

Review:

# Viral Infectious Diseases in Wild Animals in Japan

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**Even limited to mammals, there exist more than 5,000 species of wild animals. Because each wild animal is the natural host of specific viruses, the total number of viruses in wild animals is enormous. Although it is impossible to cover all the infectious diseases caused by such viruses, accumulation of data on viral infectious diseases is important. In this paper, some of the latest findings acquired from our studies on viral infectious diseases in wild animals will be introduced.**

**Keywords:** wild animal, viral infectious diseases

## 1. Introduction

The number of viruses which infect more than 5,000 species of wild mammals is enormous, as in the case of viruses in humans. Although humans are unlikely to be directly infected with the viruses which wild animals are infected with, there is a possibility that the viruses would be transmitted through other animals. However, we have little information on the viruses in wild animals. **Table 1** shows the list of infectious diseases of wild animals designated by the World Organisation for Animal Health, OIE. The circled items in the list indicate infectious diseases caused by viruses.

Rabies virus should be first referred to in considering the viral infectious diseases in wild animals. In Europe, foxes, and in the US, raccoons, bats, foxes, coyotes, skunks, etc. are infected with rabies viruses, being the source of infection to domestic animals, companion animals and humans. Japan became one of the few countries which succeeded in exterminating rabies with the Rabies Prevention Act. The extermination was achieved owing to the fact that the animals infected with rabies viruses cannot easily intrude into Japan, because it is surrounded by sea, and that import of animals has been strictly regulated under the quarantine system. However, rabies is still prevalent in Southeast Asia and thus it is considered as one of the viral infectious diseases that might intrude into Japan in the future. Rabies is the most well-known viral infectious disease which wild animals are involved in, and preventive measures such as vaccination to wild animals have been already taken.

The important issue we have to address regarding viral infectious diseases in wild animals is emerging infectious

diseases. They are infectious diseases transmittable to humans which were discovered or which causes were identified after the 1970s. Many of such diseases are caused by viruses originating in wild animals, as a result of the process where the viruses which had been parasitic on wild animals found new host by chance, consequently triggering problems for humans. Such viruses as Ebola virus, Marburg virus, SARS coronavirus, Nipah virus and Hendra virus are considered as the parasite on bats. In examining the outbreak of such emerging infectious diseases, the knowledge on viruses in wild animals is indispensable.

The motto of “One World, One Health” in the Manhattan Declaration of 2004 stresses that the health of humans, companion animals and wild animals are closely linked and should be addressed at an international level. In this review paper, we will introduce our studies on viral infectious diseases in wild animals in Japan. We hope this paper would be instrumental in working out the measures against the possible outbreak of viral infectious diseases originating in wild animals.

## 2. Infection with Japanese Encephalitis Virus (JEV)

Japanese encephalitis virus (JEV) belonging to family *Flaviviridae*, genus *Flavivirus*, causes infectious diseases transmitted via arthropod. JEV can also be grouped under the Japanese encephalitis virus serocomplex, because it is closely related genetically and antigenically to the West Nile virus (WNV) and St. Louis encephalitis virus etc.. In the life cycle of JEV in Japan, mosquitoes play a central role as shown in **Fig. 1**. A mosquito sucks blood of a pig and is infected with JEV contained in the blood of the pig. The transmitted JEV multiply in the body of the mosquito. Then, when the mosquito sucks blood of another animal, JEV is transmitted to the animal. Accordingly, a mosquito is termed vector and a pig is termed amplifier. JEV does not trigger serious damages or diseases for pigs except for abortion, but in rare cases, if the mosquito infected with JEV sucks blood of a horse or a human, the horse or human sometimes suffers from encephalitis and even die. Horses or humans do not play a central role in the life cycle of JEV because, even if they are infected with JEV, the viruses seldom appear in the blood, and thus the mosquito sucking the blood of the horses or humans does

**Table 1.** OIE listed diseases affecting wild animals.

Anthrax	Haemorrhagic septicaemia
African horse sickness <sup>o</sup>	Heartwater
African swine fever <sup>o</sup>	Highly pathogenic avian influenza <sup>o</sup>
Aujeszky's disease <sup>o</sup>	Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis <sup>o</sup>
Avian chlamydiosis	Infectious bursal disease (Gumboro disease) <sup>o</sup>
Avian infectious bronchitis <sup>o</sup>	Japanese encephalitis <sup>o</sup>
Avian infectious laryngotracheitis <sup>o</sup>	Leishmaniosis
Avian mycoplasmosis ( <i>Mycoplasma gallisepticum</i> )	Leptospirosis
Avian mycoplasmosis ( <i>Mycoplasma synoviae</i> )	Lumpy skin disease <sup>o</sup>
Bluetongue <sup>o</sup>	Maedi-visna <sup>o</sup>
Bovine anaplasmosis	Marek's disease <sup>o</sup>
Bovine babesiosis	Myxomatosis <sup>o</sup>
Bovine genital campylobacteriosis	Nairobi sheep disease <sup>o</sup>
Bovine spongiform encephalopathy	New world screwworm due to <i>Cochliomyia hominivorax</i>
Bovine tuberculosis	Newcastle disease <sup>o</sup>
Bovine viral diarrhoea <sup>o</sup>	Nipah virus encephalitis <sup>o</sup>
Brucellosis due to <i>Brucella abortus</i>	Old world screwworm due to <i>Chrysomya bezziana</i>
Brucellosis due to <i>Brucella melitensis</i>	Ovine epididymitis due to <i>Brucella ovis</i>
Brucellosis due to <i>Brucella suis</i>	Paratuberculosis
Caprine arthritis/encephalitis <sup>o</sup>	Peste des petits ruminants <sup>o</sup>
Classical swine fever <sup>o</sup>	Porcine cysticercosis
Contagious agalactia	Porcine reproductive and respiratory syndrome <sup>o</sup>
Contagious bovine pleuropneumonia	Pullorum disease
Contagious caprine pleuropneumonia	Q fever
Contagious equine metritis	Rabbit haemorrhagic disease <sup>o</sup>
Criean Congo haemorrhagic fever <sup>o</sup>	Rabies <sup>o</sup>
Dourine	Rift Valley fever <sup>o</sup>
Echinococcosis/hydatidosis	Rinderpest <sup>o</sup>
Enzootic abortion of ewes (ovine chlamydiosis)	Salmonellosis due to <i>S. abortusovis</i>
Enzootic bovine leukosis <sup>o</sup>	Scrapie
Epizootic haemorrhagic disease <sup>o</sup>	Sheep pox and goat pox <sup>o</sup>
Equine encephalomyelitis (Eastern) <sup>o</sup>	Surra ( <i>Trypanosoma evansi</i> )
Equine encephalomyelitis (Western) <sup>o</sup>	Swine vesicular disease <sup>o</sup>
Equine infectious anaemia <sup>o</sup>	Theileriosis
Equine influenza <sup>o</sup>	Transmissible gastroenteritis <sup>o</sup>
Equine piroplasmiasis	Trichinellosis
Equine rhinopneumonitis <sup>o</sup>	Trichomonosis
Equine viral arteritis <sup>o</sup>	Trypanosomiasis (tsetse-transmitted)
Foot and mouth disease <sup>o</sup>	Tularemia
Fowl cholera	Venezuelan equine encephalomyelitis <sup>o</sup>
Fowl typhoid	Vesicular stomatitis <sup>o</sup>
Glanders	West Nile fever <sup>o</sup>

not become infected with JEV. Such animals as horse and human are termed dead-end host.

