Detection of porcine reproductive and respiratory syndrome in porcine herds of Baja California, Mexico

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Abstract:

The objective of this study was to assess the presence of genotype 2 porcine reproductive and respiratory syndrome virus (PRRSV-2) in Baja California (Baja), as well as the standardization of the qRT-PCR technique. A cross-sectional study was conducted from 2016 to 2017 in farms from Baja. It was obtained 97 blood samples from clinically healthy, not-vaccinated boars and sows. Primers were designed and standardize, in order to perform qRT-PCR tests from the buffy coat. Every positive results were confirmed by sequence studies. It

was found that 9.3 % of the samples were positive. The positive samples came from 66.6 % of the sampled regions. This study demonstrates the presence of PRRSV-2 in Baja, therefore, it is necessary to conduct epidemiological studies in order to identify the magnitude of the problem and to establish preventive and control measures.

Key words: Detection, Mexico, PCR, PRRS, Swine.

Porcine reproductive and respiratory syndrome (PRRS) is a disease caused by an RNA virus, with two currently known genotypes: 1 (European) and 2 (American)[1]. It is easily transmitted by saliva, nasal secretions, urine, semen, milk and colostrum[2]. It produces reproductive failure, weak-born piglets and respiratory diseases[3]. The main route of introduction of PRRS virus (PRRSV) into previously free countries is via pig movements and with introduction of semen; therefore, protocols must be in place to reduce the risk[4]. When first introduced into an immunologically naive herd, the virus spreads to pigs of all ages in about 2 to 3 wk[2], with mortality rates in nursery pigs up to 69 %[5]. Most common diagnosis tests are commercial ELISA[6] and in recent years, molecular techniques, particularly real time reverse transcriptase polymerase chain reaction (qRT-PCR)[7].

In Mexico, PRRSV was first described in 1994 with an 8.1 % serological prevalence in imported pigs from USA and Canada[8]. Since 2002, it has been reported the presence of multiple variants of genotype 2 PRRSV (PRRSV-2) within pig farms throughout Sonora[9-10], neighbor state of Baja California (Baja), and the second biggest pork producer in Mexico. Meanwhile, in Baja, the main pork production comes from small backyard producers, with installations made basically from rustic materials, with the same areas used for the different production stages; generally fed with swill and other food waste sub-products; usually with none preventive medicine programs or veterinary's advice and in most cases not even adequate hygiene practices.

The main objective of this study was to assess the presence of genotype 2 porcine reproductive and respiratory syndrome virus in the most representative pork production regions in Baja California, Mexico, besides the standardization of the qRT-PCR test for this disease.

The study was approved by the Institutional Committee for Animal Ethics, which is represented by the Academic Group of Animal Health and the Academic Group for Diagnosis of Infectious Diseases, both of which are part of the Institute of Research in Veterinary Sciences. The owners of pigs used in this research were informed about the study and they gave their consent.

A cross-sectional study was conducted in 26 farms within six regions of Baja: Ensenada, Mexicali, Tecate and Tijuana, as well as the Mexicali and San Quintin valleys. The farms were selected from a Baja California Pig Farmers Association Database and invited to participate according to their proximity and herd size. Only those who were interested were visited. Estimation of sample size was done for one disease detection[11], considering a state swine population of 10,315[12], a 99.5% diagnostic sensitivity, 4% expected prevalence and 95% confidence level.

$$n \cong \frac{(1 - (1-\alpha)^{1/D}) \, (N - 1/2(SeD - 1))}{Se}$$

where, n= sample size; N= population size; D= number of diseased; α= confidence level; Se= test sensitivity.

Accordingly, it was needed a sample size of at least 74; however, it was possible to collect a total of 97 blood samples from the jugular vein of apparently healthy boars and sows not involved in vaccination against PRRSV. It was used sterile Vacutainer® tubes with EDTA anticoagulant (BD, Franklin Lakes, NJ, USA). Samples were transported to the Molecular Biology Laboratory of the Institute of Research in Veterinary Sciences, then there were separated 200-300 µL of the buffy coat into sterile tubes for RNA extraction. RNA extraction was made using Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The RNA was reconstituted to a final volume of 30 µL of prepared elution. RNA was stored at -70 °C until the qRT-PCR test were performed.

