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Immunization of pigs with a type 2 modified live PRRSV vaccine prevents the development of a deadly long lasting hyperpyrexia in a challenge study with highly pathogenic PRRSV JX143

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) has been confirmed to be the underlying cause of the so-called 'porcine high fever disease' (PHFD), a disease that emerged in China in 2006 and subsequently spread over South East Asia. The aim of this study was to investigate whether animals challenged with the Chinese highly pathogenic PRRSV JX143 would be protected by vaccination with single dose of a type 2 modified live virus (MLV) vaccine. Forty-four pigs 17–19 days of age were weighed and randomly assigned to either vaccination with subsequent challenge (V/C, $n = 20$), challenge only (NV/C, $n = 12$) and no vaccination and no challenge (strict controls, $n = 12$). Pigs of the challenged groups (V/C and NV/C) were inoculated intranasally 27 days post-vaccination with PRRSV JX143. Animals were monitored during the subsequent 21 days post challenge and were necropsied at the end of the experiment on day 49. Observations and measurements included body temperature, clinical scores for behavior/general condition, cough and breathing pattern, mortality, serological response and PRRSV viremia via RNA detection. Challenge in the NV/C pigs resulted in 100% morbidity and 67% mortality whereas all vaccinated pigs survived. There was a close association between hyperpyrexia (fever over 41 °C) and incidence in mortality, which was completely prevented by vaccination. Clinical symptoms were less severe, and of transient nature only, in the vaccinated pigs. Vaccination did not prevent infection, but reduced the impact of clinical disease and prevented hyperpyrexia associated mortality.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most challenging viral diseases in pigs. The causative agent of the disease, PRRS virus (PRRSV), is a member of the family *Arteriviridae* in the order *Nidovirales*. PRRSV can be divided into two genotypes: type 1, mainly comprised of viruses from Europe; and type 2, primarily comprised of viruses from North America and South East Asia.

In 2006, an unparalleled large-scale outbreak of an originally unknown, but so-called "high fever" disease with symptoms of PRRS occurred in China, affected over 2,000,000 pigs with about 400,000 fatal cases. This atypical PRRS pandemic was initially classified as a hog cholera-like disease manifesting neurological symptoms, high fever (40–42 °C), anorexia and rubefaction of the skin and ears. Necropsies combined with immunological analyses showed clearly that multiple organs were infected by highly pathogenic PRRSV isolates associated with severe pathological changes [1]. The outbreaks observed were further characterized by a fast spread within the affected regions/provinces and it was observed that pigs of all ages were affected [2].

Whole-genome analysis of the isolated viruses revealed that these PRRSV isolates could be grouped into genotype 2 and were highly homologous to HB-1, a Chinese isolate of PRRSV (96.5% nucleotide identity), and JX143 [3]. It was furthermore observed that these viral isolates comprised a unique molecular hallmark, namely a discontinuous deletion of 30 amino acids in nonstructural protein 2 (nsp2) [1]. Zhou et al. [4] investigated pigs from affected farms in 14 provinces in China and PRRSV was isolated as the single most prominent virus. To confirm that the causative agent was PRRSV, the same authors inoculated the viruses isolated into PRRSV-free pigs and were able to reproduce the clinical disease symptoms.

The "high fever disease" form of PRRS is now also referred to as "highly pathogenic PRRS", HP PRRS or porcine high fever disease (PHFD). After the initial emergence of PHFD epidemics in China in 2006, the disease was subsequently confirmed in Southeast Asian countries including Vietnam and the Philippines in 2007,

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Table 1
Clinical scores.

Parameter	Score 1	Score 2	Score 3	Score 4
Behavior/general condition	Normal physiological behavior	Animals lethargic with slight rubefaction of ears and/or extremities, but still on feed	Animals lethargic most of the time, distinct rubefaction of ears/extremities; signs of lameness or convulsion/tremor, off feed	Animals lethargic and emaciated, obvious depression, off feed
Cough	No cough	Occasionally coughing (1 episode/30 min)	Frequently coughing (2–5 episodes/30 min)	Coughing and dyspnea leading to death the same day
Breathing pattern	Normal physiological breathing pattern	Panting	Distinct thumping	Thumping with open mouth breathing

in Thailand in 2009 and Cambodia and Laos in 2010 [5]. With regard to the origin of the PHFD An et al. [6] did not find recombination or large fragment replacement, which suggests that all highly pathogenic PRRSVs originated from the same Chinese ancestor by gradual evolution.

