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Time-course of antibody and cell-mediated immune responses to Porcine Reproductive and Respiratory Syndrome virus under field conditions

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ABSTRACT

Major discrepancies are observed between experimental trials of PRRS-virus (PRRSV) infection in isolation facilities and observations made in the field on farm. Owing to the above, a cohort study was carried out in a farrow-to-finish, PRRSV-infected pig farm to characterize the time-course of the virus-specific immune response in two groups of replacement gilts. Despite the occurrence of three and two distinct waves of infection in groups 1 and 2, respectively, the large majority of animals showed little if any PRRSV-specific response in an interferon-gamma release assay on whole blood, whereas non-specific responses were consistently observed. To rule out any possible bias of our test procedure, this was used along with an ELISPOT assay for interferon-gamma-secreting cells with the same reagents on a group of PRRS-virus infected pigs in isolation facilities. A very good agreement was shown between the two sets of results. Also, as opposed to the PRRS model, plenty of Pseudorabies virus-vaccinated pigs under field conditions scored positive in another experiment in the interferon-gamma release assay, *ad hoc* modified for the Pseudorabies virus. Our results indicate that under field conditions poor or no development rather than delayed development of the PRRS virus-specific interferon-gamma response could be the rule for a long time in non-adult pigs after PRRS virus infection. Housing and hygiene conditions, as well as heavy exposure to environmental microbial payloads in intensive pig farms could adversely affect the host's immune response to PRRS virus and partly account for the discrepancies between experimental and field studies.

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) was first described in 1987 in USA and then in June 1990 in Europe. After these first reports the disease epidemically spread in Europe between 1991 and 1992 (Zimmerman et al., 2006). Two swine *Arterivirus* strains have been identified to date as etiological agents: the European (EU) strain, isolated in 1991 and named "Lelystad", and the American (US) one, isolated in 1992 with the acronym ATCC VR-2332 (Shi et al., 2010). Despite the structural correlations, the antigenic differences between the two strains imply the existence of two distinct genotypes derived from a common ancestor (Shi et al., 2010). The US strain had a large diffusion in Europe after the introduction in Denmark in 1996 of a live vaccine based on the US strain and its reversion to pathogenicity (Madsen et al., 1998).

After the first reports of highly contagious, sometimes fatal disease cases, PRRS was shown to occur in many different forms, ranging from subclinical to fatal disease with wide fluctuations of both morbility and mortality; PRRS may also present a wide range of direct and indirect economic losses (Zimmerman et al., 2006). This highlights the complex pathogenesis of the disease, which underlies the accumulation of conflicting reports related to both field trials and protocols of experimental infection. On the whole, the emerging picture outlines a multi-factor disease in which PRRS virus (PRRSV) strains show different features of pathogenicity and agonist interaction with both microbial and non-microbial environmental parameters. Apart from microbial agonist interactions, the occurrence of clinically serious cases can be referred to new virus variants escaping the neutralizing antibody response of pigs to the previous strains (Martinez-Lobo et al., 2011), and/or to the existence of outright virus "immunotypes", endowed with a different capacity to suppress and/or escape innate and adaptive immunity, by inducing a preferential cytokine profile in the infected host (Darwich et al., 2011). In this respect, the delayed and defective pattern of both innate and adaptive immune response is a hallmark observed during experimental infections, which underlies the long-term persistence of PRRS virus in the

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infected pigs (Batista et al., 2004). In particular, with regard to adaptive immunity, serum-neutralizing (SN) antibody and interferon (IFN)-γ secreting cells (SC) were proposed as correlates of protection; both of them showed a defective and erratic pattern of expression in experimental trials (Mateu and Diaz, 2008).

The complex virus/host interaction and the crucial role of the aforementioned interactions with bacterial agonists like lipopolysaccharides (LPS) (van Gucht et al., 2003) are the major reasons for the observed discrepancies between experimental trials under controlled conditions and observations made in the field on farm (Murtaugh et al., 2002). Therefore, cohort studies on PRRS virus-infected farms are badly needed to grasp the actual outcome of virus infection in clinical and productive terms, as well as the pigs' capacity to mount an effective immune response under defined environmental conditions.

In this respect, the central hypothesis of our study was that the adaptive immune responses of pigs to PRRS virus on farm could be worse than those observed under experimental conditions in age and breed-matched animals kept in isolation facilities. Therefore, it was our understanding to undertake a controlled cohort field study and to compare the findings with those generated in experimental studies in isolation facilities. To this purpose, we investigated the time-course of both humoral and cell-mediated immune responses in pigs exposed to PRRS virus under field conditions, and the results were offset against those observed in previous studies of ours (Dotti et al., 2011) and other groups.

