SHORT COMMUNICATION

Identification and genotyping of porcine circovirus type II (PCV2) in Mexico

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Received: 25 January 2018 / Accepted: 29 May 2018 / Published online: 7 June 2018
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Abstract Porcine circovirus type 2 (PCV2), family *Circoviridae*, genus *Circovirus* infection in domestic pig has been associated with several pathological conditions being the most important of them the postweaning multisystemic wasting syndrome. Many studies have demonstrated the existence of three PCV2 genotypes (a, b, and c) and recently PCV3. Until now, these genotypes or subgenotypes have not been described in Mexico. We found genetic changes in ORF2 from nine strains of PCV2 obtained from samples of Jalisco, Veracruz, Estado de México, Hidalgo and Sonora states of Mexico. Our results shown the presence of two genotypes (PCV2a and PCV2b) as well as, the presence and differences between the reported subgenotypes. The subgenotype PCV2b (1A/1B, 1A) has a higher prevalence (87.5%) in comparison with PCV2a (2C) (12.5%).

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13337-018-0460-6>) contains supplementary material, which is available to authorized users.

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Keywords Porcine circovirus type 2 genotypes · PCV2 · Subgenotype · PMWS

Porcine circovirus type 2 (PCV2) is a ubiquitous virus whose antibodies have been detected in pigs all over the world, where they have been searched [13]. PCV2 infection has been associated with various pathological conditions affecting the pig including reproductive failure (RF), porcine dermatitis and nephropathy syndrome (DPNS), and porcine respiratory disease complex (PRDC). For this reason, the term “diseases associated with porcine circovirus” was suggested to be used under the acronym PCVD or PCVAD (porcine circovirus diseases o porcine circovirus associated-diseases) [13], in order to refer to all of them. However, among all PCVAD, the PC is considered the most important of them, because it causes a significant economic impact on pig production. Thus, only within the European Union territory, annual losses associated to this disease were quantified in 600 million euros [1].

Due to the existence of different PCV2 genotypes, it has been said that they have an important role in the virulence [2, 7, 9] and even in the co-infection with different genotypes, which is considered as an aggravating circumstance in the virulence and pathogenesis of the disease [5, 8].

The sequencing of the PCV2 genome showed the existence of different genotypes. So far, five of them have been described, and on the basis of a PCVD-consortium proposal from the European Union, they are named with letters that start with a; so from now on, they will be recognize as a, b, c, d, and e types [12]. Types a and b seem to have a worldwide distribution, while type c has only been found in Denmark [4]. Other subtypes described in Asia (d and e) [7] seem to belong to the subtypes previously described (a, b, c) [3].

Segalés [12], proposed the existence of five PCV2 genotypes (a, b, c, d, e). In 2011, Cortey and collaborators reassessed the subtypes (d, e) [3], but they did not found significant differences, in order to keep them as independent subtypes, so they were reclassified into the subtypes (a, b). So far, neither of these genotypes nor their subgenotypes have been reported in our country; therefore this study represents the first report in Mexico about the genotypes found in five highly productive states, through the sequencing of the open reading frame 2 (ORF2).

In this study we consider cases of post-weaning multi-systemic wasting syndrome (PMWS) to obtain lymph nodes samples from animals of 21–40 old days with clinical signs such wasting, depression, anorexia and enlargement of subcutaneous lymph nodes, of three farms from Sonora, Jalisco, Estado de México, Hidalgo and Veracruz. In a total of 75 samples derived from pigs were processed with PCR, and the positives samples where sequenced in order to characterize and determine the PCV2 genetic variants in the Mexican swine population, by using an ORF2 reading. The PCR reaction was conducted using the DNA extracted and a commercial kit (Biotecmol)[®] following the manufacturer's instructions. The primers used for the first PCR reaction were: forward (3' CTGAT-TACCAGCAATCAGACC 5') and reverse (3' ACCGY-TACCGYTGGAGAAG 5'); with two degenerated bases, identified with Y at positions 5 and 11. This enabled the C/T adhesion in order to obtain an 831pb segment. The reaction was carried out under the following conditions in a BIO-RAD thermocycler: one cycle of 95 °C during 5; 35 cycles of 94 °C for 35 s, 60 °C for 35 s and 72 °C for 35 s. Finally, one cycle of 72 °C for 10 min was applied. Sequences and phylogenetic analysis were made in automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, USA) was used for sequence reading. Eight strains virus from 5 states were obtained and were registered in the GenBank with the following access numbers: Sonora1.Mex-2013 (KT275164), Sonora2.Mex-2013 (KT275165), Sonora3.Mex-2013 (KT275163), Jalisco1-2008 (KT275162), EdoMex-2013 (KT275160), Hgo.Mex-2014 (KT275161), Ver1.Mex-2014 (KT275166) and Ver2.Mex-2014 (KT275167), using phylogenetic analysis it includes PCV2a, PCV2b and PCV2c reference genotype and was made using MEGA 6.0 program for Windows (molecular evolutionary genetics analysis). To characterize the phylogenetic history, the criteria of maximum likelihood (ML) was applied using neighbor-joining tree (NJ) reliability was calculated using 1000 bootstrap replicates.

