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Differentiation Between American and European Strains of the Porcine Reproductive and Respiratory Syndrome Virus in Mexico by the Establishment of a Multiple PCR

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Abstract: The existence of sensitive and fast diagnostic techniques for the Porcine Reproductive and Respiratory Syndrome (PRRS) is very important because of the high presence frequency of the disease in farms causing problems in the swine industry. A commercial vaccine and organs from animals inoculated with the virus were used in order to establish a multiple Polymerase Chain Reaction (mPCR) technique. Total RNA extraction was done using Trizol® reactive. A simple RT-PCR assay established to amplify a 255 bp product from both, American and European strains. A nested multiplex method was standardized to obtain a 107 bp product from American strains and a 186 bp product from European strains. The desired 255 bp product was obtained form the positive controls as well as from other two mPCR products. The amplification of a 107 bp was achieved from serological and seminal samples, which pointed out that they corresponded to an American strain. It was possible to establish the mPCR technique is able to differentiate between American and European strains. Through out this technique, it will be possible to carry out epidemiological studies to determine the origin of PRRS virus infection, which will allow having a better control of the disease.

Key words: Swine, PRRS, diagnostic, multiple PCR, American and European strains

INTRODUCTION

One of the most important viral diseases in swine worldwide is the Porcine Respiratory and Reproductive Syndrome (PRRS). It causes reproductive failure in breeding females, respiratory clinical signs in animals of several ages, as well as a remarkable weight loss in fattening pigs (Benfield *et al*., 1992). The first clinical reports of this disease started in 1990. The virus was isolated in 1991 in the Holland, United States and Canada (Meredith, 1991; Collins *et al*., 1992; Dea *et al*., 1992). The virus is included in the Arteriviridae family (Meulenberg *et al*., 1993); genomic and antigenic differences have been described between American and European strains (Katz *et al*., 1995). It is considered that the virus is infecting pigs in most of the countries these days. The presence of the virus has been reported in the United States in up to the 80% of the herds (Zimmerman, 2003). Serological surveys done in Mexico since 1995, have notified that it is widely distributed and that it is common to find antibodies in pigs from most of the technological farms (Weirmersheimer *et al*., 1997; Morilla *et al*., 2003). Direct Immuno Fluorescence (IF) (Done *et al*., 1992), Immuno Histochemistry (IHQ) (Halbur *et al*., 1995b) and Viral Isolation (VI) have been used for detecting PRRS virus, the problem in the last case is that it only replicates in two types of cell lines (Bautista *et al*., 1993). Indirect Immuno Fluorescence (IIF), Viral Seroneutralization (VSN), Immuno Peroxidase (IP) and Immunoenzymatic Assay (ELISA) tests have been developed for detection of antibodies against PRRS. Through out serological tests, it can be determined the infection phase of a herd. Furthermore, it has been proven that inside a group of breeding females there are different subpopulations regarding the viral infection grade (Dee *et al*., 1995; Diosdado *et al*., 1998).

Because of the economical importance of this disease, several countries have implemented molecular techniques like the Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR), which has been proven to be a reliable technique for PRRS virus diagnosis on different types of samples (Suarez *et al*., 1994; Hennings *et al*.,

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1995). Other techniques, such as the nested PCR (nPCR) and the multiple PCR (mPCR) have been developed in order to increase sensitivity and to be able to differentiate among viral strains (Kono *et al*., 1996; Gilbert *et al*., 1997). It is very important to have sensitive and fast diagnostic techniques for the control and elimination of this disease due to the high frequency of the PRRS virus presence in farms, affecting the swine industry in Mexico.

Because of the aforesaid, the aim of this study was to establish a mPCR technique that allows differentiation between American and European strains of the PRRS virus, besides being highly sensitive. This will provide a confirmatory diagnostic assay for several types of samples that will be useful to perform molecular epidemiological studies.

