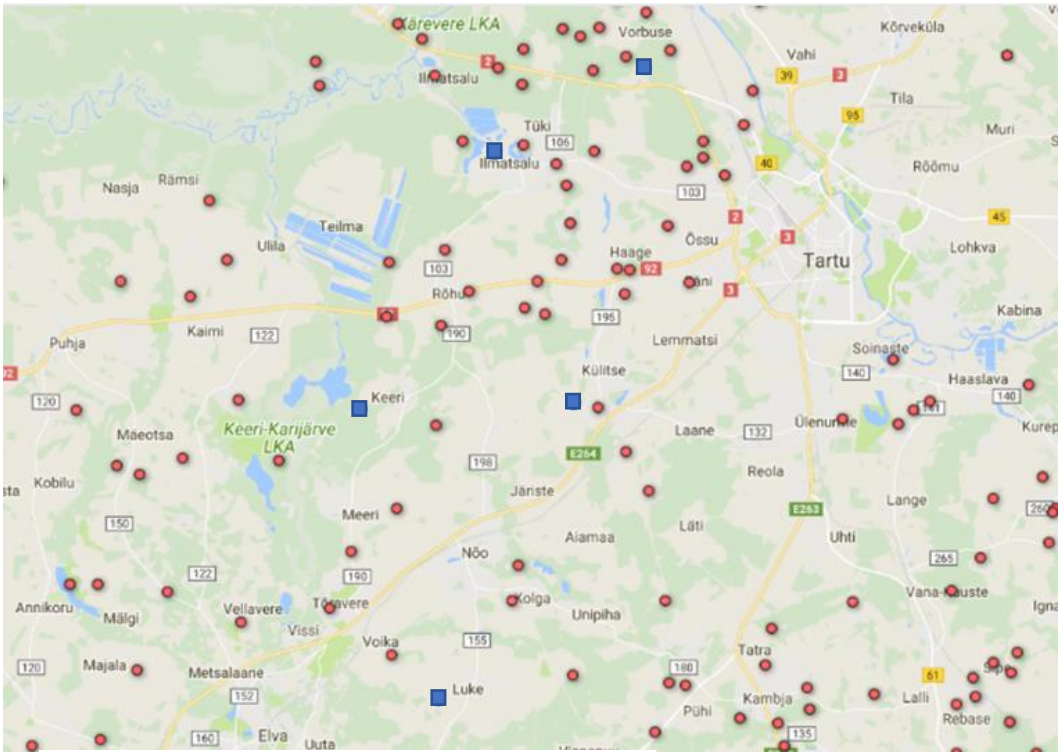


Figure 3. Distribution of the sampling sites used for collecting oral fluid samples from wild boar.



■ Sampling sites ● Cases of ASF in wild boar

Figure 4. Distribution of ASF cases in wild boar in the area of collection from oral fluid samples from October 2015 to March 2017. (The Veterinary and Food Board 2018)

3.2. PSWAB – pathogen sampling wild animals with baits

In this study, two different non-invasive sampling techniques, loose and fixed baits, were used for collecting oral fluid samples from wild boar.

3.2.1. Preparation of the baits

3.2.1.1. Loose baits

Rope-in-a-bait sampling baits described by Mouchantat *et al*. (2014a) were prepared from raw cotton ropes and cereal based bait matrix provided by a manufacturer of oral vaccine of CSF for wild boar (RIEMSER® Schweinepestoralvakzine; IDT Biologika GmbH, Germany). The exact composition of the bait is not known for author.

Bait matrix came in hard form with pieces that were 4x4x1.5 cm in size. It was stored in dark and cool at 4°C before preparation. For the preparation process, bait matrix was melted in a microwave until it became liquid form. Raw cotton ropes with a length of 10 cm and a diameter of 0.8 cm were placed in plastic test tubes, and the tubes were filled in with the

solid bait matrix. The tubes were placed in dark and cool place in order for the bait matrix to become solid again. The plastic test tubes were removed, and baits were store at 4°C until use.

3.2.1.2. Fixed baits

Fixed rope-in-a-bait sampling baits were modified from the technique used for oral fluid sampling in domestic pigs (Prickett *et al*. 2008). Fixed baits were prepared from 3-strand twisted undyed cotton ropes that were 1.6 cm in a diameter (Figure 5). 40 cm of the end of the ropes were embedded into the cereal based matrix which had been melted and stored analogically with the loose baits. The baits were stored at 4°C until use.