The typical mosquito as the vector of JEV in Japan is *Culex tritaeniorhynchus* which breeds in rice paddies. The mosquito appears when the rice paddies begin to be filled with water, and then the prevalence of JEV becomes noticeable. The incidence of JE in humans and horses reaches a peak from August to September. From the 1940s to the 1950s in Japan, more than 5,000 human JE patients and more than 3,000 horses showing the symptoms were annually reported. However, by the effect of the vaccination program against JEV, the number of human patients has decreased to less than 10. And there has been no case of infection in horses in Japan since 2003, when two horses were infected with JEV in Tottori Prefecture.

Where does JEV exist after summer, during the period from winter to the beginning of spring? The following are possible answers: 1) in the bodies of wintering mosquitoes; 2) in the bodies of wild animals; and 3) brought from the continent in some way. Recently, Dr.

Morita et al. at Nagasaki University pointed out that the JEV isolated in Japan can be divided into two groups, namely, the viruses which are assumed to have intruded from China into Japan and those indigenous to Japan [1]. Moreover, Dr. Takasaki et al. at the National Institute of Infectious Diseases succeeded in isolation of JEV from the wild boar captured on 12 December, and in detection of the JEV gene from the wild boar captured at the beginning of May [2]. The fact that JEV was detected from the wild boars captured in December and May, when there had been no report of detection of JEV from pigs, indicates the possibility that wild boars are involved in wintering of JEV. It is now thought that some of the viruses prevalent in Japan may be held in the bodies of wild animals such as wild boars, and there is a high possibility that some others may be brought in from abroad.

What species of animals are infected with JEV besides pigs and wild boars? In Japan and China, JEV was isolated from bats. Furthermore, it has been pointed out that wild birds may also play a role as amplifiers. We conducted a survey on the prevalence of JEV antibodies in

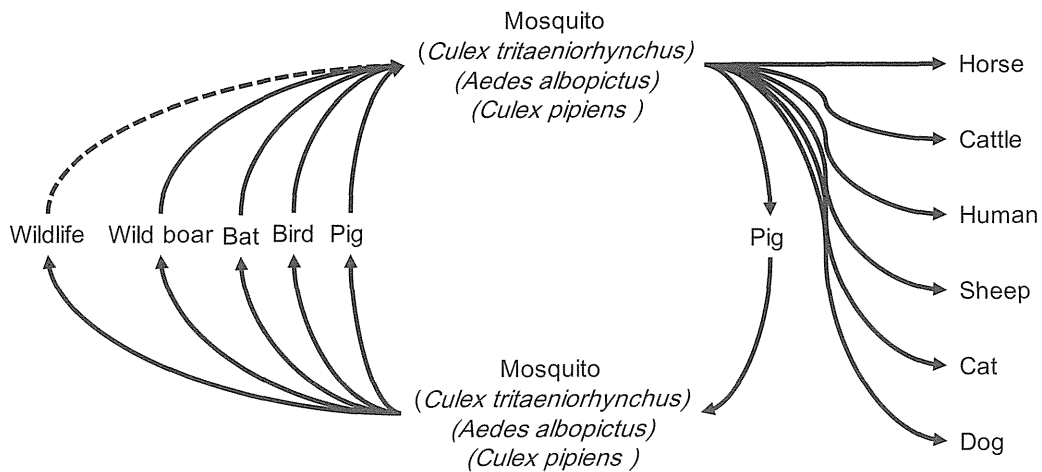


Fig. 1. Life cycle of JEV.

raccoons in the prefectures of Wakayama, Osaka, Hyogo and Hokkaido [3]. According to the findings, the antibody prevalence in western Japan was 41% to 69%, and 0% in Hokkaido, where almost JE patient had not been reported. In Wakayama Prefecture, 63% of the raccoon dogs had antibodies against JEV, while wild boars marked the antibody-prevalence of 83%, which was higher than raccoons and raccoon dogs. Moreover, as a result of surveys on the prevalence of JEV antibodies in various species of wild animals in Wakayama Prefecture, many wild animals including deer, foxes, weasels and badgers were antibody-positive, while the antibody-prevalence of bent-winged bats (*Miniopterus fuliginosus*) of one year or over inhabiting in Wakayama Prefecture was only 33%, which was lower than expected (manuscript in preparation). We also confirmed that the antibody prevalence of cats was low, while that of dogs was extremely high [4]. At the same time, we confirmed by experimental infection to dogs with JEV that dogs might not show any clinical symptoms [5]. Although in rare cases, JE has been reported to appear in animals other than horses and humans, such as cows and sheep; thus, there is a possibility that many wild animals are infected with JEV but only some of them show the symptoms. JE has the infectious cycle involving not only humans and domestic animals but also many wild animals. Wild animals may suffer from JE and die secretly, unknown to people. In particular, further surveys are needed on bats and wild boars which are regarded as amplifiers of JEV.

In Japan, because of widespread use of vaccines, the number of incidence of JE in humans and horses has decreased. However, in Korea, the annual number of patients increased to 26 in 2010, although the number had been less than 10 as with Japan. The sudden increase in the number of patients in Korea, which had been in a similar condition to Japan, means that we should continue to be attentive to JE as zoonosis. Although pigs have been exclusively focused in the discussions on host of JEV, attention must be also paid to wild boars which are close to pigs, because wild boars are more likely to appear in

residential area and near urban area. Lastly, since JEV is closely related with WNV, which is prevalent in non-Asian countries, it is difficult to distinguish the both. To prepare the intrusion of WNV into Japan, the survey on JE in wild animals and wild birds in Japan is significant.

### 3. Infection of Wild Boars with Aujeszky's Disease Virus (ADV)

Aujeszky's disease (AD) is an infectious disease caused by suid herpesvirus 1 belonging to family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. Aujeszky's disease virus (ADV) is latently infected in pigs as the natural host and triggers abortion in pregnant pigs and respiratory symptoms in new-born pigs, causing a major economic damage to pig farming. Accordingly, for the purpose of eradicating the AD, vaccines have been administered to prevent the outbreak of the disease, and ELISA to detect gE-antibody, which are induced in vaccinated pigs, has been used to detect and monitor the pigs infected with wild virus. As a result, many prefectures succeeded in eradicating the disease in Japan. It is expected that eradication of AD from pigs will be achieved all over Japan in the near future.

