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Background

Since its identification, approximately two decades ago, *Porcine circovirus 2* (PCV-2) has emerged as one of the most relevant diseases for swine industry (Segalés et al., 2013). Understandably, this has prompt remarkable efforts for its control. Nevertheless, despite several studies, substantial investments and the commercialization and wide application of effective vaccines (Segalés, 2015), PCV-2 is still a ubiquitous pathogen, damaging swine farming both directly and indirectly. One of the key of PCV-2 success is its high evolutionary rate, leading to the emergence of several genotypes and variants over time (Franzo et al., 2015a), which differ in terms of biological and immunological features and can thus adapt to an ever-changing environment. Accordingly, several studies have pointed out that host immune response (Franzo et al., 2016a) and vaccine induced immunity (Franzo et al., 2016b; Reiner et al., 2015) are shaping PCV-2 evolution. This viral plasticity could allow partially escaping the host immune response, at least at epidemiological level, and justify its survival in a mostly immune population.

However, the fine mechanisms driving PCV-2 evolution are still largely unknown, especially at individual animal level.

Of interest, most of the recently emerged livestock diseases are sustained by viruses whose origin was proven to be much more ancient than the first description of clinical syndromes, and PCV-2 is not an exception (Firth et al., 2009; Franzo et al., 2016a). The features of modern, intensive and highly interconnected swine farming have likely played a pivotal role in conditioning the raise and the epidemiology of these viruses, creating favorable conditions for a more rapid evolution and increasing the likelihood of co-infections and thus of recombination.

Although these mechanisms are theoretically plausible also for PCV-2, few experimental evidences assessing the impact of animal management on PCV-2 spreading and evolution have been provided. This lack of information is largely due to the technical challenges in the evaluation of the within-farm PCV-2 circulation and linking it with the viral dynamics at individual host level.

Aim

Having this in mind, a field study was performed to evaluate the within-animal evolutionary dynamics of PCV-2 and to investigate the effect of managerial strategies on viral circulation.

Material and methods

Three different PCV-2 positive farms were recruited in the study and, in each one, serum samples were weekly collected from 20 pigs. At 11 weeks of age, all animals were moved to different farms, however, in two cases (FARM A and FARM B) they remained in contact with pigs originating from the same farm only, while in the third (FARM C), pigs were mixed with others originating from different herds.

Serum samples were tested for PCV-2 detection using real-time PCR. Five animals were further selected after the first PCV-2 positive sample and followed for additional 4 weeks. The complete PCV-2 genome was amplified from these samples and deep sequencing was performed using the Illumina platform. For each animal and sampling week a consensus sequence was obtained and used as template for the calculation of the single nucleotide variations (SNV) at each genome position and for haplotype sequences reconstruction and prevalence estimation. Since low frequency variants can have prevalence comparable to the sequencing error occurrence, errors were preliminary identified and purged using a Poisson probabilistic approach.

Obtained haplotypes were used for recombination analysis, phylogenetic tree and haplotype network reconstruction. Finally, the action of selective pressures acting on the capsid gene was estimated.

Results and discussion

Since the phylogenetic analysis performed on reconstructed haplotypes demonstrated the presence of different genotypes in FARM C, the study was split in two parts: FARM A and B were selected to investigate the virus-host interaction and its effect on viral evolution (Study1), while FARM C was used to evaluate the within-pen spreading dynamics (Study 2).

Study 1:

All variants detected in FARM A and B were classified as PCV-2a. The study of viral subpopulations allowed demonstrating a remarkable within-animal variability, characterized by the presence of several SNV particularly in the capsid coding gene. This feature suggests that the immune derived selective pressure plays a remarkable role in driving the evolution of the huge viral population generated during viral infection. Significantly, the analysis of the haplotypes identified in a single animal (performed on the ORF2), demonstrates a quite peculiar pattern, characterized by the presence of a major haplotype, persisting for the whole duration of the study, and of minor ones

emerging over time. Some of those were detected only once while others were able to persist for a longer time period. This scenario fits well with a quasispecie structure of PCV2 viral population, characterized by the presence of several variants stemming from an "average haplotype". Since most of the mutation, particularly the non-synonymous ones, have been reported to be deleterious, these variants are likely to be purged by natural selection (Sanjuan et al., 2004). However, some of those can display a comparable or superior fitness, and thus survive and eventually spread among the animals. Significantly, the analysis of selective pressures revealed a higher tendency to diversifying selection compared with previous studies (Franzo et al., 2016a).

Considering the limited duration of the study, it is likely that most of non-synonymous were not purged by natural selection yet. Additionally, the high homogeneity of the environment, i.e. the same pig or genetically closely related animals, could have relaxed the action of selective forces.

Study 2:

The analysis of viral circulation within this herd showed a remarkable variation in the epidemiological scenario in FARM C after that animals of different origin were introduced.

While 2 animals were already infected with PCV-2b in the origin farm, the other became positive with PCV-2a and PCV-2d after the comingling. Co-infections were also detected in 4 out of 5 animals and in 3 out of those 4 pigs persisted for more than two weeks. Of note, in all co-infections where PCV-2d was identified, it appeared to prevail over other genotypes, suggesting a higher virulence of this genotype or, at least, of the particular strain.

Additionally, several recombinant strains were detected after animal mixing, a phenomenon which has been proposed to be frequent in circovirus co-infections (Franzo et al., 2015b; Lefebvre et al., 2009; Neira et al., 2017). Nevertheless, the occurrence of *in silico* recombinants cannot be excluded because of the short sequence length and the limits of the currently available software for global haplotype reconstruction.

Besides these considerations, this study demonstrates the relevance of pig management in affecting PCV-2 circulation and the mixing of different strains. This risk is particularly relevant for PCV-2 since co-infections with multiple genotypes have been proposed as a risk factor for clinical signs development (Harding et al., 2010) and recombinant clusters have been reported to display a relevant fitness and a worldwide distribution (Franzo et al., 2016a), representing a potential threat for pig farming.

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