Figure 5. Preparation of loose baits. (Photo: Hanna Männistö)

3.3. Delivery and collection

Loose baits were placed on the feeding grounds where the supplementary feed was located. Baits were settled by digging a hole in the ground and sticking the bait inside. The end of the bait was either left fully visible, covered with a 2 cm maximum of soil or obscured under supplementary feed. Some of the baits were settled on the ground and left visible or covered with maximum 2 cm of soil or supplementary feed (Figure 6). During wintertime, the baits were placed on the ground among the supplementary feed and covered with maximum 2 cm of snow or the feed. Wild boar were expected to chew on the baits while they were foraging for food at the feeding ground.

Fixed baits were tied on trees around the feeding grounds (Figure 7). They were placed 5 cm from the ground and supplementary feed was placed under the ropes. Wild boar were expected to chew on the hanging ropes.

Figure 6. Loose baits on the ground at the sampling site of Liudsepa, Luke village, Estonia. (Photo: Klaas Dietze)

Figure 7. Fixed baits tied on trees on the feeding ground of Siki, Vorbuse village, Estonia. (Photo: Hanna Männistö)

During the data collection period, the sampling sites were observed with a camera trap for 24 hours a day. The camera trap was equipped with a motion sensor. The baits and data from

the camera trap were checked regularly during the observing period. Chewed loose baits were collected into individually labelled plastic tubes which were then placed into sealable plastic bags (Figure 8). With fixed baits, chewed portion of the rope was cut off and placed into similar individually labelled plastic tubes and put in sealable plastic bags. All samples were stored at -20 °C until laboratory examination.

Figure 8. Loose bait samples collected from the sampling site of Liudsepa, Luke village, Estonia. (Photo: Hanna Männistö)

Altogether 183 loose baits and 12 fixed baits were exposed in total. The location of the sampling sites and sampling periods are presented in Table 1.

Table 1. Sampling sites, sampling period and type of bate used for wild boar oral fluid sampling

Location of the baiting site	Start date	End date	Type of bait
Kivikaevu, Nõgiaru village, Nõo municipality	19.10.2015	29.10.2015	loose
Meeri väikekoht, Meeri village, Nõo municipality	27.10.2015	09.11.2015	loose
Liudsepa, Luke village, Nõo municipality	27.10.2015	29.10.2015	loose
Liudsepa, Luke village, Nõo municipality	09.11.2015	13.11.2015	loose
Siki, Vorbuse village, Tähtvere municipality	11.01.2016	18.01.2016	loose + fixed
Siki, Vorbuse village, Tähtvere municipality	16.03.2016	21.03.2016	fixed
Ilmatsalu village, Tähtvere municipality	27.12.2016	22.03.2017	fixed

3.4. Laboratory analysis

All the samples were examined in Estonian Veterinary and Food Laboratory (VFL) for wild boar specific deoxyribonucleic acid (DNA) and ASF genome by using real-time polymerase chain reaction (RT-PCR) according to the protocol published by Tignon *et al*. (2011). Samples with threshold cycle (Ct) values below 40 were considered positive.

3.4.1. Preparation of the samples for DNA extraction

Chewed ropes were placed in 15 ml tubes with a cap. 5 ml of phosphate-buffered saline was added to the tubes, followed by an intensive vortex. The rope-containing tubes were stored at 4°C for 1 hour to let the ropes absorb the fluid. After vortexing again, the ropes were taken out of tubes with forceps and placed into 10 ml syringe. The ropes were squeezed with the plunger of the syringe and the fluid was simultaneously collected in sterile tubes, followed by centrifugation for 10 min at 3000 rpm. 200 µl of starting material was used for DNA extraction, which was performed automatically using the QIAamp cador Pathogen Mini Kit and QIACUBE DNA extractor according to the manufacturer's instructions. (Personal communication with Annika Vilem, VFL.)

