Lymph-Node-Sampler-Based Nucleic Acid Detection Helps to Diagnose and Control African Swine Fever in Pig Production

Xiaowen Li

College of Veterinary Science and Animal Husbandry: Sam Higginbottom University of Agriculture Technology and Sciences

Yang Li

Shandong New Hope Liuhe Agriculture and Animal Husbandry Technology Co.,Ltd.

Mingyu Fan

Shandong new hope liuhe agriculture and animal husbandry technology co.,ltd.

Shiran Fan

Shandong newhope liuhe group co.,ltd.

Wenchao Gao

Shandong newhope liuhe group co.,ltd.

Peng Yuan

Shandong newhope liuhe group co.,ltd.

Jing Ren

Dezhou University

Jingtao Li

Shandong new hope liuhe group co.,ltd.

Jincheng Yu

Shandong new hope liuhe group co.,ltd.

Weisheng Wu

Shandong new hope liuhe group co.,ltd.

Junxian Li

Shandong new hope liuhe group co.,ltd.

Qiannan Yu

Shandong new hope liuhe group co.,ltd.

Zhenwen Shao

Shandong new hope liuhe group co.,ltd.

Zhichun Yan (✉ zhichunyan@vip.tom.com)

Shandong New Hope Liuhe Co.,Ltd.

Keywords: African swine fever, lymph node, sampler, real-time PCR, diagnostics

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1 **Lymph-Node-Sampler-Based Nucleic Acid Detection Helps to Diagnose and**
2 **Control African Swine Fever in Pig Production**

3 **Running title: Lymph Node Sampler Helps to Diagnose ASF**

4 Xiaowen Li^{1,2}, Yang Li^{1,2}, Mingyu Fan^{1,2}, Shiran Fan^{1,2}, Wenchao Gao^{1,2}, Peng Yuan^{1,2},
5 Jing Ren³, Jingtao Li², Jincheng Yu², Weisheng Wu², Junxian Li², Qiannan Yu²,
6 Zhenwen Shao^{1,2}, Zhichun Yan^{1,2}

7 ¹ Shandong New Hope Liuhe Agriculture and Animal Husbandry Technology Co., Ltd,
8 Dezhou, China

9 ² Shandong New Hope Liuhe Group Co., Ltd, Qingdao, China

10 ³ Shandong Provincial Laboratory of Swine Health Big Data and Intelligent Monitoring,
11 Dezhou, China

12 E-mail for correspondence: Dr Zhichun Yan; zhichunyan@vip.tom.com.

Abstract

Background: African swine fever (ASF) is a highly contagious hemorrhagic and transboundary animal disease. It has rapidly spread to many regions of the world and is responsible for serious production and economic losses. However, clinical diagnosis is impractical for the similar classic symptoms of ASF and several other normal diseases. How to make a definite diagnosis is a top priority.

Results: In the present study, lymph node samples were collected by the patented “lymph node sampler”, which makes the sampling safer, more efficient and minimally invasive. The ASF virus (ASFV) contents of lymph node sample as well as serum, oral fluid, nasal and rectal swab samples from pig production line were detected by real-time PCR. The big data results demonstrated that the lymph node samples contained more stable and strongly positive ASFV nucleic acid than the porcine exudate and serum samples. Taking the lymph node sample is one of the assistant ways in diagnosing pigs suspected of having ASF. We supervised twenty farms that realized the accurate diagnosis and eliminating of the risk in the shortest time period by using lymph node sampler.

Conclusions: Our findings suggest that the lymph node sample is an ideal tissue for diagnosing ASFV infection. The lymph node sampler is a convenient tool for lymph node sampling for practitioners.

Keywords: African swine fever; lymph node; sampler; real-time PCR; diagnostics

1. Background

African swine fever (ASF) is a devastating swine viral disease that is reportable to the World Organization for Animal Health (OIE). It causes high fever, severe depression, ataxia and hemorrhages in domestic swine and results in a mortality rate approaching 100% (Ge et al., 2018). ASF is a highly contagious infectious disease and can be transmitted by exposure to infectious pigs or tissues, contaminated swills, transport vehicles, the clothing of animal workers and infected soft ticks (Penrith and Vosloo, 2009; Guinat et al., 2016; Olesen et al., 2018). Airborne transmission has been demonstrated at close range (Wilkinson and Donaldson, 1977; Olesen et al., 2017). Since its emergence in Kenya in the 1920s, ASF remains endemic in sub-Saharan Africa, the Indian Ocean region and eastern Europe (Costard et al., 2009; Oganessian et al., 2013; Gallardo et al., 2014). In 2007, ASF was confirmed in Georgia and Russia, posing the risk of further dissemination into neighboring countries (Chapman et al., 2011). Since August 2018, ASF has spread to China, the world's largest pork producer and a leading pork importer (Ma et al., 2020). Ongoing outbreaks of ASF afflicted the livestock industry and wiped out 40% of the nation's pigs, leading to severe socioeconomic losses (Li and Tian, 2018; Zhou et al., 2018; Lu et al., 2020).

