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BRIEF REPORT

Evidence of coinfection of pigs with African swine fever virus and porcine circovirus 2

William G. Dundon[1,6] · Giovanni Franzo[2] · Tirumala B. K. Settypalli[1] · N.L.P. Indi Dharmayanti[3] · Ulaankhuu Ankhanbaatar[4] · Indrawati Sendow[3] · Atik Ratnawati[3] · Tserenchimed Sainnokhoi[4] · Umberto Molini[5] · Giovanni Cattoli[1] · Charles E. Lamien[1]

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Abstract

Archival swine DNA samples from Indonesia and Mongolia, some of which were previously shown to be positive for African swine fever virus, were screened for the presence of porcine circovirus 2 (PCV-2) and porcine circovirus 3 (PCV-3) by PCR. Samples from both countries were positive for PCV-2 (three from Mongolia and two from Indonesia), while none were positive for PCV-3. The PCV-2 amplicons were sequenced, and phylogenetic analysis revealed that the PCV-2 strains belonged to four different genotypes: PCV-2a (Mongolia), PCV-2b (Mongolia and Indonesia), PCV-2d (Indonesia), and PCV-2g (Mongolia). This is the first report of ASFV/PCV-2 coinfection in pigs and the first report of the presence of PCV-2 in Mongolia.

Together with porcine parvoviruses and pseudorabies virus, porcine circoviruses (PCVs) and African swine fever virus (AFSV) are considered the four main DNA viruses that significantly affect swine health [1]. PCVs belong to the genus *Circovirus* of the family *Circoviridae*, and so far, four PCVs have been identified: PCV-1, PCV-2, PCV-3, and PCV-4, although the information available on PCV4 is still limited [2]. They all have a similar genome organization consisting of two open reading frames (ORFs) (ORF1 and ORF2) that are orientated in opposite directions on a small circular genome ranging in size from 1758 to 2001 nucleotides and encode a replication-associated protein and a capsid protein, respectively. PCV-1 is known to infect pigs but does not cause disease, while PCV-2 is the causative agent of porcine circovirus diseases (PCVDs), which are an assortment of several disease conditions including PCV-2 systemic disease (comprising postweaning multisystemic wasting syndrome and other respiratory and enteric manifestations), porcine dermatitis and nephropathy syndrome, and reproductive failure. PCV-3 has been detected in the presence of several clinical conditions, partially overlapping with PCVD. However, the clear causal nexus is still being debated. The pathogenicity of PCV-4 remains unclear [3–6].

The most recent classification methodology based on comparison of the ORF2 gene from a wide collection of PCV-2 sequences has identified nine genotypes: PCV-2a, PCV-2b, PCV-2c, PCV-2d, PCV-2e, PCV-2f, PCV-2g, PCV-2h, and PCV-2i [7, 8]. PCV-2a, PCV-2b, and PCV-2d are widespread and are similarly virulent in pigs, while the clinical significance of the remaining genotypes is unknown [3].

African swine fever (ASF) is a severe haemorrhagic disease of domestic and wild pigs characterised by high morbidity and mortality [9, 10]. The causative agent, ASFV, is a large double-stranded DNA virus of the family *Asfarviridae*. Currently, the virus is rapidly expanding through Asia, devastating the local swine industry, and it has caused the

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✉ William G. Dundon
w.dundon@iaea.org

[1] Animal Production and Health Laboratory, Animal Production and Health Section, Joint FAO/IAEA Centre, Department of Nuclear Sciences and Applications, International Atomic Energy Agency, PO Box 100, 1400 Vienna, Austria

[2] Department of Animal Medicine, Production and Health, University of Padova, Viale dell'Università 16, 35020 Legnaro, Italy

[3] Indonesian Research Center for Veterinary Science (IRCVS), Bogor, Indonesia

[4] State Veterinary Laboratory, Ulaanbatar, Mongolia

[5] Faculty of Agriculture and Natural Resources, School of Veterinary Medicine, University of Namibia, Neudamm Campus, Private Bag 13301, Windhoek, Namibia

[6] Animal Production and Health Laboratory, IAEA, Freidenstrasse, 1, 2444 Seibersdorf, Austria

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death of millions of pigs in China, Mongolia, Vietnam, Indonesia, Cambodia, Laos PDR, Myanmar, DPR Korea, South Korea, the Philippines, Timor Leste, Papua New Guinea, and Malaysia [10, 11].

