

Analysis of the Genetic Sequences of PCV2 Virus in Mexico

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Abstract—These All pig-producing countries from around the world report the presence of Postweaning multisystemic wasting syndrome (PMWS.) In America, PCV2 has been recognized in Canada, United States and Brazil. Knowledge concerning the genetic sequences of PMWS has been very important. In Mexico, there is no report describing the genetic sequences and variations of the PCV2 virus present around the country. For this reason, the main objective was to describe the homology and genetic sequences of the PCV2 virus obtained from different regions of Mexico. The results show that in Mexico are present both subgenotypes \"a\" and \"b\" of this virus and the homologies are from 89 to 99%. Regarding with the aminoacid sequence, three major heterogenic regions were present in the position 59-91, 123-136 and 185-210. This study presents the results of the first genetic characterization of PCV2 in production herds from Mexico.

Keywords—PCV-2; sequencing analysis; Mexico

I. INTRODUCTION

THE porcine circoviruses (PCV) are members of the genus *Circovirus*, family *Circoviridae*, it was originally isolated as a non-cytopathic contaminant of the porcine kidney cell line PK15, up to now no PCV infections has been consistently detected in the mammal species tested other than swine and mice [26]. These viruses are very small non-enveloped icosahedral viruses with a single stranded circular DNA genome of about 1.7 kb organized in an ambisense direction; into this family exist other 2 virus that are important for the Veterinary Medicine, chicken anemia virus and psittacine Beak and Feather Disease virus; also there are three plant virus and one that affects to human. [10], [8], [6], [30]. The PCV virus have at least six major Open Reading Frames (ORFs); and to two have been well recognized, ORF1, called the rep gene, which encodes a protein of 35.7 kDa involved in virus replication, and ORF2, called the cap gene, which encodes the major immunogenic capsid protein of 27.8 kDa [19], [12], [15], [20], [22].

Currently, two types of porcine circovirus have been described type 1 (PCV1) and type 2 (PCV2). In terms of the overall DNA sequence homology within the PCV1 or PCV2

isolates is greater than 90%, whereas the homology between PCV1 and PCV2 isolates is only 68 to 76% [5]. According with its importance, PCV1 is widespread, although it apparently caused no symptoms in pigs [8]. The other, porcine circovirus (PCV2) is considered to be an important emerging pathogen associated with a number of different syndromes and diseases in pigs as post-weaning multisystemic wasting syndrome (PWMS); dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex, reproductive failure, granulomatous enteritis, exudative epidermitis, necrotizing lymphadenitis, congenital tremor [6] and actually has been described the important impact of adverse effect on the immune system in the form of compromising the development of a protective immune response to commercial vaccines commonly used on pigs around the time of PCV2 infection [23].

On the other hand, phylogenetic researches had divided PCV2 into two major genotypic groups: PCV2- group 1 (with three clusters A, B and C) and PCV2- group 2 with 5 clusters A, B, C, D and E); the principal differences between both are the size of the genome (group-1 1767 nucleotides and group-2 1768 nucleotides, the deleted nucleotide is located at nt1040) and a difference in aminoacid sequence that may serve as a signature motif [7]. Although the group-1 had been associated in all diseased animals have not been identified molecular markers specific to a pathogenic state [2], [10]. Recently, this classification has changed to PCV2-a and PCV2-b in base on a signature motif that encodes a distinct stretch of amino acid in the viral capsid protein [33]. Also third genotype (PCV2-c) was detected in Denmark with only three sequences to date [9], [13].

Even though the reason for the variety of clinical manifestations associated with PCV2 is not well understood, currently it has been shown that the ORF3 encodes a protein that is not essential for the replication *in vitro* but is important for the viremia process and is involved in the apoptosis (activating caspase-8 and caspase-3 pathways) induced by PCV2 [15], and there is a report that describe that B lymphocytes are more important that mononuclear cells for PCV2 replication [36].