Six live attenuated PRRSV vaccines including Ingelvac® PRRS MLV, CH-1R and R98 for classical type II PRRSV, and JXA1-R, HuN4-F112 and TJM-F92 specific for PHFD, are currently marketed in China [7]. The aim of the present study was to investigate the efficacy of a commercially available PRRSV type 2 vaccine in response to a challenge with the Chinese PRRSV isolate JX143.

2. Material and methods

2.1. Experimental design

Forty-four pigs 17–19 days of age of both genders were purchased from a Chinese breeding farm (Henan Muyuan Breeding Co Ltd, China). The animals were confirmed negative for PRRSV and PCV2 viremia by RT-PCR and ELISA for PRRSV antibodies (IDEXX PRRS 2XR ELISA, IDEXX, USA) by running three tests on serum samples collected on arrival. The animals were also tested for classical swine fever virus (CSFV) by RT-PCR and pseudorabies virus (PRV) by ELISA and were confirmed negative. The pigs were randomly assigned by gender and weight blocks to either vaccination with subsequent challenge (V/C) ($n = 20$), challenge only (NV/C) ($n = 12$) and no vaccination and no challenge (controls) $n = 12$. Pigs of the V/C group were intramuscularly vaccinated with the PRRSV type 2 vaccine (Ingelvac® PRRS MLV) according to the manufacturer's recommendation at the age of 28 days (day 1 of the study). Pigs in the NV/C group were intramuscularly injected with Dulbecco's Modified Eagle's Medium (DMEM) and pigs in the strict control group remained untreated. Pigs in the V/C and NV/C groups were inoculated intranasally 27 days post-vaccination. (day 28 post-vaccination) with 3 ml PRRSV JX143 (GenBank no. EU708726) tissue culture containing $3 \times 10^{5.2}$ $TCID_{50}$/dose. PRRSV JX143 originated from the serum of a dying piglet displaying the clinical sings of PHFD in 2006 [11].

Animals were monitored during the subsequent 21 days post challenge and were necropsied at the end of the experiment. The experiment was carried out according to the guideline for care and use of experimental animals and approved by the Ethical Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.2. Clinical examinations

The animals were observed for the following three clinical signs: behavior, cough and breathing pattern. All three parameters were scored on a scale from 1 to 4 using the system outlined in Table 1. In addition, body temperature (in °C) was measured daily.

2.3. Serological response and PRRSV RNA detection

Sera were collected from pigs in the V/C group on days 0, 7, 14, 21, 28, 32, 35, 42 and from pigs in the NV/C group on days 28, 32, 35 and 42. Antibodies against PRRSV were determined by using the IDEXX PRRS 2XR ELISA according to the manufacturer's instructions. Sera were additionally analyzed to determine viremia by using RT-PCR. Viral RNA was extracted from 140 μL pig serum using the Qiagen OneStep RT-PCR kit (Qiagen, Germany). The PCR was performed using primers SF14413 and SR15497 (Supplementary Table S1). The PCR product was purified using TIANgel Mini Purification Kit (Tiangen Biotec, Beijing, PR China) and sequenced.

2.4. Statistical analysis

Data were analyzed using SAS v9.2 by applying analysis of variance procedures (ANOVA) with between-subjects factors using PROC MIXED for course of body temperature and clinical disease scores and Fisher's exact test for categorical variables, such as mortality/survival. Differences between the treatment groups were considered statistically significant if $p \leq 0.05$.

The null hypothesis was that vaccination had no effect on the course of body temperature, clinical disease scores and mortality following challenge.

3. Results

3.1. Body temperature and mortality

Prior to challenge there was no significant difference between the three groups with regard to body temperature. The results of the analysis of variance revealed that there was a significant treatment group x time interaction as displayed in Fig. 1. Mean body temperature remained within the normal range in the case of the strict control group but was markedly elevated already two days after challenge in both the V/C and the NV/C groups. The elevation was however greater in both magnitude and duration for the NV/C

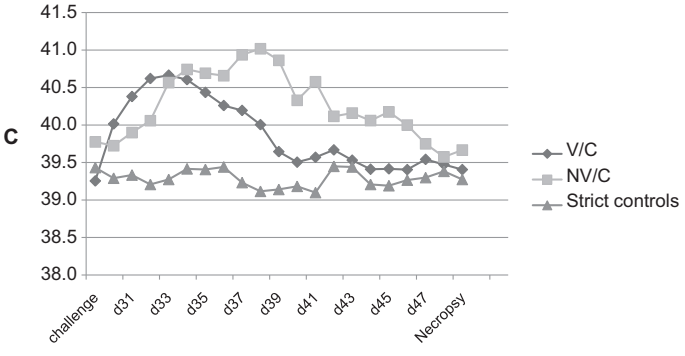

Fig. 1. Development of mean daily body temperature following challenge with PRRSV JX143.