Both PCV-2 and PCV-3 target the lymphoid tissues of the host, and circovirus infections are typically associated with lymphoid depletion and immunosuppression, so infected pigs are often more susceptible to other viruses and bacteria [12]. Coinfection with PCV-2 or PCV-3 and porcine reproductive and respiratory syndrome virus (PPRSV), classical swine fever virus (CSFV), *Mycoplasma hyopneumoniae*, and *Haemophilus parasuis* has been reported [12–16]. However, no information on coinfection with PCV-2 or PCV-3 and ASFV is currently available, so this study was undertaken to determine whether such coinfections could be detected.

Archived DNA samples (stored at -20°C) from two previous studies on ASFV in Indonesia (n = 32) [17] and Mongolia (n = 18) [18] were screened in this study. The quality of the DNA samples was confirmed by PCR using primers for the swine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene according to Duvigneau et al. [19]. To screen for PCV-2 by PCR, two primers, P5 (5'-AGAAGC TCTTTATCGGAGGA-3') and P8 (5'- GTTCGTCCTTCC TCATTACC-3'), which amplify a 687-bp segment covering the ORF2 of PCV-2, were used. The primers PCV3-1-F (5'-TTACTTAGAGAACGGACTTGTAACG-3') and PCV3-1-R (5'-AAATGAGACACAGAGCTATATTCAG-3'), covering a 650-bp region of the ORF2 of PCV-3 were used to screen for PCV-3 [20].

The PCR reaction conditions for both PCV-2 and PCV-3 were identical, and the reaction mixtures consisted of 5 µL of extracted DNA in a total volume of 20 µL containing a final concentration of 1.25 mM $MgCl_2$, 1X PCR buffer, 0.2 mM dNTPs, 10 pmol of each primer, and 2.5 U of Taq DNA polymerase. All reactions were performed with the following thermal profile: initial denaturation at 94°C for 5 min and then 35 cycles of denaturation at 95 °C for 30 s, annealing at 51 °C for 30 s for PCV-2 or at 55 °C for 30 s for PCV-3, and elongation at 72 °C for 60 s, followed by a final elongation at 72 °C for 5 min.

The PCR amplicons were purified using a Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced commercially by LGC Genomics (Berlin, Germany). The sequences of the positive samples (three from Mongolia and two from Indonesia) were submitted to the GenBank database under accession numbers MZ422380 to MZ422384 (Table 1).

The sequences were edited and assembled using the Staden software package version 2.0.0b8. Multiple sequence alignments were performed to compare and classify the PCV-2 sequences of the isolates used in the study. More specifically, the full ORF2 reference dataset described by Franzo and Segalés [7] was downloaded and aligned to the ORF2 of the sequences generated in this study at the codon level using the MAFFT method implemented in TranslatorX [21]. A neighbor-joining phylogenetic tree based on raw genetic distances (pairwise p-distances) was constructed using MEGA7. The reliability of the sequence cluster was evaluated by performing 1000 bootstrap replicates. The clustering of new sequences was assessed by both visually inspecting the phylogenetic tree and using ClusterPick to identify sequence groups with a within-cluster genetic distance lower than 13% and bootstrap support of the relative ancestral node higher than 70% [6, 22]. Additionally, a BLAST search was performed to identify closely related sequences not present in the reference dataset. Finally, a phylogenetic tree including closely related sequences and other strains representative of the Asian context was constructed using the same approach.

Of the 50 samples screened, none were positive for PCV-3. In contrast, two of the samples from Indonesia (TIA and T06) and three from Mongolia (MNG/2/BU/2019, MNG/5/BU/2019, and MNG/7/BU/2019) were positive for PCV-2 (Table 1). All of the samples had previously been shown to be positive for genotype II ASFV, and this was confirmed by qPCR according to King et al. [17, 18, 23]. The two PCV-2-positive samples from Indonesia were collected in January 2020 from the spleen and lymph nodes of two pigs purchased from a seller in the Bogor District of West Java [17], while the three PCV-2-positive samples from Mongolia originated from deceased pigs collected from farms in Bulgan Province, Northern Mongolia, in January 2019 [18]. All of the deceased pigs showed signs of ASF, including high fever (40–42 °C) and bloody diarrhoea, prior to death.