Another problem of AD is the high fatality rate in case animals other than pigs are infected with the virus. Although such infection seldom occurs, if cows, cats, dogs and so on, are infected with the virus, they suffer from neurological disorder including pruritus and almost 100% of them die. For this reason, AD disease is also termed pseudorabies, like rabies. In 1997, in Nara Prefecture, 24 hunting dogs which had eaten raw meat of wild boars died of AD [6]. It has been also confirmed that wild boars which are closely related with pigs can be the host of ADV.

The situation of infection of wild boars with ADV in Japan has been unclear so far. We conducted a survey on the situation of seroprevalence against ADV in the wild boars captured in three prefectures, where AD had

not appeared in pigs [7]. First, using a ten-fold diluted serum, which was not supposed to cause nonspecific reaction, a virus neutralization (VN) test was carried out by 80% plaque reduction test. As a result, the antibody-positive wild boars were found in two out of three prefectures surveyed. The antibody-positive rate of wild boars in each prefecture ranged from 4% to 6%. Next, those positive sera were further tested using ELISA to distinguish between infection with wild strains and vaccination. As a result, it was proven that all the positive reactions were caused not by vaccination but by infection with wild strains of ADV. In VN test, we adopted the screening method under strict conditions to raise the degree of peculiarity, in order to prevent a false positive reaction. Therefore, it is considered that more individuals are actually infected with ADV than in our findings.

Then, what kind of measures should be taken against infection of wild boars with ADV? ADV infects latently their natural host, a characteristic of herpesviruses. Thus, the antibody-positive wild boars in the above-mentioned survey may have been latently infected with ADV. Accordingly, we must keep in mind that several percent of wild boars retain ADV and they can become the source of infection to other animals including other wild boars. As a matter of course, we also must keep in mind the possibility of transmission of ADV from wild boars to pigs. Especially in the prefectures where vaccination has not been administered, attention must be paid to such possible transmission. Furthermore, raw meat of wild boards should not be given to animals including dogs as food.

#### 4. Canine Distemper (CD) in Wild Animals

Canine distemper virus (CDV) belongs to order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Morbillivirus*. Genus *Morbillivirus* includes Measles virus (MeV) causing measles in humans, and rinderpest virus (RPV) causing rinderpest in cattle. As for RPV, eradication was declared by OIE in 2011. This was a brilliant achievement following that of smallpox. The latest genetic analysis indicated that MeV diverted from RPV during the period between the 11<sup>th</sup> and 12<sup>th</sup> centuries [8]. In other words, MeV is the virus whose host range mutated from that of RPV. In my personal view, MeV, RPV and CDV are different only in the host range, but have similar strong pathogenicity.

What kind of animals are the hosts for CDV, while RPV infects cattle and water buffaloes, and MeV infects humans and monkeys? Various species of carnivora are infected with CDV, but especially canids including dogs can be the natural host of CDV. CDV has triggered fatal epidemics in many animals. As the most well-known example, about 1,000 lions out of about 3,000 inhabiting at the Serengeti National Park in Tanzania died of infection with CDV [9]. In addition, the number of island foxes inhabiting in California, USA decreased drastically to one tenth because of infection with CDV and this species of fox was designated as endangered species [10, 11]. In

2008, there was an epidemic of CDV in rhesus monkeys in China and moreover, more than 30 cynomolgus (crab-eating) monkeys imported from China were reported to have died of infection with CDV at an animal quarantine station in Japan [12, 13]. The host range of CDV may be expanding to primates.

In Japan, some cases of the CDV epidemic in wild animals have been reported. We will introduce three examples of such epidemic of CDV on which surveys have been conducted: 1) case in Tanabe City, Wakayama Prefecture [14]; 2) case in Kochi Prefecture [15, 16]; 3) case in Yamaguchi Prefecture [17].

##### 1) Case in Tanabe City, Wakayama Prefecture

In around March, 2007, raccoon dogs in the area around Tenjinzaki Cape in Tanabe City, Wakayama Prefecture, were reported to have died of asthenia. As a result of examination, it was proven that those raccoon dogs had been infected with CDV [14]. We succeeded in isolation of CDV from seven raccoon dogs and one weasel in total. According to the results of comparison of the viruses, they were identified as the same origin. After the outbreak, we conducted a survey on the retention rate of CDV antibodies in wild animals in the area around Tanabe City, Wakayama Prefecture, and confirmed that about 50% of raccoons and about 20% of raccoon dogs were CDV-positive. Besides, CDV-positive animals were also found in badgers, a weasel and martens. Among carnivora inhabiting in this area, foxes were the only species that we did not find any CDV-positive one, although only one fox was tested. The surprising findings were that 26% of wild boars and 40% of sika deer were CDV-positive. Infection of wild boars with CDV was not the first case, because the infection of peccary similar to wild boars had been already reported in the US [17], but infection of deer was the first case reported, to our knowledge.

##### 2) Case in Kochi Prefecture

In 2005, many raccoon dogs which were infected with CDV died at Katsurahama Beach in Kochi Prefecture, and thereafter, deaths of badgers and palm civets caused by infection with CDV were continuously reported in the area around Kochi City [15, 16]. We had the opportunity to isolate the viruses after 2008, and succeeded in isolation of KochiO1A strain from the affected palm civet, in determination of its complete genome sequence and also in the development of pathogenicity in dogs through experimental infection (Manuscript in preparation). The homology in the sequence of hemagglutinin genes between this virus and Onderstepoort strain used as vaccines was less than 90%. Afterward we continued the surveys on CDV in this area. In 2010, relatively many cases of infection were reported and thus the tendency of CDV epidemic was recognized again. It has been confirmed that the viruses prevalent in this area were similar to KochiO1A and kept spreading among wild animals in this area.

### 3) Case in Yamaguchi Prefecture

In Yamaguchi Prefecture, there was an epidemic of CDV in wild raccoon dogs beginning from the end of 2009 [19]. CDV was successfully isolated from those raccoon dogs. Then, in January, 2010, diarrhea, stomach-ache and coughing were recognized in 12 tigers kept at the zoo in the same area, and CDV genomes were detected from the diarrheal stools examined. Two of the tigers died of unknown reason, while many of the others recovered. However, in March, one tiger died of neurological disorder and CDV was isolated from the tiger. The result of comparison between the strains isolated from the raccoon dogs and from the tigers showed that the both viruses were closely related. These findings indicate that the CDV prevalent in wild animals including raccoon dogs were transmitted to the tigers kept at the zoo. Furthermore, as a result of examination on three lions kept at the zoo, one lion was found to be highly CDV-positive. In addition, after examining nine Asian black bears captured in Yamaguchi Prefecture, it was confirmed that one bear out of nine was CDV-positive.