The RT-PCR primers were designed to amplify a fragment with a length of 87 bp of the nucleocapsid gene contained within the open reading frame 7 (ORF7) of PRRSV-2 (GenBank AF494042.1), since this is the most conserved viral protein in PRRSV-infected pig cells[13]. The primers were designed using Primer3Plus version 2.4, GenneRunner version 6.1 and OligoCalc version 3.2, generating the primers PRRS-USA-F 5'-CGATCCAGACTGCCTTTAAC-3' and PRRS-USA-R 3'-CACTGTGGAGTTTAGTTTGC-5'.

The qRT-PCR conditions were optimized by testing primers in triplicate at 200, 400 and 800 nM with 1, 2 and 3 µL of RNA template in a total volume of 10 µL using a master mix with EvaGreen® dye (Biotium, Hayward, CA, USA). The best efficiency was achieved by using the primers at 800 nM in 2 µL of RNA template and was proved it through ten-fold dilutions to generate a melt curve analysis (Figure 1) and comparing it with agarose gel

electrophoresis. Once obtained the best concentration, it was tested by triplicate 40 control positive samples and 40 negative samples so to achieve a 95% confidence of specificity[14]. The sensitivity was determined through ten-fold dilutions by generating a standard curve of 97.6 % efficiency, R^2 of 0.996 and 3.25 slope (Figure 2).

Figure 1: Amplification peaks of different positive control dilutions in the melting temperature previously stablished (77.4-78.0 ℃)
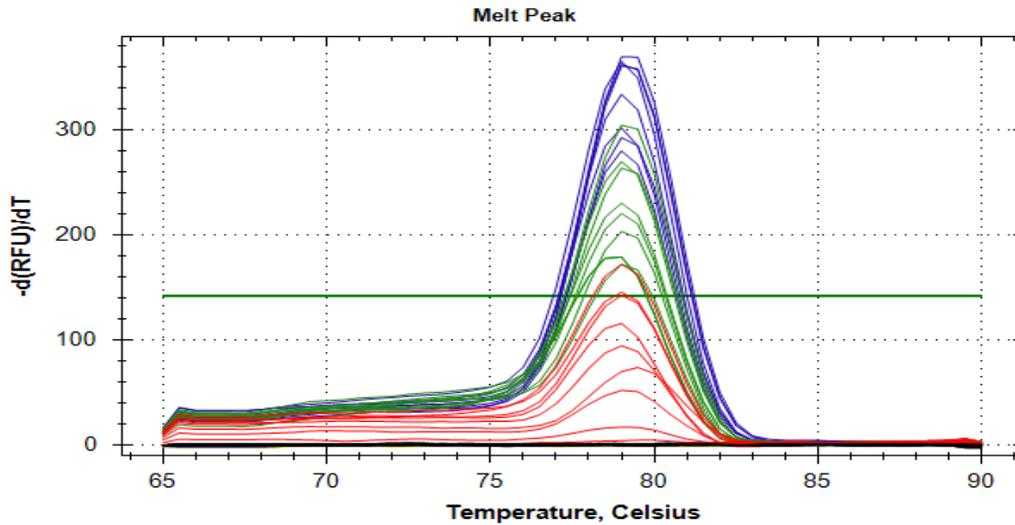

Figure 2: Standard curve of the designed primers
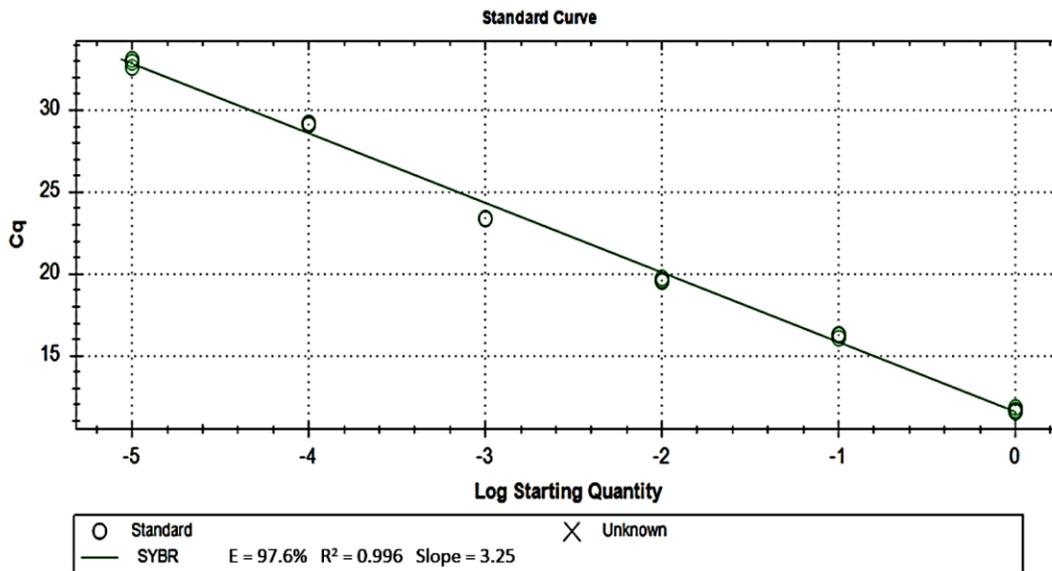
Positive control RNA was extracted from Ingelvac PRRS® MLV (Boheringer Ingelheim) vaccine. Three different negative controls were used: master mix without RNA template, molecular grade water and air. All samples were tested in duplicate. The qRT-PCR reactions were executed in a CFX96 real-time thermocycler (Bio-Rad, Hercules, CA, USA). Test reactions consisted of 1 µl of RNA, 400 nM of each primer and master mix of iScript One Step RT-PCR with EvaGreen dye and molecular grade water in a total reaction volume of 10 µL. Thermocycler conditions were calculated using CFX96 software, resulting in an initial step of reverse transcription at 50 °C for 10 min, denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 10 sec, annealing at 53.7 °C for 25 sec and extension at 72 °C for 20 sec. A melt curve analysis was performed after each run in order to confirm the melt temperature (Tm) of the amplified fragment, calculated between 77.4 and 78.0 °C. The positive samples were confirmed by sequencing at an external laboratory and these sequences, verified using BLAST tool (Figure 3).

