Sex-based Differences in the Distribution of Aujeszky’s Disease-Seropositive Japanese Wild Swine

Emi Yamaguchi  
National Agriculture and Food Research Organization

Michihiro Takagi  
National Agriculture and Food Research Organization

Makoto Osaki  
National Agriculture and Food Research Organization

Yoko Hayama  
National Agriculture and Food Research Organization

Takehisa Yamamoto (mtbook@affrc.go.jp)  
National Agriculture and Food Research Organization

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Abstract

Background: Aujeszky’s disease virus (ADV) primarily infects domestic and wild swine, causing the abortion and death of young piglets due to central nervous system disorders. In Japan, the national eradication program has been successful in most prefectures; however, ADV-infected wild swine have been concerned as a source of ADV among domestic pigs.

Results: This study assessed the nationwide seroprevalence of ADV among wild swine (Sus scrofa) in Japan. Moreover, sex-based differences in the spatial clustering of seropositive animals were investigated. In total, 1383 serum samples were obtained from wild swine caught in 41 prefectures in three fiscal years (April–March in 2014, 2015, and 2017) in Japan. Next, the seropositivity for ADV was evaluated using enzyme-linked immunosorbent assay and the latex agglutination and neutralization tests. Results showed that 29 swine were seropositive for ADV (29/1383, 2.1% [95% confidence interval, CI: 1.4%–3.0%]). Among them, 28 were caught in three prefectures located at the Kii Peninsula (28/121, 23.1% [95% CI: 16.0%–31.7%]). The degree of spatial clustering of ADV-seropositive adult swine at the Kii Peninsula according to sex was evaluated using K-function with the capture locations of 46 males including 14 seropositive and 54 females including 12 seropositive. In females, the degree of clustering was significantly higher in seropositive animals than in tested animals; however, such difference was not observed for seropositive males.

Conclusions: The spatial dynamics of ADV among adult wild swine might be characterized based on sex. This finding might be attributed to sex-based differences in behavioral patterns including dispersal among wild swine.

Background

Wild swine (Sus scrofa) are widely distributed worldwide, and they are native in Eurasia and non-indigenous in North and South American and Oceanian countries (1–3). In past several decades, wild swine population has increased (4, 5), leading to global concerns about crop predation, ecological damage, and disease transmission by wild swine (6–9). Their habitats include public and livestock areas (10, 11). Thus, various causative agents for livestock and zoonotic diseases may be transmitted at the interfaces from wild swine to domestic pigs, and vice versa, as well as to humans (9, 12, 13). Hence, to establish an effective control strategy that can promote public health and livestock hygiene, we should understand the ecology of these agents in the wild swine population.

Aujeszky’s disease virus (ADV) primarily infects Sus scrofa including domestic pigs and wild swine. It causes significant economic loss in pig farming due to the abortion and death of young piglets caused by central nervous system disorders (14). ADV had spread worldwide in the 1970s. North American, Oceanian, and European countries had officially achieved ADV-free status among domestic pigs under the eradication program (14, 15). However, serological and virological surveys revealed the presence of ADV in wild swine populations in these countries (16–18). The wide distribution of wild swine may
increase the risk of ADV invasion in domestic pig farms due to possible ADV exposure from infected wild swine (16). In Japan, more than thousands of ADV-infected domestic pigs were found in several prefectures in the late 1980s after the first case of Aujeszky's disease (AD) was detected in domestic piglets in 1981 (19). The national eradication program started in 1991 and was successful in most prefectures (20, 21). As of June 2021, only one prefecture, Ibaraki Prefecture, was reported as having ADV-infected domestic pig farms (21). Mahmoud et al. indicated that ADV-seropositive wild swine were inhabited in three prefectures in the western part of Japan (22). ADV infection in hunting dogs with the history of biting or eating wild swine had been reported in Miyazaki Prefecture (23) and Oita Prefecture (24). These results showed that wild swine can be a source of ADV among domestic pigs in ADV-eradicated areas. In addition, as wild swine have been indigenously inhabited in most prefectures, and the distribution of Japanese swine has recently expanded (3), which may increase the risk of ADV exposure to domestic pigs from wild swine. Moreover, ADV is lethal for dogs (14). Hunting dogs are at a risk of contracting ADV by biting wild infected swine, as previously reported (23, 24). Accordingly, an understanding of the factors contributing to the distribution of ADV-infected swine could help to evaluate the risk of ADV infection in domestic pig farms and hunting dogs.