The causative agent of the disease, ASF virus (ASFV), belongs to the genus *Asfivirus* within the *Asfarviridae* family (Gogin et al., 2013). ASFV is a complex enveloped virus containing a large double-stranded DNA genome that ranges in length from 170 to 193 kbp (Tulman et al., 2009; Dixon et al., 2013). Although scientists

worldwide have made great efforts to study the etiology and immunology of ASFV, no prophylactic vaccine or treatment options are commercially available due to the complexity of the virions. Fortunately, early detection and eradication are feasible to control the spread of this epidemic disease (Halasa et al., 2016). However, clinical diagnosis is impractical for the similar classic symptoms of ASF and classic swine fever, swine erysipelas and highly pathogenic porcine reproductive and respiratory syndrome. Laboratory diagnosis is indispensable to make a definite diagnosis. OIE recommends two types of laboratory diagnosis, including etiological diagnosis (e.g., polymerase chain reaction, PCR) and serological diagnosis (e.g., enzyme-linked immunosorbent assay). To date, real-time PCR has been widely used to detect ASFV nucleic acids due to its simple, rapid, highly sensitive and specific features. Nevertheless, the quantity and quality of oral, nasal and rectal swabs vary from pig to pig. False-negative results pose a concerning threat to herds. Higher ASFV content was found in blood than in oral, nasal and rectal fluid samples (Olesen et al., 2017; Zhao et al., 2019). However, blood collection is difficult, and stress in pigs cannot be neglected. Moreover, it is highly likely that obtaining blood from pigs carrying viruses will contaminate the sites, increasing the risk of ASFV infection in the herds.

Lymph nodes were collected by dissecting for viral load measurements in many studies (Palya et al., 2018; Senthilkumar et al., 2018; Liu et al., 2019). Slaughter is unavoidable to get the lymph nodes, which is unsafe and complicated. In the present study, a patent lymph node sampler was used to collect lymph node samples in a

minimally invasive way (Li et al., 2020). The viral loads of lymph nodes as well as oral fluid, nasal and rectal swab samples from the pig production line were investigated. The application of lymph node samplers in twenty farms shows great potential for the accurate diagnosis and eradication of ASFV.

2. Materials and methods

2.1 The structure and usage of the lymph node sampler.

The lymph node sampler is a simple and easy-to-operate tool. It consists of a needle, a syringe, a handle and a connection rod inside the needle (Figure 1A, 1B). The needle contains a barb, which can remove the lymphoid tissue from the muscle (Figure 1C, 1D). The connection rod can be inserted into the needle to extrude the tissue.

2.2 How to use the lymph node sampler.

First, the pig was restrained to access its inguinal lymph nodes (Figure 2A). Then, the skin was punctured vertically with the sampler to ensure that the barb entirely entered the tissue (Figure 2B). The sampler was pulled out, and the handle was pressed to push the tissue out of the needle (Figure 2C). The tissue was placed into an Eppendorf (EP) tube and submerged in nucleic acid protective fluid.

Plenty of needles should be prepared before the sampling. The sampler can be disassembled for recycling by removing the residual blood and tissue, soaking the sampler for 30 minutes in a sodium hypochlorite solution (3%), and washing the sampler with clean water. The sampler can then be reassembled and dried for the next

97 usage.

98 **2.3 Sampling methods of other tissues.**

99 For serum samples, collect the blood from the porcine anterior vena cava into an
100 anticoagulation tube. Let stand for one hour and get the upper serum. For oral fluid
101 samples, tie a cotton rope in front of the pig. Squeeze out the liquid after the sufficient
102 chew by the pig. For throat swabs, insert a long spermaduct with a sponge on the top
103 into the throat quickly. Elute the mucus with 1mL nucleic acid protective fluid (refer to
104 2.4). The cotton nasal swabs and rectal swabs were also eluted with the nucleic acid
105 protective fluid. The nasal-rectal swab and nasal-throat-rectal swab samples were the
106 mixture of the individuals.

107 **2.4 Preventing sample degradation**

108 The sample should be protected in case of degradation. In this study, a patented
109 customized nucleic acid protective fluid was used, which contains
110 ethylenediaminetetraacetic acid (EDTA) to stop DNase activity by chelating
111 magnesium, manganese and ferric ions (Yu et al., 2017; Ren et al., 2020). The
112 concentrated solution was diluted with purified water. Working solutions of nucleic acid
113 protective fluid normally last for up to 7 days, but fresh solution is recommended.

