

Phylogenetic analysis of porcine circovirus 3 circulating in Canadian pigs

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Abstract

Introduction: Porcine circovirus 3 (PCV3) has been detected in pigs worldwide and associated with several clinical signs.

Methods: To investigate the genetic diversity of PCV3 strains circulating in Canada, 44 PCV3 positive samples from Saskatchewan (2/44), Manitoba (2/44), Quebec (4/44), Alberta (11/44) and Ontario (25/44) submitted to diagnostic laboratories in Canada between 2019 and 2021 were sequenced and analyzed.

Results: Phylogenetic analysis of capsid genes showed that all of the 44 Canadian strains classified into PCV3a and segregated into seven lineages with common amino acid changes observed at A24V, R27K, N56D, T77S, Q98R, L150I (F) and R168K positions.

Conclusion: Future studies are required to determine whether the polymorphisms in capsid proteins, as revealed in this study, could be associated with differences in the pathogenicity or antigenicity of PCV3 strains. This is the first phylogenetic analysis of PCV3 strains among different provinces in Canada.

KEYWORDS

Canadian pigs, phylogenetic analysis, porcine circovirus 3

1 | INTRODUCTION

Porcine circovirus 3 (PCV3), which belongs to the family *Circoviridae* and genus *Circovirus*, was first identified in 2015 in pigs in the United States displaying myocarditis, multisystemic inflammation, porcine

dermatitis and nephropathy syndrome (PDNS) and reproductive failure (Palinski et al., 2017; Phan et al., 2016). Subsequently, PCV3 has been detected in many countries around the world raising concerns to the swine industry, although the virus has also been detected in asymptomatic pigs (Gu et al., 2020; Jiang et al., 2019; Klaumann et al., 2018).

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PCV3 is a small DNA virus with a 2000-nucleotide single-stranded circular genome containing three major open-reading frames (ORFs): ORF1 encoding replicase (rep) protein, ORF2 encoding the capsid (cap) protein and ORF3 encoding a protein with unknown functions (Assao, Santos, Pereira, et al., 2021; Klaumann et al., 2018; Palinski et al., 2017; Qi et al., 2019). The cap protein is considered to be the major structural protein, which determines the antigenic properties of the virus, showing only approximately 37% amino acid sequence identity to that of PCV2 (Nawagitgul et al., 2000; Palinski et al., 2017). PCV3 strains have shown genetic stability and low mutation rates over time and across countries, with nucleotide similarity ranging from 97% to 100% on partial or complete sequences (Assao, Santos, Pereira, et al., 2021; Assao, Santos, Rosado, et al., 2021; Franzo et al., 2020; Plut et al., 2020; Qi et al., 2019). This work aimed to investigate the genetic diversity of PCV3 strains circulating in Canada and whether a main PCV3 strain circulating across different Canadian provinces.

The investigated sample set consisted of 44 samples including oral, processing and thoracic fluids (25/44), faeces (1/44) and tissues (18/44), which were submitted to diagnostic laboratories including the Centre de Diagnostic Vétérinaire de l'Université de Montréal (CDVUM) in Quebec, Animal Health Laboratory (AHL) in Ontario and Prairie Diagnostic Services (PDS) in Saskatchewan (Table S1). Available samples enrolled in this study were from different provinces including Saskatchewan (2/44), Manitoba (2/44), Quebec (4/44), Alberta (11/44) and Ontario (25/44) between 2019 and 2021. Nucleic acid extraction was performed using MagMAX™ CORE Nucleic Acid Extraction Procedure (ThermoFisher, Austin, TX, USA) as per the manufacturer's instructions. PCV3 detection was performed using a previously described real-time PCR assay (Palinski et al., 2017) and samples with quantification cycle (Cq) values at or below 30 were selected for PCR and sequencing of the full PCV3 ORF2 sequences. Two sets of primers with the first set (Forward 5'-GTG TAC AAT TAT TGC GTT GGG-3' and Reverse 5'-AAA ACA CAG CCG TTA CTT CAC C-3') and the second set of primers (Forward 5'-GCT TTG TCC TGG GTG AGC G-3' and Reverse 5'-CCT GCG GCA TCA AAA CAC G-3') were utilised separately to generate two overlapping fragments containing the full ORF2 sequence (Arruda et al., 2019). These two PCRs were carried out using Taq DNA polymerase master mix (Qiagen, Vilnius, Lithuania). The following cycling conditions were used: initial activation phase at 94°C for 10 min; 45 two-step cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s and extension at 68°C for 45 s (Arruda et al., 2019). A 444- and 643-bp PCR product could be visualised using Qia-gen QIAxcel instrument, respectively. Then PCR products were sent to Macrogen (Seoul, South Korea) for Sanger sequencing.

