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Short communication. Differentiation of Type-I Porcine Reproductive and Respiratory Syndrome Virus vaccines and field strains by restriction fragment length polymorphism analysis

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Abstract

The use of modified live virus (MLV) vaccines is a common procedure to control porcine reproductive and respiratory syndrome virus (PRRSV) infection in the great majority of countries from America, Asia and Europe, including Spain. Current discriminatory techniques allow the detection of different MLV type-II vaccine strains. Herein we report a rapid and accurate technique aimed to discriminate between MLV type-I vaccine strains and Spanish field strains. This technique comprises a reverse transcription (RT) and nested polymerase chain reaction (nPCR) amplification of PRRSV ORF5 followed by a digestion of RT-nPCR products with two specific endonucleases, *Ita*I and *Acc*I. Combined utilization of *Ita*I and *Acc*I generates restriction fragments length polymorphisms (RFLP) patterns adequate for the differentiation of 30 Spanish field isolates, of which 12 were isolated between 1991 and 1995 and 18 between 2000 and 2003. These different RFLP patterns can be used to distinguish unequivocally between Spanish field strains of PRRSV and the three MLV type-I vaccines used in Spain: AmervacPRRS®, Pyrsvac-183® and PorcilisPRRS®.

Additional key words: epidemiology; Europe; genomic variability; heterogeneity; PRRSV; RFLP; Spain.

Resumen

Comunicación corta. Diferenciación de cepas vacunales del virus del síndrome reproductor y respiratorio porcino de tipo I y cepas de campo por análisis de polimorfismos en la longitud de fragmentos de restricción

Para controlar la infección por el virus del síndrome reproductor y respiratorio porcino (PRRSV), en la gran mayoría de países de América, Asia y Europa, incluyendo España, se usan frecuentemente vacunas basadas en virus vivos modificados (MLV). En la actualidad existen técnicas discriminatorias que permiten detectar cepas vacunales del PRRSV de tipo II. El presente trabajo describe una técnica precisa y rápida para la diferenciación de cepas vacunales de tipo I del PRRSV y cepas de campo españolas. Esta técnica se basa en la transcripción reversa y posterior amplificación de la ORF5 del genoma del PRRSV utilizando la reacción en cadena de la polimerasa anidada, seguida de la digestión de los amplicones generados con dos endonucleasas específicas: *Ita*I y *Acc*I. La utilización combinada de ambas enzimas genera patrones de polimorfismos en la longitud de fragmentos de restricción (RFLP), adecuados para la distinción de las 30 cepas de campo usadas, de las cuales 12 fueron aisladas entre 1991 y 1995 y 18 entre 2000 y 2003. Estos diferentes patrones pueden ser utilizados para distinguir entre cepas de campo españolas del PRRSV y las tres cepas vacunales de tipo I usadas en España: AmervacPRRS®, Pyrsvac-183® y PorcilisPRRS®.

Palabras clave adicionales: epidemiología; España; Europa; heterogeneidad; PRRSV; RFLP; variabilidad genómica.

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Abbreviations used: MLV (modified live virus), nPCR (nested polymerase chain reaction), PRRS (porcine reproductive and respiratory syndrome), PRRSV (porcine reproductive and respiratory syndrome virus), RFLP (restriction fragments length polymorphisms), RT-PCR (reverse transcription polymerase chain reaction).

Porcine reproductive and respiratory syndrome (PRRS) is one of the most relevant swine diseases, characterized by reproductive failure in sows and respiratory distress in pigs of all ages (Rossow, 1998). The causative agent, PRRS virus (PRRSV), is a small, enveloped RNA virus classified within the *Arteriviridae* family (Cavanagh, 1997) which is divided into type-I and type-II based on the high genetic, antigenic and pathogenic heterogeneity among different viral isolates (Meng, 2000).