Table 2
Least squares group means for duration of hyperpyrexia and body temperature and incidence of mortality following challenge.

Group	Total number of pigs	Duration of hyperpyrexia in days	Body temperature in °C	Number of deaths
Non vaccinated challenged (NV/C)	12	3.5[A]	40.26[A]	8[A]
Vaccinated challenged (V/C)	20	1.5[B]	39.77[B]	0[B]
Strict controls	12	0[B]	39.3[C]	0[B]

[A,B,C]Different superscripts within a column indicate significant differences at $p \leq 0.05$

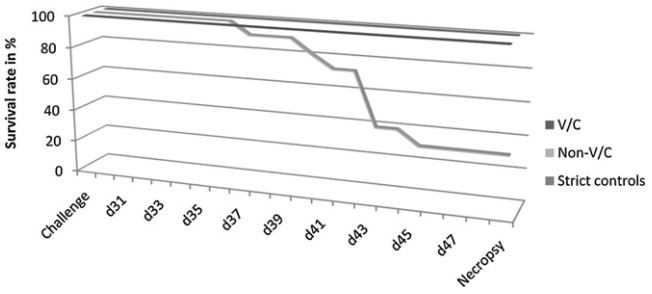

Fig. 2. Survival curve for the three treatment groups.

compared to the V/C group. The body temperature was in the fever range (equal to or more than 40 °C) for 9 and 15 days in the case of the V/C and NV/C groups, respectively.

The duration of hyperpyrexia (i.e. body temperature 41 °C or higher) was calculated for each animal and the least squares mean values for this variable and for body temperature (taken from the repeated measures ANOVA) and the incidence of mortality for each treatment groups are presented in Table 2.

The duration of hyperpyrexia was significantly longer in the NV/C group compared to both the V/C and strict control groups. There was however no evidence for any significant difference between the V/C and strict control groups. The overall mean body temperature post challenge differed significantly between the three groups with the highest value being observed in the NV/C group. There were no cases of mortality in the strict control or V/C groups. In the NV/C group 8 of the 12 pigs died after challenge. The timing of deaths was 1 on each of days 9, 12 and 13 post challenge, 3 on day 14 following challenge and 1 on each of days 15 and 17 post challenge with respective survival rates presented in Fig. 2

3.2. Clinical scores

As with body temperature there was also a significant interaction between treatment and time post challenge in the case of the three clinical scores. The results of the analysis show that whereas there was no change in the course of any of the variables in the strict control group there were marked changes in the mean values post challenge in both the V/C and NV/C groups. Shortly after challenge there was a marked increase in the mean for all three variables which was more pronounced and of longer duration in the NV/C compared to the V/C group.

The score for behavior/general condition was most affected by the challenge compared to the other two scores. All animals in the NV/C group became distinctly lethargic post challenge and showed a marked discoloration of the skin at the extremities and ears, mimicking the clinical picture. The animals in the V/C group showed a transient increase in the general condition score following challenge, but the effect was less pronounced compared to the NV/C group and animals showed normal behavior twelve days after challenge.

Coughing occurred frequently in the NV/C group following challenge, whereas the cough observed in the V/C group was less frequent and ceased before the end of the study. Interestingly the severity of coughing with dyspnea peaked 13 days post challenge in the NV/C group, whereas the scores for the V/C were already declining at this point. At end of the study the surviving animals of the NV/C group still had elevated scores with obvious coughing and dyspnea.

There was a small transient increase in respiratory pattern score post challenge in the V/C group. The animals in the NV/C group however showed a very marked increase in this score which took the form of distinct thumping commencing shortly after challenge which persisted until the end of the study. The LS means for the clinical scores for behavior, cough and respiratory pattern are presented in Table 3. The mean values in the case of behavior and cough were significantly higher in both the NV/C and V/C groups in comparison to the strict controls. The increase in the NV/C group was however significantly greater than that of the V/C group in the case of both scores. The score for respiratory pattern was similar in the strict control and V/C groups and was significantly lower in both groups in comparison to the NV/C group.

