Journal of Animal and Veterinary Advances 14 (5): 130-135, 2015
ISSN: 1680-5593
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Infection Frequency and Genetic Variability of Porcine Reproductive and Respiratory Syndrome Virus in Several States of the Mexican Republic

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Abstract: The aim of this study was to determine infection frequency and genetic variability of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in farms with suggestive clinical data located in seven states of the Mexican Republic. Sixty two farms were visited to collect blood and tissue samples, on a convenience transversal approach from animals slaughtered for decreased weight gain. Specific serum antibodies were measured by ELISA. Viruses were isolated from macerated tissues filtered and applied onto MARC-145 cells or detected by RT-PCR using specific primers to amplify 300 bp from the viral ORF7. To determine genetic variability primers amplifying 809 bp from ORF5-ORF6 were used. To differentiate between American and European strains, two sets of primers that separately amplify 337 and 241 bp from ORF7 were employed. Puebla, Veracruz, Mexico, Guanajuato, Michoacan, Queretaro and Jalisco States had PRRSV seropositive farms with 45-100% frequencies and 20-98% of positive animals. In positive farms, PRRSV was detected by RT-PCR in at least a tissue sample. Phylogenetic analysis showed high variability for PRRSV and co-existence of genetically different isolates within herds. Differential RT-PCR detected only American strains. PRRSV was isolated in three from seven sampled states. We conclude that PRRSV frequency in Mexican farms is similar to those reported for previous years and PRRSV Mexican isolated have great variability.

Key words: PRRS, diagnosis, serology, RT-PCR, isolated

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most important infectious diseases for swine industry, causing considerable economic losses worldwide (Blaha, 2000). In individual herds, direct costs relate to production losses, increased mortality and reproductive failure (Velasova *et al*., 2012). PRRS is characterized by late-term reproductive failure in pregnant sows, respiratory signs in pigs from all age groups and substantial weight losses in fattener animals (Benfield *et al*., 1992).

PRRS etiologic agent is an enveloped, singled-stranded Positive-Sense RNA Virus (PRRSV) with a diameter of 50-65 nm. It has a genome ~15 kb in size (Spilman *et al*., 2009) and is classified as a member of the order Nidovirales, family Arteriviridae, genus *Arterivirus* (Cavanagh, 1997). PRRSV genome has ten Open Reading Frames (ORF) (Conzelmann *et al*., 1993; Firth *et al*., 2011; Johnson *et al*., 2011). ORF1a and ORF1b represent from 75-80% of viral genome and encode for 14 non-structural proteins involved in viral replication and transcription in addition ORF2 to ORF7 codify for structural proteins: GP2a, E, GP3, GP4, GP5, membrane protein (M) and nucleocapsid protein (N) (Snijder and Meulenberg, 1998). Interestingly, ORF5 has the highest genetic variability whereas ORF6 and 7 are the most conserved (Nelsen *et al*., 1999).

PRRSV is divided into two distinct genotypes, genotype I (European) and genotype II (American) based both on genetic differences and geographical distribution. Both genotypes can co-circulate in geographical regions and herds (Goldberg *et al*., 2003). Lelystad and VR2332 strains are prototypes of genotype I and II, respectively

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(Benfield *et al*., 1992; Wensvoort *et al*., 1991); there is a greater genetic diversity in American strains than European ones which in turn are genetically more related (Kapur *et al*., 1996).

To detect PRRSV Virus Isolation (VI) is traditionally used, however, this technique is laborious, time consuming and has reduced sensitivity, since requires a well and quick processed sample in order to preserve virus infectivity (Benson *et al*., 2002). For diagnosis in situ hybridization and immunocytochemistry are direct methods also frequently used (Cheon and Chae, 2000). To determine specific antibodies to PRRSV Indirect Immunofluorescence Antibody (IIFA), Seroneutralization (SNT), Immunoperoxidase (IP) and Enzyme Linked Immunosorbent (ELISA) assays have been developed (Collins *et al*., 1996).

Because of its economic relevance, in several countries molecular methods such as Reverse Transcription-Polymerase Chain Reaction (RT-PCR) have been implemented. This procedure has been shown as confident, sensitive and rapid to diagnose PRRSV from different types of samples (Suarez *et al*., 1994; Christopher-Hennings *et al*., 1995; Gonzalez *et al*., 2008).

Primers targeted to ORF7, the N gen are more frequently used to detect PRRSV because it represents a conserved region. In a complementary way ORF5 is used for analysis of viral genetic variability, since its localization in the viral envelope confers it a higher variability (Andreyev *et al*., 1997).

Serological studies at United States have reported PRRSV occurrence in up to 80% of swine herds. In Mexican Republic, serological surveillances performed from 1995 have indicated that PRRSV is thoroughly distributed and seropositive pigs are often detected in most of industrialized farms, also called “technified”. However, more studies to know up to date frequency data are required.

