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2 Note

3 Title: First detection of porcine circovirus type 3 in Japan.

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14 FIRST DETECTION OF PCV3 IN JAPAN

15

16 Abstract

17 Porcine circovirus associated diseases (PCVAD) have multiple manifestations that have been
18 attributed to porcine circovirus type 2 (PCV2). Recently, a novel porcine circovirus, PCV type 3
19 (PCV3), was identified in pigs with systemic inflammation of unknown etiology. In this study, we
20 tried to detect the PCV3 genome in tissue samples collected from Japanese pig herds in 2016. The
21 PCV3 genome was detected by PCR in 7 of 73 samples. The homology between each Japanese
22 strain was 99.5% for the full-length sequence and 98.9% to 99.2% for the open reading frame 2.
23 These results suggest that PCV3 has already invaded Japanese pig farms.

24 Keywords: Porcine circovirus type 3 (PCV3), prevalence, swine

25

Circoviruses belong to the genus *Circovirus* of the family *Circoviridae* [6]. Three species of circovirus are known to infect pigs: porcine circovirus type 1 (PCV1), type 2 (PCV2), and type 3 (PCV3). While PCV1 has no pathogenicity in pigs [16], PCV2 causes pathological conditions in pigs that are referred to as porcine circovirus-associated disease (PCVAD), inducing variety of symptoms, which include post-weaning multisystemic wasting syndrome (PMWS), respiratory symptoms, breeding disorders, and porcine dermatitis and nephropathy syndrome (PDNS) [12]. However, PCV2 by itself is limited in its ability to induce the full spectrum of clinical signs and lesions associated with advanced cases of PCVAD [12]. In 2016, a novel circovirus, PCV3, was reported in the US, suggesting that PCV3 might be involved in PDNS and PMWS, which have been regarded as forms of PCVAD [13]. Phan *et al.* (2016) conducted a metagenomic analysis of the genomes of pigs with systemic inflammation and cardiac pathology of undetermined etiology and detected PCV3 [14].

After the report of PCV3 in the US, it has been reported in many countries of the world [2-5, 7, 8, 10, 15, 17, 18]. Collins *et al.* (2017) recently conducted a retrospective study of PCV3 in the UK and showed that PCV3 appeared there at least 10 years earlier than in other countries [2]. However, prior to the current study, it was unclear whether PCV3 is present in Japan. This study aimed to detect the PCV3 genome by PCR in tissue samples collected from Japanese pig herds in 2016 for the diagnosis of pig disease. We report the first identification of PCV3 in Japan.

Tissue samples, collected from 73 pigs at 27 farms in 2016, were used to detect the PCV3 genome. These samples were submitted to the Nippon Institute for Biological Science for routine diagnosis in 2016. The tissue samples originated from 9 prefectures in Japan, and the pigs ranged from fetuses to adults. The clinical history of the pigs included subclinical (n = 2), sudden death (n = 20), abortion (n = 15), diarrhea (n = 11), respiratory signs or lung abscesses (n = 9), depression (n = 6), and neurological symptoms (n = 4). No clinical history was provided for the remaining 6 cases.

Each sample was collected according to the clinical symptom. For example, five major organs (heart, lung, liver, kidney, and spleen) and lymph nodes were collected from pigs with depression or sudden death; placenta or fetal organs, from pigs with abortion; and intestine or mesenteric lymph node, from pigs with diarrhea. In total, 217 samples were collected: fetal organs (n = 55), lung (n = 47), liver (n = 32), pulmonary lymph nodes (n = 25), spleen (n = 23), intestine (n = 17), kidney (n = 14), brain (n = 12), tonsil (n = 11), mesenteric lymph nodes (n = 4), mandibular lymph nodes (n = 3), inguinal lymph nodes (n = 2), and placenta (n = 2). Samples were stored in a -80 °C freezer until used in the experiment.

The specimens were used to produce a 10% (w/v) homogenate in Eagle's minimum essential medium (Thermo Fisher Scientific, Waltham, MA, USA). The homogenate was centrifuged at 1,500 ×g for 15 min at 4 °C. The supernatant was filtered through a filter with a pore size of 0.45 μm. For DNA extraction, proteinase K (Thermo Fisher Scientific) was added to the tissue homogenate to achieve a final concentration of 200 μg/ml, and the solution was incubated at 55 °C for 2 hr. For RNA extraction, the TRIzolTM Reagent (Thermo Fisher Scientific) was used according to the manufacturer's instructions.