The Sanger sequencing chromatograms of PCR products were base-called and assembled using Tracy v0.5.9 (Rausch et al., 2020) to obtain full ORF2 sequences. Multiple alignment of ORF2 sequences was performed using Mafft v7.475 (Kato and Standley, 2013). To determine the best-fit nucleotide substitution model, jModelTest2 v2.1.10 (Darriba et al., 2012) was used to assess model fit to the sequence alignment across 88 nucleotide substitution models based on Bayesian Information Criterion estimates. The maximum likelihood tree was generated

using RAxML-NG v1.0.3 (Kozlov et al., 2019). Maximum likelihood inference was performed using the best-fit HKY nucleotide model with invariant sites (+I) and Gamma distributed rates (+G). The robustness of the phylogenetic tree nodes was determined by bootstrap analysis of 1000 replicates. The phylogenetic tree was visualised using the ggtree package v2.4.1 in R (Yu, 2020). Pairwise sequence alignment and calculation of sequence identity were conducted using Jalview v2.11.1.4 (Waterhouse et al., 2009). All 44 sequences were deposited to the National Center for Biotechnology Information (NCBI) GenBank database (accession numbers provided in Table S1).

Sequence analysis revealed that cap gene sequences of 44 Canadian strains had 96.43%–100% nucleotide identity with each other and 88.28%–100% nucleotide identity with those of 33 reference strains included in this study. According to the criteria of genetic distance of 6% at the ORF2 level (3% at the complete genome level) and over 90% bootstrap support, PCV3 is classified into two clades: clade 1 (PCV3a) and clade 2 (PCV3b) (Franzo et al., 2020). Clade 2 (PCV3b) contains only two sequences identified from Chinese farms (Franzo et al., 2020). After removing the reference sequence from clade 2 (PCV3b) (GenBank number MG372488) included in this study, the resulting pairwise nucleotide identity across all sequences ranged from 96.74% to 100%. This supports the statement of genetic stability and low mutation rates of PCV3 (Arruda et al., 2019; Assao, Santos, Pereira, et al., 2021; Qi et al., 2019). All 44 Canadian sequences in this study were classified into clade 1 (PCV3a) along with included reference strains (Figure 1), which strengthens the hypothesis that clade 2 (PCV3b) including only two sequences is considered to be either recently emerged variants or the last descendant of previously circulating genotypes (Franzo et al., 2020). The phylogenetic tree of the cap gene (Figure 1) showed that Canadian strains were subdivided into seven lineages based on nucleotide patterns. A total of 12 Canadian strains (Lineage 1) clustered with strains detected in different countries. A cluster of four Canadian strains (Lineage 2) clustered with a strain from Malaysia and a cluster of 11 Canadian strains (Lineage 3) clustered with a strain in the United States were found. Lineage 4 containing only one Canadian strain (12769/2021/MB-CAN) clustered with strains from China. Lineage 5 and 6 containing six Canadian strains respectively clustered separately. Lineage 7 containing four Canadian strains clustered with a strain from Germany. Assao, Santos, Pereira, et al. (2021) demonstrated that at least six different PCV3 lineages are circulating in North and South America based on amino acid patterns with only one available Canadian strains in that study. Further study is necessary to determine whether the nucleotide variations in cap gene, as revealed in this study, could be associated with differences in the pathogenicity of PCV3 strains.

