SHORT COMMUNICATION

The evidence of occurrence of porcine circovirus 2 isolation and characterization in Kazakhstan

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Abstract This report describes the first isolation and characterization of porcine circovirus 2 (PCV2) in the Republic of Kazakhstan. The virus was isolated from a dead piglet that did not exhibit any typical clinical symptoms of porcine circovirus disease at a pig factory in North Kazakhstan oblast (region). The isolated virus belongs to genotype 2 (PCV2) and shares 96.6% sequence homology with one isolate and two strains from China and two French strains in group 1, cluster *Iab* and 96.4% homology with two strains isolated in China and one strain from Hungary. Electron microscopy revealed the isolated virus had the typical morphological structure of PCV. This is the evidence of occurrence of porcine circovirus 2 isolation and characterization in Kazakhstan.

Keywords PCV2 · Virus isolation · MARC-145 cells · Electron microscopy · Sequencing · Phylogenetic analysis

Pig farming is affected by a number of infectious diseases that cause significant economic losses and low efficiency as a result of reduced productivity, culling, low litter sizes and requirements for counter-epizootic measures aimed at infection elimination. Porcine circovirus disease (PCVD) is one of the most significant diseases that affect industrial pig production worldwide [1].

PCVD mainly affects piglets. Pathogenic porcine circovirus type 2 (PCV2) was first isolated from sick piglets

in Canada in 1989; non-pathogenic porcine circovirus type 1 (PCV1) was detected in 1974 as a non-cytopathogenic contaminant of immortalized PK-15 piglet kidney cell cultures, and induces no clinical signs or symptoms in infected animals. PCVD associated with PCV2 is characterized by clinical signs including weight loss, growth delay, dyspnea, diarrhea, icterus and pneumonia. In pig factories, post-weaning multi-systemic wasting syndrome is most often reported in 6–15-week-old piglets, with the highest morbidity and mortality rates observed in 10-week-old piglets. PCV1 and PCV2 belong to the *Circoviridae* family and *Circovirus* genus. The members of *Circoviridae* family are small (17 nm), non-enveloped, icosahedron-shaped viruses with a single-stranded covalently closed circular negative stranded deoxyribonucleic acid (DNA) molecule. The PCV2 DNA genome is about 1767–1768 nucleotides long. The genomes of various PCV1 and PCV2 strains have been cloned and sequenced [1]. PCV genomic DNA contains two major open reading frames (ORFs). The open reading frame 1 (ORF1) nucleotide sequences of PCV1 and PCV2 share 83% homology and the amino acid sequences share 86% homology, indicating this region is highly conserved. The PCV1 and PCV2 open reading frame 2 (ORF2) nucleotide and amino acid sequences are more variable, with respective homologies of 67 and 65% [1].

Based on genetic differences, some authors have divided PCV2 into three genotypes: PCV2a, PCV2b and PCV2c, as well as PCV2ab, PCV2b and PCV2c, while other scientists have described more numerous genotypes [2]. The EU consortium on PCVD proposed three PCV2 genotypes: PCV2a, PCV2b and PCV2c [3]. The US swine population has a history of infection with PCV2a. The PCV2b genotype was first identified in North America in 2005, and the

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number of registered cases of circovirus infection among pigs subsequently increased [4].

PCV can be detected by immunohistochemistry (IHC), hybridization, electron microscopy, indirect immunofluorescence, polymerase chain reaction, polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis (PCR-RFLP), or isolated from cell cultures. Replication of PCV has been observed in primary pig kidney, pig lung, boar testicle, bull testicle, bovine kidney, lamb kidney and lamb testicle cell cultures, as well as the immortalized cell lines PK-15, Vero, HEP and HELA [5, 6].

Well-characterized PCV2 genotypes currently circulate in two countries adjacent to Kazakhstan: PCV2a, PCV2b and PCV2d in the Russian Federation, and PCV2a, PCV2b, PCV2d and PCV2e in China [7]. However, circulation of PCV has not previously been reported in Kazakhstan. The present report describes the first isolation and characterization of PCV2 in the Republic of Kazakhstan.

Pooled samples of pathological materials (mesenteric and inguinal lymph nodes, lungs) were obtained from a month-old piglet that died due to disease at a pig factory in North Kazakhstan region, Republic of Kazakhstan. Pooled lymph node samples were prepared for virological studies as described by Sjurin [8]. Briefly, the frozen pooled pathological materials (1 g) were homogenized using a laboratory dispersator (T-25 BASIC; IKA Works Inc., Wilmington, USA) for 2 min in 20 mL Hanks' balanced salt solution (HBSS) containing penicillin (100 IU/mL) and streptomycin (100 µg/mL), subjected to three freeze-thaw cycles at -40°C /room temperature to destroy the cell walls and release virus, clarified at 2000 g for 30 min, repurified at 14,926 g using a benchtop centrifuge, and the supernatant was used as seed material to infect cell cultures.

