Available online at www.sciencedirect.com
ScienceDirect
The Veterinary Journal 180 (2009) 363–370
The Veterinary Journal
www.elsevier.com/locate/tvjl

Influence of time on the genetic heterogeneity of Spanish porcine reproductive and respiratory syndrome virus isolates

Cinta Prieto [a], Ana Vázquez [a,1], José I. Núñez [b], Esther Álvarez [a], Isabel Simarro [a], José M. Castro [a,*]

[a] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda, Puerta de Hierro s/n, 28040 Madrid, Spain*
[b] *Centre de Recerca en Sanitat Animal, Campus de Bellaterra, Edifici V, 08193 Bellaterra, Barcelona, Spain*

Accepted 12 January 2008

Abstract

The aim of the present study was to establish the degree of diversity of porcine reproductive and respiratory virus (PRRSV) isolates that circulate in the same geographical area in different years. Nucleotide sequences of open reading frame (ORF) 5 were determined for 28 Spanish field PRRSV isolates from different years and three European-type modified live virus vaccines. Sequences were aligned using Clustal W software and a phylogenetic tree constructed using the neighbour joining method. The results of pairwise homology comparisons of nucleotide and deduced amino acid sequences of these PRRSV isolates indicate a tendency for heterogeneity to increase with time. The study of the phylogenetic tree revealed that Spanish PRRSV isolates constitute two well-defined clades and a group of unrelated sequences. The observed heterogeneity does not appear to be due to temporal evolution exclusively. Early and recent isolates group themselves into different clusters independently of the time of isolation, indicating the co-circulation of different variants and the maintenance of variants of the original isolates in the field.

Keywords: Porcine reproductive and respiratory syndrome virus; Genomic variability; Temporal evolution; Europe; Spain

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterised by respiratory disease in piglets and reproductive failure in sows (Rossow, 1998). The causal agent, PRRS virus (PRRSV), belongs to the family *Arteriviridae* and is a small, enveloped virus, with a single-stranded positive sense RNA genome containing nine open reading frames (ORFs) (Meulenberg et al., 1993). ORFs 1a and 1b encode non-structural proteins, including viral polymerase, while ORFs 2-7 encode the structural proteins GP2a, GP2b, GP3, GP4, GP5, M and N (Snijder and Meulenberg, 1998). GP5, encoded by ORF5, is the main envelope protein and is thought to be involved, singly or as GP5-M heterodimers, in viral attachment and penetration of target cells (Snijder et al., 2003). GP5 contains the main neutralising epitope of PRRSV (Ostrowski et al., 2002; Plagemann, 2004a,b).

PRRSV is heterogeneous and is divided into European and American subtypes on the basis of genetic, antigenic and pathogenic differences (Meng, 2000). ORF5 is one of the most variable regions of the PRRSV genome, most likely because it encodes the major envelope protein and potentially is exposed to selective pressures. The genetic variability of ORF5 was initially demonstrated within the North American genotype (Meng et al., 1995; Andreyev et al., 1997; Dea et al., 2000; Golberg et al., 2000). In contrast, early studies suggested that a low degree of genetic variability might exist among European PRRSV isolates, on the basis of ORF5 sequences (Suárez et al., 1996) or other regions of the PRRSV genome, mostly ORF7 (Suárez et al., 1996; Drew et al., 1997; Le Gall et al.,

* Corresponding author. Tel.: +34 91 3943714; fax: +34 91 3943908.
E-mail address: chemaca@vet.ucm.es (J.M. Castro).
[1] Present address: Instituto de Salud Carlos III, Crta de Majadahonda a Pozuelo, Km 2, 28220 Majadahonda, Madrid, Spain.

1090-0233/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2008.01.005

1998). However, recent reports, based on ORF5 sequences, have shown that genetic diversity of European PRRSV is at least as high as that of North American types (Indik et al., 2000; Forsberg et al., 2002; Stadejek et al., 2002, 2006; Mateu et al., 2003, 2006).

Moreover, Forsberg et al. (2002) defined a marked geographical pattern after analysing PRRSV isolates from different European countries, namely a cluster of Lelystad-like isolates, a cluster of purely Danish isolates and a cluster of highly diverse Italian-like isolates. More recently, Stadejek et al. (2006), studying isolates of eastern European countries, divided PRRSV European genotype into four subtypes, with all reference European-type sequences included in subtype 1 and several genetic subtypes of Belarusian and Lithuanian sequences comprising subtypes 2, 3 and 4. The authors also concluded that a marked geographical pattern could be observed along the Eastern Polish border, with a great diversity of strains east of that border.

Nonetheless, most of the above mentioned studies did not include information about the temporal evolution of European strains of PRRSV. Only limited information has been published in relation to evolution of PRRSV strains in the same geographical area, with inconclusive results (Mateu et al., 2003, 2006). The main objective of the present study was to determine the influence of time on the evolution of Spanish PRRSV isolates, with the purpose of establishing the degree of diversity of PRRSV circulating in the same geographical area in different years. Additionally, to improve knowledge of PRRSV evolution, a second objective was to establish the rate of fixation of mutations per site and year.