2. Materials and methods

2.1. Pig farm

According to our objectives, we decided to carry out the study in a reputedly PRRSV-infected farm, allowing for a wide cohort study on replacement gilts at different time points. The chosen pig operation was a partly closed, farrow-to-finish herd, including 500, internally replaced sows. The animals were housed in a main farm (site 1) containing sows, boars, weaned piglets and a recently built fattening pen, and also in two satellite farms: one (a site 3) hosting a pen for 800 head, the other (sites 2 and 3) including housing facilities for 1000 weaned piglets, the growing gilts and a pen for some 1000 fatteners. The numbers 1, 2 and 3 of the sites refer to the time order of occupancy in the production cycle: 1 for pregnant and nursing sows, and suckling piglets; 2 for weaned piglets (up to 30 kg b.w.); 3 for fattening pigs (30–170 kg b.w.). Hypor sows were reared in the farm as both grand-parents (about 50 with one boar) and parents, associated to terminal Rocky line boars. Most piglets were sold at 30 kg b.w. to fattening farms. Feeds for sows, growers and fatteners were produced on farm. Feeds for suckling piglets (first and second period) were purchased from external sources as ready-to-use products, usually as drug-supplemented feeds according to the actual needs. Prophylaxis measures for piglets included injection of inactivated vaccines against Porcine *Circovirus 2* at 18–20 days, *Mycoplasma hyopneumoniae* at 35–40 days (one week after weaning) and of a live attenuated Aujeszky's disease vaccine (compulsory treatment according to the Italian national control plan). All sows were vaccinated against Aujeszky's disease, as well as against porcine *Parvovirus* and Erysipelas. Gilts were injected with a live attenuated PRRS vaccine at a body weight of 60–70 kg, when they were moved to the pen intended for growing gilts inside site 2–3. Gilts were brought back to the main site at a body weight of 140 kg after testing some animals for PRRS virus (PCR on serum) and antibodies, as well as for antibody to Pseudorabies virus (PRV). They were housed in a gestation compartment and vaccinated against *Escherichia coli*, porcine *Parvovirus* and Erysipelas.

2.2. Animal groups and samplings

Two groups of replacement gilts were introduced into the farm nurseries and weaned at a mean age of 30 days. At weaning, they were ear-tagged and a cohort study was started based on serial blood samplings in vacuum tubes (Vacuette, Greiner, Austria) containing either no anti-coagulant (for antibody and PCR assays), or lithium-heparin (for cell-mediated immunity assays). Pigs of group 1 (numbers 1–30) were clinically inspected and blood samples were collected on trial day (TD) 0, 30, 60, 90, 120, 150 and 180 (30, 60, 90, 120, 150, 180, 210 days of age, respectively). Pigs of group 2 (60 days younger, numbers 31–60) were enrolled in the trial on TD 60 at thirty days of age (weaning); blood samples were collected on TD 60, 90, 120, 150 and 180 (30, 60, 90, 120, 150 days of age, respectively). PRRS vaccination was carried out on TD 100 and 160 in groups 1 and 2, respectively.

2.3. Real-time RT-PCR for detection of PRRSV in serum samples

This was performed using the "TaqMan® AgPath-IDTM NA and EU PRRSV Multiplex Reagents" test kit (Applied Biosystem, Foster City, CA, USA) on a ABI 7500 apparatus (Applied Biosystem), according to the manufacturer's directions. Serum samples were scored positive for EU strain PRRSV with Ct $\leqslant$37, corresponding to Ct $\geqslant$40 with the NA strain-specific primers. The reading was validated if the Ct of both EU and NA positive controls ranged between 32 and 35, and that of the negative control was $\geqslant$40.

2.4. ELISA for IgG antibody to PRRS virus

The antibody response of pigs was evaluated by a commercial kit (Herdchek Porcine Reproductive and Respiratory Syndrome Antibody test Kit, IDEXX Laboratories) according to the manufacturer's directions. The threshold for low-positive sera was set at a sample to positive (s/p) ratio of 0.4 according to the following formula: (Sample:PRRSV)-(Sample:NHC)/(Positive Control:PRRS)-(Positive Control:NHC). NHC: normal host cell antigen.

2.5. PRRSV-specific interferon-γ release assay

The assay, validated in a previous study (Dotti et al., 2011), was carried out on heparinized venous blood transported to the laboratory within 8 h after collection. Blood was distributed in triplicate in 1-ml aliquots in 24-well microtiter plates. The 3 wells of each sample were supplemented with 100 μl of PBS, cell-adapted EU PRRSV BS114 strain grown in MARC-145 cells (7,00,000 $TCID_{50}$/well) and a cryolysate of MARC-145 cells, respectively. This (mock virus) was prepared by freezing (−80 °C) and thawing three times a cell suspension at 4×10^6/ml in the same medium employed for PRRS virus propagation (RPMI 1640 + 2% fetal calf serum, FCS). After clarification (5000 rpm, 15 min), mock virus was frozen in aliquots at −80 °C and used in the assays reported hereunder at a dilution corresponding to the cell concentration of the virus stocks. As a result, both PRRS and mock virus contained the same concentration of MARC-145 lysed cells at their respective working dilutions. Plates were incubated at 37 °C for 18 h in a 5% CO_2 incubator. After centrifugation (2000 rpm, 10 min, 5 °C) plasma of each well was collected and frozen in aliquots at −20 °C. These were used in the measurement of interferon (IFN)-γ by sandwich ELISA. Briefly, Maxi Sorp NUNC ELISA plates (Nunc™, Serving Life Science, Denmark) were adsorbed with anti-swine IFN-γ capture monoclonal antibody (mAb) P2F6 (Thermo Scientific, Rockford, IL) at 5 μg/ml in 0.1 M carbonate/bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. After blocking with 4% bovine serum albumin (BSA) in PBS (assay buffer), plates were washed thrice with 0.2% Tween 20 in PBS (PBS-Tween). Then, 50 μl/well of undiluted test