Considering cap as the major structural protein and the main antigen of PCV3 (Klaumann et al., 2018), the amino acid sequences of the 44 Canadian PCV3 strains included in this study were compared to the reference strain NC_031753. Sequence analysis showed the presence of 17 amino acid substitutions in the cap protein (Table 1). Common amino acid changes at A24V, R27K, N56D, T77S, Q98R, L150I (F) and R168K positions were observed (Table 1). It has been speculated that

TABLE 1 Molecular features of the PCV3 capsid protein from Canada

Sequence_ID	R 4	A 5	F 7	A 24	R 27	N 56	A 75	T 77	Q 98	F 104	V 134	K 140	L 150	S 156	R 168	L 173	G 196
84-0001/2020/ON-CAN	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39787/2020/AB-CAN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17-0006/2020/ON-CAN	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-
52-0005/2020/ON-CAN	-	-	-	-	K	D	-	N	R	-	-	-	F	-	K	-	-
21843/2021/QC-CAN	-	-	-	-	K	-	-	N	-	-	-	-	-	-	-	V	-
89-0001/2021/ON-CAN	-	-	-	-	-	-	-	S	-	-	-	-	I	-	-	-	-
77-0003/2020/ON-CAN	-	P	-	-	-	-	-	S	-	-	-	-	I	-	-	-	-
6918-4/2021/AB-CAN	-	T	-	-	-	-	-	S	-	Y	-	-	I	-	-	-	-
6918-3/2021/AB-CAN	-	-	-	-	-	-	-	S	-	Y	-	-	I	-	-	-	-
6918-2/2021/AB-CAN	-	-	-	-	-	-	-	S	-	Y	-	-	I	-	-	-	-
6918-1/2021/AB-CAN	-	-	-	-	-	-	-	S	-	Y	-	-	I	-	-	-	-
43-0004/2021/ON-CAN	-	-	-	-	-	-	-	S	-	-	-	-	I	-	-	-	-
40-0006/2021/ON-CAN	-	-	-	-	-	-	-	S	-	-	-	-	I	-	-	-	-
39423/2020/AB-CAN	-	-	-	-	-	-	-	S	-	-	-	-	I	-	-	-	-
32-0001/2020/ON-CAN	-	-	-	-	-	-	-	S	-	-	-	-	I	-	-	-	-
22838/2021/QC-CAN	-	-	-	-	-	-	T	S	-	-	-	-	-	-	-	-	-
8886/2021/QC-CAN	-	-	-	-	K	D	-	S	R	-	-	-	I	G	K	-	-
70-0004/2021/ON-CAN	-	-	-	-	K	D	-	S	R	-	-	-	F	-	K	-	-
66782/2021/QC-CAN	-	-	-	-	K	-	-	S	-	-	-	-	-	-	-	-	-
60-0001/2020/ON-CAN	-	-	-	-	K	D	-	S	R	-	-	-	F	-	K	-	-
42-0003/2021/ON-CAN	-	-	-	-	K	D	-	S	R	-	-	-	F	-	K	-	-
34-0001/2020/ON-CAN	-	-	-	-	K	-	-	S	-	-	-	-	I	-	-	-	-
3-0001/2021/ON-CAN	-	-	-	-	K	-	-	S	R	-	-	-	F	-	K	-	-
12769/2021/MB-CAN	-	-	-	-	K	-	-	S	-	-	-	-	I	-	-	V	-
99-0011/2020/ON-CAN	-	S	-	V	K	-	-	S	-	-	-	-	I	T	-	-	-
95-0008/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
95-0005/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
9322/2021/MB-CAN	-	-	-	V	K	-	-	S	-	-	-	T	I	-	-	-	-
91-0005/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
82-0015/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
75-0064/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
72-0005/2021/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
68-0001/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
59-0002/2020/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
54-0007/2021/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
39422/2020/AB-CAN	-	-	-	V	K	D	-	S	R	-	-	-	I	-	K	-	-
39420/2020/AB-CAN	-	G	-	V	K	D	-	S	R	-	I	-	I	-	K	-	-
37-0002/2020/ON-CAN	-	-	Y	V	K	-	-	S	-	-	-	-	I	-	-	-	-
30768/2019/SK-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	E
29102/2019/SK-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
24-0009/2021/ON-CAN	-	-	-	V	K	-	-	S	-	-	-	-	I	-	-	-	-
20232/2021/AB-CAN	-	-	Y	V	K	-	-	S	-	-	-	-	I	-	-	-	-
1972-5/2021/AB-CAN	-	-	-	V	K	D	-	S	R	-	-	-	I	-	K	-	-
1972-4/2021/AB-CAN	-	-	-	V	K	D	-	S	R	-	-	-	I	-	K	V	-