MATERIALS AND METHODS

Samples: A commercial vaccine and organ samples from animals experimentally inoculated with the PRRS virus were used for the establishment of this technique. Samples were kindly given by Dr. Maria Antonia Coba Ayala, researcher at the Virology Laboratory of the National Research Center for Animal Microbiology, National Research Institute for Forestry, Agricultural and Animal Production. In addition, serological and seminal samples from animals previously tested by ELISA were obtained. Sera and semen were provided by the Serological Laboratory of the Regional Union of Swine Producers of the State of Guanajuato.

RNA extraction: Total RNA was isolated from different kinds of samples with the Trizol reactive, according to manufacturer recommendations including some modifications (Chomczynski and Sacchi, 1987; Socci *et al*., 2003). In the case of tissues, (lymph node, lung and tonsil) 1 g of sample was weighed and macerated in PBS to a final 1:10 dilution. For seminal RNA extraction, 15 mL were previously mixed with 10% Kaolin volume/volume and left at room temperature for 15 min. After that, samples were centrifuged at 15000 rpm for 20 min. Extraction was done from the cellular fraction. For the vaccine and sera samples, 250 μL were taken, 1 mL of Trizol was added to all kind of samples and left still for 15 min at room temperature. Then 300 μL of chloroform were added, incubated for 10 min at room temperature and centrifuged for 15 min at 12000 rpm at 4°C. The watery phase was transferred into a clean tube; 500 μL of isopropanol were added to the sample and incubated at -20°C for 1 h in order to precipitate RNA. It was centrifuged for 15 min at 12000 rpm a 4°C. The precipitated phase was washed with 1 mL of 75% cold ethanol and centrifuged for 5 min at 7500 rpm at 4°C. Then it was left to dry at room temperature and resuspended in water free from RNAses, incubated 10 min at 60°C and frozen at -20°C until used.

PCR: A one step RT-PCR was carried out to obtain complementary DNA and its amplification (Socci *et al*., 2003) using the oligonucleotides described by Gilbert *et al*. (1997). The sequence of these oligonucleotides is: 5'-CCTCCTGTATGAACTTGC-3'; 5'-AGGTCCTCGAAC TTGAGCTG that amplify a 255 bp fragment of the gene coding for the ORF 1b region of the PRRS virus. A mPCR was done to differentiate between American and European strains from a 255 bp product using the following oligonucleotides: 5'-GGCGCAGTGACTAA GAGA-3'; 5'-GTAACTGAACACCATATGCTG-3' that amplify a 107 bp fragment for American strains and 5'-GTATGAACTTGCAGGATG-3';5'-GCCGACAATACCAT GTGCTG-3 of 186 bp for European strains (Gilbert *et al*., 1997). For the reaction mixture 2.5 mM of 0.2 mM $MgCl_2$ of each desoxinucleotide, 5.2 U of RNAse inhibitor, 12 U of Taq polymerase, 1X of reaction buffer, 10 pmol of each of the oligonucleotides and 1 mL of target RNA, were used in a final volume of 25 μL. One cycle of 48°C 30 min and 94°C 2 min and 35 cycles at 94°C 30 sec, 55°C 45 sec and 72°C 60 sec and one cycle at 72°C 5 min were applied to the mixture. The same reactive concentrations were used to perform the mPCR, except that the concentration of the two pairs of oligonucleotides were diminished at 5 pmol each, taking 1 μL of the product from the first reaction. The amplification program was: one 95°C 5 min cycle, 35 cycles at 94°C 20 sec, 47°C, 30 sec and 72°C 30 sec and 1 cycle at 72°C 15 min. Eight micro liter of each amplification product were analyzed by electrophoresis in 1.5% agarose gel in buffering Tris-Acetate-Edta solution (TAE) dyed with ethidium bromide. RNA from a commercial vaccine of the PRRS virus, containing the 2 types of strains, was used as positive control, as well as tissues from inoculated animals. Water free from RNAses was the negative control.