Consequently, the main objective of this study was to determine the PRRSV current frequency as well as its genetic variability, in several states of the Mexican Republic.

MATERIALS AND METHODS

Sampling: A convenience transversal sampling was done selecting complete-cycle industrialized farms, from cooperating producers where the occurrence of disease was clinically suspected. Sixty two farms located in Puebla (3), Veracruz (5), Mexico (10), Guanajuato (6), Michoacan (16), Queretaro (11) and Jalisco (11) States were sampled.

Samples: For serology, blood samples from 30 fattener animals, between 4 and 6 months aged and 30 sows, from first to sixth delivery were taken. For virus isolation and molecular tests, one or two animals with considerable growth retardation and respiratory problems were slaughtered taking tissue samples from lymphatic nodes, tonsils and lungs. Samples were stored at -70°C until used.

Serology: Specific antibodies to PRRSV were detected by ELISA (HerdCheck, IDEXX Laboratories, Maine, USA) which is routinely used as a gold standard to screen the serological status of herds since is a test with 97.4% sensitivity and 99.6% specificity (Ferrin *et al*., 2004).

RT-PCR

ARN isolation: RNA was extracted from cell culture supernatants, blood and tissues as following. From each animal equivalent fragments of selected tissues: lymphatic node, tonsil and lung were pooled, macerated in physiologic saline solution 1:5 w/v and centrifuged at 14,000 g for 5 min; supernatants were recovered. The 200 μL from each sample were processed using the high pure PCR Template kit (Roche) as indicated by the manufacturer but in the final step RNA was eluted in 50 μL of sterile water for injection.

cDNA and amplification: RT and amplification were done in the same tube. Primers used to identify PRRSV, amplifying a ~300 bp from ORF7 were 5'-CCA GCC AGT CAA TCA RCT GTG-3' and 5'-GCG AAT CAG GCG CAC WGT ATG-3' (Donadeu *et al*., 1999). Positive samples were also amplified with primers targeted to ORF5-6: 5'-TTG ACG CTA TGT GAG CTG AAT G-3' and 5'-ACT TTC RAC GTG GTG GGC-3' (Ogawa *et al*., 2009), generating a 809 bp amplicon. To differentiate between American and European strains two sets of primers were used: 5'-AGT CCA GAG GCA AGG GAC CG-3' and 5'-TCA ATC AGT GCC ATT CAC CAC-3' for American strains; and 5'-ATG ATA AAG TCC CAG CGC CAG-3' and 5'-CTG TAT GAG CAA CCG GCA GCA-3' for European strains which generate amplicons of 337 and 241 bp, respectively from ORF 7 (Truyen *et al*., 2006).

PCR mixture contained 1x reaction buffer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer, 1.25 U of Taq polymerase, 12 U of reverse transcriptase, 5 U of RNase inhibitor, 3 μg of bovine serum albumin and 1 μL of RNA template into a final volume of 25 μL. PCR was done with an initial cDNA synthesis and pre-denaturation step of 48°C for 30 min and 94°C for 10 min followed by then 35 cycles of denature, annealing and extension (1 min at 94°C, 1 min at 58°C, 1 min at 72°C)

and a final step of 7 min at 72°C, for complete extension. The 8 μL of each amplified product were analyzed by electrophoresis on 1.5% agarose in Tris-Acetate-EDTA buffer (TAE) containing ethidium bromide.

Sequencing: For sequencing, ORF5 and 6 amplified products were purified by the Wizard® SV Gel kit and PCR Clean-Up System (Promega), following the manufacturer protocol.

Phylogenetic analysis: Nucleotide obtained sequences were analyzed to construct a phylogenetic tree by the Software MEGA 5.05 using the Maximum Likelihood Method and a 500 replicates bootstrapping. Sequences of reference strains VR2332 and vaccine Ingelvac were comparatively included.

Virus isolation: When a pig resulted positive for RT-PCR, its tissues were individually macerated, approximately 1 g in 5 mL of physiologic saline solution and centrifuged; supernatants were recovered, 0.45 μm filtered and stored at -70°C until used. Filtrates, 200 μL from each one were inoculated in duplicate onto 50-60% confluent MARC-145 cell monolayers seeded in 24 well plates (Nunc). Four wells were mock inoculated with medium alone. VR2332 strain was inoculated onto two cell monolayers as positive controls. Viruses were adsorbed for 1 h at 37°C in a 5% CO_2 humid atmosphere, discarded and new maintenance medium was added. Multiwell plates were incubated at 37°C as before and read daily for 6 days searching the presence of Cytopathic Effect (CPE). After the 6 day period, medium and cells were harvested, scraped if necessary and stored at -70°C until used. First passage was examined by RT-PCR to confirm viral infection of samples.

