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Observation of high recombination occurrence of Porcine Reproductive and Respiratory Syndrome Virus in field condition

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ABSTRACT

Recombination in *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV) is a well-documented phenomenon. A high recombination frequency has been reported in experimental conditions both *in vitro* and *in vivo*, and its role in driving viral evolution has been postulated by several authors. However field evidences are rare, mainly obtained from large-scale sampling and typically represented by single sequences rather than by groups of circulating "recombinant progenies". The present work was aimed to investigate the gray area between experimental studies and large-scale epidemiological investigations. The study was performed on ORF5, ORF7 and concatenated sequences obtained in our laboratory or available in GenBank collected between 2009 and 2012 in northern Italy. Six independent recombinant strains out of 66 concatenated sequences (~9%) were found, demonstrating a high recombination frequency respect to previous field studies but comparable to *in vitro* experiments. *In silico* analysis let speculate that this new strain displayed physicochemical features diverse enough to potentially alter its immunological properties. Taken altogether, the results of our study support previous experimental evidences that depict PRRSV to be extremely prone to recombination. The limited temporal and geographical spread of recombinant strains however states in favor of a limited fitness of the recombinant progeny compared to parental strains and the marginal role of this phenomenon in PRRSV evolution.

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) was first recognized quite contemporaneously in the U.S. and in Europe between the end of 1980s and the early 1990s. Since then PRRS has emerged as the most prevalent disease of swine in the world, causing remarkable economic losses (Neumann et al., 2005; Nieuwenhuis et al., 2012). The agent of the disease, *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV), classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*, is an enveloped, single-stranded positive-sense RNA virus. The viral genome is approximately 15 kb in length and contains nine open reading frames (ORFs) (Firth et al., 2011; Meulenberg, 2000). Two main genotypes, Type I (European-like) and Type II (North American-like) have been identified sharing 50–70% nucleotides and 50–80% amino acids (Shi et al., 2010a). A great and progressively increasing (Mateu et al., 2006; Pesch et al., 2005) genetic variability has been observed: mean nucleotide diversity within European and American genotypes has been estimated to be about 15% and 12.5%, respectively (Cho and Dee, 2006; Shi et al., 2010a,b). Genetic distance, calculated on ORF5, has reached a maximum of about 30% in Type I and 21% in Type II (Murtaugh et al., 2010). RNA virus evolution is assumed to result primarily from RNA polymerase infidelity. Indeed the PRRSV nucleotide substitution rate has been estimated to vary between 4.7×10^{-2} and 1.55×10^{-3} (Murtaugh et al., 2010; Yoon et al., 2012). Although the role of recombination in evolution of RNA viruses is still debated (Simon-Loriere and Holmes, 2011), several authors assert that recombination is an important mechanism of genetic diversity generation in PRRSV (Liu et al., 2011; Mengeling, 2002; Murtaugh et al., 2010), playing a potential role in conditioning virulence, antigenic escape and diagnostic failure. Several studies have demonstrated recombination in both *in vitro* (van Vugt et al., 2001; Yuan et al., 1999) and *in vivo*, in experimental (Liu et al., 2011) and field conditions (Fang et al., 2007; Forsberg et al., 2002; Li et al., 2009; Shi et al., 2010a; Stadejek

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et al., 2008). In the latter case, results where typically obtained comparing sequences obtained from large-scale (*i.e.* country level) sampling. The aim of this study was to investigate recombination on a smaller scale in terms of geographic distance and time window (Forsberg et al., 2002).

2. Materials and methods

2.1. Samples

The samples used in this study were drawn from the Istituto Zooprofilattico Sperimentale delle Venezie's historical archive, a regional public veterinary laboratory collecting passive field samples brought by practitioners for diagnostic purposes. All of the 163 samples (serum and lung), coming from 52 pig farms among 12 provinces in northeastern Italy (enclosing a geographic area of about 28,000 km^2), found positive at routine RT-PCR to PRRSV between 2010 and 2012 and stored at −80 °C, were analyzed. RNA had been extracted from 200 μl of serum or 200 μl of lung homogenate using the High Pure viral RNA kit and High Pure RNA tissue kit, respectively (Roche Diagnostics, Monza, Italy). Each sample had been routinely tested using a classical two step RT-PCR targeting a genomic fragment within the ORF7 region and allowing the differentiation between the Type I and Type II strains through electrophoresis on acrylamide gels (Persia et al., 2001).

2.2. Sequencing

ORF5 and ORF7 of each sample were amplified using a one-step RT-PCR as described by Oleksiewicz et al. (1998). Briefly, ORF5 sequence was amplified using the primer ORF5F (5′ CAA TGA GGT GGG CIA CAA CC 3′) and ORF5R (5′ TAT GTI ATG CTA AAG GCT AGC AC 3′) while ORF7 was amplified using the primer pair ORF7F (5′ GCC CCT GCC CAI CAC G 3′) and ORF7R (5′ TCG CCC TAA TTG AAT AGG TGA 3′), obtaining an amplicon of 719 bp and 637 bp respectively.