**Figure 3:** Sequence of positive samples and verified using BLAST tool
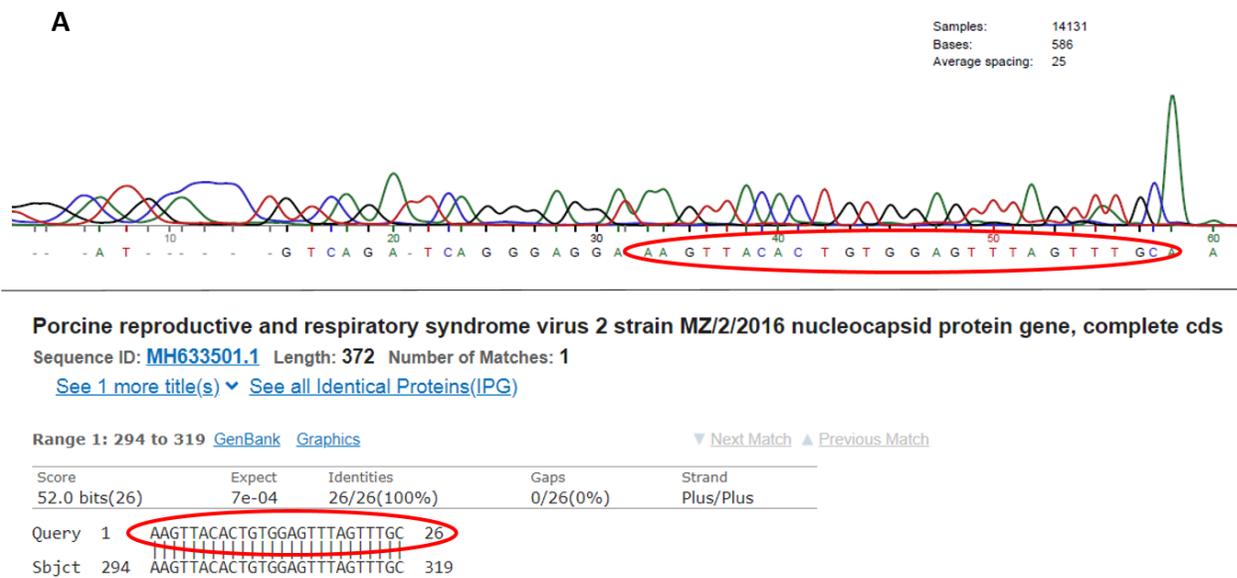
Panel A: Sequencing results of one of the positive samples, highlighting a sequence of 26 nucleotides. Panel B: BLAST database screenshot, showing the 26 nucleotides of the panel A, highlighted and matching a type 2 porcine reproductive and respiratory syndrome virus strain in the nucleocapsid protein gene. The database casts 100 results (not shown), all of them PRRSV-2 strains.