Previous studies revealed that various biological factors (e.g., reproductive behavior, food habitat, and immunosuppression) associated with sex-based differences cause sex-biased dynamics of infectious diseases among wild mammals (25–27). For ADV in wild swine, experimental and field evidences showed that venereal contact might be the major ADV transmission route between wild swine (16, 28, 29). In wild swine populations, males disperse long distances (30, 31) and mate polygynously (32), whereas females stay around the birthplace with their maternal group (32). These behaviors, which are characteristic of each sex, may cause sex-based differences in the spread of the infection. A previous field study showed that ADV seroprevalence was higher in females and changed with the seasons in males among wild swine, which was suggested to be caused due to sex-related differences in behaviors (33).

Hence, the current study reported the seroprevalence of ADV among wild swine in Japan and investigated the sex-based differences in the spatial clustering of seropositive animals. The research results could help provide an understanding of the mechanism of ADV spreading among wild swine and useful information about the planning control measures for AD and other contact-transmissible diseases.

Results

In total, 1383 serum samples were collected from swine in 41 prefectures in 2014, 2015, and 2017, including 699, 312, 372 samples each year (Table 1). In the tested samples, 312 (22.6% [95% confidence interval (CI): 20.4%–24.9%]) and 48 (3.5% [95% CI: 2.6%–4.6%]) swine were positive based on the S enzyme-linked immunosorbent assay (ELISA) and gI ELISA, respectively. There were 41 swine (3.0% [95% CI: 2.1%–4.0%]) that tested positive in both ELISAs. In 320 swine that tested positive for either of ELISAs, 29 (2.1% [95% CI: 1.4%–3.0%]) swine were positive for both latex agglutination test (LAT) and the serum neutralization test (NT) and were confirmed as ADV seropositive. All of seropositive swine were
positive in both ELISAs. The remaining 291 swine that tested positive for either of ELISAs were ADV seronegative, with consideration of the risky of non-specific reaction.

In total, 28 of 29 seropositive swine were caught in the ADV endemic area consisted of three neighboring prefectures, Mie, Nara, and Wakayama, located at the Kii Peninsula, (Table 1, Figure 1, and Figure 2), except for one that was detected at Miyazaki Prefecture. The seroprevalence for ADV among swine captured in these prefectures was 23.1% (28/121 [95% CI: 16.0%–31.7%]). According to sex, the seroprevalence rates were 24.6% (15/61 [95% CI: 14.5%–37.3%]) in male swine and 20.3% (12/59 [95% CI: 11.0%–32.8%]) in female swine. Hence, the results did not significantly differ (p = 0.73, chi-square test). The sex of an adult swine could not be determined. Adult swine had a significantly higher seropositivity than juvenile swine (27/101, 26.7% [95% CI: 18.4%–36.5%] vs 1/20, 5.0% [95% CI: 0.1%–24.9%]; p = 0.04, Fisher's exact test).