Some studies on PCV2 and syndromes causing by it have been directed in order to obtain a better knowledge focused on the dynamics and behavior of PCV2 infections within farms. Major clinical signs evident are wasting and respiratory or digestive disorders, the low weight is evident at 7 and 12 weeks of age, and there is high mortality [29]. The

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seroconversion pattern is equivalent to that of typical antibody dynamics for most viral agents that affect swine and both humoral immune response and cell-mediated immune response are required for full protection [21]; and has been established (by Real Time quantitative PCR) that the peak of the virus load is between 14 and 21 days PI [28]; as well is documented that the injection directly into tissue of SPF pigs of cloned PCV2 genomic DNA is capable of developing and infection and disease resembling those induced by infection via intranasal route, with gross and histopathological lesions in multiple tissues [11].

Nowadays researches around the world have developed molecular diagnostic techniques more sensible and sensitive in order to identify the herds infected [3], [14], [25], [4], [16], and based in serology the porcine circovirus type 2 has been described as a important virus in Europe (Germany, United Kingdom, Spain, France, Czech Republic, Sweden, and Denmark), Asia (China, Japan and Taiwan) and America (Canada, Costa Rica, Mexico, Brazil and the United States) with seroprevalences between 50% to 100% of positive farms [37], [1], [5], [33], [34], [17], [32]; additionally, in all these countries the knowledge of the genetic sequences have been very important in order to determinate the level of the homology between the different isolations and with this to learn more about of the disease. In Mexico pigs with antibodies against PCV2 was identified in 2001 [32], also it has been showed the presence of antibodies against PCV2 in 98% of backyard pigs from Mexico, this is indicative that PCV is present around all the Mexican territory [27]; but at own knowledge there is not a preview report that described the epidemiological and genetic behavior of PCV2 in Mexico, because of this the main objective of this study was to contribute to the understanding of the genetic characterization of PCV2 virus in Mexico.

II. MATERIALS AND METHODS

A. Collection and type of samples

This study was conducted in 34 swine farms distributed in 10 different regions of Mexico, which are important for its pig production; in 20 of these farms the pigs showed clinical PMWS besides that histopathological findings related with PMWS was founded in pigs from these farms. The sampling period was from January to September of 2006 collecting serum (n=3094), organs (n=122) including lymph nodes, lungs, ileum, semen (n=22), and fecal swaps (n=437); the samples were pooled according with the type of sample to a total of 888 pools (Table 2).

B. DNA isolation

The DNAeasy kit (QIAGEN) was used for the DNA extraction from the tissue and serum samples according with the manufacturer's instructions. For tissue 5-25 mg were weighed and incubated at 55°C over night with lysis buffer plus Proteinase K (20mg/ml), while each 200 µl of serum

samples was used for the process. For both, DNA was eluted with 60 µl buffer and 1 µl was used for the PCR reaction.

C. PCR and cloning of ORF2 fragment

A set of PCV2 specific primers (the sequence was kindly provided by Li Wang- VIDO University) was directed to amplify a fragment of 506 nucleotides. These primers amplify the sequences between the position 1570 - 1100 of the ORF2 (*cap* gene), the PCR was performed in a 25 µl reaction using 10- 20 ng template DNA, 10 µMol each primer, 0.5 Units of Taq polymerase Recombinant (INVITROGEN), 1.5 mM magnesium chloride, 0.2 mM each (10mM) dNTP's and the solution buffer recommended by the manufacture. The PCR thermocycling conditions were: initial denaturation 2 min at 94°C, then 35 cycles of denaturation 40 s 94°C, annealing 30 s 55°C, elongation 30s 72°C and finally one terminal extension of 72°C 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel dye with Ethidium Bromide at 100 volts/ 45 min.

Ten positive samples (corresponding at different geographical regions) were purified with PCR Kit purify (INVITROGEN) and the amplified was cloned into TOPO TA cloning for sequencing (INVITROGEN) according with the manufacture instructions and the plasmidic DNA was extracted with MiniPrepKit SNAP (INVITROGEN), finally the samples were sequenced using an ABI PRISM 377 DNA sequencer (APPLIED). In order to get the percentages of sequences identity among different PCV2 sequences around the world; the sequences were compared pair-wise at both the nucleotide and amino acid levels using GENE BANK Blast program, multiple alignments and the phylogenetic tree were performed using the CLUSTAL W program using the sequence of the capsid protein (ORF2).

III. RESULTS

The number of samplings carried out in all these farms was 89 with 72 of the 89 samplings found to be positive (80.89%). The analysis for each the farms showed that 33 of 34 farms were PCV2 PCR positive; which is equal to 97.06%.