Table 1 Description of samples investigated in this study

Sample	Country	Date	Organ	Location	PCV-2 genotype	GenBank no.
MNG/2/BU/2019	Mongolia	11/1/2019	Kidney	Bulgan Province	a	MZ422380
MNG/5/BU/2019	Mongolia	11/1/2019	Kidney	Bulgan Province	g	MZ422381
MNG/7/BU/2019	Mongolia	11/1/2019	Submandibular lymph node	Bulgan Province	b	MZ422383
T1B	Indonesia	1/2020	Spleen	Bogor District	d	MZ422382
T06	Indonesia	1/2020	Lymph node	Bogor District	b	MZ422384

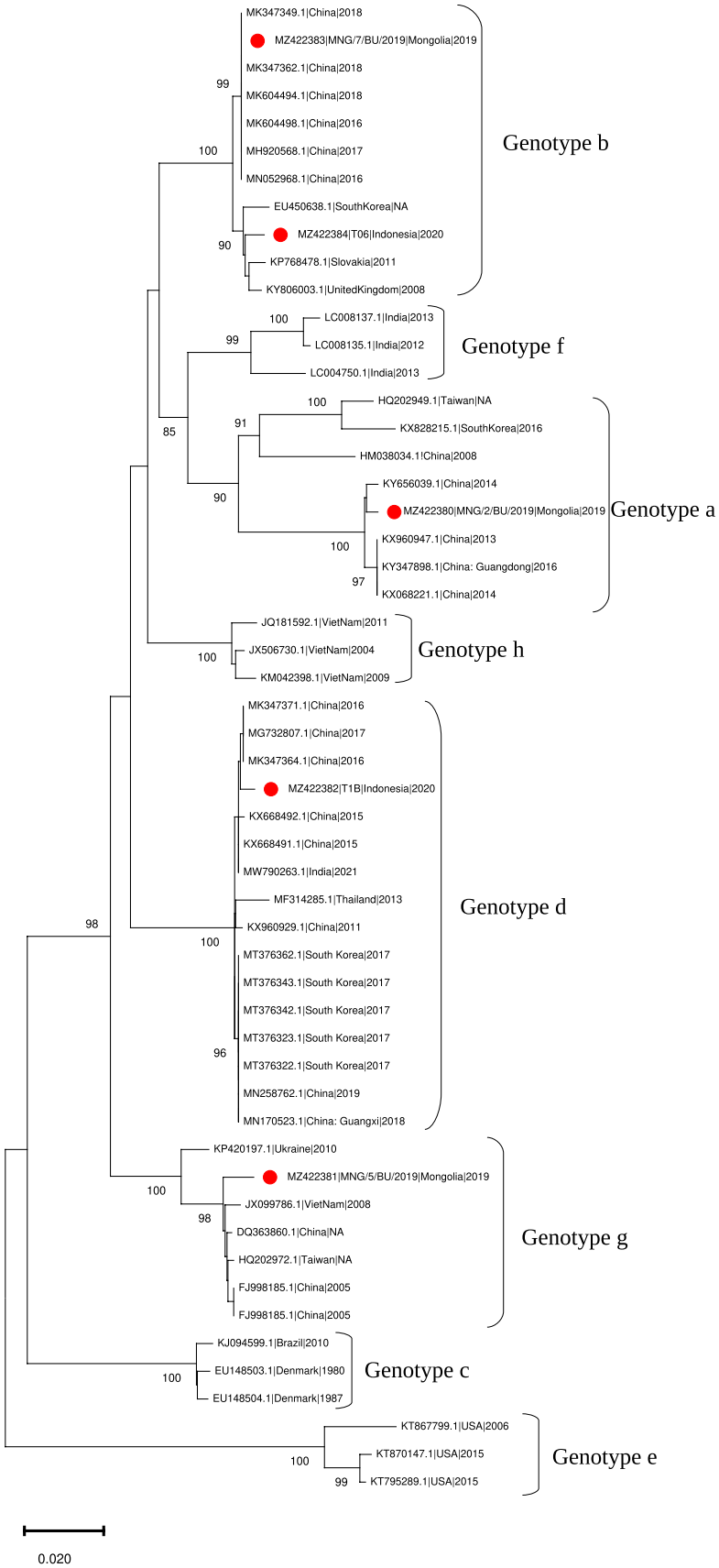

Fig. 1 Neighbour-joining phylogenetic tree constructed based on a subset of representative ORF2 sequences. Strains for which sequences were obtained in the present study are indicated by a red circle. A more comprehensive tree, including several genetically and geographically related sequences, is provided as Supplementary Fig. S1.