After centrifugation, the supernatant, obtained as a DNA solution, was subjected to PCR for amplification of the PCV3 open reading frame (ORF) 2. PCR was performed using GoTaq® Green Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. Forward and reverse primers were designed according to a previous report [13].

Concerning clinical signs, the general viral pathogens for pigs, which include PCV2, swine herpesvirus 1 (SuHV-1), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), Japanese encephalitis virus (JEV), porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus A (PoRV-A), and Getah virus (GETV), were subjected to RT-PCR or simple PCR analysis [11]. Isolation of general bacterial pathogens

74 was attempted on blood agar plates and chocolate agar plates. Colonies were picked, and the 16S
75 rRNA gene was amplified by PCR using Ex Taq® (TAKARA BIO INC., Kusatsu, Japan) according
76 to the manufacturer's instructions. Isolates were identified by 16S rRNA gene sequencing.

77 The full-length sequence of the genome was determined for the samples that were found to be
78 positive for the PCV3 ORF2 by PCR. Primer sets were designed according to a previous report, and
79 their respective annealing temperatures were determined [13] (Table 1). The full-length PCV3
80 genomic sequence was assembled from sequences of each of the amplified products. Each
81 nucleotide sequence was aligned with previously reported sequences of the members of the genus
82 *Circovirus* and PCV3 ORF2, and was input into the Clustal W multiple sequence alignment
83 program to produce the phylogenetic tree. Bootstrap values were calculated on 1000 replicates of
84 the alignment, using MEGA ver. 7.0 software [9].

85 The details of the PCV3-positive samples are shown in Table 2. The PCV3-positive rate at the
86 farm level was 22.2% (6/27). The positive rate of PCV3 in all the samples was 9.6% (7/73). The
87 detected PCV3 ORF2 genomes were designated as TG1923-2/2016, KGS1945-2/2016,
88 KGS1945-3/2016, MZ1950-1/2016, KGS2065-4/2016, KGS2098-1/2016, and GM2124-2/2016
89 strains. The positive rates of PCV3 by prefecture were 0/5 (0%) in Toyama, 0/5 (0%) in Chiba, 1/6
90 (16.7%) in Gunma, 1/3 (33.3%) in Tochigi, 0/2 (0%) in Ehime, 0/5 (0%) in Kochi, 0/2 (0%) in
91 Kumamoto, 1/17 (5.9%) in Miyazaki, and 4/28 (14.2%) in Kagoshima. The detection rate of PCV3
92 by age group was 6.7% (1/15) for fetuses, 13.3% (2/15) for suckling piglets (3–4 weeks of age),
93 9.0% (1/11) for weaned pigs (5–8 weeks of age), and 9.4% (3/32) for fattening pigs (9 weeks of age
94 and older). The positive rates of PCV3 by organ were 1/55 (1.8%) in fetal organs, 3/47 (6.4%) in
95 lung, 1/32 (3.1%) in liver, 2/25 (8.0%) in pulmonary lymph nodes, 1/23 (4.3%) in spleen, 2/17
96 (11.8%) in intestine, 4/14 (28.6%) in kidney, 1/12 (8.3%) in brain, 0/11 (0%) in tonsil, 1/4 (25.0%)
97 in mesenteric lymph nodes, 1/3 (33.3%) in mandibular lymph nodes, 0/2 (0%) in inguinal lymph

98 nodes, and 0/2 (0%) in placenta. Although the PCV3 genome was detected in various organs of the
99 whole body, we could not confirm a specific target organ for PCV3 infection. Similar to our results,
100 Ku *et al.* (2017) reported that the PCV3 genome was detected in various organs of the whole body
101 [8]. The PCV3 genome was also detected in fetal organs, suggesting that PCV3 may be transmitted
102 vertically from dams to fetuses. Previous epidemiological studies in the US and China reported that
103 the PCV3 genome was also detected in fetal organs, and the authors refer to the possibility that
104 PCV3 is transmitted vertically, and is involved in abortion [8, 13]. Pigs with the PCV3 genome had
105 shown some clinical symptoms, such as sudden death (n = 4), abortion (n = 1), depression (n = 1),
106 and neurological symptoms (n = 1). Co-infection with other pathogens, such as PPV, PRRSV,
107 *Streptococcus suis*, *Escherichia coli*, and *Staphylococcus haemolyticus*, was detected in the
108 PCV3-positive samples.