In addition, samples from each one of the regions were further characterized by sequencing. Ten distinct sequences were identified, and the results showed that the homologies of nucleotides from Mexican PCV2 virus are between 89% until 99%. The PCV2 sequence obtained in Guanajuato (MX LEON) differs most compared to the the other Mexican sequences (89 to 91%) just has 99% the homology with PCV2 from Queretaro (MX QRO) that is the second sequence with different homology (89 to 91%). The others sequences apparently are more closely related (it is showed in Table I).

TABLE I
GENETIC HOMOLOGY BETWEEN THE MEXICAN PCV2.

homo-logy, %	PCV2MX LEON 1	PCV2MX JAL 2	PCV2MX MICH 3	PCV2MX NL 4	PCV2MX PBC_SC 5	PCV2MX PBC_C 6	PCV2MX QRO 7	PCV2MX SON_N 8	PCV2MX SON_G 9
PCV2MX JAL 2	90 (4.57/506)								
PCV2MX MICH 3	89 (4.55/506)	90 (304/306)							
PCV2MX NL 4	90 (4.57/506)	90 (304/306)	99 (502/506)						
PCV2MX PBC_SC 5	91 (4.61/506)	94 (479/506)	94 (477/506)	94 (477/506)					
PCV2MX PBC_C 6	89 (4.55/506)	90 (304/306)	99 (502/506)	99 (502/506)	94 (477/506)				
PCV2MX QRO 7	99 (504/506)	90 (4.57/506)	89 (4.55/506)	90 (4.57/506)	91 (4.61/506)	89 (4.53/506)			
PCV2MX SON_N 8	89 (4.55/506)	90 (304/306)	99 (502/506)	99 (504/506)	94 (477/506)	90 (303/306)	89 (4.55/506)		
PCV2MX SON_G 9	90 (4.58/506)	90 (303/306)	99 (503/506)	99 (505/506)	94 (479/506)	90 (303/306)	90 (4.58/506)	99 (505/506)	
PCV2MX YUC II	90 (4.56/506)	90 (303/306)	99 (503/506)	99 (503/506)	94 (478/506)	90 (303/306)	90 (4.56/506)	99 (503/506)	99 (304/306)

The phylogenetic analysis (Figure 1) shows that the Mexican PCV2 isolates are divided in 3 principal clusters: the first has 2 sub-clusters that correspond to the Central and Southeast regions with 3 sequences; the second is formed only by one sequence of the Central Region; and the third cluster includes the majority of the sequences, and is sub-divided in 3 sub-clusters with a subcluster with two sequences from the North of Mexico; one more subcluster with just one sequence from the north of Mexico too; and one cluster with the 2 sequences that should the greatest heterologies obtained from the low laying area region.

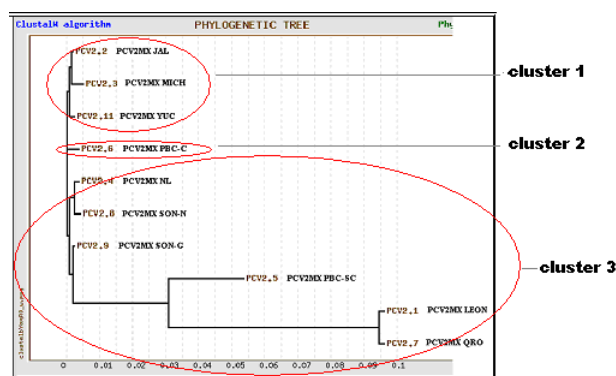


Fig. 1 Phylogenetic tree of Mexican PCV2 isolates.

The analysis of the amino acids sequences shows that there was a range from 88% until 100% amino acid identify among Mexican PCV2 isolates. Again, the sequences obtained from Guanajuato y Queretaro were the most different compared to those obtained in the others regions (see Table II). When the Mexican virus amino acids sequences were compared with the PCV2 isolates recovered around the world (Figure 2), it was possible to identify that in Mexico there are virus of PVC2- a and PCV2- b; in the phylogenetic tree (Figure 3) the sequences Guanajuato (MX LEON) and Queretaro (MX QRO) are into the group a and the others belong to group b, this is the reason why there is a big differences. Also the majority of sequences are closely related with European virus, except for Puebla (MXPBC) that is more related with one virus from China and Yucatan (MXYUC) that is closely related with Canadian virus.