**Table 1.** Seroprevalence of Aujeszky’s disease virus among wild swine in 41 prefectures in Japan in 2014, 2015, and 2017. Pref IDs refer to the location of each prefecture in Figure 1.
<table>
<thead>
<tr>
<th>Prefecture ID</th>
<th>Prefecture</th>
<th>2014</th>
<th>2015</th>
<th>2017</th>
<th>Total</th>
<th>Prevalence (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Miyagi</td>
<td>0/0</td>
<td>0/0</td>
<td>0/39</td>
<td>0/39</td>
<td>0% (0%–9.03%)</td>
</tr>
<tr>
<td>7</td>
<td>Fukushima</td>
<td>0/0</td>
<td>0/0</td>
<td>0/32</td>
<td>0/32</td>
<td>0% (0%–10.89%)</td>
</tr>
<tr>
<td>8</td>
<td>Ibaraki</td>
<td>0/0</td>
<td>0/0</td>
<td>0/40</td>
<td>0/40</td>
<td>0% (0%–8.81%)</td>
</tr>
<tr>
<td>9</td>
<td>Tochigi</td>
<td>0/0</td>
<td>0/2</td>
<td>0/29</td>
<td>0/31</td>
<td>0% (0%–11.22%)</td>
</tr>
<tr>
<td>10</td>
<td>Gunma</td>
<td>0/0</td>
<td>0/45</td>
<td>0/0</td>
<td>0/45</td>
<td>0% (0%–7.87%)</td>
</tr>
<tr>
<td>11</td>
<td>Saitama</td>
<td>0/0</td>
<td>0/0</td>
<td>0/37</td>
<td>0/37</td>
<td>0% (0%–9.49%)</td>
</tr>
<tr>
<td>12</td>
<td>Chiba</td>
<td>0/0</td>
<td>0/0</td>
<td>0/34</td>
<td>0/34</td>
<td>0% (0%–10.28%)</td>
</tr>
<tr>
<td>14</td>
<td>Kanagawa</td>
<td>0/0</td>
<td>0/0</td>
<td>0/36</td>
<td>0/36</td>
<td>0% (0%–9.74%)</td>
</tr>
<tr>
<td>15</td>
<td>Niigata</td>
<td>0/25</td>
<td>0/0</td>
<td>0/0</td>
<td>0/25</td>
<td>0% (0%–13.72%)</td>
</tr>
<tr>
<td>16</td>
<td>Toyama</td>
<td>0/17</td>
<td>0/0</td>
<td>0/0</td>
<td>0/17</td>
<td>0% (0%–19.51%)</td>
</tr>
<tr>
<td>17</td>
<td>Ishikawa</td>
<td>0/0</td>
<td>0/0</td>
<td>0/34</td>
<td>0/34</td>
<td>0% (0%–10.28%)</td>
</tr>
<tr>
<td>18</td>
<td>Fukui</td>
<td>0/6</td>
<td>0/0</td>
<td>0/0</td>
<td>0/6</td>
<td>0% (0%–45.93%)</td>
</tr>
<tr>
<td>19</td>
<td>Yamanashi</td>
<td>0/24</td>
<td>0/0</td>
<td>0/0</td>
<td>0/24</td>
<td>0% (0%–14.25%)</td>
</tr>
<tr>
<td>20</td>
<td>Nagano</td>
<td>0/16</td>
<td>0/0</td>
<td>0/0</td>
<td>0/16</td>
<td>0% (0%–20.59%)</td>
</tr>
<tr>
<td>21</td>
<td>Gifu</td>
<td>0/38</td>
<td>0/0</td>
<td>0/0</td>
<td>0/38</td>
<td>0% (0%–9.25%)</td>
</tr>
<tr>
<td>22</td>
<td>Shizuoka</td>
<td>0/24</td>
<td>0/38</td>
<td>0/0</td>
<td>0/62</td>
<td>0% (0%–5.78%)</td>
</tr>
<tr>
<td>23</td>
<td>Aichi</td>
<td>0/24</td>
<td>0/0</td>
<td>0/0</td>
<td>0/24</td>
<td>0% (0%–14.25%)</td>
</tr>
<tr>
<td>24</td>
<td>Mie</td>
<td>6/27</td>
<td>11/49</td>
<td>0/0</td>
<td>17/76</td>
<td>22.37% (13.6%–33.38%)</td>
</tr>
<tr>
<td>25</td>
<td>Shiga</td>
<td>0/20</td>
<td>0/0</td>
<td>0/0</td>
<td>0/20</td>
<td>0% (0%–16.84%)</td>
</tr>
<tr>
<td>26</td>
<td>Kyoto</td>
<td>0/16</td>
<td>0/0</td>
<td>0/0</td>
<td>0/16</td>
<td>0% (0%–20.59%)</td>
</tr>
<tr>
<td>27</td>
<td>Osaka</td>
<td>0/0</td>
<td>0/0</td>
<td>0/50</td>
<td>0/50</td>
<td>0% (0%–7.11%)</td>
</tr>
<tr>
<td>28</td>
<td>Hyogo</td>
<td>0/20</td>
<td>0/0</td>
<td>0/0</td>
<td>0/20</td>
<td>0% (0%–16.84%)</td>
</tr>
<tr>
<td>29</td>
<td>Nara</td>
<td>4/28</td>
<td>0/0</td>
<td>0/0</td>
<td>4/28</td>
<td>14.29% (4.03%–32.67%)</td>
</tr>
<tr>
<td>30</td>
<td>Wakayama</td>
<td>7/17</td>
<td>0/0</td>
<td>0/0</td>
<td>7/17</td>
<td>41.18% (18.44%–67.08%)</td>
</tr>
<tr>
<td>31</td>
<td>Tottori</td>
<td>0/25</td>
<td>0/0</td>
<td>0/0</td>
<td>0/25</td>
<td>0% (0%–13.72%)</td>
</tr>
<tr>
<td>32</td>
<td>Shimane</td>
<td>0/21</td>
<td>0/43</td>
<td>0/0</td>
<td>0/64</td>
<td>0% (0%–5.6%)</td>
</tr>
<tr>
<td>33</td>
<td>Okayama</td>
<td>0/19</td>
<td>0/50</td>
<td>0/0</td>
<td>0/69</td>
<td>0% (0%–5.21%)</td>
</tr>
<tr>
<td>34</td>
<td>Hiroshima</td>
<td>0/33</td>
<td>0/0</td>
<td>0/0</td>
<td>0/33</td>
<td>0% (0%–10.58%)</td>
</tr>
<tr>
<td></td>
<td>Prefecture</td>
<td>Adult Male</td>
<td>Adult Female</td>
<td>Total</td>
<td>Seropositivity</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Yamaguchi</td>
<td>0/25</td>
<td>0/0</td>
<td>0/25</td>
<td>0% (0%–13.72%)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Tokushima</td>
<td>0/26</td>
<td>0/35</td>
<td>0/61</td>
<td>0% (0%–5.87%)</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>Kagawa</td>
<td>0/35</td>
<td>0/0</td>
<td>0/36</td>
<td>0% (0%–9.74%)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Ehime</td>
<td>0/15</td>
<td>0/0</td>
<td>0/15</td>
<td>0% (0%–21.8%)</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Kouchi</td>
<td>0/22</td>
<td>0/0</td>
<td>0/22</td>
<td>0% (0%–15.44%)</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Fukuoka</td>
<td>0/19</td>
<td>0/0</td>
<td>0/19</td>
<td>0% (0%–17.65%)</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Saga</td>
<td>0/23</td>
<td>0/0</td>
<td>0/23</td>
<td>0% (0%–14.82%)</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Nagasaki</td>
<td>0/25</td>
<td>0/50</td>
<td>0/75</td>
<td>0% (0%–4.8%)</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Kagoshima</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0% (0%–8.81%)</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Oita</td>
<td>0/16</td>
<td>0/0</td>
<td>0/16</td>
<td>0% (0%–20.59%)</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Miyazaki</td>
<td>1/29</td>
<td>0/0</td>
<td>1/29</td>
<td>3.45% (0.09%–17.76%)</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Okinawa</td>
<td>0/28</td>
<td>0/0</td>
<td>0/28</td>
<td>0% (0%–12.34%)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18/699</strong></td>
<td><strong>11/312</strong></td>
<td><strong>0/372</strong></td>
<td><strong>29/1383</strong></td>
<td><strong>2.1% (1.41%–3%)</strong></td>
<td></td>
</tr>
</tbody>
</table>