109 Previous epidemiological studies in the US, China, and Poland showed that the positive rates
110 of PCV3 in individual samples were 12.5% (34/271), 34.7% (77/222), and from 0 to 65.0% (the
111 median was 22.2%), respectively [8, 13, 15]. Kwon *et al.* (2017) reported that the positive rates of
112 PCV3 in pen-based oral fluid samples and at the farm level were 44.2% (159/360) and 72.6%
113 (53/73), respectively, in South Korea [10]. The PCV3 genome-positive rate in this study was within
114 the range of detection rate reported in other countries.

115 In previous reports, the PCV3 genome was detected in samples from young pigs aged 14-21
116 days and 63-70 days, and in aborted fetuses [13, 14]. Stadejek *et al.* (2017) reported that PCV3 was
117 most common among weaned pigs, finishers, and sows (26.1%, 28.0%, and 29% of serum pools,
118 respectively); however, only 5% of serum pools from suckling piglets were PCV3 genome-positive
119 [15]. The authors suggested that the difference in the detection rate among the age groups is due to
120 the protective effect of maternal immunity. However, in this study, the PCV3 genome was detected
121 in all age groups.

122 PCV3 infection might be associated with PDNS, reproductive failure [8, 13], multisystemic
123 inflammation [14], and congenital tremors [1]. Additionally, in this study, PCV3 was detected in an
124 abortive fetus. PCV3 genomes were also identified from subclinical cases [19]. PCV2 likely
125 requires other cofactors to induce the full spectrum of clinical signs and lesions associated with
126 advanced cases of PCVAD [12]. As other pathogens were detected in PCV3-positive samples, it is
127 possible that co-infection with PCV3 and other pathogens induced the complicated symptoms
128 observed, such as PCVAD. To investigate the pathogenicity of PCV3, it is necessary to further
129 investigate the pathogenicity of both PCV3 singular infection cases and co-infection cases.

130 Of the seven PCV3 positive samples, three whole genome sequences were obtained, and the
131 genomes were found to consist of 2 kb single circular DNA. The sequences of the three strains,
132 GM2124-2/2016, KGS2098-1/2016, and TG1923-2/2016, were subjected to phylogenetic analysis.
133 The three strains grouped in a clade previously reported as PCV3, and which was separated from all
134 other members of the genus *Circovirus* (Fig. 1). The homology between each Japanese strain was
135 99.5% for the full-length sequence, and 98.9% to 99.2% for the ORF 2. Fux *et al.* showed that the
136 sequences of PCV3 could be divided into two main groups (a and b) and several subclusters (a1, a2,
137 b1 and b2) [5]. A phylogenetic tree was constructed based on the nucleotide sequence of the ORF2
138 (Fig. 2). In this study, all the genotypes of PCV3 detected in Japan (GM2124-2/2016,
139 KGS2098-1/2016, and TG1923-2/2016) belonged to the a1 subgroup. However, PCV3 reported
140 from the other countries (Brazil, China, Germany, Italy, and South Korea) belonged to various
141 clusters, and did not present any genotype and geographical correlation. The fact that all three
142 strains obtained in this study classified into the same subcluster could be attributed to the small
143 number of strains examined. Thus, further investigations using a large number of Japanese PCV3
144 strains are necessary.

145 In conclusion, this is the first report on the prevalence of PCV3 in Japan. We investigated the

146 occurrence of the PCV3 genome in tissue samples collected from Japanese pig herds in 2016 using
147 PCR. In the examination of the 73 samples collected from 27 farms, the PCV3 genome was
148 detected in 7 samples from 6 farms in the Kanto and Kyusyu regions of Japan. Our results indicate
149 that PCV3 is prevalent in commercial pig herds in Japan. To elucidate the influences of PCV3 on
150 the pig industry, further studies involving virus isolation and elucidation of PCV3 pathogenicity in
151 pigs are needed.