3.3. Serological response and PRRSV RNA detection

The group ELISA S/P ratio was used as to measure the serological response to PRRSV infection. The negative-control pigs remained negative for PRRSV antibodies throughout the study. In the V/C group, the antibody was first detected between 10 and 14 days post-vaccination, S/P ratio $\geq$0.4 occurred at 14 days post challenge with 8 of 20 pigs positive, at 21 days post-vaccination, 13 of 20 pigs were positive. The highest S/P ratio in the V/C group was observed after challenge and remained high to the end of the study (ELISA S/P approximately 2). The NV/C pigs seroconverted quickly following challenge; at 7 days post challenge 9 of the 12 pigs were positive with S/P ratio $\geq$0.4.

Following vaccination 60% of the animals of the V/C group turned PCR positive. Thereafter the percentage of positive animals declined gradually. Upon challenge 65% of the animals in the V/C group turned PCR positive, whereas all animals in the NV/C group turned positive. The number of viremic pigs following challenge was significantly lower compared to the NV/C group ($p \leq 0.05$) until end of the experiment. There was a strong association between the percentages of PCR positive animals and development of high body

Table 3
Least squares treatment group means for scores behavior/condition, cough and respiratory pattern following challenge.

Group	Total number of pigs	Behavior	Cough	Respiratory pattern
Non vaccinated challenged (NV/C)	12	2.2[A]	1.7[A]	1.8 [A]
Vaccinated challenged (V/C)	20	1.3[B]	1.3[B]	1.1[B]
Strict controls	12	1.0[C]	1.0[C]	1.0[B]

[A,B,C]Different superscripts within a column indicate significant differences at $p \leq 0.05$.

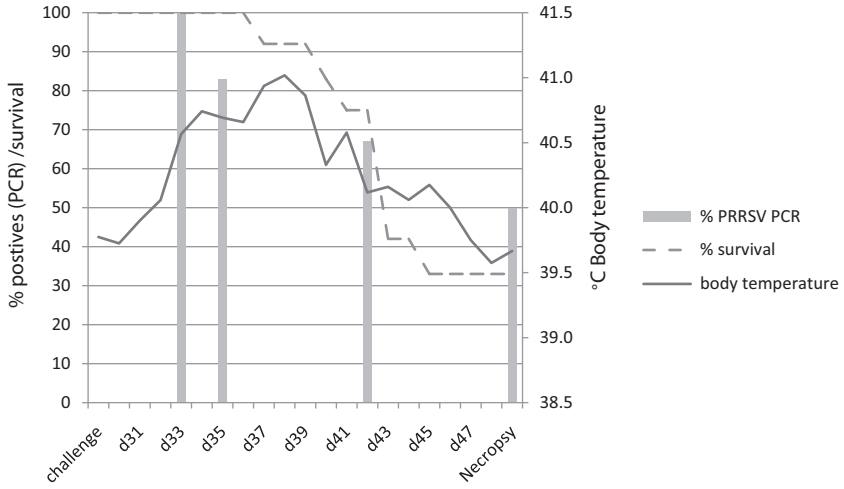

Fig. 3. Association of body temperature, mortality and percent PCR positives in the NV/C group.

temperatures. At study days 42 and 49 only 6/12 and 4/12 animals of the NV/C group were still alive, however of the few surviving animals the majority were still viremic at study termination. There was a linear close association between the incidence of hyperpyrexia, mortality and number of PRRSV PCR positives for the NV/C group as evident from Fig. 3.

4. Discussion

Recent publications [9,10] have shown that PHFD remains pandemic in China, and that the viruses isolated after 2008 still have a close relationship with each other although PRRSV is one of the most rapidly evolving viruses and its evolutionary rate of $4.7–9.8 \times 10^{-2}$/site/year is the highest among RNA viruses reported so far [11].

Compared with the initial outbreak period of PHFD, the number of outbreaks still remains high [10] and the PRRSV isolates circulating still maintain a high degree of pathogenicity. [12].

It should be recognized in this context that considerable changes have occurred in pig husbandry practices over the last decade and that some of them have been associated with an entirely new epidemiology of the infectious diseases of swine. In the case of PRRS, larger herds and increased movement of pigs and semen has facilitated the spread of the virus within and between countries [13].

The nucleotide sequence of PRRSV JX143 shares an overall identity of 61.6% and 89.5%, respectively, with the prototype virus being the PRRS Lelystad virus isolate (Type 1, GenBank no. M96262) and the parental isolate of the type 2 modified life vaccineVR-2332 isolate (Type 2, GenBank no. DQ176021), and is 99.3% identical to the JXA1sequence (GenBank no. EF112445), another Chinese PRRSV isolate isolated during the initial PHFD outbreak [1,14].