3.4.2. PCR assays

First PCR was carried out with the primers according to Forth (2015) to detect the wild boar specific mitochondrial DNA cytochrome b gene (mtCytB). RT-PCR was performed by using commercially available QuantiTect Multiplex PCR Kit (Qiagen) in a total volume of 25 µl. Briefly, 5.5 µl of DNase/RNase-free water, 12.5 µl 2x QuantiTect Multiplex mastermix, 2 µl mtCytB specific primer mix (10 µM of each primer and 1.25 µM of FAM-BHQ-2 probe) were pooled as mastermix. Finally, a 5 µl aliquot of DNA extracted from samples was added to 20 µl of PCR master mix. The cycling protocol was as follows: one cycle of 95°C for 15 min to activate Thermus aquaticus (Taq) polymerase, followed by 45 cycles consisting of denaturation for 1 min at 95°C and annealing/elongation for 1 min at 60°C. (Personal communication with Annika Vilem, VFL.)

Samples positive for mtCytB were further investigated for ASFV using the primers described by Tignon *et al*. (2011). RT-PCR was performed using commercially available 5xHOT FIREPol Probe qPCR Mix Plus, no ROX kit (Solis BioDyne) in total volume of 20 µl. Briefly, 7 µl of DNase/RNase-free water, 5 µl of 5xHOT FIREPol Probe qPCR mix, 0.8 µl of both ASFV p72 specific primers (final concentration 0.4 µM), 0.4 µl of ASF p72

specific FAM-TAMRA probe (final concentration 0.2 μM), 0.8 μl of both endogenous control primers (beta actin, final concentration 0.4 μM) and 0.4 μM of endogenous control HEX-BHQ-1 probe (beta actin) were pooled as a mastermix and finally 5 μl of DNA was added to 15 μl of PCR master mix. The cycling protocol was as follows: one cycle of 95°C for 15min to activate Taq, followed by 45 cycles consisting of denaturation for 20 seconds at 95°C and annealing/elongation for 1 min at 60°C. (Personal communication with Annika Vilem, VFL.)

4. RESULTS

According to the camera trap recordings, wild boar were visiting the feeding grounds mainly during night time. Chewed loose baits were found in 15-meter radius from where they were distributed.

In Kivikaevu, village of Nõgiaru, the study was carried out with loose baits in October (Table 1). In addition to wild boar, the baiting site was visited also by European roe deer (*Capreolus capreolus*), raccoon dogs (*Nyctereutes procyonoides*), and moose (*Alces alces*). Only one loose bait sample (3%) (Table 2 and 6) was collected during the 10 days period. Other loose baits on the ground had either disappeared or had not been attractive to wild boar.

Table 2. Sampling period, number of loose baits distributed, and samples collected in Kivikaevu, Nõgiaru village, Estonia

Kivikaevu, Nõgiaru village, Nõo municipality		
Visit date	**Baits distributed**	**Samples collected**
	n	**n**
19.10.2015	25	0
20.10.2015	0	0
21.10.2015	0	0
22.10.2015	0	0
23.10.2015	0	0
26.10.2015	0	1
27.10.2015	5	0
28.10.2015	8	0
29.10.2015	0	0
Total	38	1

In Meeri väikekoht, village of Meeri, the study was also carried out with loose baits in October (Table 1). According to the camera trap recordings, no wild boar visited the baiting site. Altogether 5 samples (10%) were collected (Table 3 and 6), and despite the camera trap recordings, wild boar DNA was detected from 2 out of 5 samples. According to the camera trap recordings, baits with negative results were assumed to be consumed by raccoon dogs and red foxes (*Vulpes vulpes*) which were, including European roe deer, visiting the baiting site.

Table 3. Sampling period, number of loose baits distributed, and samples collected in Meeri väikekoht, Meeri village, Estonia

Meeri väikekoht, Meeri village, Nõo municipality		
Visit date	**Baits distributed**	**Samples collected**
	n	**n**
27.10.2015	15	0
28.10.2015	10	0
02.11.2015	15	0
03.11.2015	0	2
04.11.2015	10	1
05.11.2015	0	1
06.11.2015	0	1
07.11.2015	0	0
09.11.2015	0	0
Total	50	5

The two experiments held in Liudsepa, village of Luke, gave altogether 13 samples (23%) (Table 4 and 6). 4 out of 13 samples were negative for wild boar DNA. In the first experiment which was held at the same time with Kivikaevu and Meeri väikekoht, 25 loose baits were placed underground and 2 of those had been dug up and chewed (Figure 8). 5 samples got collected from the 10 baits placed on the ground. Some of the baits were disappeared. In the second experiment that took place a week after, all the baits were placed underground. Altogether 6 samples were found. The first two samples were from the baits which had been left to the feeding ground from the first experiment. The baiting site was visited by wild boar, a red fox and European roe deer.