Most seropositive swine were detected in three neighboring prefectures (Mie, Nara, and Wakayama) in the Kii Peninsula; therefore, these prefectures were regarded as the ADV endemic area, and the swine captured in this area were subjected to spatial analyses. Out of 121 tested swine in the endemic area, the 124 spatial analysis used the data of 100 adult swine captured, which comprised 46 males, including 14 125 seropositive, and 54 females, including 12 seropositive. Figure 2 shows the distribution of tested adult 126 swine according to ADV seropositivity based on each sex. As shown in Figure 3, K tested M (h) – K tested F (h) 127 was plotted within the 95% CI of simulated K tested M (h) – K tested F (h), which indicated that the degree of 128 spatial clustering for the tested male and female distributions did not significantly differ (Figure 3). In the 129 analyses of the spatial clustering of seropositive swine, K positive M (h) was plotted within the 95% CI of 130 simulated K positive M(h) in male swine (Figure 4). Meanwhile, K positive F (h) was plotted outside of the 95% 131 CI of simulated K positive F (h) at a distance of approximately 15 and 25 km in female swine (Figure 4).

**Discussion**

The current study showed the nationwide ADV seroprevalence and sex-biased spatial clustering of seropositive wild swine in 2014, 2015, and 2017 in Japan. The seroprevalence was 2.1% (29/1383) at the national level, although the prevalence might be underestimated because of the lower sensitivity of NT. In addition, because ADV viral DNA can be detected from seronegative wild swine (18) and we did not conduct viral detection test, disease prevalence might also be underestimated. Most seropositive swine
were localized at Kii Peninsula (23.1%, 28/121), which indicated that ADV was endemic among the swine population in the Kii Peninsula.