The diagnosis of PRRSV infection is frequently based on the detection of a portion of PRRSV genome by reverse transcription polymerase chain reaction (RT-PCR) in clinical samples (Suárez *et al.*, 1994; Christopher-Henning *et al.*, 1995). The extensive use of modified live virus (MLV) vaccines for the control of PRRS, both in growing and breeding pigs, might hinder the diagnosis of the disease. Furthermore, all available MLV vaccines based either on type-I or type-II strains induce a long-lasting viremia in vaccinated pigs similar to that produced by field viruses (Mengeling *et al.*, 1999; Scortti *et al.*, 2006). Therefore, a rapid, sensitive and accurate technique that allows the discrimination between vaccine and field strains of PRRSV is necessary. In those lines, a test based on the patterns produced by digestion of PCR products with different restriction enzymes has been developed for the differentiation of type-II field strains and Ingelvac PRRS MLV/Repro™, a MLV type-II vaccine frequently used for the control of the disease (Wesley *et al.*, 1998). This method has frequently been used to typify PRRSV type-II isolates (Cai *et al.*, 2002; Brar *et al*., 2011). Nonetheless, the extensive genetic differences between type-I and type-II strains of PRRSV make the application of this technique useless in the European situation.

The present study describes an easy and fast technique that discriminates MLV type-I vaccine strains from field strains, by means of RT and nested polymerase chain reaction (nPCR) amplification of PRRSV ORF5 and restriction fragment length polymorphisms (RFLP) analysis of RT-nPCR products digested with specific endonucleases. To determine which restriction enzymes would differentiate MLV type-I vaccine strains from field strains, PRRSV type-I ORF5 sequences were obtained from nucleotide data bases (EMBL/GenBank libraries) and restriction patterns for different enzymes examined using WebCutter 2.0 software (http://rna.lundberg.gu.se/cutter2). *Ita*I was selected on the basis of predicted cutting patterns. Later on, a second restriction enzyme —*Acc*I— was chosen to determine a second RFLP pattern for a particular group of strains that shared the same RFLP pattern than one of the MLV type-I vaccine.

To validate the technique, RFLP patterns of 30 Spanish PRRSV isolates were compared to those of the three MLV type-I vaccines (AmervacPRRS®, Pyrsvac-183® and PorcilisPRRS®). For this purpose, RNA was extracted using Chelex® 100 resin (BioRad, Hercules, CA, USA) as described Walsh *et al.* (1991). The RT-PCR was performed using a commercial one-step RT-PCR kit (GeneAmp® Gold RNA PCR Core kit, Applied Biosystems, Foster City, CA, USA), following the manufacturer´s instructions. Primers used to amplify the whole ORF5 and the flanking regions of ORF4 and ORF6 were previously reported by Prieto *et al*. (2009). Second round PCR amplification was performed following a preceding technique (Suárez *et al.*, 1994). An amplicon of 606 bp than comprises the whole ORF5 was obtained. The restriction enzyme *ItaI* (Roche, Basel, Switzerland) was used to digest the products of the second round PCR. For this purpose, 14 µL of each PCR product were incubated at 37ºC overnight in a digestion mixture containing 3 U of the enzyme and 2 µL of the corresponding buffer in a final volume of 20 µL. The products of digestion were visualized by electrophoresis at 50 V for 3 hours in 3% agarose gels (AG5, Ecogen, Spain) prepared in TBE 1x. Quantity One BioRad software version 4.2.1. (BioRad, Hercules, CA, USA) was used to predict the size of the bands on agarose gels.When RFLP patterns obtained after digestion with *ItaI* were the same for field isolates and one of the MLV vaccine strains used —PorcilisPRRS®—, a second digestion with the restriction enzyme *Acc*I (Roche, Basel, Switzerland) was performed in the above mentioned conditions.

The restriction enzyme *ItaI* cut ORF5 of all strains tested, including field and vaccine strains, at least once (Fig. 1a and 1b). A total of 18 different RFLP patterns were obtained. Some of them were very alike —*i.e.* patterns 2, 9 and 11. Of the 18 patterns found, pattern 1 corresponded to two Spanish MLV vaccines —AmervacPRRS® and Pyrsvac-183®—, pattern 2 corresponded to PorcilisPRRS® vaccine strain, and the sixteen additional RFLP patterns corresponded to the Spanish field PRRSV strains. The number of RFLP patterns obtained for recently isolated field viruses (Fig. 1a) —15 patterns in 18 strains tested— is higher than the number of RFLP patterns for isolates obtained in the first few years after the description of the disease (Fig. 1b) —6 in 12 strains tested—.

