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BRIEF REPORT

Pseudorabies in farmed foxes fed pig offal in Shandong province, China

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Abstract Pseudorabies (PR, Aujeszky's disease) is an acute, highly contagious viral disease resulting in major economic losses to the swine industry. PR is endemic in wild and domestic animals, although its natural host is the pig. Here, we report an outbreak of PR in foxes on a fur-producing farm in Yuncheng county, Shandong, China, that were fed pig offal. The diagnosis of PR was based on nervous signs and standard PCR methods and by isolation of PRV from fox brain tissue in Vero cells. The diagnosis was confirmed by an indirect immunofluorescence assay and electron microscopy. Phylogenetic analysis of a partial (804 nt) viral glycoprotein gC gene sequence indicated that it was likely to be a field strain closely related to a cluster of PRV previously identified in China.

Aujeszky's disease (AD) is prevalent in pig populations in most parts of the world, leading to heavy economic losses in the swine industry [1]. The disease, also named pseudorabies (PR), is caused by porcine pseudorabies virus (PRV) or suid herpesvirus 1 (SuHV-1), a neuroinvasive virus belonging to the genus *Varicellovirus,* subfamily *Alphaherpesvirinae,* family *Herpesviridae* [2]. A broad range of symptoms in wild and domestic animals can be caused by PRV, but pigs are regarded as the only natural host. Clinical manifestations are dependent on age at exposure, immune status, route of infection, and strain virulence. Young pigs usually develop neurological disorders and finally may succumb to severe encephalomyelitis. In contrast, respiratory symptoms involving sneezing and pneumonia commonly occur in older pigs and in pregnant gilts or sows. PRV infection frequently results in abortion and birth of mummified fetuses or stillborn neonates [3, 4]. Pigs that survive productive infection may become latently infected and become a source of virus spread.

PRV has been successfully eradicated in domestic pigs in many countries because of large-scale vaccination programs [4]. However, it has been reported that the virus still circulates in domestic swine herds in China [5, 6, 7]. The presence of PRV in domestic pigs poses a potential for transmission of the virus to carnivores and can also give rise to infection of wild species. A growing body of evidence suggests that infection of non-porcine species with wild-type PRV is characterized by disturbances of the nervous system and severe localized pruritus, with death generally occurring within several hours after onset of clinical symptoms. Fatal PRV infection has also been noted in several carnivorous species such as dogs, cats, bears, wolves, foxes, coyotes, raccoons, and panthers after consuming virus-contaminated meat, offal or carcasses [8]. Clinical cases of infection of foxes with pseudorabies have been reported in China [9], but none of them have been intensively analyzed.

In August 2014, over 1,200 captive foxes of all ages and of both sexes on a fur-bearing-animal farm in Yuncheng county, Shandong, China,, died after consuming mainly domestic raw pork liver. The infection spread rapidly, with over 200 foxes out of a total population of 1500 animals dying daily. Clinical symptoms included initial fever, anorexia, vomiting and dyspnea, developing into intense pruritus, frequent snarling and repeated lying down and

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rising, with death occurring within a few hours to 3 days from the onset of clinical symptoms. The outbreak was associated with 80 % (1,200/1,500) morbidity, and 100 % of the animals that developed symptoms died. No necropsies were conducted, but brain tissue samples were collected from animals suspected of having succumbed to PRV infection. Conventional PCR targeting a highly conserved viral gD gene fragment was conducted to diagnose the infection [10].

Homogenates were prepared from fresh brain tissue samples taken from dead foxes suspected of having succumbed to PRV infection. These were centrifuged at 6000 × *g* for 10 min at 4 °C, and the supernatants were passed through sterile 0.45-μm Millipore filters. Aliquots of the filtrates were added to confluent monolayers of Vero cells grown in 6-well plates in Dulbecco's minimum essential medium (DMEM) with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Gibco). The inocula were allowed to adsorb for 1 h at room temperature. The monolayers were then washed once, fresh DMEM containing 2 % FBS and 1 % penicillin-streptomycin was added, and incubation was continued at 37 °C in a humidified atmosphere of 5 % CO_2 in air. At 24 h postinfection, cells were washed 3 times with PBS and fixed in 4 % formaldehyde for 10 min at 4 °C. After blocking in PBS containing 1 % (wt/vol) bovine serum albumin at 37 °C for 30 min, the cultures were incubated for 1 h with mouse monoclonal anti-gE antibody at a dilution of 1:100, and for another 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (MBL) at 1:5,000 dilution. Fluorescence was observed using a fluorescence microscope. Some cell cultures left unfixed were harvested when cytopathic effects (CPE) developed. After three freeze-thaw cycles, 1-ml aliquots of supernatants were centrifuged at 12,000 × *g* for 1 min at 4 °C. The resulting cell sediments were negatively stained with 2 % phosphotungstic acid and observed by electron microscopy (EM). Positive cultures (i.e., those containing virus-like particles) were stored frozen until further study.