Materials and methods

Virus isolates

Field isolates of PRRSV ($n = 28$) used in this study originated from unrelated farms showing clinical signs compatible with PRRS in sows or growing pigs and from different regions of Spain, although the majority of them came from the north east. As the purpose of the study was to compare early and recent sequences, the isolates were randomly selected among those obtained in one of two different periods of time: 1991–1995 ($n = 12$) and 2000–2003 ($n = 16$). Six of the early isolates included in this study are the same as those used by Suárez et al. (1996); the use of a different set of primers that amplifies ORF5 and the flanking regions of ORF4 and 6 allowed us to sequence the complete ORF5 and justify the inclusion of these samples. Three European-type modified-live-virus (MLV) vaccines (AmervacPRRS, Laboratorios Hipra; Pyrsvac-183, Laboratorios Syva; PorcilisPRRS, Intervet Laboratories) were also included in the study.

RNA extraction and RT-PCR

Total RNA was obtained from all samples by a boiling procedure using Chelex 100 resin (BioRad) (Walsh et al., 1991). The fifth passage in porcine alveolar macrophages (PAM) of PRRSV strain 5710 isolated in the north of Spain in 1992 (Suárez et al., 1994) was used as a positive control for RT-PCR. PRRSV-negative pig serum and fetal bovine serum were used as negative controls.

For reverse transcriptase polymerase chain reaction (RT-PCR), 10 μL of total RNA were used as template. The reaction was performed using a commercial one step RT-PCR kit (GeneAmp Gold RNA PCR Core Kit, Applied Biosystems), following the manufacturer's instructions. Sense (5′-ACATTCGGTTGCTGCATTTCCTGA-3′) and antisense (5′-ACGAGCTTTTGTGCGGCGATA-3′) primers were used that resulted in a final amplicon of 719 base pairs (bp), comprising all ORF5 and the flanking regions of ORF4 and 6. In cases in which these primers did not render an amplicon of the expected size (two isolates belonging to the first few years after the emergence of PRRS), a different pair of primers was used to amplify ORF5 (Suárez et al., 1996). The amplicon obtained with the second pair of primers was 606 bp long and comprised the complete ORF5.

RT-PCR product purification and nucleotide sequencing

RT-PCR products were purified using a commercial kit (GeneClean, Bio 101, Q-BIO Gene, MP Biomedicals), following the manufacturer's instructions. Individual sequences of both strands of the ORF5 PCR products were determined with the same pair of primers used for RT-PCR, amplifying the samples by asymmetric PCR with fluorescent terminators and analysing the products by electrophoresis on an ABI prism 310 Genetic Analyzer (Applied Biosystems). At least two different RT-PCR products were sequenced to verify that no errors had occurred during DNA amplification and that the sequences obtained were correct.

Genetic variability and phylogenetic analysis

Sequences were aligned using Clustal W software (Higgins et al., 1994) and were manually corrected. All sequences were analysed in pairwise comparisons. The reference strain of European-type PRRSV, Lelystad, was included in the comparisons. Protein conservation was analysed with the Blocks programme (http://blocks.fhcrc.org/) (Henikoff et al., 2000).

Distance estimation for nucleotide sequences was performed according to the Jukes and Cantor method (Nei and Kumar, 2000), as well as Kimura's two parameter approach (Kimura, 1980). The phylogenetic tree was constructed by means of the neighbour joining method using VR-2332, the prototype of the American genotype, as the outgroup (Saitou and Nei, 1987). To assess the statistical reliability of the dendrograms, bootstrapping values were calculated (random number seed: 123; 1,000 replicates) (Felsenstein, 1985). The analyses were performed with MEGA 3.1 software (Kumar et al., 2004). For comparative purposes, other ORF5 sequences of PRRSV from different European countries were retrieved from the EMBL data base and included in the phylogenetic analysis, based on the year of isolation and the country of origin (Table 1).

Statistical analyses

One way analysis of variance was used to evaluate differences between groups and Duncan's multiple range test was used to delimit categories of statistically significant ($P < 0.05$) differences between groups. All statistical tests were carried out with SAS software.

Results

Genetic diversity

All ORF5 sequences analysed were 606 bp long (GenBank accessions DQ345725-DQ345755). Pairwise comparisons revealed a decrease in similarity over time, with higher percentage similarity among early isolates (1991–1995) and lower percentage similarity among isolates from recent years (2000–2003) (Supplementary Table 1). The nucleotide similarity among PRRSV isolates from the first few years after the appearance of the disease (1991–1995) was 91.4–99.1% (mean 94.7 ± standard error 2.1%). When