Table 4. Sampling period, number of loose baits distributed, and samples collected in Liudsepa, Luke village, Estonia

Liudsepa, Luke village, Nõo municipality		
Visit date	**Baits distributed**	**Samples collected**
	n	**n**
27.10.2015	35	0
28.10.2015	0	5
29.10.2015	0	2
Total	35	7
09.11.2015	16	2
10.11.2015	0	0
11.11.2015	10	2
12.11.2015	0	0
13.11.2015	0	2
Total	26	6

Figure 9. Partially chewed loose bait in Liudsepa, Luke village, Estonia. (Photo: Klaas Dietze)

In Siki, village of Vorbuse, the first experiment was held in January (Table 1) while there was still snow on the ground. Both loose and fixed baits were used. According to the camera trap recordings, wild boar and European roe deer had been visiting the baiting site every night. All the loose baits were placed on the ground among the supplementary feed due to frozen ground. Most of them had disappeared and altogether five samples out of 39 baits (13%) were collected (Table 5 and 6). During the one-week experiment period, wild boar did not seem to be interested in the fixed bait. Nevertheless, in the end of the observation period, 6 samples were collected from the fixed rope (Table 7). Wild boar were not recorded to chew on the rope, as the battery of the camera trap had run out during the weekend. However, all the samples were confirmed to have been chewed by wild boar by RT-PCR in the laboratory (Table 8).

The second experiment held in Siki in March was performed only with fixed baits (Table 1). In a one-week period, wild boar were visiting the feeding ground every night but were not interested in the baits. European roe deer were also recorded by the camera trap. The location of the baits in the feeding ground was changed to other trees halfway through the observation period. Still no wild boar approaching was recorded and samples were not collected.

Table 5. Sampling period, number of loose baits distributed, and samples collected in Siki, Vorbuse village, Estonia

Siki, Vorbuse village, Tähtvere municipality		
Visit date	**Loose baits distributed**	**Loose bait samples collected**
	n	**n**
11.01.2016	15	0
12.01.2016	9	1
13.01.2016	5	1
14.01.2016	5	2
15.01.2016	5	1
18.01.2016	0	0
Total	39	5

Table 6. Loose bait sampling sites, number of baits and collected samples

Loose baits			
Location of the baiting site	**Exposed baits in total**	**Samples collected**	
	n	**n**	**%**
Kivikaevu, Nõgiaru village, Nõo municipality	38	1	3
Meeri väikekoht, Meeri village, Nõo municipality	50	5	10
Liudsepa, Luke village, Nõo municipality	56	13	23
Siki, Vorbuse village, Tähtvere municipality	39	5	13
Total	183	24	13

Table 7. Fixed bait sampling sites, number of baits and collected samples

Fixed baits		
Location of the baiting site	**Exposed baits in total**	**Samples collected**
	n	**n**
Siki, Vorbuse village, Tähtvere municipality	5	6
Ilmatsalu village, Tähtvere municipality	6	11
Total	11	17

The last experiment held in village of Ilmatsalu, was carried out a year later, from the end of December until the end of March (Table 1). Altogether 11 samples were collected out of 6 exposed fixed baits (Table 7). The feeding ground was regularly visited by wild boar (Figure 10) but also by European roe deer, raccoon dogs, foxes and a moose. In the last month of the observation period, wild boar visited the site less often.

Figure 10. Camera trap picture of wild boar chewing on rope-in-a-bate baits tied on trees on the feeding ground of Ilmatsalu village, Estonia.

Laboratory results of the samples are shown in Table 8. Altogether 24 loose bait samples (13%) were obtained out of 183 exposed baits (Table 6) over 31 nights. With fixed baits, 17 samples were obtained with 11 baits (Table 7) over 100 nights of the observing period. All the fixed bait samples and 71% of the loose bait samples were wild boar DNA (RT-PCR) positive (Table 9). All the samples were negative to ASFV (RT-PCR).