Nucleotide sequences were compared using a pairwise comparison with distances lower than 0.005 (this meant, that they corresponded to nearly identical or identical

sequences limited to a maximum of one amino acid and three nucleotide differences).

Our results shown a total of 51 changes on the bases were identified regarding to the reference sequence, with 22 mutations that produced an amino acid change. The most important changes observed between the samples and the reference, were in positions: 1375, 1378, 1395, 1402, 1441, 1484, 1531, 1534, 1564, 1573, 1582, 1591, 1600, 1601, 1612, 1663, 1727, and 1734. A high similarity between the JaliscoMex-2008 field sample and the reference was found that corroborated the phylogenetic tree results, where it was classified as PCV2a. In other field samples, changes that apparently generated the genotype variation were evident, and supported the classification given by the PCV2b phylogenetic tree (Fig. 1a).

Table 1 shows the codon position and the change that it generated in the amino acids, with respect to the reference sample AF027217.1 Can-2009; as well as insertions, deletions and stop codon location. The main amino acid polymorphisms, which created the classification change within the various existing genotypes regarding to the PCV2a reference, were located in positions: 121, 151, 169, 190, 206, 210, 232 and 234. These positions had a great similarity with the JaliscoMex-2008 field sample and differed drastically with the PCV2b and PCV2c genotypes. Moreover, similarity was shown between field and reference samples for each subgenotype, especially for PCV2b (1A/1B), (1B) and PCV2a with the (2C) subgenotype.

The obtained results from the phylogenetic tree, classified the PCV2b 1C reference samples as a different genotype. When the amino acid alignment of these samples - regarding to the reference and other references- was checked, 11 changes were observed: 4 were equal to PCV2b at positions 181, 190, 191, 232; another 4 were equal to PCV2a at positions 121, 151, 210, 234; and only 3 changes were observed for the subgenotype 1C at positions 134, 169, 215. Something in particular observed in the analysis for each genotype, was that only PCV2b 1C samples had the deletion in position 232 and the stop codon at position 234 (Table 1).

Sequence analysis showed similarity in different genotypes, where the subgenotype PCV2b (1A/1B) predominated with an 85.7% (6/7), and 14.3% (1/7) was registered for PCV2b (1B). The 12.5% (1/8) that belonged to PCV2a was classified as the subgenotype PCV2a (2C). Furthermore, it also pointed out the difference between PCV2 and the nonpathogenic PCV1 (Fig. 1a).

In this study, the similarity percentages between different genotypes, when compared to the reference sequence, were consistent with those reported by different authors [6, 7, 10]; where a and b genotypes had a similarity percentage between 95 and 97% (97.1% in sequenced samples), 92% for genotype C (89% in our analysis), and

Fig. 1 **a** Phylogenetic tree constructed with the nucleotide sequence derived from 8 field sequences and ORF2 references taken from genebank (identified by their reference numbers). Elaborated with (MEGA 6.0) program, using the Tamura-Nei method [14]. **b** Distance between PCV2a subgenotypes, and difference between PCV2a/PCV2b genotypes and the subgenotype PCV2b (1C)

Table 1 Codon position and aminoacid change with respect to the reference for PCV2b

Aminoacid substitutions	121	134	151	169	181	190	191	210	215	232	233	234
AF027217.1 Can-2009	T	T	P	S	T	S	R	D	V	K	P	*
KT275162 Jal.Mex-08	.	.	.	.	N	.	A	.	.	.	.	.
KT275160 EdoMex-2013	S	.	T	.	N	T	G	E	.	-	.	L
KT275165 Son2.Mex-2013	S	.	T	.	N	T	G	E	.	-	.	L
KT275163 Son3.Mex-2013	S	.	T	.	N	T	G	E	.	-	.	L
KT275166 Ver1.Mex-2014	S	.	T	.	N	A	G	E	.	-	T	I
KT275167 Ver2.Mex/2014	S	.	T	.	N	T	G	E	.	-	.	L
KT275164 Son1.Mex-2013	S	.	T	.	N	T	G	E	.	-	.	L
KT275161 Hgo.Mex-2014	S	.	T	.	N	T	G	E	.	-	.	L
AF112862.1 Can-05	.	.	.	R	N	.	.	.	.	.	.	.
AY181948.1 Chi-09	.	.	.	.	.	.	.	.	.	.	.	.
AB072301.1 Jap-09	.	P	.	.	N	.	.	.	.	.	.	.
HQ395028.1 Chi-12	S	.	T	.	N	T	G	E	.	-	.	L
AY596823.1 Chi-04	S	.	T	.	N	A	G	E	.	-	A	L
HQ395027.1 Chi-12	S	.	T	.	N	T	G	E	.	-	T	L
AY682993.1 Chi-04	S	.	T	.	N	A	G	E	.	-	.	L
AY682994.1 Chi-04	.	N	.	R	N	T	G	.	I	-	.	.
EF524539.1 Chi-09	.	N	.	R	N	T	G	.	I	-	.	.
HM038030.1 Chi-10	.	N	T	R	N	T	G	.	I	-	.	.
AY484410.1 Hol-04	.	N	.	R	N	T	A	.	.	-	T	.
AY484416.1 Hol-04	S	.	T	.	N	A	G	E	.	-	.	L
AY424405.1 Aus-03	S	.	T	.	N	A	G	E	.	-	.	L
AY691169.1 Chi-04	S	.	T	.	N	A	G	E	.	-	.	L
AY713470.1 Ale-05	.	.	.	R	N	.	A	.	I	-	.	.
AF109398.1 Can-01	S	.	T	G	N	T	G	.	.	.	.	.
AY146991.1 Tai-02	.	.	.	.	N	.	A	.	.	.	.	.
AY256459.1 Hun-03	.	.	T	.	N	.	A	.	.	.	.	.
AY424402.1 Aus-03	S	.	.	.	N	.	.	.	.	.	.	.
AF109399.1 Can-00	S	.	.	.	N	.	.	.	.	.	.	.
AY094619.1 Usa-03	.	N	.	.	N	.	.	.	I	N	.	.