RESULTS AND DISCUSSION

Serology: It was initially determined the prevalence of antibodies to PRRSV in Mexican complete-cycle industrialized farms. Sixty two industrialized farms were sampled in seven states of the Mexican Republic. We found that 79% (49/62) of these farms had PRRSV seropositive animals. From 1,461 collected sera, 46% (671/1461) had detectable antibody levels. Table 1 shows serology results per farm and state.

RT-PCR: Complementary results were seen by RT-PCR for all studied states but Jalisco. From 33 swine farms, 191 samples were collected. Results are summarized in the Table 2.

Table 1: Frequency of pigs having antibodies against the PRRSV determined by ELISA in swine farms from seven states of the Mexican Republic

States	Farms	Positive/ Total farms	Percentage	Positive/ Total sera	Percentage
Michoacan	16	16/16	100	150/152	98
Jalisco	11	11/11	100	94/107	88
Guanajuato	6	6/6	100	73/84	87
Veracruz	5	3/5	60	78/165	47
Mexico	10	6/10	60	187/536	35
Puebla	3	2/3	67	25/95	26
Queretaro	11	5/11	45	64/322	20

Table 2: PRRSV detection by RT-PCR targeted to ORF7, in tissues, sera and blood of animals from industrialized farms in six states of our country

States	Sampled herds	Positive/Total herds	Positive/Total samples
Veracruz	4	3/4	4/14
Mexico	9	3/9	6/112
Guanajuato	6	6/6	23/35
Puebla	3	3/3	4/5
Queretaro	10	1/10	1/21
Michoacan	1	1/1	2/4
Total	33	17/33	40/191

Phylogenetic analysis: All 191 samples were analyzed by RT-PCR (ORF5-6) for the presence of PRRSV viral RNA. Eleven positive samples were amplified and sequenced to construct their phylogenetic tree (Fig. 1).

Virus isolation: Finally, a total of 97 tissue samples (lymphatic nodes, tonsils and lungs) from 17 RT-PCR positive herds were inoculated onto MARC-145 cells. Virus isolates were obtained just from herds in Guanajuato, Veracruz and Puebla States. Figure 2a shows the distinctive CPE of PRRSV.

PRRS directly causes huge economic losses in swine industry worldwide but in Mexico infection frequency and genetic variability current data are scarce. In this study, we found that all seven sampled Mexican states were positive for PRRSV infection with positive farm frequencies between 45 and 100% and positive animals percentages of 20-98%; these findings are consistent with studies done since 1995 for some regions in our country. In other study, Rovelo (2010) found that 100% of sampled farms were positive in Yucatan State with 3-97% of animals being PRRSV seropositive.

Our data indicate that states with higher frequencies of seropositive farms were Michoacan, Jalisco and Guanajuato. This is easily explained because of elevated density and transit of pigs in this region which surely drives a faster PRRSV spreading. On the contrary, Queretaro State showed the lowest infection frequency which in turn could be related to a lower swine density, significantly lesser than those of States above referred.

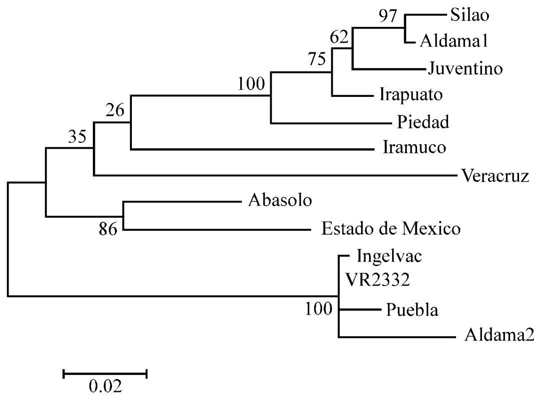

Fig. 1: Phylogenetic tree of eleven PRRSV isolates from Mexican Republic and reference strains. The tree is based on 809 bp fragment encoded by a region comprising ORF5 and 6. Phylogenetic analysis was conducted by using Mega5.05. Percentages of replicate trees in which the associated isolates clustered in the bootstrap test (500 replicates) are shown next to the branches. Scale bar indicates nucleotide substitutions per site

