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Vaccination of pigs reduces Torque teno sus virus viremia during natural infection

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ABSTRACT

Anelloviruses are a group of single-stranded circular DNA viruses infecting several vertebrate species. Four species have been found to infect swine, namely Torque teno sus virus (TTSuV) 1a and 1b (TTSuV1a, TTSuV1b; genus *Iotatorquevirus*), TTSuVk2a and TTSuVk2b (genus *Kappatorquevirus*). TTSuV infection in pigs is distributed worldwide, and is characterized by a persistent viremia. However, the real impact, if any, on the pig health is still under debate. In the present study, the impact of pig immunization on TTSuVk2a loads was evaluated. For this, three-week old conventional pigs were primed with DNA vaccines encoding the ORF2 gene and the ORF1-A, ORF1-B, and ORF1-C splicing variants and boosted with purified ORF1-A and ORF2 *Escherichia coli* proteins, while another group served as unvaccinated control animals, and the viral load dynamics during natural infection was observed. Immunization led to delayed onset of TTSuVk2a infection and at the end of the study when the animals were 15 weeks of age, a number of animals in the immunized group had cleared the TTSuVk2a viremia, which was not the case in the control group. This study demonstrated for the first time that TTSuV viremia can be controlled by a combined DNA and protein immunization, especially apparent two weeks after the first DNA immunization before seroconversion was observed. Further studies are needed to understand the mechanisms behind this and its impact for pig producers.

1. Introduction

Torque teno viruses (TTVs) are small, non-enveloped viruses with a circular single-stranded DNA genome, belonging to the family *Anelloviridae* [1]. Currently, four species have been found to infect swine, namely Torque teno sus virus (TTSuV) 1a and 1b (TTSuV1a, TTSuV1b; genus *Iotatorquevirus*), TTSuVk2a and TTSuVk2b (genus *Kappatorquevirus*). TTSuV infection in pigs is distributed worldwide and characterized by a persistent viremia with high prevalence of TTSuV1 and TTSuVk2a species [2], while prevalence of TTSuVk2b is lower [3]. For the most prevalent TTSuVs, circulating levels of up to 10^6 DNA copies per ml of sera for TTSuV1 and up to 10^7 for TTSuVk2a have been detected [4].

The TTSuV genome contains four open reading frames (ORF), ORF1, ORF1/1 (also known as ORF1-A), ORF2, and ORF3 (also known as ORF2/2). ORF3 can only be translated after splicing of the mRNA precursor, and splicing of the ORF1 mRNA transcript results in different protein isoforms [5,6], as is also indicated in human TTV [7,8]. The predominant transcripts detected in vitro and in vivo of ORF1 are spliced and full-length ORF1 transcripts have not been detected [5,6]. Splicing of TTSuVk2a ORF1 results in three protein isoforms (ORF1-A, -B and -C) and the amino acid composition between them varies, depending on the splicing site used [5,6].

ORF1 encodes the largest TTSuV protein with a predicted viral capsid domain in the N-terminal half of the protein and a putative replication-associated domain in its C-terminal half [9]. It has been suggested that the ORF2 protein is involved in viral replication [10,11]. At present, the role of the ORF3 protein is unknown.

The pathogenic potential of anelloviruses is still controversial. Torque teno sus viruses persistently infect a high proportion of animals that are apparently healthy [12]. Thus, infection by itself does not cause immediate disease and therefore TTSuV by itself is not considered pathogenic. However, it is believed that TTSuVs can influence the development of some diseases or even affect their outcome [1]. Evidence for TTSuV disease association has been accumulating, especially with regard to porcine circovirus type 2

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(PCV2) infection and its associated pathological conditions, namely porcine circovirus diseases (PCVDs) [13,14]. In fact, the TTSuV2 load in serum was significantly higher in pigs suffering from post-weaning multisystemic wasting syndrome (PMWS), which in turn had significantly higher PCV2 loads in serum [15]. A possibility is that TTSuV2 is up-regulated in the framework of immunosuppression associated with PMWS, the most important PCVD [16]. Indeed, this has been found in humans where chemically and virally induced immunosuppression can lead to an increase in TTV viral load and prevalence [17–21].

Although TTSuVs are currently considered non-pathogenic, it is hard to believe that a viral infection characterized by high viral loads and virus persistence would not have any consequences to its host. Indeed, a similar situation exists for the related virus PCV2, which is ubiquitous in the swine population and is necessary but not sufficient for the onset of PMWS. Currently, vaccines against PCV2 can reduce the serum viral titres, prevent the disease, and improve the performance of the farm [22].

In the present study, the impact of pig immunization against TTSuVk2a was evaluated by following the specific seroconversion and the viral load dynamics during natural infection. Our strategy was to use a combination of DNA and protein immunizations. Quantitative PCR techniques were used to detect and quantify the viremia levels of each TTSuV species separately and together while the induction of specific antibodies where followed by indirect ELISA. This showed that vaccination against TTSuVk2a resulted in a specific antibody induction and in a significant reduction of the TTSuVk2a loads, while DNA levels of the other TTSuV species were not affected. Further studies are needed to elucidate the intrinsic mechanisms involved in protection.

