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Identification of African swine fever virus genomic DNAs in wild boar habitats within outbreak regions in South Korea

Kyung-Lak Lee [†], Yongjun Choi [†], Jongchan Yoo , Jusun Hwang ,
Hyun-Gi Jeong , Weon-Hwa Jheong , Seon-Hee Kim ^{*}

National Institute of Environmental Research, Incheon 22689, Korea

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*Corresponding author:

Seon-Hee Kim

Biosafety Research Team, Environmental Health Research Department, National Institute of Environmental Research, 42 Hwangyeong-ro, Seo-gu, Incheon 22689, Korea.

E-mail: sunnykim14@gmail.com

[†]Kyung-Lak Lee and Yongjun Choi contributed equally to this study.


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ORCID iDs

Kyung-Lak Lee 

<https://orcid.org/0000-0001-7276-0445>

Yongjun Choi 

<https://orcid.org/0000-0002-5077-7999>

Jongchan Yoo 

<https://orcid.org/0000-0002-6425-4793>

Jusun Hwang 

<https://orcid.org/0000-0002-9628-9595>

Hyun-Gi Jeong 

<https://orcid.org/0000-0002-5162-7175>

Weon-Hwa Jheong 

<https://orcid.org/0000-0001-8752-607X>

Seon-Hee Kim 

<https://orcid.org/0000-0002-6937-0646>

<https://vetsci.org>

ABSTRACT

An African swine fever (ASF) outbreak in wild boars was first reported on October 2, 2019, in South Korea. Since then, additional cases were reported in South Korea's border areas. We here report the identification of ASF virus (ASFV) DNAs from two out of eight environmental abiotic matter samples collected from areas where ASF-positive wild boar carcasses were found. Comparative genomic investigations suggested that the contaminating ASFV DNAs originated from the wild boar whose carcass had been found near the positive sample sites. This is the first report on the identification of ASF viral material in wild boar habitats.

Keywords: African swine fever virus; environmental surveillance; microclimate; South Korea; wild boar

INTRODUCTION

Since June 2007, African swine fever (ASF) has been gradually spreading from Georgia to Asian countries, being first reported in China in August 2018, followed by continuous outbreaks emerging in other countries including Vietnam, North Korea, and Mongolia. In South Korea, ASF was first identified in a pig farm on September 17, 2019 [1,2] and in a wild boar on October 2, 2019 [3]. All ASF cases in Korea have been restricted to the northern part of the country, with the discovery of the first ASF-positive wild boar carcass at approximately 1.4 km distance from the South Korea border [3]. Until June 17, 2020, a total of 630 ASF cases were identified in wild boars within the northern area delineated by the regional fence.

ASF virus (ASFV; the causative agent of ASF) is highly resistant to low temperatures and low humidity, and remains infectious outside the host for a long time [4,5]. Therefore, it has been supposed that any abiotic material, such as soil and water contaminated by the body fluids of ASF-infected individuals, may serve as a primary source for indirect ASFV transmission in local wild boars. Common behaviors of wild boars, such as frequent wallowing and bathing, may increase the probability of opportunistic contact with natural fomites contaminated with viral particles [5]. Recent investigations have detected ASFV DNAs in the feed and drinking water supplied to pig farms, suggesting that ingestion of such contaminated materials can also serve as an effective route for indirect ASFV transmission in domestic pigs [6]. However,

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Lee KY, Yoo J, Kim SH;
Data curation: Lee KY, Hwang J, Kim SH;
Investigation: Choi Y, Jeong HG; Methodology:
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J; Writing - original draft: Lee KL, Hwang J;
Writing - review & editing: Kim SH.

reports focusing on wild boars and/or their natural habitat are scarce, and ASF-infected regional studies are critical to establish epidemiological measures for the control of ASF.

MATERIALS AND METHODS**Study site**

The present ASFV environmental survey was conducted on wild boar habitats in South Korean border regions (Gyeonggi province: Paju, Yeoncheon; Gangwon province: Cheorwon). A total of 19 outbreaks of ASFV were reported in Paju in 2019.