114 For the lymph node samples, 1 mL of nucleic acid protective fluid was added to
115 soak the tissue. For the swab samples, the sticks were broken and 1 mL of nucleic acid
116 protective fluid was added. The samples were sent for detection at room temperature
117 within one day.

2.5 Quantitative real-time PCR (qPCR).

ASFV DNA was quantified with qPCR analysis in a “one step” way without the process of nucleic acid extraction. The kit from Beijing MingRiDa Technology Development Co., Ltd. (010688870) was used according to the user guide. Briefly, lymph node samples were homogenized. Swab samples were oscillated and centrifuged at 8000 g for 2 min. Five microliters of supernatant, negative control or positive control fluid was mixed with 20 μ L of operating fluid. Twenty microliters of mixture was taken for qPCR detection.

3. Results

3.1 The ASFV content of lymph nodes was higher than that of other collected sample tissues.

Suspected ASF samples from the pig production line in Northwestern Shandong Province from January 2020 to March 2021 were collected for nucleic acid detection by qPCR. The results showed that 37 serum samples, 243 lymph node samples, 237 oral fluid, 17 throat swab, 102 nasal swab, 25 rectal swab, 1341 nasal-rectal swab and 26 nasal-throat-rectal swab samples were true positive for ASFV. The CT values were collected and are shown in Figure 3. Serum samples are widely used for many pathogenic diagnoses. The nasal, nasal-rectal and nasal-throat-rectal swab samples had almost the same CT value as the serum samples, suggesting that the virus contents of nasal and serum samples were comparable. Saliva is other common sampling point.

However, our results showed that the mean CT value of saliva samples was much higher than those of other sites, suggesting that the viral loads in saliva were lower than those in other samples. The lymph node samples were the only samples with lower CT values than the serum samples. The standard deviation (SD) value of the lymph node sample CT value was much smaller than that of serum samples. These data suggest that the ASFV content of lymph node samples is more stable than that of serum samples and is higher than that of other samples.

3.2 A larger proportion of strongly positive results was found in lymph node samples.

The distribution of CT values between the samples was also analyzed. Generally, CT values over 35 are considered hard to judge, while values less than 30 can be identified as strongly positive results. The percentage of strongly positive lymph node samples was 70.9%, while the relative percentages of strongly positive serum, nasal swab and oral-nasal-rectal swab samples were 45.9%, 37.3%, 37.4%, respectively (Figure 4). These data suggest that the ASFV content of lymph nodes is much higher than that of other sites, which has great potential for the accurate diagnosis of ASFV.

3.3 ASFV-positive pigs were removed within 3 weeks with the help of lymph node samplers.

To check whether lymph node samples can help to eliminate ASFV-positive pigs in production, lymph node samplers were immediately applied on farms that tested positive for ASFV nucleic acid. Once abnormal symptoms appeared, such as fever,

inappetence, blood spots, emesis or death, the surrounding samples and porcine lymph node samples were collected and detected for the first time. The positive pigs were removed from the herd according to the biosafety requirements. The surroundings were disinfected repeatedly using 3% sodium hypochlorite until free from nucleic acid. Among all twenty farms we monitored, the time from ASFV positivity to negativity ranged from 1 day to 18 days (Figure 5). These results demonstrated that lymph node sampling is workful for the removal of ASFV-positive pigs.

4. Discussion

ASF is an acute viral infection that has a major impact on global pig production. Stockbreeders displayed passivity in the diffusion of practices without effective vaccines or drugs. However, the highly contagious characteristic of ASF transmission indicates the potential for possible interventions to manage the risk of ASF introduction and transmission (Lichoti et al., 2016; Govoeiyi et al., 2020). Biosecurity is a high priority for pig farmers due to its high efficiency in controlling this disease (De Lorenzi et al., 2020; Jackman et al., 2020). It contains at least two parts: how to prevent ASF infection entirely and how to remove infected pigs rapidly and accurately (Álvarez et al., 2019).

Immunology and nucleic acid detection are two main methods to diagnose ASF. qPCR assays have many advantages compared with immunology methods, such as simple operation processes, high sensitivity, high specificity and excellent repeatability.

Thus, qPCR is the most commonly used laboratory test method to diagnose ASFV at present, along with the ASFV antigen detection method recommended by the OIE (King et al., 2003). However, it is difficult to determine CT values larger than 35. The main reason for the high CT values is the low viral content of the samples. The degradation of nucleic acids due to improper preservation and transportation methods also results in confusing data. The present study took advantage of a patented lymph node sampler to puncture the skin in a minimally invasive manner to efficiently obtain lymph node tissue. Moreover, an innovative nucleic acid protective fluid was used to prevent the degradation of ASFV DNA. The qPCR data revealed that the ASFV content of the lymph node samples was higher than that of other common kinds of samples, including serum, oral fluid and nasal-rectal swabs, suggesting that the lymph node is an ideal tissue for the diagnosis of ASF.