The above findings suggest that, although the infection with CDV was previously thought to be limited to canids or felids belonging to *Carnivora*, the host range of CDV is now spreading to other animals.

Then, what kind of measures should be taken against CD which has been prevalent among wild animals? Based on our findings, vaccines are effective for dogs and vaccination might be also recommended to the other companion animals. However, as for such animals highly sensitive to CDV as ferrets, the safer method of vaccination should be considered. In addition, as for such rare species of animals as tigers in the CDV-infected area, vaccination is strongly recommended. And there might be a possibility that humans are also infected with CDV, because CDV has been already transmitted to non-human primates. Thus, it would be necessary for those who may have frequent contacts with wild animals to confirm whether or not they have been vaccinated against MeV. The vaccination against MeV is strongly recommended for those who have not been vaccinated against MeV nor infected with MeV, because the immune response to the MeV vaccine may be also effective to CDV. Moreover, based on the fact that CDV infects wild boars and deer, we can assume that CDV can also infect pigs and cattle. Therefore, prevention against invasion of wild animals into livestock farms is important, although it is also needed to prevent viruses other than CDV. Moreover, in protecting weakening wild animals, separation from other animals is necessary, because such wild animals are highly suspicious to have been infected with CDV. There is a report of possible transmission of CDV from a protected palm civet to a pet dog (manuscript in preparation).

### 5. Viruses Originated from Bats

As mentioned above, bats are the natural host of viruses causing emerging infectious diseases. In the case of Nipah virus, it is believed that virus transmitted from bats to pigs because pig farms were kept away from urban areas for various reasons and were obliged to relocate near the wood where bats originally inhabited. Nipah virus excreted from bats were transmitted to pigs and consequently spread from the infected pigs to humans. Meanwhile, in the case of Hendra virus, when bats were forced to leave their original habitats and moved near the stables for horses, the virus was transmitted from bats to horses, and consequently from the infected horses to humans. This was the result of the increased opportunities for humans, pets and livestock to have contacts with bats due to progressing deforestation etc., although there had been few opportunities for such contacts before. The viruses infected bats as the natural host had coexisted with bats quietly in forests before, however, since humans or other species of animals intruded into the area, some viruses caused emerging infectious diseases as new problems for humans. Thus, bats are not to blame as the cause. We should consider why so many viruses as the cause of the emerging infectious diseases originate in bats. Domestic or companion animals have had many opportunities to have contacts with humans in their long history of domestication process. Therefore, even if such domestic animals had viruses which could cause emerging infectious diseases, those viruses must have already been transmitted to humans in the long history, triggering some problems to humans. Contrarily, wild animals have had fewer opportunities to have contacts with humans and the viruses parasitic on the wild animals have also had fewer opportunities for transmission to humans. This is the reason why so many emerging infectious diseases originate in wild animals. In mammals, there exist more than 1,000 species of bats belonging to *Chiroptera*, which is the second largest number of species after *Rodentia*. If each species of bat is natural host of specific viruses, the total number of viruses should be enormous. A tiny part of them possesses infectious capacity to humans and appears as the viruses to cause emerging infectious diseases. More important point is that bats are regarded as relatively close to horses according to the genetic classification [20]. Thus, the viruses which can be transmitted to bats may be likely to be transmitted to horses. On the other hand, because rodents are considerably far from humans genetically, infection of humans and other animals with the viruses carried by rodents is thought to be difficult.

It has been made clear that the viruses carried by bats are likely to be the cause of emerging infectious diseases. Then, what kind of viruses do bats carry? We have had too little knowledge on bats; there has been almost no cultured cell to isolate the viruses originating in bats. We succeeded in the establishment of cultured cells from the following species of bats: *Rhinolophus ferrumequinum*, *Pteropus dasymallus yayeyamae*, and *Miniopterus fuliginosus*, and isolated some viruses in the process of estab-

lishing such cultured cells. These examples are introduced below.

### 1) Isolation of a novel gamma-herpesvirus from a horseshoe bat, *Rhinolophus ferrumequinum*

Horseshoe bat, *Rhinolophus ferrumequinum*, is regarded as the natural host of SARS coronavirus. For this reason, we established cultured cell line originating from *Rhinolophus ferrumequinum*, BKT1, which was expected to be useful for isolation and analysis of the viruses originating in *Rhinolophus ferrumequinum*. During the passage culture of the adherent cells originating in the spleen of another *Rhinolophus ferrumequinum*, a cytopathic effect (CPE) was confirmed. Large viruses were found by observation with an electron microscope, and as a result of the genetical analysis using the Rapid Determination of Viral RNA (RDV) method developed by Mizutani et al. (the National Institute of Infectious Diseases) [21], the virus was identified as a novel gamma-herpesviruses. This virus was close to equine herpesvirus 2 carried by horses, which may be because of the close genetic relation between bats and horses (manuscript in preparation).

### 2) Isolation of a novel adenovirus from Japanese fruit bat, *Pteropus dasymallus yayeyamae*

Fruit bats are the natural hosts of such viruses as Ebola virus, Marburg virus, Nipah virus and Hendra virus. *Pteropus dasymallus yayeyamae* is the only specie of fruit bats which inhabits in Japan and has not been designated as endangered species, thus is permitted to be captured. We succeeded in the establishment of the cultured cell, FBKT1, originating in *Pteropus dasymallus yayeyamae*. However, the virus was isolated during the passage culture of the spleen cells, as in the case of Horseshoe bat. As a result of identification, the isolated viruses were confirmed to be a novel adenoviruses. The isolation of adenovirus from bats was reported for the first time [22].

### 3) Isolation of a novel beta-herpesvirus from a bent-winged bat, *Miniopterus fuliginosus*

We also succeeded in the establishment of cultured cells and isolation of a novel beta-herpesviruses from a bent-winged bat, *Miniopterus fuliginosus*. Because we had already found out the presence of adenovirus and herpesvirus in bats, we first tried to detect the both viruses using the consensus primers for adenovirus and that for herpesvirus. However, the isolated virus was PCR-negative, accordingly we next tried to identify the viruses using the above-mentioned RDV-method. As a result, they were identified as a novel betaherpesvirus [23]. The virus existed in the spleen of 4% of the bats. The virus was detected in kidney, lung, liver and spleen, but not detected in intestines, trachea, urinary bladder and brain. The important point in this survey was that the virus could not be detected by the consensus primers which had been previously considered to be able to detect all kinds of herpesviruses. The similar result was acquired in analyzing

the virus originating in bats in the Philippines [24]. The results of analyses showed the importance of isolation of viruses and genetic identification, and indicated that overconfidence in consensus primers would be risky.