It was found 9.3 % (9/97) positive samples to PRRSV-2, present in 66 % (4/6) of the regions where the study was carried out. The frequency of positive cases was 33 % (2/6) in Tijuana, 16.6 % (3/18) in San Quintin Valley, 11.1 % (3/27) in Mexicali and 3.7 % (1/27) in Ensenada (Figure 4). In Tecate (0/13) and Mexicali Valley (0/6) there was no positive samples. Signs of PRRS were not found or reported on any farm.

Figure 4: Geographical distribution of PRRSV in Baja California and the frequency in each tested area
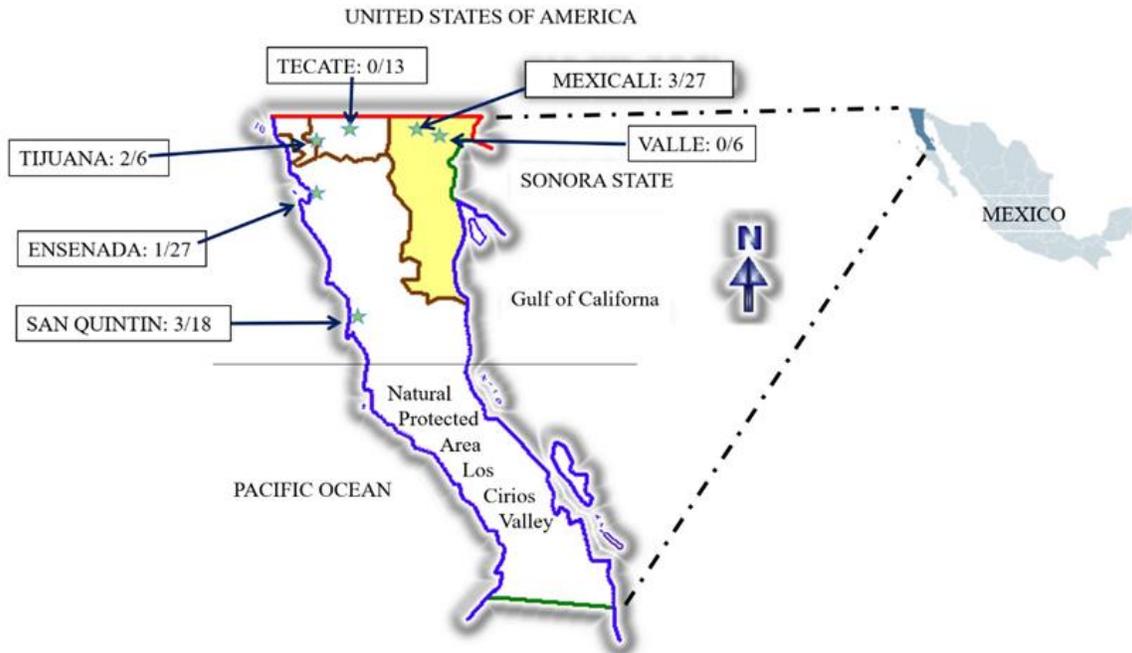
The main objective of this study was to assess the presence of type 2 porcine reproductive and respiratory syndrome virus in the most representative pork production regions in Baja California, therefore, considering the epidemiological design of the study can state that PRRSV-2 is present in Baja, with a prevalence of at least 4 %.

This study represents the first report of PRRSV-2 in Baja[15], despite the previous report of its presence in the northwest area of the country[16] which includes, besides the state of Baja, the state of Sonora and four other states. It is important to highlight the geographical proximity with Sonora as well as the characteristics of their pork industry, since Sonora introduces pork meat as well as live pigs and semen into Baja[17], thus the possibility of PRRS contagion is present considering that vaccination against PRRSV has never been implemented in Baja since it is supposed to be free of the disease and furthermore, the biggest proportion of small backyard producers, who do not usually use primary preventive medicine measures.

In this study were also designed primers capable of detecting PRRSV-2 in the ORF7 region, and these results were confirmed by sequencing, proving the effectiveness of the test and the presence of PRRSV-2 in Baja, regardless of the lack of clinical signs[18]. This might be owed to the presence of low-virulence strains of PRRSV within Mexican territory[19]; or a low viral concentration within the samples[20] given the amplification of the positive curves was observed after cycle 30.

This study demonstrates the broad presence of PRRSV-2 in Baja, even in absence of clinical signs that indicate the presence of the disease; therefore, it is necessary to make prospective epidemiological studies aiming at determining the prevalence and the possible associated risk factors in order to identify the magnitude of the problem as well as to establish preventive and control measures.

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Conflict of interest statement

The authors have no financial or personal relationship with people or organizations that could inappropriately influence or bias the content of the paper.

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