Table 8. Results of RT-PCR analysis of pSWAB samples for ASFV and wild boar specific DNA

A. Loose baits

Location of the baiting site	Animals visiting the feeding ground	Date	Sample ID	ASFV DNA PCR result pos/neg	beta-actine[a] routine PCR Ct value[b]	beta-actine mtCyB[c] PCR Ct value
Kivikaevu	wild boar raccoon dog moose roe deer	26.10.2015	1	neg[d]	neg	32.58
Meeri	raccoon dog	3.11.2015	1	neg	neg	neg
	red fox	3.11.2015	2	neg	neg	neg
	roe deer	4.11.2015	2/1	neg	neg	neg
		5.11.2015	3/1	neg	30.76	37.52
		6.11.2015	4/1	neg	38.31	neg
Liudsepa	wild boar	28.10.2015	1	neg	neg	34.19
	red fox	28.10.2015	2	neg	neg	37.81
	roe deer	28.10.2015	3	neg	neg	35,83
		28.10.2015	4	neg	neg	neg
		28.10.2015	5	neg	38.54	33.87
		29.10.2015	2.1	neg	36.16	37.63
		29.10.2015	2.2	neg	neg	neg
		9.11.2015	NA	neg	neg	30.15
		9.11.2015	NA	neg	neg	35.82
		11.11.2015	2-1	neg	neg	36.27
		11.11.2015	2-2	neg	neg	neg
		13.11.2015	1	neg	neg	neg
		13.11.2015	2	neg	neg	34.76
Siki	wild boar	12.1.2016	1/12.1.	neg	34.27	30.59
	roe deer	13.1.2016	1/13.1.	neg	30.01	27.47
		14.1.2016	1/14.1.	neg	34.84	31.14
		15.1.2016	1/15.1.	neg	30.09	28.59
		18.1.2016	1/18.1.	neg	27.92	25.90

B. Fixed baits

Location of the baiting site	Animals visiting the feeding ground	Date	No. of samples (sample ID)	ASFV DNA PCR result pos/neg	beta-actine routine No. positive (range of Ct values)	beta-actine mtCyB No. positive (range of Ct values)
Siki	wild boar roe deer	18.1.2016	6 (2-7/18.1)	neg	6 (29.18–33.22)	6 (29.35–31.04)
Ilmatsalu	wild boar raccoon dog red fox roe deer moose	12.1.2017	4 (1.IL-4.IL)	neg	2 (34.07–37.08)	4 (29.82–35.31)
		16.1.2017	4 (5.IL-8.IL)	neg	2 (35.75–36.49)	4 (30.59–36.45)
		31.1.2017	3 (9.IL-11.IL)	neg	2 (34.88–37.12)	3 (33.91–35.54)

[a] Wild boar specific gene marker; [b] Ct – threshold cycle; [c] mtCyB – mitochondrial DNA cytochrome b gene; [d] neg – Ct value ≥ 40 – negative result

Table 9. Summary of laboratory results of non-invasive rope-in-a-bait oral fluid samples from wild boar

Bait type	Samples collected	Positive wild boar DNA (RT-PCR)		Positive ASFV genome (RT-PCR)	
	n	n	%	n	%
Loose baits	24	17	71	0	0
Fixed baits	17	17	100	0	0

5. DISCUSSION

There are no published results on the collection of oral fluid samples from wild boar in field conditions. The idea of using loose baits is based on wild boar's natural behaviour to forage on the ground. The potential of fixed baits may be explained by wild boar's natural curiosity, and therefore their will to explore, chew and play on things. Although, compared to domestic pigs and wild boar living under experimental conditions, wild boar living in natural surroundings are provided with more stimulus and activity which might affect negatively on their interest into baits organized by human.

Collection of the loose bait samples was a time-consuming process. The samples were found over a wide area and undergrowth of forest and freshly fallen snow in the winter time made it difficult and sometimes even impossible to find them. Most of the baits placed on the feeding ground disappeared. According to the camera trap recordings, some of them were most likely picked up by other animals, such as red foxes, raccoon dogs or birds. Alternatively, they may have been collected by external visitors such as hunters. It is also possible that the wild boar have consumed the baits, or that animals have carried them too far to be found.

The problem of losing the baits was solved by fixing the baits to trees. Routine check-ups of the sampling sites and sample collection did not take as much time compared to the sampling with loose baits. However, wild boar did not show much interest in the fixed baits, and therefore a longer sampling period would be needed. In conclusion, oral fluid sampling with fixed baits has its own limitations to be considered.