As mentioned above, we succeeded in isolation and identification of novel viruses originating from bats. The possibility may be low that these viruses will cause emerging infectious diseases. However, accumulated knowledge on such viruses infecting bats is anticipated to be useful at the time of outbreak of the emerging infectious diseases originating from bats in the future.

## 6. A Novel Rhabdovirus Transmittable to Wild boars, Deer, Cattle and Pigs

In the trial to isolate JEV from the serum of wild boars, CPE was recognized in the Vero cell. As a result of identification, rhabdovirus-like particles were found by observation with an electron microscope, and it was proven by the RDV method that the virus was the novel rhabdovirus. According to viral neutralization tests, it was found that more than 50% of wild boars in the area had been infected with the virus, and that there were some positive individuals in deer, cattle and pigs, although the number of individuals were few. The pathogenicity and the infectious cycle of this virus are still unknown, but attention may be needed for infection of humans with the virus, because it has the potential to be transmitted to many animals (manuscript in preparation).

The above finding is an example which indicates that unknown infectious diseases are secretly prevalent in wild animals in Japan. The possibility cannot be denied that new emerging infectious diseases will appear in Japan.

## 7. Infection of Raccoons with Highly-Pathogenic Avian Influenza Virus

In considering the intrusion route of the viruses to cause highly-pathogenic avian influenza (HPAI) brought from the Eurasian Continent by wild birds into poultry farms, we thought about the possibility of wild animals being the mediators. Focusing on raccoons, which were likely to prey on wild birds on the shore and intrude into poultry farms, we investigated whether or not they had antibodies against HPAI virus [25]. As a result of the survey, HPAI-positive raccoons were found in three prefectures out of four. And in two prefectures out of the three, there had been no report of the outbreak of HPAI so far. The findings of the survey indicate that wild birds infected with the HPAI virus would intrude into Japan with a considerably high frequency and raccoons would prey on them. It remains unknown whether or not these raccoons can become the source of infection with HPAI virus for livestock. However, through the survey on the situation of the antibody-prevalence of raccoons against HPAI virus, it has been made clear that more HPAI virus has intruded into Japan than we expected. According to a study abroad,

it was reported that red foxes which ate the birds infected with HPAI virus excreted the viruses later without showing the symptom of the disease [24]. Raccoons may show the same pattern as red foxes. Although our study focused on raccoons, it may be possible that other wild animals such as weasels, martens and rodents are infected with HPAI virus and that the virus intrudes into poultry farms via such wild animals.

Raccoons are invasive alien species which has recently intruded into Japan. Such alien species may become the natural host of viruses of new emerging infectious diseases, and trigger unexpected spread of such diseases. Especially in Japan, where no large carnivorous animal inhabits, humans are the only natural enemy of the alien species like raccoons. Accordingly, humans should take appropriate measures against the alien species.

The possibility of intrusion of wild animals into pig farms or poultry farms is still unknown. However, by preventing the intrusion of wild animals, we can prevent various kinds of infectious diseases, because wild animals may carry many viruses to cause infectious diseases.

## 8. Conclusions

We have introduced some viral infectious diseases in wild animals, but these are a tiny part of numerous viruses; there are still many other viruses including unknown ones. It is thought that the measures for wild animals against viral infectious diseases might be not necessary, but prevention of intrusion of wild animals into the breeding site of domestic animals is important. As for rare species of animals, some measures should be taken, because the number of such species can decrease drastically due to the infectious diseases. Humans should avoid approaching wild animals, although in Japan people have little awareness because rabies does not occur. In addition, raw meat of wild animals should not be eaten. The viral infectious diseases originating in wild animals may continue to break out in the future. Thus, it would be desirable to accumulate information on the viruses in wild animals, based on the principle of "One World, One Health."

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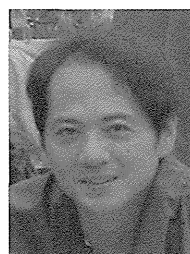
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# Journal of ZOO and WILDLIFE MEDICINE



## COMPLEX CARCINOMA OF THE MAMMARY GLAND IN A FREE-LIVING JAPANESE RACCOON DOG (*NYCTEREUTES PROCYONOIDES VIVERRINUS*)

Tomomi Nakashima, D.V.M., Masahito Kubo, D.V.M., Ph.D., Ai Oshita, Akiho Katayama, Kazuo Suzuki, and Ken Maeda, D.V.M., Ph.D.

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**Abstract:** A complex carcinoma of the mammary gland was diagnosed in a free-living old female Japanese raccoon dog (*Nyctereutes procyonoides viverrinus*). Invasion into lymphatic vessels and metastasis in the inguinal lymph node were observed. This is the first report of complex carcinoma of the mammary gland in a raccoon dog.

**Key words:** Complex carcinoma, free-living, mammary gland, *Nyctereutes procyonoides viverrinus*, raccoon dog.

### BRIEF COMMUNICATION

Mammary-gland tumors are the most common neoplasm in female dogs, and the third in frequency after skin tumors and lymphoma in female cats.<sup>10</sup> On the other hand, mammary-gland tumors are rarely observed in wild or exotic animals.<sup>1,2,4,5,7,15,17</sup> There have been a few reports of the mammary-gland tumors in nondomestic carnivores.<sup>1,2,4,5,7,11,15,17</sup> Especially in exotic Canidae, there have been few case reports, e.g., adenocarcinoma of the mammary gland in a free-ranging red fox (*Vulpes vulpes*);<sup>7</sup> mixed mammary tumor composed of infiltrating ductal carcinoma with centers of mucinous, epidermoid, and chondrosarcoma (chondrosarcoma carcinoma) in a captive Mexican gray wolf (*Canis lupus baileyi*);<sup>4</sup> and simple ductal mammary papilloma<sup>2</sup> and tubulopapillary carcinoma of the mammary gland<sup>5</sup> in captive maned wolves (*Chrysocyon brachyurus*).