As shown in Fig. 4, the degree of spatial clustering of ADV-seropositive swine was significantly higher than that of tested swine in females at a distance of approximately 15 and 25 km but not in males at any distance. Meanwhile, the sex-based difference was not observed in the distribution of tested swine. Such sexually characterized spatial clustering might be attributed to the sex-based differences in behavioral patterns among wild swine. Maternal groups comprising female relatives and their offspring (32) may have close contacts within the group, and this may increase ADV transmission via oral and respiratory routes. As piglets mature sexually, females commonly stay around the birthplace with their maternal group, whereas males leave their group and become solitary (32). Such a difference can contribute to the spatial clustering of seropositive female swine. In addition, experimental infection studies showed that ADV is primarily transmitted via venereal contact among cohabiting swine (28). Male swine roam actively to search for breeding opportunities during breeding periods (34) and mate with several females (32). Hence, multiple female swine might be infected from the same infected male in its roaming area, which could produce the clustering of seropositive females.

Although more than 20% of wild swine were ADV seropositive in the Kii Peninsula, there was no seropositive swine in prefectures surrounding the peninsula. The Kii Peninsula is surrounded by the metropolitan area of Osaka and Kyoto and Lake Biwa, the largest lake in Japan (Fig. 2). This may act as a geographical barrier to the translocation of wild swine in this peninsula and prevent the spillover of ADV-infected swine to the outside area. The genetic information of the swine population in and around the Kii Peninsula could help assess the possibility that the localized distribution of ADV-seropositive swine was caused by limited swine movement.

In Ibaraki Prefecture, where domestic pigs are still infected with ADV, no wild swine with ADV antibodies were detected in this study. Meanwhile, in the Kii Peninsula, where ADV has already been eradicated among domestic pigs (21), ADV infection in wild swine was identified in this study. Thus, the ADV endemic areas of domestic pigs and wild swine do not coincide with each other although we should investigate to make sure that the domestic swine remain ADV-free in Kii peninsula. Venereal contact is the major ADV transmission route among wild swine (16, 28). Since domestic pigs are mainly raised inside sheds in Japan, it is unlikely that wild swine transmit ADV to these animals via direct contact. However, as the wild swine population in this area still presents with ADV, direct and indirect contact between wild swine and domestic pigs in pig farms should be prevented to maintain the ADV-free status in the Kii Peninsula.

In Kii Peninsula and Miyazaki Prefecture, since ADV-seropositive swine are inhabited, hunters should be aware of the risk of ADV infection in hunting dogs from wild swine in these areas. ADV was isolated from a hunting dog that had bitten a wild swine in Miyazaki Prefecture (23). On the other hand, in this study, no ADV-seropositive swine was detected in Oita Prefecture, where hunting dogs that ate wild swine died of AD in 2018 (24), which was later than when the present survey was performed (2014). Because this study
only included the results from surveillance conducted for 1 or 2 years, monitoring ADV infections among wild swine should be continued to identify areas with seropositive wild swine.

As a limitation of this study, juvenile animals were not included in the spatial analysis to prevent the influence of maternal antibodies. Spatial analysis including juvenile animals can be helpful to consider the possibility of ADV transmission within maternal group. Genetic relationship of tested animals would also contribute to assessing the mechanisms of ADV transmission within and between maternal groups.

**Conclusion**

Our study showed the sex-based difference in the spatial clustering of AD-seropositive adult wild swine in Japan. This difference might be caused by the sedentariness among female swine and the polygynous mating system of wild swine. Therefore, the distribution of other diseases among wild swine can possibly be characterized according to sex. The obtained results will contribute to examining how the disease agents spread within and between groups of wild swine. Recently, wild swine is important carrier of causative agents for human and domestic animals (9, 12, 13). Wild swine has been suggested to play essential roles in expanding the area with animals infected with classical swine fever viruses and African swine fever viruses, resulting in serious economic loss in pig farming (35, 36). Hence, preventing infectious disease transmission from wild swine is a global challenge. To establish an effective control strategy, we should understand the characteristics of wild infected swine and investigate the biological factors of host animals, such as sex, contributing to infectious disease dynamics among wild swine.