RESULTS AND DISCUSSION

The expected amplification of a 255 bp product was achieved from the positive controls (Fig. 1). Regarding mPCR, it was possible to observe 186 and 107 bp products in the vaccine and tissues from inoculated animals (Fig. 2). An amplified product from serological and seminal samples was successfully obtained by mPCR. Comparison between results yielded by ELISA and mPCR tests is depicted in Table 1. It was observed that the regular RT-PCR that was standardized was capable to identify PRRS virus from vaccines and tissues from animals

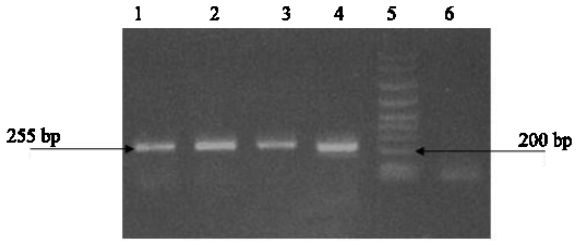

Fig. 1: Results obtained form a commercial vaccine and animal inoculated with the PRRS virus. Lanes 1, vaccine; lane 2-4, tissues (tonsil lymph node and spleen); lane 5, size marker; lane 6, control (-)

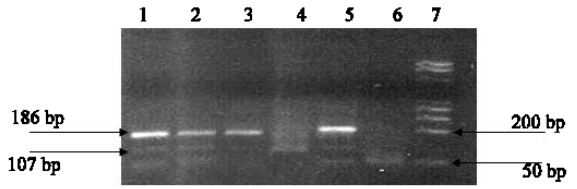

Fig. 2: Results obtained by mPCR form a vaccine and one tissue form an animal inoculated with the PRRS virus. Lanes 1-3, vaccine; lane 4, organ (lymph node); lane 5 vaccine; lane 6, control (-); lane 7 size marker

inoculated with the virus, according to the results obtained here. However, it is not possible to differentiate the type of strain infecting the animals through out this assay, reason why it is necessary to apply a multiple PCR. On the other hand, the second technique increases considerably the sensitivity (Paton *et al*., 2000). mPCR results showed that this assay was capable to differentiate strains of the PRSS virus in the commercial vaccine and in tissues form inoculated animals. A 187 bp amplification product was obtained from the European strains, while a 107 bp product was obtained from the American strains, as previously reported (Gilbert *et al*., 1997). The strain that was identified in the animals inoculated with the PRRS virus and in all the serological and seminal samples corresponded to the American type. The huge importance of this result emerges from the fact that the identification of the type of strain will help to determine the origin of an outbreak. Considering the aforesaid, it will be possible to carry out molecular epidemiology studies in this country, allowing a better control of the disease. It has been reported that the ELISA test is an effective filter to detect animals that have been exposed to the PRRS virus (Morilla *et al*., 2003). However, a mPCR assay (either on serum or semen) is needed to confirm if an animal carries the virus. The mPCR technique was capable of detecting the PRRS virus in seminal samples, even when the serum sample of the same animal

Table 1: Analysis of serological and seminal samples by ELISA and mPCR techniques

	Techniques		
	ELISA[a]	mPCR	
Animals	Serum	Serum	Semen
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	-	-
19	-	-	-
20	-	-	+
21	-	ND	-
22	-	ND	-
23	+	ND	+
24	+	+	+
25	+	+	+
26	-	-	-
27	-	-	+

[a]The presence or absence of antibodies was assessed calculating the S/P coefficient of each sample. If the coefficient was equal or higher than 0.4, the simple was classified as positive. ND = Not determined

was negative by ELISA test, as it is showed in Table 1. This result is relevant because of the fact that even if an animal is negative to the ELISA test, it does not guarantee that the animal is free from the virus. In light of this, mPCR becomes a valuable tool to identify infected animals and then avoid PRRS virus entrance to farms by semen. This technique will allow a faster and more accurate diagnosis of the PRRS virus in Mexico. Nonetheless, it must be validated on field samples and comparing results with those of the standard tests for PRRS.

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