Abiotic matter sampling and viral DNA analysis

Mud and water samples were collected from areas located < 0.5 km from previously identified ASFV-positive carcasses (2 mud samples from sites 1 and 2; 1 mud and 1 water sample each from sites 3 and 4; **Fig. 1**). The mud samples (200 g) were suspended in phosphate-buffered saline solution (1:5 v/v), sonicated for 3 min, and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants, as well as the water samples (1 L) from which debris had been removed by primary sedimentation, were subjected to precipitation using PEG-NaCl [7], and the DNAs were extracted using the Maxwell tissue kit (Promega, USA) according to the manufacturer's instructions. A partial segment of ASFV *B646L* gene encoding the capsid protein p72 (257 bp) was amplified by real-time and conventional polymerase chain reactions, as recommended by the World Organization for Animal Health [6], using the PPA1 (5'-AGTTATGGGAAACCCGACCC-3') and PPA2 (5'-CCCTGAATCGGAGCATCCT-3') primers. Another region of the *B646L* gene was amplified using the p72-D (5'-GTACTGTAACGCAGCACAG-3') and p72-U (5'-GGCACAAGTTCGGACATGT-3') primers as previously described [8]. After sequencing, the nucleotide sequences of the *B646L* segment (402 bp) were used in a phylogenetic analysis using Mega 6.0 (neighbor-joining algorithm, Kimura 2-parameter model, a bootstrap analysis with 1,000 replicates) [9].

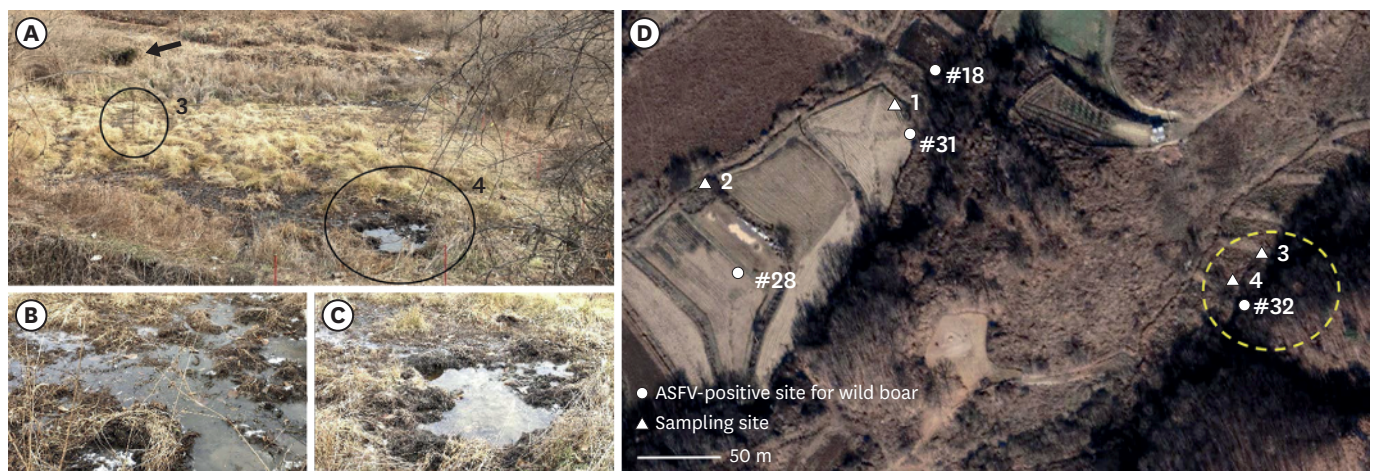


Fig. 1. Locations of the ASF cases and environmental matter sampling. (A) Landscape of ASF-positive sites. Black arrow indicates a nest. Circles indicate two waterholes where ASFV-positive samples were collected. (B) Sampling site 3: a puddle close to the nest with wild boar tracks. (C) Sampling site 4: a bathing place used by wild boars. (D) Satellite image (Google Earth) showing where ASFV-positive wild boar carcasses were found (white dots) and environmental samples were collected (white triangles). Sites from where ASFV-positive environmental samples were collected are indicated with dotted circles. #18, #28, #31, and #32 in the figure indicate carcass case numbers. ASF, African swine fever.

RESULTS

Between October 30 and November 30, 2019, four ASFV-positive wild boar carcasses were identified within a 0.15-km radius area in Jeongja-ri, Gunnae-myeon, Paju, a city located in the northwestern part of South Korea (cases #18, #28, #31, and #32; **Fig. 1**). The area was located within a civilian-controlled area (total area, 5.6 km²) where limited small-scale farming activities were allowed. The average rainfall a month and the normal average temperature were 3 days and 5°C, respectively, during those days. We conducted a field investigation on December 3, 2019, nearby where the ASF-infected carcasses were found, and identified nests and waterholes with tracks and traces suggestive of wild boars (**Fig. 1A-C**). Water and/or mud samples were collected from waterholes in each of the selected sites to assess ASFV presence (**Fig. 1D**).