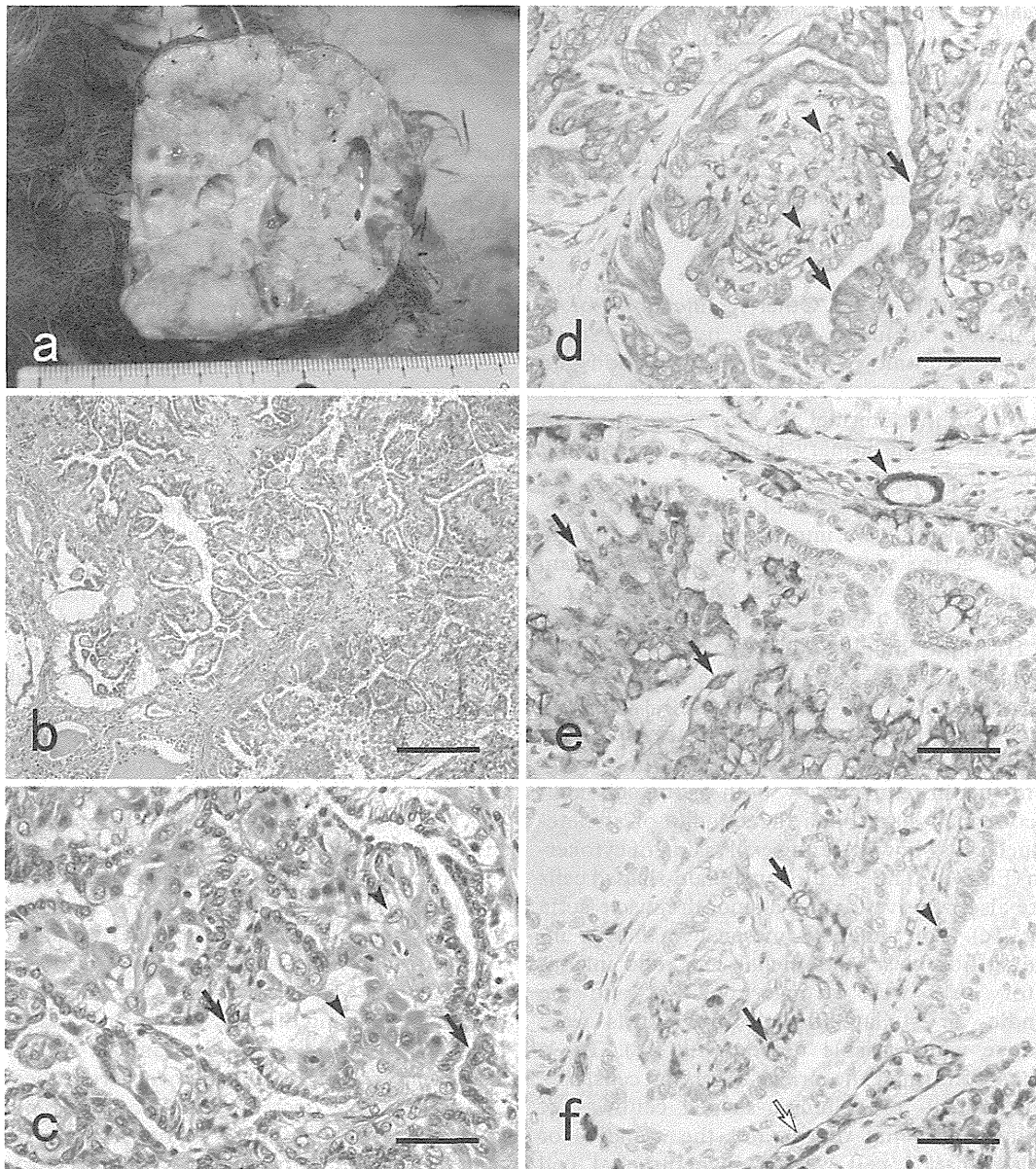
In December 2011, the carcass of a female Japanese raccoon dog (*Nyctereutes procyonoides viverrinus*) was submitted to the Laboratory of Veterinary Pathology, Yamaguchi University for postmortem examination because there was a nodular lesion on the ventral skin. The animal had been found in Nagano, Tanabe, Wakayama, Japan (33°46'N, 135°27'E), and head trauma was considered to be the cause of death. Many teeth had been lost and remaining teeth were wearing

and fractured; this suggested that the animal was very old. On the caudal abdominal region, there was a firm, moderately well demarcated cutaneous mass approximately 6 × 3 × 3 cm in size. The mass was not fixed to underlying muscles. The skin on the lesion was hairless and partly ulcerated. The cut surface of the mass was yellowish-white and lobulated, and many cysts with variable sizes were present (Fig. 1a). The inguinal lymph nodes were slightly enlarged. Other gross findings included firm pale shrunken kidneys, calcification of the walls of the celiac, cranial, and caudal mesenteric arteries and their branches, partial sclerosis of the pancreas, and roundworms in the small intestine. The ovaries and uterus had a normal appearance.

Tissue samples of the mass and major organs were fixed in a 10% neutral-buffered formalin solution, embedded in paraffin wax, sectioned at 4 μm and stained with hematoxylin and eosin (H&E).

Microscopically, the mass was composed of a proliferation of two cell types (Fig. 1b). One cell type was cuboidal to columnar in shape and had moderately eosinophilic cytoplasm and a round-to-oval nucleus (Fig. 1c). Cells of this type formed tubular and papillary structures and resembled luminal epithelial cells. Another cell type was spindle or stellate in shape and had strongly eosinophilic cytoplasm and a round-to-oval nucleus (Fig. 1c). Cells of this type intermingled with luminal epithelial cells and resembled myoepithelial cells. Both types of cells showed moderate nuclear pleomorphism, but mitotic figures were rarely observed. Tumor cells invaded the dermis, and the surface of mass was partially ulcerated with purulent inflammation. Normal mammary tissue was observed around the tumor. Lymphatic invasion was seen and metastasis was noted in the inguinal lymph node but not in other histologi-

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**Figure 1.** (a) Cut surface of the cutaneous mass on the lower abdominal region. Note the lobulated structure and variable-sized cysts. (b) Proliferation composed of luminal epithelial and myoepithelial-like cells is shown. Note tubular and papillary structures. Hematoxylin and eosin (H&E) stain. Bar, 200  $\mu$ m. (c) Higher magnification of Figure 1b. Arrows indicate luminal epithelial cells. Arrowheads point to myoepithelial-like cells being spindle or stellate in shape and having intensely eosinophilic cytoplasm. H&E stain. Bar, 50  $\mu$ m. (d) Both luminal epithelial (arrows) and myoepithelial-like cells (arrowheads) express cytokeratin. Immunohistochemistry, counterstained with Meyer's hematoxylin. Bar, 50  $\mu$ m. (e) Myoepithelial-like cells (arrows) are strongly positive for alpha-smooth muscle actin, but luminal epithelial cells are negative. Arrowhead indicates vascular smooth muscle cell. Immunohistochemistry, counterstained with Meyer's hematoxylin. Bar, 50  $\mu$ m. (f) Some myoepithelial-like cells are positive for vimentin (black arrows), but luminal epithelial cells are negative. White arrow and arrowhead indicate endothelial cell and inflammatory cell, respectively. Immunohistochemistry, counterstained with Meyer's hematoxylin. Bar, 50  $\mu$ m.

**Table 1.** Primary antibodies used for immunohistochemistry.

Antigen	Type	Clone	Dilution	Pretreatment <sup>a</sup>	Source
Cytokeratin	pAb <sup>b</sup>	—	1:1000	+	Dako Japan Inc., Tokyo, Japan
Vimentin	mAb <sup>c</sup>	V9	1:100	+	YLEM, Rome, Italy
Alpha-smooth muscle actin	mAb <sup>c</sup>	1A4	Prediluted	—	Dako Japan Inc.