Our study was carried out during the time of the year when supplementary feed was provided to wild boar. The summer period was framed out of the study as natural food availability is higher during this time of the year and wild boar visits to the feeding grounds were estimated to be lower. The quality of the feed in the feeding grounds changed during the year – by visual estimation it was poorer in the late winter. That may explain why in the end of the experiment held in Ilmatsalu, wild boar were not visiting the feeding ground that often. Also, according to the study presented by Thurfjell *et al*. (2014), wild boar decrease movement in bad weather conditions including low temperatures and deep snow.

In this study, no ASFV could be detected from wild boar oral fluid samples. This may be due to the fact that, as Grau *et al*. (2015) presented in their study, pigs who present clinical signs of the disease lose their appetite, and their interest to chew on the ropes decreases. The ASFV strain circulating in Estonia causes an onset of the clinical signs usually within a week post infection (Nurmoja *et al*. 2017a). In experimental inoculation studies, ASFV have been detected from oral fluid samples 5–7 dpi (Gabriel *et al*. 2011; Guinat *et al*. 2014). Although those studies were done by intramuscular inoculation and therefore may not present the natural infection pattern, according to those results we may suspect that in our study, the infected wild boar did not have an appetite anymore by the time they were excreting the virus genome via oral fluid. That might be one of the reasons why all the collected samples were negative for ASFV genome. Therefore, probability to get an infected wild boar who is excreting the virus via oral fluid to chew on the pSWABs would be small.

On the other hand, it might be possible that by the time the study was conducted, no ASF was circulating among the animals we were sampling. We cannot exclude that the herds visiting the sampling sites may not have become infected during the study and therefore the infection was not detected. To exclude that possibility, other samples should have been taken simultaneously with oral fluid sampling to confirm if ASFV was present.

With pSWABs it is not possible to get information about individual animal disease status. One sample might be chewed by more than one wild boar (Mouchantat *et al*. 2014), and it is not likely that all the animals chew on the baits. However, it may give us information about epidemiological status in certain areas.

While the ASF spread among wild boar is slow, the sampling must be extensive. In order to detect the virus, all feeding grounds in an area at risk should be covered. Collection of the samples is a time-consuming process and requires a lot of effort to get a reliable number of samples. All that make the use of pSWABs probably unpractical for ASF surveillance.

As different pathogens are excreted into oral fluid, in theory pSWABs could also be used to determine other diseases, such as porcine reproductive and respiratory syndrome (PRRS), CSF and FMD. Additionally, the method could be modified to collect oral fluid samples from other wild ungulates as well. It would also enable researchers to carry out sampling in areas where hunting is prohibited, such as in national parks. However, the practical use of the baits requires further research, especially within infield conditions.

6. CONCLUSIONS

It is possible to use pSWABs to collect oral fluid samples from wild boar to detect ASFV, although the method is not very practical in field conditions. It takes time to get a reliable number of samples and by the time infected wild boar start to excrete the virus, they might not be willing to chew on the baits anymore. PSWABs are not suitable for individual animal sampling but could possibly be used to detect infected herds.

SUMMARY

Collection of oral fluid samples from wild boar in the field conditions to detect African swine fever virus (ASFV)

African swine fever (ASF) is a contagious viral disease that causes a lethal, hemorrhagic fever in domestic pigs and wild boar. The virus is shed in blood, tissues, secretions and excretions of sick and dead animals. It is highly resistant in environment depending on the virus strain. Transmission of the disease may occur in three different ways; direct contact between infected and susceptible animals, indirect contact through contaminated objects, and with tick vector.

The disease has been circulating in the Eastern European Union area since 2014.

ASF virus infection can be detected by virus isolation, detecting ASFV genome by polymerase chain reaction (PCR), or detecting ASF antigen from blood or tissues as well as by antibody detection form blood or tissue fluids. It has also been demonstrated in experimental conditions that oral fluid may be used in diagnostics of the disease by detecting the virus or its DNA. Therefore, our aim was to investigate whether oral fluid samples could be collected from wild boar in field conditions by using non-invasive rope-in -a-bait (pSWAB) oral fluid sampling baits and evaluate the suitability of pSWABs for detecting ASFV infection in wild boar population in infected area.