Studies of the sensitivity of PK-15, MARC-145 (clone of MA-104 cells derived from monkey kidney) and SEK (swine embryonic kidney) cell lines to PCV2 (based on cytopathic effect of the virus) revealed MARC-145 and SEK cell cultures were most sensitive to the PCV-2; therefore, MARC-145 cells were used for all experiments.

To isolate virus, MARC-145 cells free of PCV2 were cultured in 50 mL flasks (Nest Scientific, Rahway, NJ, USA) in Eagle's minimal essential medium (EMEM) containing 5% fetal bovine serum (Sigma, St. Louis, MO, USA), 0.2 mM *L*-glutamine and 2% sodium bicarbonate for 48–72 h, the growth medium was decanted, then the monolayer was rinsed with sterile Hanks' balanced salt solution, seeded with 1.0 mL of suspension prepared from the pathological materials and incubated at 37°C for 1 h. Then, 6 mL EMEM complete media was added and cultures were observed daily for 3–7 days using an inverted light microscope (Olympus CK2, Tokyo, Japan).

Cytopathic changes within the cell monolayer were taken as evidence of viral presence. Culture flasks with distinct cytopathic changes were frozen at -40°C . Infected cell cultures that did not exhibit cytopathic changes were stored at -40°C , freeze-thawed, and the supernatant was used to infect fresh cell cultures as described above; if no cytopathic changes were evident in the second culture, the freeze-thaw/infection cycle was carried out once more.

Virus-containing cell culture media was clarified by centrifugation at 2100 g for 15 min at 4°C (Eppendorf 5810R centrifuge, Hamburg, Germany), then 5 mL of the clarified suspension was sedimented by ultracentrifugation using a Optima L-90 K ultracentrifuge (Beckman Coulter, Brea, CA, USA) using a SW-50.1 hanging rotor at 192,000 g for 1 h at 4°C . The pellet was resuspended in 200 µL single-phase phosphate-buffered saline pH 7.2 (Sigma), dripped into the wells of a Teflon plate, then a grid with formvar carbon-coated support film was applied onto each drop of the viral suspension. The grids were transferred onto drops of water containing 2% phosphotungstic acid (pH 6.8) for 1–2 min, then onto drops of phosphotungstic acid (pH 7.0) for 5 min. After contrasting and removing the excess contrast with filter paper, the film was air-dried and specimens were examined using a JEM-100 CX II transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating electron voltage of 80 kV and 20,000 $\times$ to 40,000 $\times$ magnification. Morphometry of virions was carried out on reversed images with the same magnification using a 0.1 mm graded magnifying glass to determine the arithmetic mean ($\pm$ standard deviation) size of the virions.

DNA was extracted from purified, concentrated virus-containing material using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All laboratory procedures were performed according to published guiding principles for ensuring the quality of PCR in diagnostic laboratories.

PCV2-specific primers were selected from published data [9], additionally tested for compliance using the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and synthesized using a Synthesizer H-16 (K & A Laborgeraete, Schaaflheim, Germany) using a DMT-*dA*, *dC*, *dG*, *dG* (*bz*)-CPG Column (Sigma). PCV2 genome segments were amplified by RT-PCR on a GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA) using AccuPrime *Taq* DNA Polymerase High Fidelity kit (Invitrogen) with denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C and 2 min, followed by 8 min elongation at 72°C .

The amplified PCR products were separated on agarose gels and extracted using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA) following the

manufacturer's instructions. Specimens were prepared for sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and sequencing was conducted on an automatic 16-capillary sequencer (3130xl Genetic Analyzer; Applied Biosystems/Hitachi, Foster, CA, USA). Nucleotide sequences were analyzed and assembled using Sequencher v 5.0 software (Gene Codes Corporation).

Comparative analysis of nucleotide sequences was carried out using the NCBI website (<http://www.ncbi.nlm.nih.gov/genbank/>). Phylogenetic sequence analysis was performed using Mega 6.06 (<http://www.megasoftware.net/>). Phylogenetic trees were generated using the neighbor-joining method and Kimura 2-parameter model [10] with inclusion of substitutions d: transitions + transversions, as well as inclusion of first + second + third + non-coding codons over 500 bootstrap replications.

PCV2 viral DNA was detected in organ/tissue samples and cell culture suspensions by real-time PCR using the PCV2 Real-Time Detection Kit (Bionote, Inc., Hwaseong, Korea) and real-time PCR for detection of PCV2 kit (Vetbiokhim Ltd., Moscow, Russian Federation) according to the manufacturers' guidelines.

All experiments were conducted in triplicate to ensure the reliability of the results. Data were analyzed using Prism version 5.01 (Graph Pad Software, Inc., San Diego, CA, USA); values are expressed as mean $\pm$ standard deviation. Statistical significance was examined using t-tests and defined as $P < 0.05$.

Real-time PCR analysis of pathological materials collected from the dead piglet in North Kazakhstan region, Republic of Kazakhstan, demonstrated the presence of PCV2 DNA (data not shown). The PCV2-positive specimens were used to infect MARC-145 cells to further isolation and characterization of the virus. On days 3–4 of culture, destructive changes were observed in the cell monolayers and 4 days after the second passage, the cells rounded up and detached from the culture flask leaving cavities within the monolayers.