Note: Polymorphisms identified in the amino acid sequences are shown by the columns with their respective amino acid positions. The reference sequence used for the comparison of polymorphisms is NC_031753.1.

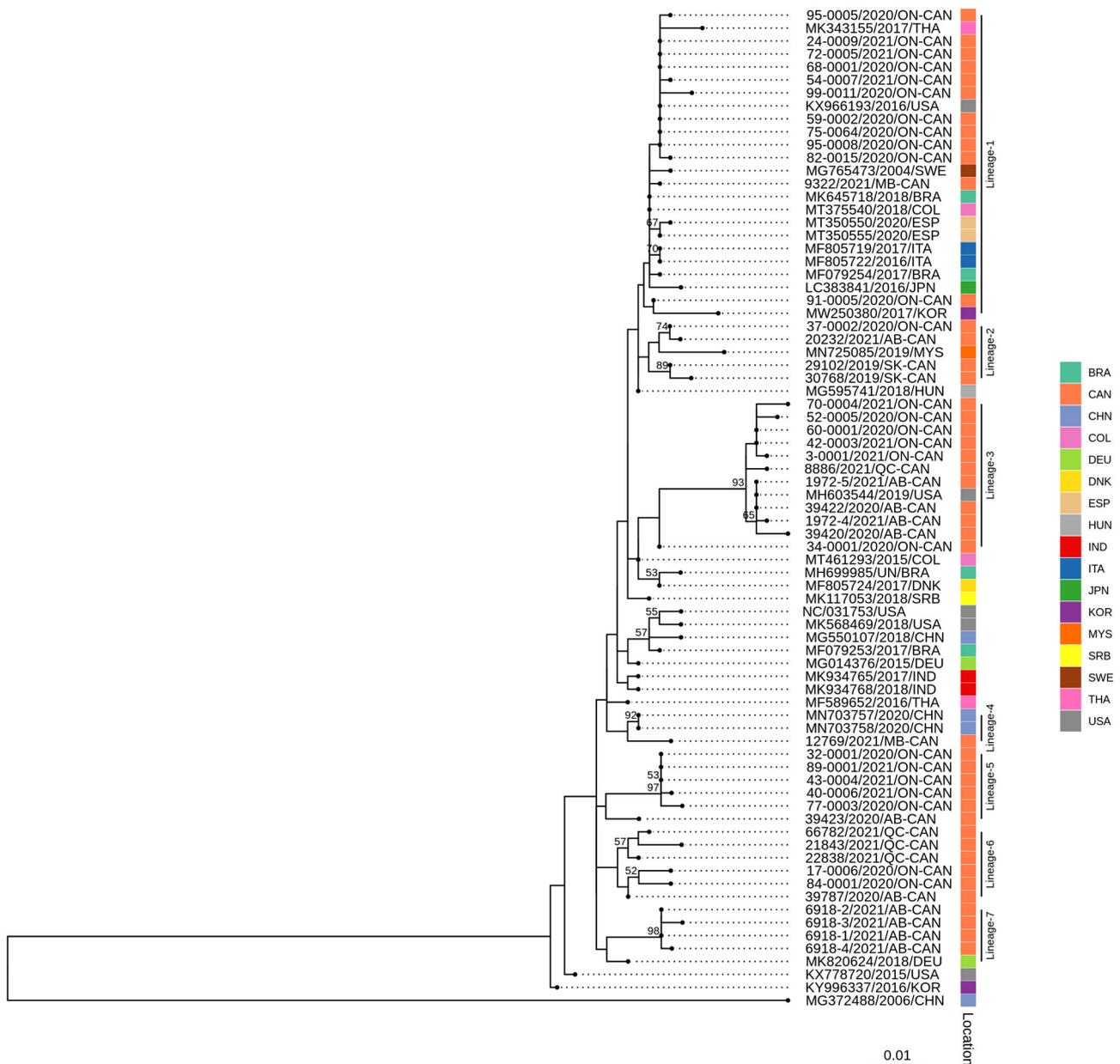


FIGURE 1 Phylogenetic analysis of PCV3 cap genes based on the nucleotides' sequences. All reference strain sequences included in this study were downloaded from the NCBI GenBank database. The tree was constructed by the maximum-likelihood method using the HKY nucleotide model with invariant sites (+I) and Gamma distributed rates (+G) and 1000 bootstrap replicates. Bootstrap values are indicated for nodes with values >50%. Canadian PCV3 strains are indicated by light orange colour.

A24V and R27K might be related to immune escape and were used for lineage or subclade classification of PCV3 (Assao, Santos, Pereira, et al., 2021; Gu et al., 2020; Fu et al., 2018). In this study, mutation of $^{24}ARRR^{27}$, $^{24}ARRK^{27}$ and $^{24}VRRK^{27}$ was found without mutation of $^{24}VRRR^{27}$, which was reported previously (Sun et al., 2018). Amino acid change at T77N position was observed on both sequences from AB and ON, along with other amino acids changes at R4K, A5P (T, S, G), F7Y, A75T, V134Y, K140T, S156G (T), L173V and G196E positions across different provinces (Table 1), suggesting the importance of these differences for understanding the evolutions of these strains.

2 | CONCLUSION