Practice has indicated that our synthetic solutions work well in the prevention and control of ASF according to the contingency plan (Ministry-of-Agriculture-and-Rural-Affairs, 2020). In general, we require all the pigs, materials and workers that intend to enter the farm to be ASFV free by taking corresponding measures. Breeders should inspect the herds daily and determine abnormal symptoms, including depression, fever, inappetence, purple skin, blood spots, emesis, nosebleed, abortion, hematochezia and death. The nasal swab, rectal swab and lymph node samples were collected immediately and sent to a testing laboratory nearby. The laboratory was continuously running for 24-hour full-time service to give feedback to the decision makers within 8 hours. The

positive pigs were removed with minimum contamination. The oral fluid and nasal-rectal swab samples of the whole herd, as well as the whole divisional ground, were collected at once. The positive pigs were removed again. The disinfection of the whole farm was immediately executed using potent disinfectors. Seven days later, the next cycle of census, elimination and disinfection were carried out until no positive results occurred. Supported by these powerful biosecurity measures, lymph-node-sample-based rapid and accurate definite diagnosis plays a dominant role in the systematic projects.

In conclusion, this study took full advantage of the lymph node sampler to collect lymph node samples from pigs in a minimally invasive way. The viral content of lymph nodes, rather than serum or other swab samples of porcine secretions, was proven to be the highest. The lymph-node-sampler-based sampling method helps to realize the accurate diagnosis and elimination of ASF.

Declarations

Ethics approval and consent to participate

The authors confirm that all the procedures were conducted in adherence with the the guidelines of New Hope Group Subcommittee of Experimental Animal Ethics with no. of 151920.

Consent for publication

Not applicable.

Availability of data and materials

The dataset generated in this study is available from the first author and corresponding author on reasonable request.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

Study design and planning: Zhichun Yan, Xiaowen Li; animal experiments: Wenchao Gao, Peng Yuan, Jingtao Li, Jincheng Yu, Weisheng Wu, Junxian Li; virological work: Zhenwen Shao, Qiannan Yu, Yang Li; data processing and analysis: Jing Ren, Mingyu Fan, Shiran Fan; drafting manuscript: Xiaowen Li, Yang Li; critically revising manuscript prior to the submission: Zhichun Yan. All authors read, edited, and approved the manuscript.

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Figure legends

Figure 1. The structure of inguinal lymph node sampler. **a** The appearance of the assembled lymph node sampler. **b** Component disassembly of the lymph node sampler. **c** The barb of needle. **d** The connection rod can be inserted into the needle to extrude the tissue.

Figure 2. Three steps to obtain inguinal lymph node samples using a sampler. **a** Find and pinch the inguinal lymph node. **b** Puncture the skin and tissue. **c** Pull out the

sampler and press the handle several times to squeeze the tissue out. d Enlarged view
of squeezed lymph node tissue.

Figure 3. The ASFV content of lymph nodes and other kinds of collected
samples. Eight kinds of porcine samples were collected, and their ASFV content was
detected by qPCR. Individual CT values are presented.

Figure 4. A larger proportion of strongly positive results was found in lymph
node samples. The CT values were sorted into different ranges. The distribution of
each 5 CT ranges is shown in the figure. Values smaller than 30 were identified as
strongly positive results.

Figure 5. ASFV-positive pigs were removed within 3 weeks according to the
viral content of lymph nodes. The ASFV-positive farms judged the suspected pigs by
the viral content of the lymph nodes. The positive pigs were removed from the herd,
and the surroundings were disinfected thoroughly by irrigation with 3% sodium
hypochlorite until ASFV nucleic acid was undetectable. The results of nucleic acid
detection are shown. Red: positivity; green: negativity.

Figures

Figure 1

The structure of inguinal lymph node sampler. a The appearance of the assembled lymph node sampler. b Component disassembly of the lymph node sampler. c The barb of needle. d The connection rod can be inserted into the needle to extrude the tissue.

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ASFV-positive pigs were removed within 3 weeks according to the viral content of lymph nodes. The ASFV-positive farms judged the suspected pigs by the viral content of the lymph nodes. The positive pigs were removed from the herd, and the surroundings were disinfected thoroughly by irrigation with 3% sodium hypochlorite until ASFV nucleic acid was undetectable. The results of nucleic acid detection are shown. Red: positivity; green: negativity.