<sup>a</sup> Pretreatment = microwaving (500 W, 5 min) in 10-mM citrate buffer (pH 6.0).

<sup>b</sup> pAb = rabbit polyclonal antibody.

<sup>c</sup> mAb = mouse monoclonal antibody.

cally examined organs. Other significant histological lesions included chronic tubulointerstitial nephritis, chronic pyelitis, stone formation and fibrosis in the pancreas, chronic cystitis, hyalinization of the uterine arteries, a fragment of nematode in the urinary bladder, and sarcocyst in the tongue.

To determine the types of cells composing the tumor, sections were immunostained for smooth muscle actin, vimentin, and pan-cytokeratin. Briefly, sections were treated with primary antibodies shown in Table 1, and then incubated with the Histofine Simple Stain MAX-PO (R) or (M) (Nichirei Biosciences Inc., Tokyo, Japan). Positive reactions were visualized with 3'3-diaminobenzidine tetrahydrochloride and sections were counterstained with Mayer's hematoxylin.

Immunohistochemically, both of the cuboidal-to columnar-shaped and the spindle- or stellate-shaped cells showed strong expression of cytokeratin (Fig. 1d). The spindle- or stellate-shaped cells were also positive for alpha-smooth muscle actin (Fig. 1e). For vimentin, mesenchymal cells including fibroblast, endothelial cells, and inflammatory cells were strongly positive, and the spindle- or stellate-shaped tumor cells were positive with variable degree (Fig. 1f). These results confirmed the origin of tumor cells, i.e., the cuboidal- to columnar-shaped cell was of luminal epithelial origin and the spindle- or stellate-shaped cell was of myoepithelial cell origin.

Histopathologically and immunohistochemically, the present tumor was diagnosed as complex carcinoma of the mammary gland according to the World Health Organization classification.<sup>14</sup> Mammary-gland tumors in dogs and cats are composed of three lines, i.e. luminal epithelial, myoepithelial, and connective-tissue components, and the definition of complex carcinoma is combined proliferation of malignant luminal epithelial and myoepithelial cells.<sup>14</sup> Differentiation of mammary-gland tumors from apocrine gland tumors is important because these two tumors are histopathologically very

similar.<sup>6</sup> In the present case, normal mammary-gland tissue was observed around the tumor; therefore tumor origin is considered to be the mammary gland.

Very few cases of complex type of mammary-gland tumors have been reported in nondomestic carnivores, e.g., a case in a captive aged California sea lion (*Zalophus californianus*);<sup>11</sup> however, no case of complex type of mammary-gland tumors has been reported in free-living Canidae. This is surprising because the prevalence of mammary-gland tumors is highest in the bitch and complex carcinoma is relatively common in the dog.<sup>13</sup> Complex carcinomas of the mammary gland in free-living Canidae are very rare, probably because there are few mammary-gland tumors overall in these animals. Some authors propose that most free-ranging animals may not be able to survive long enough to develop mammary-gland tumors,<sup>7</sup> but there are few reports of mammary tumors in captive canidae despite the longevity compared to free-ranging animals. These findings may suggest that there may be other factors in the development of mammary-gland tumors. Reproductive experiences may be one of the factors influencing the low incidence of mammary tumors in wild animals. In humans, early first full-term pregnancy and prolonged breastfeeding are suggested to decrease the risk of developing breast cancer.<sup>3,9</sup> The majority of free-living animals are thought to have reproductive experiences at relatively younger ages. The present raccoon dog was thought to have had at least one reproductive experience, because hyalinization of the uterine arteries, a vascular change regularly seen in pregnant and postpartum animals,<sup>12</sup> was observed. In addition, in humans multiparous women are at a lower risk of breast cancer than women who have had only one or two births.<sup>16</sup> On the other hand, women who have only one child are at higher risk of breast cancer than nulliparous women for up to 15 yr after childbirth and at lower risk thereafter.<sup>8</sup> These varied effects of childbirth on the development of mammary-gland tumors with the interval since

birth suggests that there may be a variation in the incidence of mammary tumors in wild animals by the number of births. However, there is also the possibility that free-living animals decompose and the tumors may not be found.

To the authors' knowledge, this is the first report of the mammary-gland tumor in raccoon dogs and also the first report of complex carcinoma of the mammary gland in nondomestic Canidae.

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26 **ABSTRACT**

27           Hepatitis E virus (HEV) causes a food- and water-borne disease in humans  
28           and wild boar (*Sus scrofa leucomystax*) meat is one of the most important sources of  
29           infection in Japan. The levels of seroconversion to HEV were tested in 113 serum  
30           samples from wild boar captured in Shimonoseki City, Yamaguchi Prefecture, Japan  
31           from 2010 to 2012 by enzyme-linked immunosorbent assay (ELISA) using virus-like  
32           particles as antigen, and nested reverse transcription-polymerase chain reaction  
33           (RT-PCR). Anti-HEV IgG was detected in 47 of the 113 wild boar serum samples  
34           (42%) and HEV RNA was detected in five samples (4%). Sequence analysis showed  
35           that the five HEV isolates belonged to genotype 4, forming a cluster with a previous  
36           isolate from a human hepatitis E case in this region in 2011. These results indicate  
37           that wild boar in this region are infected with potentially pathogenic HEV at a high  
38           frequency.

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40   Keywords: Hepatitis E virus, Japan, Wild boar, Zoonosis

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42           Hepatitis E virus (HEV) is the causative agent of acute or fulminant hepatitis E  
43 in humans. In Asia, Mexico, the Middle East and North Africa, transmission of HEV  
44 occurs by the fecal-oral route through contaminated water supplies. In developed  
45 countries, HEV has previously been thought to be imported by travelers from countries  
46 where the disease is prevalent. However, there are many reports of patients who were  
47 infected whilst in the USA, Europe and Japan (Emerson et al., 2007). In 2003, people  
48 who had consumed uncooked deer meat were found to be infected with HEV in Japan  
49 (Tei et al., 2003). HEV infection is now a recognized zoonosis with deer, pigs and wild  
50 boar acting as reservoirs for human infection in developed countries (Takahashi and  
51 Okamoto, 2013).