Our results demonstrated that Canadian PCV3 strains submitted to diagnostic laboratories and sequenced in this study were not diverse. All of the Canadian strains were classified into PCV3a and segregated into different clusters with strains from North America, South America, Asia and Europe. Amino acid polymorphisms found in this study indicated the importance of these differences for understanding the evolutions of these strains. This is the first phylogenetic analysis of PCV3 strains among different provinces in Canada. The new PCV3

sequences will allow further investigations to explore the evolutions of PCV3 and future studies are required to better determine the polymorphisms in cap proteins that could be associated with differences in the pathogenicity or antigenicity of PCV3 strains.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

M.Z., Y.H. and J.E.H. conceptualised the idea of the study. M.Z., C.C.L., D.O. and C.A.G. curated the data. M.Z. and C.C.L. performed formal analysis and visualisation and provided software. M.Z. and J.E.H. performed investigation. M.Z. and M.B.A. designed methodology and performed validation. M.Z. and Y.H. administered the project. M.Z., Y.H., D.O. and C.A.G. provide resources. M.Z., Y.H. and J.E.H. performed supervision. M.Z. wrote the original draft. All authors reviewed and edited the manuscript. Y.H. acquired funding.

DATA AVAILABILITY STATEMENT

All 44 sequences were deposited to the National Center for Biotechnology Information (NCBI) GenBank database (accession numbers provided in Supplementary Table 1).

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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