Total viral DNA from first-passage fox isolates was prepared as a template for PCR amplification using a viral DNA extraction kit (Axygen) according to manufacturer's protocol and stored at −70 °C. An 804-bp fragment of the pseudorabies gC gene was amplified using LA Taq DNA polymerase with GC buffer (TaKaRa) and previously described primers [8].

The PCR products were purified from agarose gels using DNA gel extraction kits (Axygen), automated DNA sequencing and with forward and reverse primers was carried out by Comate Bioscience Co., Ltd., China for. BLAST analysis of sequence data was performed to search for significant similarity of sequences in the GenBank database. Sequence assembly and multiple alignments were conducted with the MegAlign program of DNAStar software using ClustalW. A phylogenetic tree was constructed with MEGA version 5.2 software by the neighbor-joining method with 500 bootstrap replicates, using the maximum composite likelihood model.

The following are the GenBank accession numbers and origins of the sequences used for construction of the phylogenetic tree: Ea (AF158090, China), Fa (AF403051, China), JY (KF997104, China), YY (KF997101, China), MZ1 (KF997097, China), MZ2 (KF997099, China), BJ/YT (KC981239, China), WY (KF997094, China), LXB6 (GQ926931,China), SDWF1-China-2012 (KJ526446, China), YamagataS-81 (D49435, Japan), Becker (JF797219, USA), Kaplan (JQ809328, Germany), Bartha (JF797217, Hungary), PRV2908 (AF176480, USA), P-PrV-Iudr5 (KF060292, Malaysia), A/swine/Italy/3779-1/1997 (JQ768114, Italy), CL/15 (JF460030, Argentina).

Shandong province, in eastern China, is the country's main fox-breeding region. In this study, an outbreak of PR on a fox farm was diagnosed, and molecular characterization of the isolated PRV was conducted based on partial analysis of the gC gene.

Many pathogens and toxic reagents can produce clinical symptoms that are similar to those of PR; hence, a diagnosis of PR cannot be made based on clinical symptoms alone. Virus isolation and identification, considered the gold standard for diagnosis [11], was therefore undertaken. RNA and DNA extracted from brain tissue homogenates were assayed for rabies virus (RABV) and PRV by RT-PCR and PCR, respectively, revealing the presence of PRV, but not RABV. Incubation of brain tissue homogenates with Vero cells resulted in typical herpesviral focal cytopathic effects, with cell aggregation, refraction and large syncytia, within 2 days. Indirect immunofluorescence assay using mouse monoclonal anti-PR gE as primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody as secondary antibody confirmed

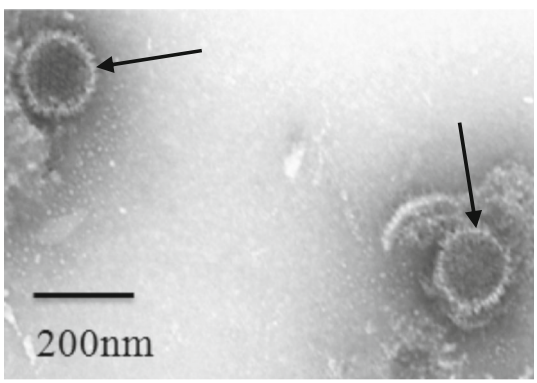

Fig. 1 Electron micrograph of negatively stained cell debris from freeze-thawed infected Vero cells, showing PRV particles