52           HEV is a non-enveloped, single-stranded positive-sense RNA virus and it is the  
53 only member of the genus *Hepevirus* in the family *Hepeviridae* (Emerson et al., 2007).  
54 There is only one HEV serotype, which is genetically divided into four genotypes  
55 (Okamoto et al., 2007). Genotypes 1 and 2 are restricted to humans and are often  
56 associated with outbreaks in developing countries. Genotypes 3 and 4 cause zoonotic  
57 infections in both developing and developed countries (Aggarwal et al., 2011).  
58 Genotypes 3 and 4 have been isolated from many species, including pigs, wild boar,  
59 deer, rats, rabbits, mongooses and horses (Meng et al., 1997; Nakamura et al., 2006;



60 Saad et al., 2007; Geng et al., 2010; Johne et al., 2010; Cossaboom et al., 2011).

61 Hepatitis E occurs sporadically in Japan, but the number of infected patients is  
62 increasing (Takahashi and Okamoto, 2013). The source of the infection is unknown in  
63 many cases because the incubation period can be as long as 6 weeks. Confirmed cases  
64 were recently reported following consumption of pig or wild boar meat (Takahashi and  
65 Okamoto, 2013). In Yamaguchi Prefecture, in western Honshu, two of the four cases of  
66 hepatitis E since 2006 are thought to have resulted from consumption of undercooked  
67 wild boar meat. In 2011, one case of hepatitis E was reported in Shimonoseki City,  
68 Yamaguchi Prefecture. The patient consumed raw wild boar's liver 1 month before the  
69 onset of symptoms and it is presumed that this was the source of the virus (Okita et al.,  
70 2012). However, the prevalence of HEV in wild boar in the city is unknown.

71 In this study, the prevalence of anti-HEV IgG detected by enzyme-linked  
72 immunosorbent assay (ELISA) and HEV RNA detected by reverse  
73 transcription-polymerase chain reaction (RT-PCR) was determined in samples taken  
74 from wild boar in Shimonoseki City, Yamaguchi Prefecture, Japan.

75 A total of 113 serum samples were collected from wild boar in mountainous  
76 regions of Shimonoseki City, Yamaguchi Prefecture, Japan (Latitude 34° 18' 6",  
77 Longitude 130° 58' 52"). Most animals were hunted with government permission

78 during the winter season and blood was collected from their hearts by 21G needle  
79 (Nipro, Osaka, Japan) and 50ml syringe (Nipro). Seventy-one serum samples were  
80 collected from wild boar captured in the mountainous region in Wakayama Prefecture  
81 (Latitude 33° 45' 36, Longitude 135° 23' 11") between 2007 and 2010. After  
82 centrifugation, sera were collected and then kept at -20°C until use. Liver samples were  
83 also collected from most wild boar, carried to laboratory at 4°C and then kept at -80°C  
84 until use.

85           Anti-HEV IgG was detected in sera by ELISA, using virus-like particles  
86 (VLP) as the coating antigen (Yamashita et al., 2009). VLP were diluted in 0.1 M  
87 carbonate buffer (pH 9.6) to 1 µg/ml and 100 µl per well was added to 96-well  
88 microplates (Nunc, Roskilde, Denmark). The wells were blocked with 0.1% bovine  
89 serum albumin in phosphate-buffered saline (PBS) for 1 hour at 37°C. After three  
90 washes with PBS containing 0.05% tween-20 (PBS-T), wild boar serum at a dilution of  
91 1:100 in PBS-T containing 10% fetal calf serum (FCS) was added for 1 hour at 37°C.  
92 After the plate was washed with PBS-T three times, horseradish peroxidase-conjugated  
93 rabbit anti-swine IgG (MP Biomedicals, Carlsbad, CA, USA), diluted 1:1000 in PBS-T  
94 containing 10% FCS was added for 1 hour at 37°C. After a further three washes with  
95 PBS-T, horseradish peroxidase substrate (BioRad, Hercules, CA, USA) was added to

96 each well for 30 minutes at room temperature. After stopping the reaction with 2%  
97 oxalic acid, the absorbance at 415 nm was measured using a spectrophotometer  
98 (Bio-Rad).

99 RT-PCR was used to detect HEV RNA in sera. Viral RNA was extracted from  
100 140 µl of each serum sample using the QIAamp Viral RNA Mini kit (Qiagen, Hilden,  
101 Germany). To amplify the partial gene of ORF2, RT-PCR was carried out with primers  
102 HEV-F1 (5'-TAY CGH AAY CAA GGH TGG CG-3') and HEV-R2 (5'-TGY TGG TTR  
103 TCR TAR TCC TG-3') using a One Step RT-PCR kit (Qiagen). Then, nested PCR was  
104 performed with the internal primers HEV-F2 (5'-GGB GTB GCN GAG GAG-3') and  
105 HEV-R1 (5'-CGA CGA AAT YAA TTC TGT CG-3') using a KOD-Plus-Ver.2 (Toyobo,  
106 Osaka, Japan) (Yamamoto et al., 2008). The amplified PCR products were extracted  
107 from the gel using a QIAEX II Gel Extraction Kit (Qiagen). The nucleotide sequence  
108 was determined using BigDye Terminator v.3.1 technology (Applied Biosystems, Foster  
109 City, CA, USA) with primers HEV-F2 and HEV-R1. The five nucleotide sequences  
110 were deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers  
111 AB746334, AB746335, AB746336, AB746337 and AB746338. Furthermore, the  
112 junction between ORF1 and ORF2 was amplified by RT-PCR from liver samples of  
113 three HEV-RNA-positive wild boar (Shimonoseki-WB72, -WB97, and WB99) using a

114 different set of primers; HE-008 (5'-GGG GTT GGT TGG ATG AAT ATA GGG GA-3')  
115 and HE-015 (5'-TGG AAG AAR CAY TCY GGT GAG CC-3') (Takahashi et al., 2002).  
116 Japanese HEV isolates have previously been detected successfully using this method.

117           One hundred and thirteen serum samples from wild boar in Shimonoseki City  
118 and 71 from boars in Wakayama Prefecture were assayed for anti-HEV IgG by ELISA.  
119 The results showed that all wild boar samples in Wakayama Prefecture were negative  
120 for HEV (Fig. 1). Mean and standard deviation (SD) of the OD values obtained from the  
121 sera of wild boar from Wakayama Prefecture were 0.099 and 0.075, respectively (Fig. 1).  
122 The cut-off value was determined as 0.324 (mean + 3 × SD). When the ELISA results  
123 were analyzed using a cut-off value of 0.324, none of 71 (0%) and 47 of 113 wild boars  
124 (42%) from Wakayama Prefecture and Shimonoseki City, respectively, were positive for  
125 HEV. There was no significant difference in HEV seroprevalence by sex or body weight  
126 ( $p>0.05$ ) (Table 1).

127           HEV-RNA was detected in sera from five of 112 wild boar (4%) captured in  
128 Shimonoseki City (Shimonoseki-WB52, -WB72, -WB74, -WB97 and -WB99). All five  
129 HEV-RNA-positive samples were from boars captured in Kanda or Imade, in which the  
130 seroprevalence of HEV (68% and 56%, respectively) was higher than that seen in boars  
131 captured at the other sites. Sequence analysis of the amplified products (338 bp)