2. Materials and methods

2.1. Experimental design of the animal trial

Thirty six (36) male piglets (Landrace × Piétrain genetic origin) that were tested TTSuVk2a negative by PCR [23] were selected for this study. The 3-week old animals were randomly distributed over group A, immunized animals ($n = 18$), and group B, control animals ($n = 18$), but taking into account the piglets' weight, sow, and PCR positivity to TTSuV1 (3 animals tested PCR positive for TTSuV1 at the starting time point). Throughout the study animals from both groups were housed together. Pigs in Group A were DNA immunized twice (when 3 and 5 weeks old) followed by a protein boost (at 7 weeks of age). Pig weight was recorded at 3, 7 and 15 weeks of age and serum samples were collected every two weeks until pigs reached 15 weeks of age. Treatments, housing and husbandry conditions conformed to the European Union Guide lines for animal welfare and the study was performed according to *Good Experimental Practices*. At the end of the experiment (when the animals were 15-weeks old), after final blood sampling and weighing, all animals were euthanized using an intravenous (jugular vein) lethal dose of sodium pentobarbital.

The assays to detect antibodies against Porcine circovirus 2, Porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae* (MH) and *Actinobacillus pleuropneumoniae* (APP) were performed by the R&D Service Laboratory at MSD Animal Health, Boxmeer, according to standard procedures.

2.2. Preparation and application of DNA vaccine

Constructs, based on the pcDNA3.1 plasmid, containing the three ORF1 isoform proteins to cover as much of the antigenic regions variability as possible, being TTSuVk2a ORF1 splicing variants ORF1-A, ORF1-B, and ORF1-C, as well as ORF2 genes under the control of the cytomegalovirus (CMV) promoter were used for the first two immunizations. Plasmid DNA purification was carried out using the Endofree Plasmid Purification Maxi Kit (QIAGEN) following the manufacturer's instructions. Each animal from group A received 400 μg of pooled plasmids (i.e. 100 μg per plasmid) in both the first and second immunization. Control animals in group B received the empty pcDNA3.1 plasmid. The DNA in a total volume of 1.5 ml of saline solution (0.9% w/v of NaCl) was administered intramuscularly in the neck, alternating right and left side in subsequent immunizations.

2.3. Preparation and application of protein vaccine

TTSuVk2a ORF1-A and ORF2 genes were cloned into *Escherichia coli* expression plasmid pET24a in frame with a 6xHistidine tag (Novagen). For protein production, *E. coli* Rosetta 2 (DE3) cells were used. The pET24a-transformed bacteria were cultured in 5 ml of Luria broth (LB) medium with kanamycin (30 mg/ml) and chloramphenicol (34 mg/ml) for 8 h at 37 °C. This starter culture was subsequently inoculated into 1 l of LB containing kanamycin (30 mg/ml) and chloramphenicol (34 mg/ml) and grown until the optical density reading at 600 nm (OD_{600}) reached 0.6. At this point, protein expression was induced by adding 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) for 4 h. Cell pellets were collected by centrifugation at 4000 × *g* at 4 °C for 30 min and stored at −80 °C until use. Protein purification was done using His SpinTrap columns (GE Healthcare Life Science) according to the recommended protocol. The ORF1-A protein was purified under denaturing conditions using 8 M urea and the ORF2 protein under native conditions. Purified proteins were further dialysed 4 times with PBS using dialysis cassettes with 20 kDa molecular weight cut-off (Pierce). Protein preparations were analysed for purity by gel electrophoresis followed by coomassie staining and for identity by Western blot analysis using an anti-His antibody. Protein concentrations were determined using a BCA^{tm} Protein Assay Kit (Pierce) according to the manufacturer's instructions. Purified ORF1-A and ORF2 proteins were pooled and 27 μg/animal mixed 1:1 with an oil-in-water adjuvant (XSolve; MSD Animal Health) was used in the 3rd immunization. The eighteen pigs in control group B were each injected with saline solution mixed with adjuvant (1:1).

2.4. Detection of antibodies against TTSuVk2a ORF1-A and ORF2 by ELISA

Seroconversion of animals was tested by ELISA using either purified ORF1-A or ORF2 proteins as antigen. Low binding 96-well microtitre plates were coated with 50 μl per well containing 680 ng/ml protein in carbonate-bicarbonate buffer pH 9.6. After overnight incubation at 4 °C, plates were washed 3 times with TBS-T (Tris-buffered saline, 0.1% Tween20) and then blocked with blocking buffer (1× phosphate-buffered saline, 1% casein, 0.05% Tween20). After incubation at 37 °C for 1 h, each well was washed 3 times with TBS-T. Serum samples were diluted 1:200 in blocking buffer and 50 μl was transferred to the corresponding well. After incubation at 37 °C for 2 h, plates were washed 3 times with TBS-T. Then 50 μl per well the diluted HRP-conjugated anti-swine whole IgG (1:40,000 in blocking buffer; Sigma) was added and plates were incubated at 37 °C for 1 h. Finally, 50 μl of TMB substrate (3,3,5,5-tetramethylbenzidine liquid, supersensitive, Sigma) was added and the plates were incubated in the dark at room temperature until the OD_{650} of the negative controls reached about 0.05. At this time-point the reaction was stopped by adding 50 μl/well of 2 M H_2SO_4 and the OD_{450} of each sample was determined. All serum samples were run in duplicate. Each plate contained one positive and one negative control serum. The ELISA cut-off value was calculated as