Amplification and band specificity were visualized using a SYBR safe stained 2% agarose gel, after electrophoresis. Amplicons were sequenced with the same primers, in both senses, using the BygDye terminator v.3.1 Cycle Sequencing Kit (Applied Biosystem®, Monza, Italy). Sequences were obtained using ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem®, Monza, Italy). Chromatograms were evaluated by FinchTV (http://www.geospiza.com) and consensus sequences were reconstructed using CromasPro (CromasPro Version 1.5). When both ORFs were available, concatenated sequences were constructed using Mesquite (Maddison and Maddison, 2011).

2.3. Sequence analysis

Sequences obtained plus those (*i.e.* 11 ORF7 and 64 ORF5) derived from Pesente et al. (2006) were aligned by Guidance (using PRANK as alignment method) (Penn et al., 2010) and score evaluated. For clarification purposes, all ORF7 sequences published by Pesente et al. (2006) were renamed with the accession number assigned to ORF5. JModelTest 2.1.2 (Darriba et al., 2012) was used to select the model of evolution according to Akaike Information Criterion (AIC). Phylogenetic trees based on ORF5 and ORF7 were reconstructed applying the Maximum Likelihood method implemented in PhyML 3.0 (Guindon et al., 2010) assuming the GTR + Γ4 + I nucleotide substitution model. Phylogenetic tree reliability was evaluated using a fast nonparametric version of the aLRT (Shimodaira–Hasegawa [SH]-aLRT), which was developed and implemented in the PhyML 3.0 (Anisimova et al., 2011).

ORF5, ORF7 and concatenated sequence alignments were tested for evidence of recombination using RDP3 (Martin et al., 2010). In order to obtain a conservative estimate, a recombination event was accepted only when detected by two or more methods implemented in the program with a *p*-value lower than 5×10^{-5}. A collection of partitions without recombination was obtained dividing the original alignment at the recombination breakpoint. Phylogenetic trees were reconstructed for each partition using RAXML (Silvestro and Michalak, 2012) and used to calculate per site log likelihoods for each alignment partition. Statistical significance of topological incongruence between segments separated by recombination breakpoints were assessed through SH, KH, ELW and AU tests implemented in CONSEL (Shimodaira and Hasegawa, 2002). A p-value < 0.05 was assumed to indicate statistical significance.

A discrete states phylogeographic reconstruction of PRRSV strains migration pattern was performed using BEAST 1.7.5 (Drummond et al., 2012) as described by Lemey et al. (2009). The 12 provinces where the samples had been collected were considered to be discrete states. An asymmetric substitution model, coupled with the Bayesian Stochastic Search Variable Selection (BSSVS), was implemented. Non-recombinant, concatenated ORF5–ORF7 sequences, for which sampling data was known, were analyzed for this purpose. Bayesian Factor (BF) was calculated in order to define well supported diffusion rates using SPREAD (Bielejec et al., 2011). Rates yielding a BF > 10 were considered adequately supported (Kass and Raftery, 1995). The same software was used to generate the KML file compatible with Google Earth displaying migration history.

2.4. In silico structural analysis

Structural consequences of recombination on GP5 were considered for a recombinant cluster that demonstrated circulation over time in a farm. Nucleotide and amino acid *p*-distance of recombinant strains from their parents were calculated using MEGA5 (Tamura et al., 2011). Hydrophobicity profile was calculated using ProtScale (Wilkins et al., 1999) assuming the Kyte & Doolittle scale. Secondary structure and transmembrane topology of GP5 were predicted using Psipred (http://bioinf.cs.ucl.ac.uk/psipred/). N-linked glycosylation sites were estimated using NetNGlyc 1.0 Server (Gupta et al., 2004). The possible role of recombination in generating strains with different immunological properties was evaluated through *in silico* prediction of T- and B-epitopes. Linear B-cell epitopes were predicted using the BepiPred 1.0 Server (Larsen et al., 2006).

For cytotoxic T lymphocytes epitopes, NetCTLpan 1.1 Server (Stranzl et al., 2010) a pan-specific major histocompatibility complex class I epitope predictor, integrating prediction of proteasomal cleavage, antigen transport efficiency and MHC-I binding affinity, was used. All swine MHC-I alleles deposited in the program database were selected to predict 8-,9-,10-,11-mer peptides. MHC-II binders were predicted using NetMHCII 2.2 server (Nielsen and Lund, 2009), searching 15-mer peptides that bound the collection of human MHC-II (loci DR and DQ). Considering that the stronger the binding, the more likely the peptide to become T-cell epitopes (Gustiananda, 2011), highly stringent cut-offs were applied. Peptides were accepted as possible epitopes when their rank score was <1% and IC50nM < 500 (including high and intermediate affinity binder of SLA-I). To limit the presence of false positive results using HLA-II based software, only strong binder (IC50nM < 50) predicted by NetMHCII were accepted, according to Díaz et al. (2009) and Gustiananda (2011).

3. Results

3.1. Recombination analysis

A total of 131 ORF5 and 111 ORF7 were obtained, including those achieved from Pesente et al. (2006). The accession numbers