Phylogenetic analysis revealed that the samples contained PCV-2 DNA belonging to four separate genotypes, namely, PCV-2a, PCV-2b, PCV-2d and PCV-2g (Fig. 1 and Supplementary Fig. S1). More specifically, the three viruses identified in Mongolia belonged to different genotypes (PCV-2a, PCV-2d, and PCV-2g), while the two viruses from Indonesia belonged to genotypes PCV-2b and PCV-2d.

The population dynamics of PCV-2 are constantly changing [24]. Until 2000, PCV-2a was the dominant genotype in the domestic pig population globally, but a shift occurred in 2003/2004, resulting in the predominance of PCV-2b. In 2010, another shift was observed, resulting in a global predominance of PCV-2d. Studies in East Asia and Southeast Asia have identified PCV-2 genotypes in pigs in Indonesia (PCV-2f and PCV-2h) [25], Malaysia (PCV-2b) [26], Thailand (PCV-2a, PCV-2b, PCV-2d, and PCV-2h) [27], Vietnam (PCV-d, PCV-d, and PCV-2h) [16, 28], Korea (PCV-2a, PCV-2b, and PCV-2d) [13, 29], and China (PCV-2a, PCV-2b, PCV-2d, PCV-2f, and PCV-2g) [15, 30–33].

Although PCV-3 was not identified in this study, the virus has been identified in several countries of the region, including China, Malaysia, Thailand, South Korea, and Vietnam [20, 34–38]. Indeed, PCV-3 prevalence of 34.7% and 44.2% has been reported in China and South Korea, respectively [20, 34]. Therefore, larger sample sizes and more-robust epidemiological investigations may be required to determine whether PCV-3 is actually present in swine populations in Mongolia or Indonesia.

This is the first report of PCV-2 in Mongolia, and we confirm the overall epidemiological situation of PCV-2 in Asia, which is characterised by remarkable genetic heterogenicity. Genotypes already known to be part of the circulating viral population in the region were detected (Fig. 1). Indeed, it was not unexpected to find genotypes PCV-2a, PCV-2b, or PCV-2d in either Mongolia or Indonesia. However, the identification of PCV-2g in Mongolia is noteworthy, as this genotype has only been described in a few countries to date, having been reported in pigs in northern Vietnam [28], China [30], and Taiwan (GenBank no. HQ202972) and in wild boar in Ukraine (GenBank no. KP420197). From the phylogenetic tree in Fig. 1 it can be seen that the Mongolian PCV-2g virus sequence is more closely related (99% nucleotide sequence identity) to those of isolates from China, Vietnam, and Taiwan (Supplementary Fig. S1), indicating an epidemiological link between these Asian countries. Local transboundary movement and trade of infected animals is thus the most plausible explanation for this observation. Similar animal movements could explain the presence of PCV-2b and PCV-2d in Indonesia.

Because of the acute nature of ASF and the high mortality associated with infection, this was the most likely cause of death of the sampled pigs. Therefore, the infection with PCV-2 most probably occurred prior to ASFV infection. Whether PCV-2 infection and the associated immunosuppression predisposed the pigs to the ASFV secondary infection is unclear due to the limited number of positive samples and a lack of supporting epidemiological evidence. Nevertheless, this study should encourage further investigations with larger sample sizes to determine whether there is any correlation between PCV-2 occurrence and ASFV infection, epidemiology, and pathogenesis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05312-7.

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Data availability statement The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nih.gov/genbank/, reference numbers MZ422380 to MZ422384.

Declarations

Conflict of interest W.G. Dundon is on the Editorial Board of Archives of Virology.

Ethical approval No animal work was performed for this study.

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