Specific DNA fragments of African swine fever virus detected in hard ticks from cattle and sheep

Authors: Chen, Ze, Xu, Xiaofeng, Yang, Xiaojun, Dou, Weihao, Jin, Xiufeng, Ji, Haishuo, Liu, Guangyuan, Luo, Jianxun, Yin, Hong, Gao, Shan, Yin, Hong

Date: 2019-01-29T00:00:00+00:00

Abstract

The primary objective of this study was to detect viruses carried by hard ticks. As an unexpected finding, a 235 bp specific fragment from the African swine fever virus genome was detected. Subsequent sequencing revealed that both ticks and sheep harbor this fragment, particularly sharing a specific mutation C38T that is absent in the corresponding fragment of any currently published African swine fever virus genomes.

Full Text

Preamble

Systematic & Applied Acarology 24(1): 180–184 (2019)
http://doi.org/10.11158/saa.24.1.13
ISSN 1362-1971 (print) ISSN 2056-6069 (online)

DNA Segments of African Swine Fever Virus Detected for the First Time in Hard Ticks from Sheep and Bovines

ZE CHEN[1], XIAOFENG XU[1], YUFENG WANG[2], JINLONG BEI[3], XIUFENG JIN[1], WEIHAO DOU[1], HAISHUO JI[1], YUJIE DUAN[1], XIAOJUN YANG[2]* & SHAN GAO[1]*

[1]College of Life Sciences, Nankai University, Tianjin 300071, P.R. China
[2]Southwest Forestry University, Kunming 650224, P.R. China
[3]AGRO-Biological Gene Research Center, Guangdong Academy of Agricultural Science, Guangzhou 510640, P.R. China

*Correspondence to: Shan Gao (gao_shan@mail.nankai.edu.cn); Xiaojun Yang (yangxj86@163.com)

Abstract

In this study, we detected African Swine Fever Virus (ASFV) in *Dermacentor* ticks (Ixodidae) from sheep and bovines using small RNA sequencing. To validate this finding, we targeted a 235-bp DNA segment in multiple DNA samples from *D. silvarum* and sheep blood. This segment showed 99% identity to a corresponding 235-bp region of ASFV and contained three single nucleotide mutations (C38T, C76T, and A108C). The C38T mutation results in a single amino acid substitution at position 66 (G66D), suggesting the existence of a novel ASFV strain distinct from all previously reported strains in the NCBI GenBank database, including the ASFV strain reported in China in 2018 (GenBank: MH713612.1). To further confirm ASFV presence in *Dermacentor* ticks, we detected three ASFV DNA segments in *D. niveus* females collected from bovines and in their first-generation offspring reared in our laboratory. These results demonstrate transovarian transmission of ASFV in hard ticks. Our study reveals for the first time that ASFV infects a broader range of hosts (e.g., sheep and bovines) and vectors (e.g., hard ticks) beyond the previously known Suidae family and Argasidae (soft ticks). These findings pave the way for further investigations into ASFV transmission dynamics and the development of novel prevention and control strategies.

Keywords: ASFV; hard tick; virus detection; transmission; transovarian

---

African Swine Fever Virus (ASFV) is a large (~190 kbp) double-stranded DNA virus with a linear genome containing at least 150 genes. ASFV causes African Swine Fever (ASF), a highly contagious viral disease of swine with mortality approaching 100% (Galindo & Alonso 2017).

ASF first emerged in Africa in the early 1900s (Montgomery 1921) and remained confined to the continent until 1957, when it was reported in Portugal. Subsequently, ASF spread geographically, causing substantial economic losses in the swine industry. In 2018, ASF appeared in Liaoning province, China, and rapidly disseminated to 20 provinces and 4 municipalities. Although PCR with specific primers enables rapid ASFV detection (Yang et al. 2011), current understanding of ASFV hosts and vectors remains limited to the Suidae family and soft ticks, respectively (Chen et al. 2011).

It is well established that ASFV infects Suidae members, including domestic pigs, warthogs, and bushpigs (Denyer & Wilkinson 1998), and that soft ticks of the genus *Ornithodoros* serve as biological vectors. In China, only 19 Ixodidae species (including *D. nuttalli*, *D. silvarum*, and *Amblyomma testudinarium*) have been reported infesting Suidae (Chen et al. 2019). However, none of these tick species have been documented as ASFV vectors to date (Chen et al. 2011; Yu et al. 2015). Notably, many *Dermacentor* ticks share host ranges similar to *Ornithodoros* ticks but exhibit higher mobility and broader geographical distribution. Compared to *Ornithodoros* and other Ixodidae, *A. testudinarium* has a

wider distribution in southern China, where ASF was reported in 2018, whereas *Ornithodoros* occurrences have not been documented. *A. testudinarium* also parasitizes all Suidae members. Furthermore, *Dermacentor* and *Amblyomma* ticks produce substantially more eggs than *Ornithodoros* ticks. Based on our previous high-throughput sequencing studies, viruses transmitted by *D. nuttalli*, *D. silvarum*, and *A. testudinarium* have been underestimated compared to insect-borne viruses (Zheng et al. 2017).