Of the eight environmental samples collected, viral DNAs were detected in mud and water from sites 3 and 4, respectively (**Fig. 1B and C**). The partial *B646L* gene sequences of these viral DNAs (GenBank accession numbers, MT771054 and MT771055) were identical to those obtained from the four wild boar carcasses. A neighbor-joining tree showed that these ASFV isolates were categorized within genotype II, together with the previously reported Korean (Korea/19S804/wb/2019/MN81797 and Korea/Pig/Paju1/2019/MN60396) and Chinese (China/Jilin/2018/boar and China/Guangxi/2019/domestic pig) isolates (**Fig. 2**). Additionally, the length of a C-stretch near the 13,267 genomic region, which was found to be highly polymorphic among ASFV isolates [10], was also tightly conserved in all the ASFV genomes isolated from the environmental specimens and the four wild boar carcasses (data not shown).

DISCUSSION

In this study, we detected ASFV DNAs in abiotic specimens, which had been collected in the natural habitats of ASFV-positive wild boars with wallowing pool. Analysis of the viral DNAs strongly suggested that these viral materials originated from the diseased wild boars, presumably via excreted body fluids, including saliva, feces, and urine. As ASFV is highly resistant to harsh environmental conditions, natural fomites may serve as a source of indirect ASFV transmission in wild boar populations.

After the first emergence of ASF in wild boars in South Korea, many efforts have been conducted to prevent spread of the fatal disease, including the strict separation between infected and ASF-free areas by border fences, culling, and carcass removal. Similarly, to what occurred in European countries affected by the sylvatic cycle of ASF, natural predators of wild boar have disappeared from Korean ecosystems, and this situation likely contributed to the high population density of wild boars. As ASF transmission occurs through direct contact between susceptible and infectious individuals within a population [11], a high wild boar population density can potentially facilitate the spread of porcine diseases. Active country-wide population control is in process within and around the outbreak regions to prevent further spread of ASF in wild boars by lowering the population density of wild boars [3]. Meanwhile, wild boars obtain a considerable fraction of their diet by grubbing the soil to search for edible plants and animals [12]. It is also well known that the wild animals frequently wallow in mud for cooling, sunburn protection, and removal of ectoparasites [13]. Therefore, the soil and mud contaminated with the body fluids/materials of ASFV-positive wild boars can be considered major sources of indirect transmission of ASF.