TABLE II
AMINO ACIDS HOMOLOGY BETWEEN THE MEXICAN PCV2 ISOLATES.

homo-logy, %	PCV2MX LEON 1	PCV2MX JAL 2	PCV2MX MICH 3	PCV2MX NL 4	PCV2MX PBC_SC 5	PCV2MX PBC_C 6	PCV2MX QRO 7	PCV2MX SON_N 8	PCV2MX SON_G 9
PCV2MX JAL 2	88								
PCV2MX MICH 3	88	100							
PCV2MX NL 4	88	100	100						
PCV2MX PBC_SC 5	89	94	94	94					
PCV2MX PBC_C 6	88	99	99	99	94				
PCV2MX QRO 7	100	88	88	88	89	88			
PCV2MX SON_N 8	88	100	100	100	94	99	88		
PCV2MX SON_G 9	88	99	99	99	94	98	88	99	
PCV2MX YUC II	88	100	100	100	94	99	88	100	99

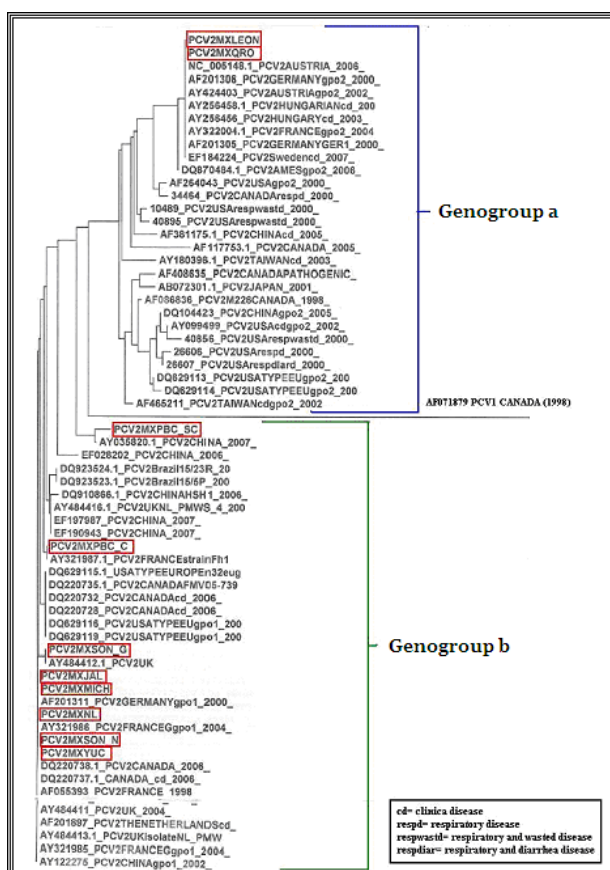


Fig. 3 Phylogenetic tree of Mexican PCV2 isolates and others PCV2 virus around the world. In the red boxes are indicated the sequences from Mexico. Subgenotype A and Subgenotype B.

The analysis of the main changes between amino acid sequences of both PCV2 genogroups found in Mexico shows that there are 20 changes in the capsid protein; the most of these changes are in the domain region situated in the position from aa59 to aa91. In the table III is showed in detail.

The couple of Mexican sequences that are into genogroup a do not show changes in the amino acid sequence and their sequences are closely related with European sequences. Regarding genogroup b, the sequences obtained from Puebla region show change from R₆₃ to K₆₃ in PCV2MX PB_C; and

R₅₉ to K₅₉, R₈₉ to L₈₉, L₁₂₃ to I₁₂₃, T₁₅₁ to P₁₅₁ and A₁₉₀ to S₁₉₀ in PCV2MX_PB_SC.