Small RNA sequencing (sRNA-seq) generates thousands of short RNA sequences (<50 bp) and has been successfully applied for virus detection in plants (Li et al. 2012), invertebrates, and humans (Wang et al. 2016). In 2016, an automated bioinformatics pipeline called VirusDetect was developed to facilitate large-scale virus detection using sRNA-seq (Zheng et al. 2017). This study aimed to detect viruses in *Dermacentor* ticks using sRNA-seq. Unexpectedly, VirusDetect identified ASFV in *D. nuttalli* from sheep and bovines. To confirm this result, we detected a 235-bp ASFV DNA segment (AY261365.1:103819-104053) in *D. nuttalli*, *D. silvarum*, and sheep, but not in *A. testudinarium* or bovines. To further validate ASFV presence in *Dermacentor* ticks, we detected three ASFV DNA segments in *D. niveus* females from bovines and their first-generation (F1) offspring reared in our laboratory.

Amblyomma testudinarium ticks were collected from buffalo in Yunnan province, China. *D. nuttalli*, *D. silvarum*, and *D. niveus* ticks were collected from sheep and bovines in Xinjiang Autonomous Region, China. We pooled several ticks to create four samples representing *A. testudinarium* adults, *D. nuttalli* adults, *D. niveus* larvae, and *D. niveus* nymphs, and constructed four sRNA-seq libraries that were sequenced using 50-bp single-end strategy on the Illumina HiSeq 2500 platform (Chen et al. 2017). Because the *D. nuttalli* adult library was sequenced twice, five sRNA-seq datasets were deposited in the NCBI SRA database under project accession numbers SRP084097 and SRP178347.

Data cleaning and quality control were performed using the Fastq_clean pipeline (Zhang et al. 2014), optimized for Illumina raw reads. Virus detection was conducted using VirusDetect (Zheng et al. 2017). Detection of siRNA duplexes was performed using duplexfinder (Niu et al. 2017). Statistical computations and plotting were performed using R v2.15.3 with Bioconductor packages (Gao et al. 2014). The ASFV reference genome (GenBank: AY261365.1) was used for all data analyses.

RNA extraction from *D. nuttalli* ticks and cDNA synthesis were performed using protocols from our previous study (Gao et al. 2018). DNA extraction from *D. nuttalli*, *D. silvarum*, and *D. niveus* ticks, as well as from sheep and bovine blood, followed protocols from our previous study (Cheng et al. 2017). PCR was performed using Thermo Scientific DreamTaq Green PCR Master Mix (2×) with initial denaturation at 95°C for 3 min, followed by 34 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with final extension at 72°C for 5 min. Primers for amplifying three ASFV DNA segments are listed in Table 1 .

After cleaning and quality control, 13,496,191; 25,194,632; 37,888,277; 12,302,335; and 15,077,054 clean reads were used for virus detection in *A. testudinarium* adults, *D. nuttalli* adults, *D. nuttalli* adults (replicate), *D. niveus* larvae, and *D. niveus* nymphs, respectively. VirusDetect identified ASFV in all four *Dermacentor* samples but not in the *A. testudinarium* sample. VirusDetect reports the closest reference sequence for each detected virus. The closest ASFV reference genome (GenBank: AY261365.1) was derived from ticks collected in Warmbaths, South Africa, in a previous study. Alignment of clean reads to the ASFV reference genome yielded mapping rates of 0.06% (15,585/25,194,632), 0.06% (23,330/37,888,277), 0.08% (10,241/12,302,335), and 0.08% (12,807/15,077,054) for the four *Dermacentor* samples—significantly higher than the 0.01% (905/13,496,191) mapping rate for the *A. testudinarium* sample. The length distribution of virus-derived small RNAs (vsRNAs) in the four *Dermacentor* samples peaked at 15–19 bp rather than 21–24 bp, differing from vsRNA patterns in plants (Niu et al. 2017) and other invertebrates (Liu et al. 2018). We did not detect small interfering RNA (siRNA) duplexes from ASFV in any of the four *Dermacentor* samples.