**Methods**

**Sample collection**

We obtained blood samples from wild swine caught for game hunting or pest control in Japan. The target area for sample collection included prefectures that were identified to be inhabited by wild swine in an investigation in 2014 (37) during three fiscal years (April–March in 2014, 2015, and 2017) in Japan (Fig. 1). Blood samples were sent to our laboratory, while maintained at a temperature of 4°C, and centrifuged for serum extraction. Next, serum samples were stored at −20°C before performing diagnostic tests. Using the samples, data about individual characteristics including sex, bodyweight estimated visually by capturers, and date and location of capture and capture method were recorded. Ages were determined using their estimated body weights (juveniles: <30 kg, adults: ≥30 kg) (38).

**ADV antibody detection**

The serum samples were initially screened using two commercial ELISA kits (ADV(S) ELISA kit and ADV (gI) ELISA kit [IDEXX Laboratories, Inc., Westbrook, ME]), according to the manufacturer’s recommendation. ADV(S) ELISA kit based on Shope strain can detect the antibodies to field and vaccine ADV strains. Meanwhile, the ADV (gI) ELISA kit can identify the antibodies to the gE antigen of field ADV strains excluding gE-negative vaccine strains such as that applied in Japan. The samples that tested
positive in either of the two ELISA procedures were analyzed with the LAT (Scientific Feed Laboratory Co., LTD., Japan) with serial dilutions. The samples that agglutinated with more than 40 times dilution were further analyzed using NT to determine antibody titers, as described in a previous study with some modifications (39). Briefly, sera were inactivated at 56°C for 30 min. Each serum was diluted twofold in serum-free minimum essential medium (MEM) (Life Technologies, Grand Island, NY) in 96-well U-bottomed tissue culture plates and was mixed with ADV suspension, Yamagata S81 strain isolated in Japan, containing $4 \times 10^3$ TCID$_{50}$/mL. After incubation at 37°C for 1 h, the diluted serum–virus mixture was added in duplicates to the confluent monolayers of CPK cells (40) grown in 96-well flat tissue culture plates. The mixture was allowed to absorb at 37°C for 1 h. After viral absorption, cells were washed with PBS and incubated with serum-free MEM for 3–5 days. The plates were monitored for CPE via microscopy. Since the samples in the present study were collected from wildlife, and are expected to contain foreign substances, ELISAs may diagnose pseudo-positivity due to nonspecific reactions. Therefore, LAT and NT were also conducted, and only samples that were positive in both tests were considered ADV-seropositive in identifying only those samples that were highly truly positive. The seroprevalence was summarized according to prefectures and plotted on a map at an individual level. Since ADV is latent in the trigeminal ganglia in infected hosts and these animals have life-long antibody reactions (14), seropositive swine were considered infected with ADV.

**Detection of sex-based differences in the spatial clustering of ADV-seropositive swine**

The association between sex and age as well as seropositivity for ADV among swine was evaluated using the chi-square test or the Fisher’s exact test. Sex-based differences in the distribution of ADV-seropositive wild swine were assessed based on the degree of spatial clustering of seropositive animals according to sex. In the spatial clustering analysis, the data of adult swine were used to prevent the effects of maternal antibodies among juveniles (41). Firstly, we examined the sex-based differences in the spatial clustering for the distribution of tested swine because the sex-based difference in the clustering of seropositive individuals could be biased if the clustering of tested swine differed between sex.

Ripley’s K-function analysis was performed to evaluate the degree of spatial clustering (42). The K-function analysis measures the degree of spatial clustering in a point pattern by counting the average number of neighbors of each point within a certain distance $h$. The K-function was estimated as

$$
\hat{K}(h) = \frac{\hat{N}^{-1}}{n} \sum_{i=1}^{n} \sum_{j=1, i \neq j}^{n} \frac{I_h(d_{ij})}{w_{ij}}, \ h > 0
$$
where \( h \) is the given distance, \( n \) is the number of tested or seropositive swine, \( d_{ij} \) is the Euclidian distance between animals \( i \) and \( j \), and \( \lambda \) is the intensity of events (i.e., the average number of tested or positive swine per unit area). \( I_h (d_{ij}) \) is a function that takes value 1 if \( d_{ij} < h \); otherwise, it is 0. \( w_{ij} \) is the proportion of the study area within the circle of radius \( d_{ij} \) centered at point \( i \). Distance \( h \) ranged from 0.5 to 50 km by 0.5 km. By comparing K-functions between the empirical and simulated events, this analysis can indicate whether the empirical events are spatially clustered at a certain distance.