TABLE III
MOST FREQUENT CHANGES OF AMINO ACIDS IN PCV2 SEQUENCES FROM MEXICO

sample	amino acid position																			
	59	63	72	76	77	89	90	98	99	101	123	125	131	136	151	155	189	190	191	206
PCV2MEXO	A	S	L	L	D	V	T	K	I	S	I	I	Q	P	N	S	R	K	D	
PCV2MEXON	A	S	L	L	D	V	T	K	I	S	I	I	Q	P	N	S	R	K	D	
PCV2MEXAL	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOL	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXON_M	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_2	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_3	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_4	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_5	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_6	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_7	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_8	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_9	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E
PCV2MEXOCH_10	R	R	M	I	N	L	S	P	R	V	S	V	T	L	T	L	A	G	I	E

IV. DISCUSSION AND CONCLUSIONS

PCR is a sensitive diagnostic method and is widely used for pathogen detection, including PCV2. Since PMWS was described in Canada (1997), PCV2 has been isolated from different samples in Germany, France, Spain, Korea, Japan and other important countries producers of pigs [6]. In America there are reports of the presence of PCV2 in United States, Canada, Costa Rica and Brazil; but in Mexico there is just one report of the presence of antibody and few cases reports isolated. This is the first report of the distribution of PCV2 in Mexican herds (with PCR method), and show that PCV2 virus is distributed around all the Mexican territory. Nine of ten Mexican regions were positives for PCV2 which indicates that PCV2 virus is present in the majority of the Mexican territory.

In the current study, the genomics sequences of ten PCV2 viruses from the pigs shared 92-99% identity with PCV2 strains from GenBank, and did not display homology with PCV1 strains. The phylogenetic analysis showed that the sequences of Mexican PCV2 viruses are distributed into 3 clusters and the homology between the amino acids sequences is displaying of 88% until 100%.

According with the results from this work apparently exist a geographical relationship in which subgenotype is present. Is important to mention that in Mexico there are the two subgenotypes groups (a and b) of PCV2 virus that have been already described in different studies but using different nomenclature [2], [7], [9], [13], [29], [31], but the most prevalent is the subgenotype b (with more that 80%) in contrast with the situation in USA where the subgenotype a was the only one reported until 2007 [7]; however we could not establish the clinical significance of this divergence due in all the cases the farms showed clinical disease compatible with PDNS; this is according with previous reports (Opriessnig *et al.*, 2008) describing that no significant differences in virulence between isolates from PCV2a and PCV2b clusters are observed.

Other important observation is that the majority of Mexican virus sequences are closely related with European PCV2s, for the PCV2- group a with Austria, Germany, France, Hungarian

and Sweden, and the virus inside of PCV2- group b have relation with European, Asian and Canadian virus.

The amino acid alignment identified three major heterogenic regions between different ORF2 PCV2 sequences in regions 59-91, 123-136 and 185-210, this finding is according to similar observations that have been already described [13] and also corresponding to the antigenic domains (65-87, 113-139 and 193-207) described previously by Mahe *et al.* (2000). Our results show that up to 20 amino acid positions located in positions 59- 210 were constantly different between sequences included in genotype a in respect those included in genotype b.

The principal differences (according with the type of amino acid) in the two genogroups of Mexican PCV2 virus, are the changes in 11 aa of the ORF2 with Ala₅₉/ Ser₆₃/ Asp₇₇/ Lys₈₈/ Ile₈₉/ Ile₁₃₁/ Gln₁₃₆/ Pro₁₅₁/ Ser₁₉₀/ Arg₁₉₁/ Lys₂₀₆ in PCV2 group a and Arg₅₉/ Arg₆₃/ Asn₇₇/ Pro₈₈/ Arg₈₉/ Thr₁₃₁/ Leu₁₃₆/ Thr₁₅₁/ Ala₁₉₀/ Gly₁₉₁/ Ile₂₀₆ in PCV2 group b (the numbers following the amino acid indicates the position in the amino acid sequence). On the other hand, the most important difference of the amino acid sequences within the genogroup b is located in the position 89, 151 and 190 where there is a substitution from an aminoacid basic polar to a nonpolar, from a uncharger polar to nonpolar and from nonpolar to uncharger polar, respectively.

In conclusion, these results have demonstrated that both subgenotypes of PCV2 are present in at least in 90% of the principal zones for swine industry in Mexico and also has contributed to the understanding of PCV2 epidemiology in Mexico. Is important to conduct future research in order to understand the economic impact of this pathogen in the Mexican swine industry and demonstrate if one subgenotype is more pathogenic than other (as has been reported recently in USA and Canada by Cheung *et al.*, 2007). One more time it was confirmed that PCV2 virus is present around the entire world.

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