To validate ASFV presence, we used PCR with specific primers to amplify the 235-bp ASFV segment (AY261365.1:103819-104053) from total RNA of *D. nuttalli* adults that had tested positive by sRNA-seq. Gel electrophoresis revealed a clear 235-bp band as expected, but Sanger sequencing failed due to low cDNA concentration. We therefore used PCR to detect this segment in a larger panel of DNA samples from *D. silvarum* ticks and sheep and bovine blood. Gel electrophoresis showed 235-bp bands in 33 of 80 *D. silvarum* samples and 12 of 100 sheep blood samples (Figure 1A [Figure 1: see original paper]). Two 235-bp DNA sequences obtained by Sanger sequencing from *D. silvarum* and sheep confirmed that these bands represented the target ASFV segment (Figure 1B [Figure 1: see original paper]). Additionally, gel electrophoresis showed 560-bp bands in 41 of 100 bovine samples. Sanger sequencing of two 560-bp sequences and BLAST analysis indicated these bands likely originated from the bovine genome.

Sequence analysis of the two 235-bp segments from *D. silvarum* and sheep revealed 100% identity between them (Figure 1B [Figure 1: see original paper]). Comparison with the ASFV reference genome (GenBank: AY261365.1) identified three single nucleotide mutations at positions 38, 76, and 108 (C38T, C76T, and A108C). Since the 235-bp ASFV segment encodes 78 amino acids, the C38T mutation causes a single amino acid substitution at position 66 (G66D). This C38T mutation indicates a novel ASFV strain distinct from all reported strains in NCBI GenBank (version 197) and from the 2018 Chinese ASFV strain (GenBank: MH713612.1). BLAST analysis confirmed that this newly detected 235-bp segment shows no high similarity to sequences from other viruses, bacteria, or animal genomes, demonstrating high specificity for ASFV. These results confirm ASFV presence in *D. nuttalli*, *D. silvarum*, and sheep. Because *D. silvarum* ticks and sheep blood were collected from two different locations in Xinjiang Autonomous Region, this novel strain can infect sheep and be transmitted by *D.*

silvarum ticks.

To further confirm ASFV presence in *Dermacentor* ticks, we reared *D. niveus* females from bovines on New Zealand white rabbits maintained at 18–20°C with 50% relative humidity (RH) under natural daylight cycles. After detachment, ticks were collected and incubated in cotton-plugged glass tubes containing folded filter paper in an incubator (20 ± 1°C, 50% RH, natural daylight cycles). We then used PCR to amplify three ASFV segments—the 235-bp segment (AY261365.1:103819-104053), a 257-bp segment (AY261365.1:105122-105378), and a 322-bp segment (AY261365.1:124995-125316)—in *D. niveus* females and their F1 generation offspring. Sanger sequencing confirmed ASFV presence and demonstrated transovarian transmission in *D. niveus*. Comparison with the ASFV reference genome (GenBank: AY261365.1) revealed a total of 10 single nucleotide mutations and three single amino acid mutations across these three segments, suggesting another novel ASFV strain capable of infecting bovines and being transmitted by *D. niveus* ticks.

In conclusion, we detected a 235-bp ASFV segment in hard ticks from sheep and bovines, providing evidence for a novel ASFV strain distinct from all reported strains in Suidae and soft ticks. The 100% identity between the two 235-bp sequences from *D. silvarum* and sheep warrants further sequencing of ASFV genomes from these sources to determine their relationship. Additional experiments confirmed ASFV presence and transovarian transmission in *D. niveus*, indicating yet another novel strain. Beyond establishing a foundation for future research, we provide laboratory-reared *D. niveus* ticks for studies on ASFV transmission and control measure development.

Funding. This study was financially supported by the National Natural Science Foundation of China (31471967) to Ze Chen and the National Natural Science Foundation of China (31260106) to Xiaojun Yang.

TABLE 1. Primers to amplify three DNA segments of ASFV.

Location	Forward Primer	Reverse Primer
103819-104053	GCAGAACTTTGATGGAAACTTA	TCCTCATCAACACCGAGATTGGCAC
105122-105378	CCCTGAATCGGAGCATCCT	AGTTATGGGAAACCCGACCC
124995-125316	AAGGAGGATGTGCATTCATT	AGGTCATCTTCAAAACGGAT

Three DNA segments are located on the ASFV reference genome (GenBank: AY261365.1). The first and second segments belong to the capsid protein p72 gene. The third segment belongs to the helicase superfamily II group gene.