70% for PCV1 genotype (69% in our analysis). Olvera et al. [10] have been reported that PCV2b is the predominant genotype in most world countries. We didn't found evidence of the presence of genotype PCV2c within the analyzed samples.

Considering the sequences of the reported genotypes and comparing them with our results, the presence in Mexico of two of the genotypes and several subgenotypes was demonstrated. In addition, regions where the genotype change was produced are shown, taking into account that only 61% of ORF2 was obtained, but the result was the same as the one reported by other authors [7]. Because the phylogenetic analysis revealed that Mexican sequences have high nt. identity with Asian (China) genotype "b" sequences. Given the close commercial link of Mexico with that country, such similarity might account for the origin of PCV2b strains currently present in the Mexican

pig population, however the deletion in 232 position has not been reported before.

Although variations were distributed throughout the ORF2 sequence, the protein alignment revealed the areas of higher heterogeneity among samples and the reference genotypes. The EdoMex.Mex-2013, Son1.Mex-2013, Ver1.Mex-2014, Ver2.Mex-2014, Son2.Mex-2013, Son3.-Mex-2013, and Hgo.Mex-2014 samples, with respect to the PCV2b subgenotypes in positions 121–136, 169–191, 206–215, 232–234, were similar to the ones reported by Savic in Serbia [11].

In the Phylogenetic analysis the PCV2b strain was classify as a different genotype, generated by deletion and stop codon. Also, this might suggest that the changes found in the sample alignment, could be the result from a recombination between genotypes PCV2a and PCV2b, as has been proposed by many authors. In our case, we could not corroborate this because the analyzed segment

was very small (61% of the ORF2), and it is required to have the two main ORFs to determine whether or not this recombination occurred [6, 10, 11]. For PCV2a, the positions that generated more similarity with respect to the reference were 121, 151, 169, 190, 210, 232, and 234. These positions showed the same amino acid for the Jalisco-08 sample, which classified it as PCV2a and corroborated the distribution given in the phylogenetic tree.

Conversely, in PCV2a, PCV2b genotypes and PCV2b 1C subgenotype, a marked difference between each of the aligned subgenotypes was shown, where distances of 0.01, 0.02, 0.03 among the different subgroups were observed (Fig. 1b).

This tree showed a high prevalence of PCV2b and its subgenotypes 1A–1B, demonstrating their presence in 4 of the 5 evaluated states, and only one state for PCV2a (Fig. 1a). With respect to PCV2b, the phylogenetic tree showed a wide distribution in the main clade that included the majority of Mexican sequences which might suggest the presence of endemic Mexican strains.

The results obtained in this study showed similarities with several authors, especially in areas of higher heterogeneity, as reported by Savic [11] and Huang [7]. This supports the importance of our results and opens a new path for further research, in order to clarify the uncertainties that exist about the virus behavior; as well as, the disease current health status and the obtaining of the complete viral genome, in order to accurately determine the presence of different subgenotypes and the possible presence of recombinant viruses between PCV1, PCV2a and PCV2b.

From the obtained results and the discussion about them, we can conclude that the ORF2 sequences of porcine circovirus type 2 from pigs of the different states, showed considerable genetic differences that allowed us to report for the first time, more than one PCV2 genotype in Mexico.

In our country, it remains necessary to gather more information about the behavior, presentation and clinical manifestations of PCV2. This study is the first report in Mexico about PCV2 genotypes, but many factors need to be reviewed concerning the agent, management and the environmental conditions, in order to obtain a clearer view about present genotypes, their clinical manifestations in the different states, the sanitary state against the disease and economic losses, among other factors that may be overlooked, but that directly affect the economy of large and small pig producers throughout the country.

Acknowledgements This study was partially funded by the following project: PAPIIT IN223514 and Consejo Nacional de Ciencia y Tecnología Number: CB-2015-01/00254244. We would like to show our gratitude to Juvencio García, MVZ for the samples provided throughout the study.

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