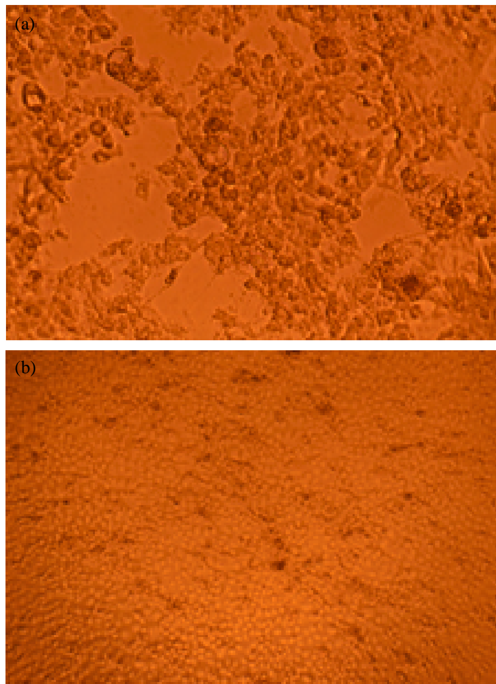

Fig. 2: a) MARC-145 cells inoculated with a PCR-positive sample showing the PRRSV CPE on the 7th day post-infection and b) MARC-145 cells mock inoculated

Interestingly, in Mexico State with the greater number of serum samples (536), a relatively high percentage (60%) of farms had PRRSV seropositive animals, although, a relatively low percentage (35%) of animals resulted seropositive, especially if compared to Veracruz. This paradoxical data may be explained since in Mexico State farms are distributed in municipalities with great territories and swine factories are far away from each other, so effective control and preventive measures are more easily to take.

In Veracruz and Puebla with lower numbers of sampled farms, relatively high frequencies (60 and 67%, respectively) of seropositive farms were observed. A previous study reported that 100% of farms in Puebla State were seropositive. Then we think that more extensive studies in these neighboring regions are required in order to estimate more accurate PRRSV infection frequencies.

Detection of viruses frequently relies on the detection of viral nucleic acids in clinical samples and RT-PCR for detection of PRRSV RNA has been found to have greater advantages than serology and viral culture (Benson *et al*., 2002). By RT-PCR, PRRSV was detected in at least one sample from every positive farm, excluding Jalisco farms where tissue sampling were not allowed. PRRSV was identified in every sampled farm in Guanajuato (6/6) and Puebla (3/3) with 66% (23/25) and 80% (4/5) of positive samples, respectively. Strikingly, in Puebla virus was detected in all three sampled farms, meanwhile serology only detected specific antibodies in two out of three farms. This observation suggests that the virus identified only by RT-PCR could be antigenically different from others; alternate explanations would be that antibodies were not recognized by ELISA or an infection was recently acquired by herd. By the way, antigenic variability of PRRSV field strains as an RNA virus, explains the ineffectiveness of current vaccines which in turn makes disease control difficult such as has been described elsewhere for PRRSV (Li *et al*., 2011) and other RNA viruses.

On the other hand, using differential RT-PCR, we identified only American strains as reported before. However, it is urgent to study more comprehensively different regions in our country to confirm the presence of only American strains of PRRSV. We also investigated the genetic variability of the PRRSV isolated strains and have shown that isolates from several Mexican states present a high degree of variability related to vaccine virus (Ingelvac) and reference strain (VR2332) and genetically different isolates may co-exist within a herd as observed in the phylogenetic tree for the isolates Aldama 1 and 2 from the same region. This explains furthermore why the approved vaccine is not efficient for controlling PRRS in our country.

Alveolar macrophages have been described as the best host cell for isolating PRRSV. Nevertheless, their production is complicated and time-consuming (Wensvoort *et al*., 1991) and permissiveness to PRRSV substantially varies in different macrophage lineages. In this study, MARC-145 cells were used because of its suitability to isolate PRRSV from different tissues (Kim *et al*., 1993). Tonsils, lymphatic nodes and lungs were chosen to be examined due to the known tropism of PRRSV for these organs. PRRSV isolates were obtained from farms located in Guanajuato, Puebla and Veracruz. Isolates were not obtained from farms in Michoacan and Queretaro, however, here virus was detected by RT-PCR. It is likely that VPRRS was not yet viable in clinical samples due to harsh conditions of the field work: temperature variations, long periods of transportation to lab and delay in sample processing.

The sensitivity and specificity of diagnostic tests are generally based on a "gold standard". Unfortunately, there is currently no recognized gold standard for detection of PRRSV in some cases (Benson *et al*., 2002). So results from this study, supports the approach that always is the best to do more than one test to confirm the presence of PRRSV in swine herds.

CONCLUSION

Puebla, Veracruz, Mexico, Guanajuato, Michoacan, Queretaro and Jalisco States of Mexican Republic had farms PRRSV seropositive. Frequencies of PRRSV seropositive animals in Mexican farms were similar to those informed since 1995. By RT-PCR, PRRSV was detected in at least one sample of every seropositive farm sampled. Using differential RT-PCR, only American strains were detected in Mexican farms. Phylogenetic analysis indicates a great PRRSV variability and shows that different virus can co-circulate in swine farm which evidently difficult the disease control by only using the approved vaccine. In our hands, MARC-145 cells were useful for isolation of PRRSV.

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