References

Chen, Z., Luo, J. & Yin, H. (2011) Biological vectors of African swine fever. *Acta Veterinaria et Zootechnica Sinica*, 42, 605–612. (in Chinese)

Chen, Z., Sun, Y., Yang, X., Wu, Z., Guo, K., Niu, X., Wang, Q., Ruan, J., Bu, W. & Gao, S. (2017) Two featured series of rRNA-derived RNA fragments (rRFs) constitute a novel class of small RNAs. *PLoS One*, 12(4), e0176458. https://doi.org/10.1371/journal.pone.0176458

Chen, Z. & Yang, X.J. (2019) *Systematics and Taxonomy of Ixodida*, Beijing, Science Press. (in Chinese, in press)

Cheng, Z., Shang, Y., Gao, S. & Zhang, T. (2017) Overexpression of U1 snRNA induces decrease of U1 spliceosome function associated with Alzheimer's disease. *Journal of Neurogenetics*, 31, 337–343. https://doi.org/10.1080/01677063.2017.1395425

Denyer, M. & Wilkinson, P. (1998) African swine fever. In: Delves, P.I. & Roitt, I.M.C.E. (Eds.) *Encyclopedia of immunology* (Second Edition). San Diego, Academic Press, pp. 54–56

Galindo, I. & Alonso, C. (2017) African swine fever virus: A review. *Viruses*, 9, 103. https://doi.org/10.3390/v9050103

Gao, S., Ou, J. & Xiao, K. (2014) *R language and Bioconductor in bioinformatics applications*. Tianjin, Tianjin Science and Technology Translation Publishing Ltd, 224 pp. (in Chinese)

Gao, S., Tian, X., Chang, H., Sun, Y., Wu, Z., Cheng, Z., Dong, P., Zhao, Q., Ruan, J. & Bu, W. (2018) Two novel lncRNAs discovered in human mitochondrial DNA using PacBio full-length transcriptome data. *Mitochondrion*, 38, 41–47. https://doi.org/10.1016/j.mito.2017.08.002

Li, R., Gao, S., Hernandez, A.G., Wechter, W.P., Fei, Z. & Ling, K.S. (2012) Deep sequencing of small RNAs in tomato for virus and viroid identification and strain differentiation. *PLoS One*, 7(5), e37127. https://doi.org/10.1371/journal.pone.0037127

Liu, C., Chen, Z., Hu, Y., Ji, H., Yu, D., Shen, W., Li, S., Ruan, J., Bu, W. & Gao, S. (2018) Complemented palindromic small RNAs first discovered from SARS coronavirus. *Genes*, 9, 442. https://doi.org/10.3390/genes9090442

Montgomery, R. (1921) On a form of swine fever occurring in British East Africa (Kenya colony). *Journal of Comparative Pathology & Therapeutics*, 34, 159–191.

Niu, X., Sun, Y., Chen, Z., Li, R., Padmanabhan, C., Ruan, J., Kreuze, J.F., Ling, K., Fei, Z. & Gao, S. (2017) Using small RNA-seq data to detect siRNA duplexes induced by plant viruses. *Genes*, 8, 163. http://dx.doi.org/10.3390/genes8060163

Wang, F., Sun, Y., Ruan, J., Chen, R., Chen, X., Chen, C., Kreuze, J.F., Fei, Z., Zhu, X. & Gao, S. (2016) Using small RNA deep sequenc-

ing data to detect human viruses. *BioMed Research International*, 2016, http://dx.doi.org/10.1155/2016/2596782

Yang, J., Guan, G., Liu, Z., Wang,Y., Li, Y., Ma, M., Liu, A., Ren, Q., Chen, Z., Gou, H., Du, P., Luo, J. & Yin, H. (2011) One PCR method used for detection of African swine fever virus. *Acta Veterinaria et Zootechnica Sinica*, 42, 1201-1206. (in Chinese)

Yu, Z., Wang, H., Wang, T., Sun, W., Yang, X. & Liu, J. (2015) Tick-borne pathogens and the vector potential of ticks in China. *Parasites & Vectors*, 8, 24. https://doi.org/10.1186/s13071-014-0628-x

Zhang, M., Sun, H., Fei, Z., Zhan, F., Gong, X. & Gao, S. (2014) Fastq clean: An optimized pipeline to clean the Illumina sequencing data with quality control. *International conference on bioinformatics and biomedicine*, 44-48.

Zheng, Y., Gao, S., Padmanabhan, C., Li, R., Galvez, M., Gutierrez, D., Fuentes, S., Ling, K., Kreuze, J.F. & Fei, Z. (2017) VirusDetect: An automated pipeline for efficient virus discovery using deep sequencing of small RNAs. *Virology*, 500, 130-138. https://doi.org/10.1016/j.virol.2016.10.017

Submitted: 14 Jan. 2019; accepted by Zhi-Qiang Zhang: 18 Jan. 2019; published: 29 Jan. 2019

Note: Figure translations are in progress. See original paper for figures.