The aim of the present study was to investigate the efficacy of the commercially available PRRSV type 2 vaccine in a challenge study with the PRRSV isolate JX143. This study confirms Tian's findings [1] that the typical clinical signs of PHDF can be reproduced in non vaccinated animals by challenging them with this virus even after cell culture propagation. Non vaccinated challenged pigs exhibited the typical signs of PHFD. Although the animals were negative for PRRSV and PCV2 by RT-PCR and ELISA for PRRSV, CSF by RT-PCR and pseudorabies virus by ELISA at the start of the study the animals were not screened for African Swine Fever virus (ASF) and bacterial infections like *Salmonella spp*. However, due to the fact that the strict control animals did not show any clinical signs of disease, increase in body temperature or mortality, it is justified to attribute the effects observed to the challenge. Furthermore, our results are in accordance with the findings of Ni et al. [15] who inoculated pigs with a highly pathogenic PRRSV from Lao PDR (Lao 1.13) which resulted in a subsequent morbidity of 100% and mortality of 60%.

The results of our study show the correlation of hyperpyrexia (41 °C or greater) and mortality as it could be shown that an animal with pronounced hyperpyrexia would die with a probability of 80%. In the NV/C group 8 out of 12 pigs died and all of the pigs had hyperpyrexia, providing profound evidence for the correlation of hyperpyrexia and mortality. Viremia preceded high body temperatures and clinical signs of disease. In vaccinated animals, viremia was relatively short, reflecting very likely the fast response of the immune system. The results obtained for viremia are in accordance with the findings of Wang et al. [16], who found the same pattern of response. Unfortunately, only a qualitative PCR has been carried out and thus no information on viral load is available. The qualitative data however show the relevance of pre-existing immunity in controlling initial viremia after challenge. Type 2 PRRSV appears to attain higher levels of viremia, which may account for its more virulent phenotype [17]. Our findings with challenging non-vaccinated pigs are similar to those obtained by Wu et al. [18], who infected pigs experimentally with the highly virulent PRRSV SD-JN.

In a study published by Roca et al. [19] pigs were challenged with another Asian type 2 PRRSV isolate that has been claimed by the authors to be "virulent" and vaccination with a type 1 modified live vaccine provided some degree of protection. However, as the isolate used in this study (HP-PRRS21) has not been further identified with regard to where it had been isolated and the clinical symptoms produced in the field, it is questionable whether this virus belongs to the group of highly pathogenic PRRS viruses responsible for PHFD and may thus be a type 2 PRRSV that causes typical clinical symptoms of PRRS not belonging to the group of viruses causing PHFD.

Vaccination did not prevent infection; however, it prevented the occurrence of hyperpyrexia which resulted in a survival rate of 100%. Moreover, vaccination resulted in less pigs being viremic, which corresponds also with the finding of having less clinical symptoms over a shorter period of time when compared to the non vaccinated challenged animals. Many vaccines have been produced to combat PRRSV [20]. Documentation of the immunological

properties to elicit protective immunity has only been established for a few of these products. In this study the vaccine used efficaciously induced a serological response against PRRSV and even more important induced a very fast anamnestic response after challenge infection.

It has been confirmed that vaccination against PRRS reduces the impact of disease in commercial nurseries and reduces virulent virus transmission in a model finisher [21].

This study is very relevant as it confirms efficacy in reducing clinical symptoms after single vaccination, which is sufficient to induce a strong immunity effective enough to prevent mortality and facilitate control of challenge virus spread in piglets being exposed to a heterologous and extremely pathogenic PRRSV isolate. Fundamental to the control of the disease is the achievement of stabilization of the field virus circulation in the breeding herd by applying mass vaccination using modified live vaccines followed by control programs in the growing pigs that include all in/all out pig flow strategies, partial depopulation as well as improved biosecurity programs [22]. It is also known that younger pigs suffer more severely than adult pigs [23], which is probably caused by a poorly developed immune system or an immune evasion strategy adapted by the virus.

In summary, this study demonstrated that the experimental challenge of pigs with PRRSV JX143 resulted in typical clinical signs of PHFD in pigs with morbidity of 100% and mortality of 67%, whereas vaccination with a type 2 based MLV resulted in 100% survival and associated prevention of hyperpyrexia as well as reduction of clinical disease scores. However, the underlying mechanism for this effect warrants further immunological investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.02.012.

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