Comparison of RFLP patterns obtained by digestion of vaccine strains and field isolates with *Ita*I shows that most of the field isolates tested exhibited RFLP patterns

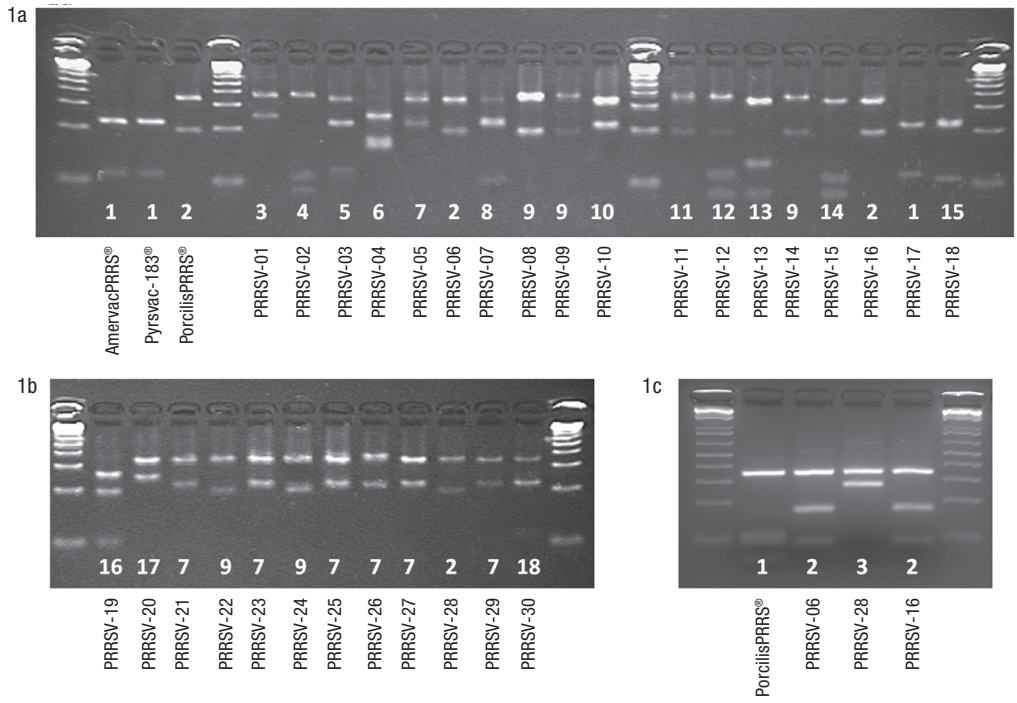

Figure 1. Restriction fragment length polymorphism (RFLP) patterns obtained after digestion with *Ita*I of vaccine strains and Spanish PRRSV strains isolated between 2000 and 2003 (1a) or between 1991 and 1995 (1b). RFLP patterns obtained after digestion with *Acc*I of PorcilisPRRS® and field isolates that showed RFLP pattern 2 after cut with *Ita*I (1c). The numbers indicate the different patterns obtained for these PRRSV strains. The total number of RFLP patterns was 15 for strains isolated between 2000 and 2003 and 6 for strains isolated between 1991 and 1995. The first patterns correspond to vaccine strains, AmervacPRRS®, Pyrsvac-183® and PorcilisPRRS®, respectively.

distinguishable from vaccine strains. However, one field isolate exhibited RFLP pattern 1 while three of them showed RFLP pattern 2. The only isolate that exhibited the same RFLP pattern than the Spanish vaccine strains (pattern 1) was isolated from a herd that has started vaccination with a MLV Spanish vaccine a few months earlier. To study whether this isolate had a vaccine origin, the PCR product of ORF5 was sequenced and compared to ORF5 of the Spanish vaccine strain used in the farm. A percentage of similarity of 99.5% seems to confirm that this isolate derivates from the vaccine strain used in the farm (Fig. 2). A similar result has been previously reported between Porcilis PRRS® strain and field isolates showing close phylogenetic relationships (Pesente *et al.*, 2006). However, the digestion of RT-PCR products of isolates that shared the RFLP pattern 2 when cut with *Ita*I with a second restriction enzyme, *Acc*I, rendered different patterns indicating that those field isolates are different from the vaccine strain. Accordingly, pattern 1 corresponded to the vaccine strain while pattern 2 was shared by two recent field isolates and pattern 3 was found in one early isolate (Fig. 1c).