The sex-based difference in the spatial clustering of tested samples was assessed with the difference between the K-function of male (\( K_{\text{tested M}}(h) \)) and female (\( K_{\text{tested F}}(h) \)) animals. The significant difference in spatial clustering between sex was evaluated by comparing empirical \( K_{\text{tested M}}(h) - K_{\text{tested F}}(h) \) and simulated \( \hat{K}_{\text{tested M}}(h) - \hat{K}_{\text{tested F}}(h) \) in each distance \( h \) (43, 44). Simulated \( \hat{K}_{\text{tested M/F}}(h) \) was generated as 10000 samples of randomly selecting male sample with the same number of that of the empirical data and annotating the rest samples as female samples.

Consequently, the degree of spatial clustering of ADV-seropositive swine was evaluated. Since the capture locations of ADV-seropositive swine are dependent on the locations of tested swine, the degree of spatial clustering of ADV-seropositive swine was assessed by comparing the K-function of ADV-seropositive swine (\( K_{\text{positive}}(h) \)) with that of tested swine (\( K_{\text{tested}}(h) \)) for each sex. The significance of spatial clustering was evaluated by comparing empirical \( K_{\text{positive}}(h) \) and simulated \( \hat{K}_{\text{positive}}(h) \) values for each distance \( h \) for each sex. The simulated \( \hat{K}_{\text{positive}}(h) \) was generated as the 10000 samples of randomly selected ADV seropositive swine from all tested swine with the same number of seropositive swine in the empirical data, for each sex. The differences in each function between the empirical and simulated values with 95% CI were plotted. The empirical values with lower or higher than the 95% CI were considered significantly different in the test of the degree of spatial clustering. Geographical and statistical analyses were performed using QGIS version 3.10.1 and R version 4.0.2 (45), respectively. To calculate K-function, splancs R-package (46) was used.

**Abbreviations**

ADV: Aujeszky’s disease virus

AD: Aujeszky’s disease

CI: confidence interval

ELISA: enzyme-linked immunosorbent assay

LAT: latex agglutination test

NT: neutralization

MEM: minimum essential medium
Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors. However, as mentioned in the text, all applicable institutional and/or national guidelines for the care and use of animals were followed in accordance with the relevant law (Act No. 88 of 2002).

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the current study are the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EY, YH and TY contributed to the conception, design, data analysis and writing of the manuscript. MT and MO contributed to sample and data collection, laboratory work and writing of the manuscript. All authors read and approved the final manuscript.

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E.Y., Y.H., and T.Y. contributed to the conception, design, data analysis, and writing of the manuscript. M.T. and M.O. contributed to sample and data collection, laboratory work, and writing of the manuscript. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Distribution of Aujeszky’s disease virus (ADV)-seronegative and ADV-seropositive wild swine tested in the national serosurvey in Japan in 2014, 2015, and 2017. The numbers on map are Pref IDs in Table 1.
Figure 2

Distribution of Aujeszky's disease virus (ADV)-seropositive and ADV-seronegative adult swine according to sex in three prefectures, including Mie, Nara, and Wakayama, in the Kii Peninsula (gray area) in 2014 and 2015.

Figure 3
Plot of empirical $K$-function $K_{tested\ M}(h) - K_{tested\ F}(h)$ (solid line) with the 95% confidence interval (CI) of simulated $K_{tested\ M}(h) - K_{tested\ F}(h)$ (dashed line). $K_{tested\ M}(h)$ and $K_{tested\ F}(h)$ mean $K$-functions of tested male and female swine, respectively. $K_{tested\ M}(h) - K_{tested\ F}(h)$ plotted outside of 95% CI of simulated $K_{tested\ M}(h) - K_{tested\ F}(h)$ at a scale of distance $h$ means that the degree of spatial clustering of

![Graph showing male and female swine K-functions](image)

**Figure 4**

Plot of empirical $K$-function (solid line) in adult male swine ($K_{positive\ M}(h)$) and female swine ($K_{posi\_F}(h)$) with the 95% CI of $K_{positive\ M}(h)$ (dashed line). $K_{positive\ M}(h)$ plotted outside of 95% CI of simulated $K_{positive\ M}(h)$ at a scale of distance $h$ means that ADV-seropositive male swine are significantly clustered at the scale. The significance of spatial clustering in ADV-seropositive swine is identified in the same way.