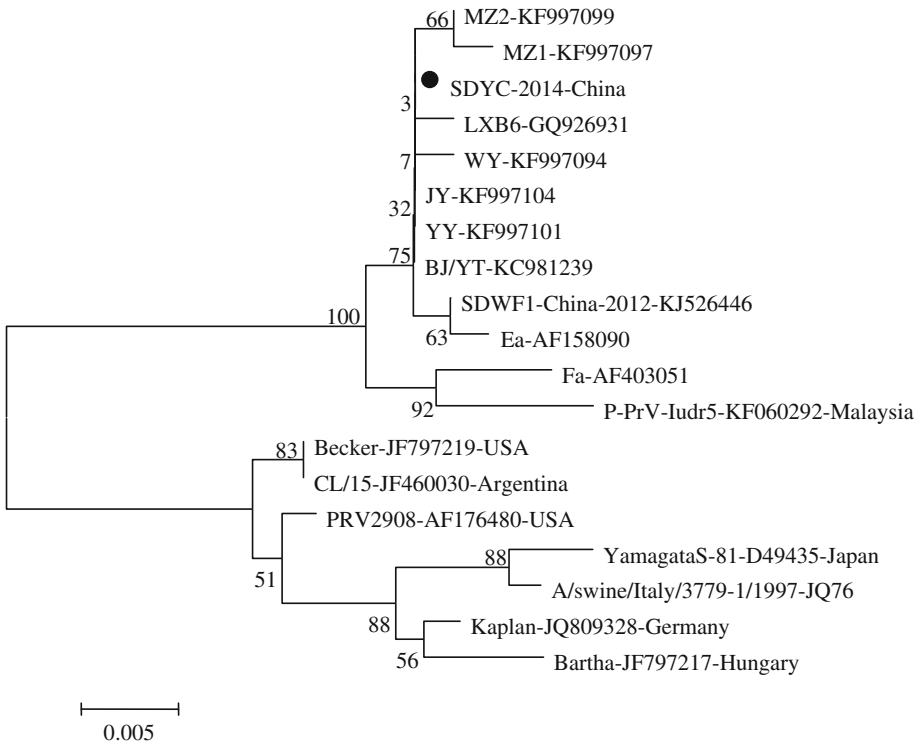

Fig. 2 Phylogenetic analysis based on the deduced amino acid sequence of the partial gC gene. The phylogenetic tree was constructed by the neighbor-joining method in MEGA 5.2, and bootstrap analysis was performed with 500 trials. The black dot indicates SDYC-2014-China, which was identified in this study

the presence of PRV. In addition, PRV-like particles were observed in the freeze-thawed cell debris by EM. The particles were circular in shape and approximately 150 nm in diameter (Fig. 1). The virus isolate was designated SDYC-2014-China.

The gC gene of PRV has been widely used for molecular characterization of PRV because it shows considerable genetic variation among isolates. A partial sequence of the gC gene of SDYC-2014-China, 804 bp in length and encoding 268 amino acids, was recovered and identified by BLAST analysis. Phylogenetic comparison with sequences obtained from GenBank showed that SDYC-2014-China clustered closely with PRV strains previously identified in Chinese swine herds while demonstrating notable genetic divergence from PRV strains from other countries (Fig. 2).

PR live attenuated vaccines generally contain partial TK or gE gene deletions, which permits discrimination between vaccine and wild-type viruses. PRV live attenuated vaccine strains have not been reported to induce PR in foxes, and the pathogenicity of PRV in foxes remains unknown. To rule out the possibility that the SDYC-2014-China strain originated from a live attenuated vaccine, we amplified the complete sequences of the TK and gE genes of the SDYC-2014-China strain and found that both were complete (data not shown), indicating that the foxes were infected with a wild-type PRV strain.

In this study, PR outbreaks occurred on a farm where captive foxes were fed mainly raw pork livers. Analysis of this material by PCR and sequencing revealed sequences that were 100 % identical to those from fox brain tissues. It therefore appears that SDYC-2014-China originated in domestic swine, and therefore, there is a potential risk of infection of other carnivorous species that have pork products in their diet. PRV of domestic swine has been shown to play a pivotal role in transmission of this disease to other susceptible mammals [11]. The present findings therefore underscore the need for enhanced surveillance of swine herds and for appropriate means of disposal of dead or infected pigs to prevent spillover of PRV to carnivorous animals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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