The study of RFLP patterns of field PRRSV isolates could be a first approach to discard the vaccine origin of a strain implicated in an outbreak of the disease. The usefulness of this approach has been confirmed for type-II strains using 3 different restriction enzymes (Wesley *et al*., 1998). The present study confirms that an analogous technique can be applied to type-I strains. Thus, the digestion of PCR products of PRRSV type-I ORF5 with only one or, occasionally, two restriction enzymes (*ItaI* and *Acc*I), differentiates efficiently vaccines and field strains. Increasing the number of strains with representatives of different countries would confirm the usefulness of this technique in epidemiologic studies. Furthermore, the fact that the number of RFLP patterns found in isolates of recent years is higher than in those from early years, after the description of the disease, indicates a continuous genetic drift that increases genetic diversity. This scenario has been previously documented (Wesley *et al.*, 1999; Prieto *et al*., 2009).

In conclusion, RFLP analyses of PRRSV type-I isolates can be considered an useful, fast and convenient method to discriminate between field and vaccine

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001 ATGAGATGTT CTCACAAATT GGAGCGTTTC TTGACTCCTC ACTCTTGCTT 050
001 ---------- ---------- ---------- ---------- ---------- 050

051 CTGGTGGCTT TTTTTGCTTT GTACCGGCTT GTCTTGGTCC TTTGTCGATG 100
051 ---------- --------G- ---------- ---------- ---------- 100

101 GCAACGACAG CAGCTCGACA TACCAATACA TATATAATTT GACGATATGC 150
101 ---------- ---------- ---------- ---------- ---------- 150

151 GAGCTGAATG GGACCGAATG GTTGCCCAGC CATTTTGACT GGGCAGTCGA 200
151 ---------- ---------- ----T----- ---------- ---------- 200

201 GACCTTTGTG CTTTACCCGG TTGCCACTCA TATCCTTTCA CTGGGTTTTC 250
201 ---------- ---------- ---------- ---------- ---------- 250

251 TCACAACAAG CCATTTTTTT GATGCGCTCG GTCTCGGCGC TGTGTCCACT 300
251 ---------- ---------- ---------- ---------- ---------- 300

301 ACAGGATTTG TTGGCGGGCG GTATGTACTC AGCAGCGTGT ACGGCGCTTG 350
301 ---------- ---------- ---------- ---------- ---------- 350

351 TGCTTTCGCA GCGCTCGTAT GTTTTGTCAT CCGCGCTGCT AAAAATTGCA 400
351 ---------- -------C-- ---------- ---------- ---------- 400

401 TGGCTTGCCG TTATGCCCGT ACCCGGTTTA CCAACTTCAT TGTGGACGAC 450
401 ---------- ---------- ---------- ---------- ---------- 450

451 CGGGGGAGGA TCCATCGATG GAAGTCTCCA ATAGTGGTAG AGAAATTGGG 500
451 ---------- ---------- ---------- ---------- ---------- 500

501 CAAAGCTGAA GTCGGTGGCG ACCTCGTCAC CATCAAACAT GTCGTCCTCG 550
501 ---------- ---------- ---------- ---------- ---------- 550

551 AAGGGGTTAA AGCTCAACCC TTGACGAGGA CTTCGGCTGA GCAATGGGAA 600
551 ---------- ---------- ---------- ---------- ---------- 600

601 GCCTAG                                               606
601 ------                                               606
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Figure 2. Comparison of the sequences of AmervacPRRS® ORF5 (above) and PRRSV-16 ORF5 (below), respectively. The 99.5% sequence similarity would confirm that the field isolate PRRSV-16 derivates from the vaccine strain used a few months earlier for the vaccination of the same herd.

viruses, although additional studies comprising more isolates should be performed to give strength to the technique. Additionally, the study of RFLP patterns could be useful in epidemiological studies, although a thoughtful study should include sequencing approaches to characterize unequivocally every PRRSV strain.

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