The viral suspension from second passage cultures was further examined by electron microscopy (Fig. 1). Morphometry of the virions revealed small, non-enveloped isometric particles with a uniform structure and shape, typical of circoviruses. The hexagonal shape of the capsomer layer indicates icosahedral symmetry. Some virions had supercapside envelopes. The mean diameter of the virus particles was 20.13 ± 0.08 nm. Virions tend to form clusters in cellular vesicles, and typical aggregate clusters were observed by electron microscopy.

RT-PCR was performed using the specific primers PCV2_MCV1 and PCV2_MCV2 to detect PCV2. Only one (#17 in the series of samples taken during the survey of

different farms in North Kazakhstan) of the four tested samples (from a single dead animal) was positive.

The complete genome of the PCV2 isolate 17 was sequenced using the primers PCV-1NF/PCV-1NR and PCV-2NF/PCV-2NR for phylogenetic analysis and genotyping. The phylogenetic tree constructed using MEGA 6.06 and genotyping analysis are shown in Fig. 2. Comparison with other PCV2 sequences in the GenBank database revealed the isolated virus (No. 17; named KZ PCV2 isolate 17) belongs to group 1, cluster *lab* of PCV genotype 2. BLAST comparisons with closely related strains in PCV2 group 1 revealed the isolated virus (No. 17) has 99.6% sequence homology with the ZHZ1 (KX161698 PCV2) isolate (China, 2006); Chinese strains SXTY14 (KX352156 PCV2) and ZhuJi 2003 (AY579893 PCV2), and French strains Fh22 (AY322002 PCV2) and Fd1 (AY322000 PCV2) in cluster *lab*; 99.4% homology with the Chinese strains GX (AY556475 PCV2) and CHST (AY682992 PCV2) and Hungarian strain 304 (AY256457 PCV2); and 99.0% homology with the Chinese strain QY (AY 682995 PCV2). Moreover, PCV2 No. 17 isolate shares 93–95% homology with the strains in cluster *c*.

PCVD is one of the most significant diseases in industrial pig-breeding worldwide [1]. Monitoring and detection of PCV infection in pig populations in Kazakhstan, as well as identification of the dominant circulating genotypes, is of interest to both scientists and farm workers. However, even though PCV is not officially recorded in Kazakhstan as it has not been well-studied, PCVD is widespread in adjacent countries.

China and Russia share common borders with Kazakhstan (1782.75 and 7512.8 km long, respectively) are established trading partners, and have well-developed pig industries. Phylogenetic analysis by Wang et al. [7] indicated 40 Chinese PCV2 strains isolated between 2004 and 2008 could be classified into four genotypes: PCV2a (eight strains), PCV2b (30 strains), PCV2d (four strains) and PCV2e (six strains). On the contrary, Guo et al. [2] showed 19 Chinese PCV2 strains isolated between 2004 and 2008 could be divided into two groups and three clusters with genomes of 1766 bp (15.8%), 1767 bp (73.7%) and 1768 bp (10.5%), respectively. Moreover, 17 PCV2 strains belonged to group 1: 1A (52.6%), 1B (21.1%), 1C (15.8%), but only two strains were attributed to group 2 (10.5%). Moreover, all three known PCV2 genotypes, 2a, 2b and 2c, are circulating in the Russian Federation [4].

Electron microscopy revealed the isolated virus had the typical morphological structure of PCV described by Allan and Ellis [1]. PCV2 isolate No. 17 appears to be 96.6% identical to one isolate and two strains from China and two French strains that belong to group 1, cluster "*lab*" (Figs. 1, 2). PCV2 isolate No. 17 is also 96.4% identical to

Fig. 1 Electron microscopy of the isolated PCV2 virus (negative contrast with 2% phosphotungstic acid). **a** 200,000 $\times$ magnification, **b** 150,000 $\times$ magnification

Fig. 2 Phylogenetic tree based on the complete nucleotide sequences of the genomes of the isolated virus (KZ PCV2 isolate 17) and other strains of PCV2 constructed using the MEGA 6.06 program via the neighbor-joining method (500 bootstrap replicates)

two Chinese strains and one strain from Hungary. Moreover, our phylogenetic analysis agrees with the genotyping by Opriessnig [11], who divided PCV2 into three genotypes: PCV2_{ab}, PCV2_b and PCV2_c.

This study provides the first confirmation that PCV2 is also circulating in Kazakhstan, especially in the region of the pig-breeding farm in North Kazakhstan where the samples for this study were obtained. Further experiments need to be performed to determine the detrimental effects of PCV2 for pig breeding herds and to elucidate its role in the pathogenesis of PCVD. An epidemiological survey of PCV2 in Kazakh herds is scheduled, in order to determine the prevalence of PCV2 and hence the spread of the virus among Kazakh pigs.

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