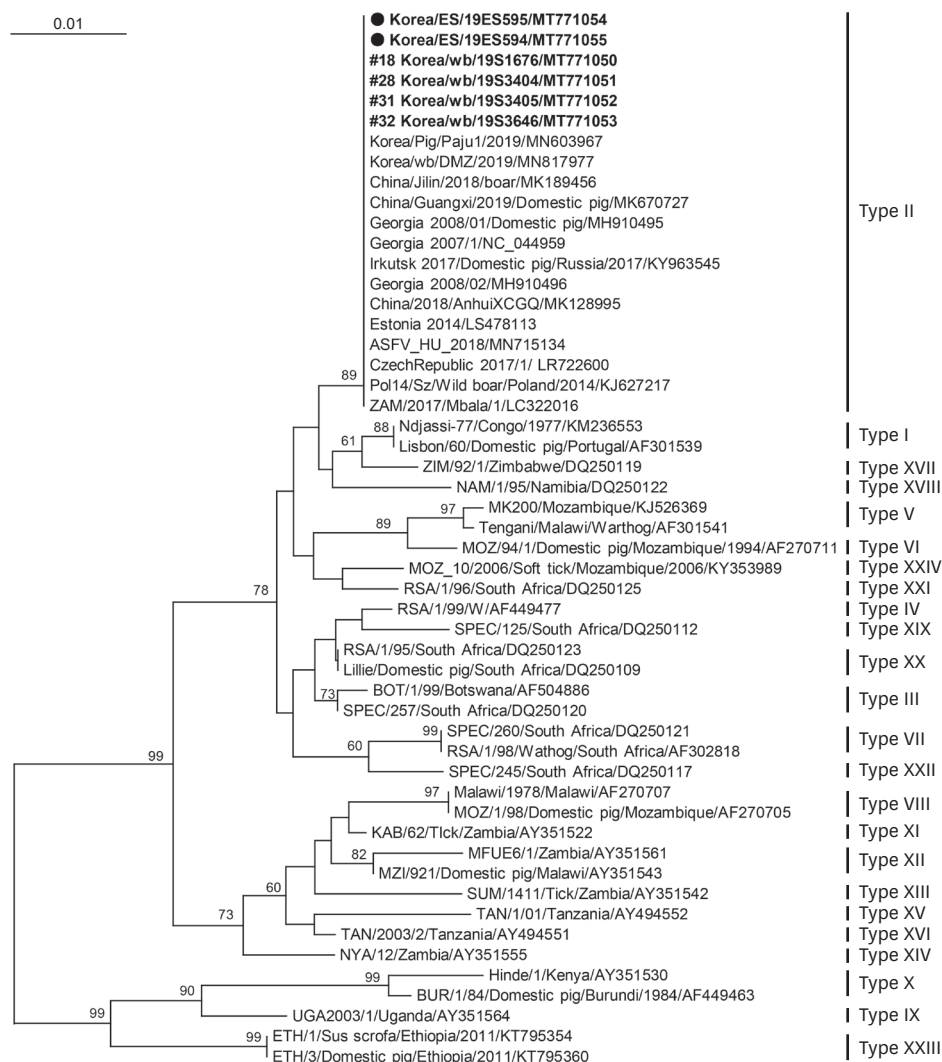


Fig. 2. Genotypes of ASFV isolates investigated in this study. The neighbor-joining tree was constructed based on an alignment of partial *B646L* sequences. ASFV isolates from wild boar carcasses and environmental samples identified in this study are presented in bold font. Numerals at branching nodes indicate bootstrap percentages of 1,000 replicates (> 50%). Scale bar indicates the number of nucleotide substitutions per site. ASFV, African swine fever virus.

Together with reduced home range, the overlapping food sources and waterholes due to the increased wild boar population density [14] would increase the possibility of the soil/mud-mediated viral transmission.

ASFV viability outside a host is dependent on temperature and media type [5,11]. ASFV can maintain its infectious potential in soil and water for up to 112 and 176 days, respectively, during the cold season [15]. In natural habitats, ASFV transmissibility through contaminated abiotic matter can be further influenced by various environmental and/or biological factors [4] and by specific behavioral patterns of wild boars [11]. For instance, most contacts among wild boar individuals within a group occur within a range of 0.5–1 km, which is in accordance with the recent prediction that 80% of the ASF events in wild boars are likely to occur through within-group transmissions [11].

Microclimate conditions may have further influenced on the results of this study. The collection sites of negative samples (sites 1 and 2) were located in open agricultural areas directly exposed to sunlight during daytime. In contrast, two positive samples (sites 3 and 4) were collected from an area immediately adjacent to a patch of forest that was consistently covered in shade, where the cooler temperature could have contributed to the prolonged stability of the viral material. Alternatively, the lack of viral material in the negative samples could be partly attributable to the strong disinfection activity conducted in the surrounding area or to the relatively long delay in sample collection after the discovery of the carcasses (35, 8, and 5 days delay for carcasses #18, #28, and #31/#32, respectively). Ct values of the positive samples were 31.2 (site 3) and 32.5 (site 4), while that of the nearby wild boar carcass sample (#32) was 18.9. No infectious ASFV particles could be detected in these positive samples in the *in vitro* infection assays performed using porcine alveolar macrophages (data not shown). A similar situation was recently reported in an investigation using buried wild boar carcass-related materials [16]. Considering the greatly reduced Ct values of positive samples, the negative infectivity is likely a result of the low viral burden. It is also possible that there are no infectious viral particles in the natural samples, since the types of soil components, such as humic acids, are known to abolish the infectivity of ASFV [17].

In conclusion, our study confirmed the presence of viral genomes in the abiotic matters obtained within ASFV-positive wild boar habitat. This finding suggests a potential function of the environmental matters as a source for indirect transmission of ASF in wild boars with spatiotemporally overlapping habitats. Further studies focusing on the effects of natural factors on the persistence of viral infectivity will be necessary to gain further insight into the epidemiological role(s) of abiotic matters related to the indirect transmission and/or sylvatic cycle of ASFV in wild boar populations.

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