152 **Nucleotide sequence accession numbers**

153 The GenBank accession numbers for the full PCV3 genomes sequence are LC269727, LC383840,
154 and LC383841.

155

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210

211

212 Figure caption

213 Fig. 1. Phylogenetic tree of full-length circovirus genome. The percentage of replicate trees in
214 which the associated taxa clustered together in the bootstrap test is displayed next to the branches.
215 GenBank accession numbers are written at the end. Previously published sequences used as
216 reference sequences for this phylogenetic analysis included PCV3, KT869077, KX458235,
217 KX778720, KX898030, KX966193, KY075986, KY075988, KY075989, KY075991, KY778776,

218 KY996338, KY996340, MF162298, MF162299, MF079253, and MF079254; Barbel circovirus
 219 (BarCV), GU799606; Bat circovirus-1 (BtCV-1), JX863737; BtCV-2, KC339249; BtCV-3,
 220 JQ814849; Beak and feather disease virus (BFDV), AF080560; Canary circovirus (CaCV),
 221 AJ301633; Canine circovirus (CanineCV), KC241983; Chimpanzee associated circovirus 1
 222 (CgfaCV), GQ404851; Duck circovirus (DuCV), AY228555; European catfish circovirus
 223 (EcatfishCV), JQ011377; Finch circovirus (FiCV), DQ845075; Goose circovirus (GoCV),
 224 AJ304456; Gull circovirus (GuCV), DQ845074; Human associated circovirus 1 (HufaCV),
 225 GQ404856; Mink circovirus (MiCV), KJ020099; Pigeon circovirus (PiCV), AF252610; PCV1,
 226 Y09921; PCV2, AF027217; PorkNW2/USA/2009, HQ738638; Raven circovirus (RaCV),
 227 DQ146997; Starling circovirus (StCV), DQ172906; Swan circovirus (SwCV), EU056309; and
 228 Zebra finch circovirus (ZfiCV), KP793918. The PCV3 strain analyzed in this study was indicated
 229 by an asterisk.

230 Fig. 2. Phylogenetic tree of PCV3 ORF2 regions. The percentage of replicate trees in which the
 231 associated taxa clustered together in the bootstrap test is displayed next to the branches. The country
 232 of origin and GenBank accession numbers are written at the end. Previously published sequences
 233 used as reference sequences for this phylogenetic analysis included PCV3 from Brazil, China,
 234 Denmark, Germany, Italy, South Korea, Spain, Sweden, Thailand, and the US. The PCV3 strains
 235 analyzed in this study were indicated by an asterisk.

Table 1. Primer sets for sequencing of PCV3 genome

Primer Sets	Primer name	Position	Primer sequences (5'→3')	Tm (°C)	Annealing temperature	Amplification size	Intended purpose	Reference
1	Full F1	74-93	CACCGTGTGAGTGGATATAC	57.8	55	854	To obtain PCV3 genome sequences.	[14]
	Full R1	927-910	CAAACCCACCCTTAACAG	57.9				
2	Full F2	1609-1627	GTCGTCTTGGAGCCAAGTG	63.5	60	825		[14]
	Full R2	433-415	CGACCAAATCCGGGTAAGC	66.4				
3	Full F3	594-611	GAAGTTGCGGAGAAGATG	58.4	55	550		original
	Full R3	1143-1122	CGCAATAATTGTACACAAGTGC	61.9				
4	Full F4	1004-1024	ATATTCGTGGAAAGTTGGAGG	61.6	60	778		original
	Full R4	1781-1763	CCACTTCATTACCCGCCTA	62.4				

Table 2. Details of samples detected PCV3 genome

Viral strain	Days of age	Clinical symptoms^{a)}	Other detected pathogens^{b)}	Organs^{c)}	Prefecture	GenBank accession No.
KGS2065-4/2016	Abortus	Abo	PPV, <i>Ec</i>	Lu	Kagoshima	
KGS1945-2/2016	14-21	SD	PPV	Ki	Kagoshima	
KGS1945-3/2016	14-21	SD	PPV, <i>Sh</i>	Ki	Kagoshima	
GM2124-2/2016	50	NS	PRRSV, <i>Ec</i>	Lu, HLN, BI	Gunma	LC269727
KGS2098-1/2016	72	SD	<i>Ss</i>	He, Lu, Li, Ki, Sp, MLN, JI, Co	Kagoshima	LC383841
TG1923-2/2016	130	Dep, AB	PRRSV	Lu, HLN, ILN	Tochigi	LC383840
MZ1950-1/2016	180	SD	<i>Ss</i>	Ki	Miyazaki	