Sampling was spanned a period of 1.5 years from October 2015 until March 2017. The sampling was conducted in five different feeding grounds in County of Tartu, Estonia. Altogether 24 loose bait samples (13%) were obtained out of 183 exposed baits over 31 nights. With fixed baits, 17 samples were obtained with 11 baits over 100 nights of observing period. All the fixed bait samples and 71% of the loose bait samples were positive to wild boar DNA (RT-PCR). No ASFV DNA was detected from any of the samples. In conclusion, oral fluid sampling with pSWABs is possible, but it is laborious and therefore not very practical. Collection of the loose bait samples from the feeding ground and its surroundings is a time-consuming process and requires a lot of effort.

In the present study, no ASFV was detected in wild boar, although the virus was circulating in the wild boar population in the immediate vicinity during the period when the study was

conducted. We may suspect that infected wild boar lose their appetite, and therefore their interest to chew on the ropes by the time they excrete the virus genome via oral fluid.

More studies are needed to investigate the practical use of pSWABs for detecting other infectious diseases in field conditions.

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KOKKUVÕTE

Suuõõne vedeliku proovide kogumine metssigadelt välitingimustes sigade Aafrika katku viiruse määramiseks

Sigade Aafrika katk (SAK) on kontagioosne viirushaigus mis tekitab letaalset hemorraagilist palavikku kodusigadel ja metssigadel. SAK viirus levib haigete ja surnud loomade vere, kudede,sekreetide ja ekskreetide vahendusel. SAK viirus on väga resistentne väliskeskkonnas. Haiguse ülekanne võib toimuda kolmel erineval viisil – otsesel kontaktil haigete ja vastuvõtlikute loomade vahel, kaudse kontakti tulemusel viirusega saastunud esemete või keskkonnaga, ning puuksiirutajate vahendusel.

SAK on levinud Euroopa Liidu idaosas alates 2014. aastast.

SAK viiruse nakkust loomadel on võimalik tuvastada viiruse isoleerimise või antigeenide määramise teel, samuti viiruse DNA-määramisega PCR meetodil verest või kudedest, samuti viiruse vastaste antikehade määramisega verest või koevedelikest. Laboratoorsetes tingimustes on demonstreeritud, et haiguse diagnostikas, täpsemini viiruse või selle DNA tuvastamiseks saab kasutada ka sigade suuõõne vedeliku. Käesoleva uuringu eesmärgiks oli uurida kas suuõõnevedeliku proove on võimalik koguda elus metssigadelt välitingimustes kasutades selleks köispeibutisi (ingl. k. *rope-in-a-bait* – pSWAB), ning hinnata meetodi sobivust SAK viirusinfektsiooni tuvastamiseks metssigade populatsioonis nakatunud piirkonnas.

Uuringuks koguti proove 1.5 aasta jooksul oktoobrist 2015 märtsini 2017. Proove koguti viiel erineval söödaplatsil Tartu maakonnas. Kokku 183-st söötmisplatsidele paigutatud lahtisest peibutiseste hulgast 31 ööpäeva jooksul leiti üles 24 (13%) peibutist. 11 fikseeritud peibutisest hulgast saadivõeti vaatlusperioodil kätte vältel 17 proovi 100 ööpäeva jooksul. Kõik fikseeritud peibutistest võetud proovid ja 71% lahtiste peibutiste proovidest olid positiivsed metssea DNA suhtes. Kõik proovid olid negatiivsed SAK viiruse DNA suhtes. Uuringu tulemustest võib järeldada, et suuõõnevedeliku proovide kogumine köispeibutiste abil on võimalik, kuid on töömahukas ning seetõttu mitte väga praktiline. Lahtiste proovide otsimine söödaplatsilt ja selle ümbrusest nõuab märkimisväärselt aega ja pingutust.

Käesoleva uuringu käigus SAK viirust metssigadel tuvastada ei õnnestunud, ehkki viirus ringles lähipiirkonna metsseapopulatsioonis uuringu läbiviimise perioodil. Võimalik, et metssead ei ole enam peibutistest huvitatud sellel ajal kui viirus hakkab erituma suuõõne vedeliku kaudu, kuna on kaotanud haiguse tõttu söögiisu.

Kõispeibutiste kasutamise sobivus teiste infektsioonhaiguste tuvastamiseks vajab edasist uurimist.

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