^{a)} Dep: Depression, AB: Abdominal breathing, SD: Sudden death, Abo: abortion, NS: Neurological symptoms

^{b)} *Ec* : *Escherichia coli* , *Sh* : *Staphylococcus haemolyticus* , *Ss* : *Streptococcus suis*, PPV: porcine parvovirus, PRRSV: porcine reproductive and respiratory syndrome virus

^{c)} Lu: Lung, He: Heart, Li: Liver, Sp: Spleen, Ki: Kidney, JI: Jejunum Ileum, Co: Colon, BI: Blain, MLN: Mesenteric lymph nodes, ILN: Inguinal lymph nodes, HLN: Hilar lymph nodes

Fig. 1

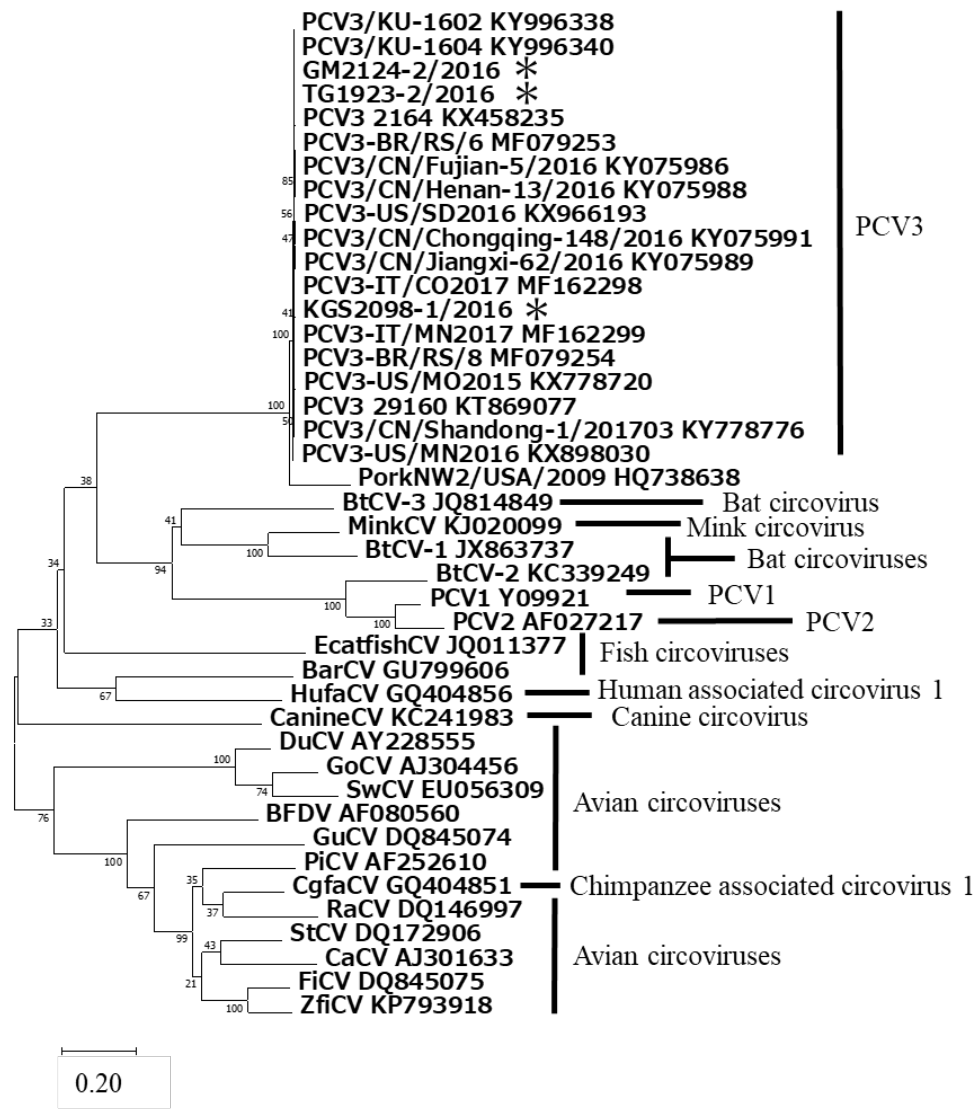


Fig. 2

