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Effect of challenge dose and route on porcine reproductive and respiratory syndrome virus (PRRSV) infection in young swine

Kyoung-Jin Yoon\textsuperscript{a}\textsuperscript{*}, Jeffrey J. Zimmerman\textsuperscript{a}, Chih-Cheng Chang\textsuperscript{a}, Sol Cancel-Tirado\textsuperscript{a}, Karen M. Harmon\textsuperscript{a}, Michael J. McGinley\textsuperscript{b}

\textsuperscript{a}Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
\textsuperscript{b}Diamond Animal Health Inc., Des Moines, IA 50317, USA

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Abstract — Porcine reproductive and respiratory syndrome virus (PRRSV) is perceived to be highly infectious because of the rapid spread of the virus through populations of domestic swine throughout the world. However, no information has been published on the minimum infectious dose of PRRSV and the effect of challenge dose on clinical response. In this experiment, ten groups of pigs ($n = 3$ per group) were inoculated with one of five different quantities ($10^1-10^5$ fluorescent foci units per millilitre) of PRRSV (isolate ISU-P) by either intramuscular or intranasal routes. Clinical signs and body temperature were monitored for 21 days. Serum was collected periodically throughout the study period to monitor the presence of virus in serum and the early immune response of pigs. A 2-mL inoculum containing $10^1$ fluorescent foci units of virus per millilitre was found sufficient to achieve infection by either route. Time to onset of clinical signs was highly associated with challenge dose ($P < 0.01$), regardless of route of exposure. However, no dose- or route-dependent differences in the severity of clinical manifestation were observed. No significant differences in the time of onset or degree of humoral immune response to PRRSV infection were observed between different treatment groups. However, intramuscular exposure appeared to induce a more uniform antibody response compared to intranasal exposure. These results confirmed that PRRSV is highly infectious; a fact that should be taken into consideration when designing strategies for the prevention and control of PRRSV. © Inra/Elsevier, Paris.

PRRSV / animal challenge / minimum infectious dose / nasal route / intramuscular route

\textsuperscript{*} Correspondence and reprints
Tel.: (1) 515 294 1083; fax: (1) 515 294 6619; kyoung@iastate.edu
Résumé – Effet d'une dose d'épreuve et de la voie d'administration sur l'infection par le virus du syndrome dysgénésique et respiratoire porcin chez le porcelet. Le virus du syndrome dysgénésique et respiratoire du porc (PRRSV) est considéré extrêmement infectieux à cause de la propagation rapide du virus à travers le cheptel porcin mondial. Toutefois, il n'y a pas d'information dans la littérature sur la dose infectieuse minimale du PRRSV et sur l'effet d'une dose d'épreuve sur la réponse clinique. Nos expériences portent sur dix groupes de porcs (n = 3 par groupe) inoculés avec une des cinq quantités de la souche ISU-P de PRRSV (10^1 à 10^5 unités de fluorescence par millilitre) soit par voie intramusculaire soit par voie intranasale. Les symptômes cliniques et les températures ont été enregistrés périodiquement pendant les 21 j de l'étude. Des échantillons de sérum ont été collectés pour déterminer la virémie et pour évaluer la réponse immunologique des porcs. Une inoculation de 2 mL de virus, contenant 10^1 unités de fluorescence par millilitre a été suffisante pour causer une infection. Le début des symptômes cliniques était hautement lié à la dose d'épreuve, quelle que soit la voie d'administration (p < 0.01). Cependant, la gravité des manifestations cliniques était indépendante de la dose et de la voie d'administration du virus. Il n'y a pas eu de différences significatives quant au début d'infection ou au degré de la réponse immunologique parmi les groupes de porcs. Toutefois, l'inoculation intramusculaire paraissait produire une réponse d'anticorps plus uniforme que l'inoculation intranasale. Les résultats confirment que le PRRSV est hautement infectieux ; ce fait devrait être pris en considération en concevant des stratégies de contrôle du PRRSV. © Inra/Elsevier, Paris.

PRRSV / dose infectieuse minimale / voie nasale / voie intramusculaire

1. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a disease of swine caused by a RNA virus belonging to the Family Arteriviridae [4]. Other viruses in the family include lactate dehydrogenase elevating virus (LDV) of mice, equine arteritis virus, and simian haemorrhagic fever virus [17]. These viruses replicate primarily in host macrophages and are known to establish asymptomatic persistent infections in their hosts. Clinical signs of PRRS virus (PRRSV) infection include infertility, late-term abortions, increased numbers of mummified foetuses and stillborn pigs in breeding stock, and respiratory distress in young pigs [10, 12]. In the United States (USA) economic losses due to acute PRRSV infection in breeding and nursery animals were estimated to be $236 per inventoried female and $18.21 per pig placed [18, 19].

Porcine reproductive and respiratory syndrome virus entered the domestic swine population relatively recently. The earliest evidence of PRRSV infection comes from a retrospective serologic study of herds in Ontario, Canada. Carman et al. [3] found that none of 50 herds sampled in 1978 were serologically positive for PRRSV, but PRRSV antibodies were detected in serum samples from two of 51 (3.9 %) herds collected in 1979. In the USA, a retrospective serologic survey found no evidence of infection in 1 425 serum samples collected from 118 Iowa swine herds in 1980, but by 1988, 17 of 27 herds (63.0 %) and 313 of 658 (47.6 %) animals were seropositive to PRRSV [29]. In Asia, antibodies against PRRSV were retrospectively documented in serum from pigs imported into South Korea in October 1985 [20] and in samples collected in June 1988 in Japan [9]. In Europe, clinical outbreaks of PRRS were first reported in November 1990 near Münster in Germany [14].

Following its entry into the domestic swine population, PRRSV spread rapidly throughout the world. Currently, PRRSV infection is endemic in all pork producing areas of the world, with the exception of Australia, New Caledonia, Norway,
Sweden and Switzerland [2, 30]. From these field data, it was reasonable to hypothesize that PRRSV was highly infectious, but no measures of infectivity were available in the literature. For that reason, we carried out the following study to assess the minimum infectious dose (i.e., minimum amount of virus required to establish infection) of PRRSV and evaluate the response of young swine to challenge at different exposure levels and by different routes.

2. MATERIALS AND METHODS

2.1. Experimental design

Thirty-six 4- to 5-week-old pigs were obtained from a swine herd known to be free of PRRSV on the basis of clinical, serological and virological evidence and randomly assigned to one of 12 treatment groups, each treatment group consisting of three pigs. Treatment groups were defined by the route of inoculation and quantity of virus in the inoculum. Five groups were inoculated intranasally (IN) with PRRSV and five groups were injected intramuscularly (IM). Although the IM route was not recognized as a natural route of infection in pig with respect to PRRSV, the IM route challenge was chosen as a point for comparison since previous studies on the minimum infectious dose (MID) for several viruses, including LDV, demonstrated that the MID may vary depending upon the route of exposure [1, 25, 28]. Within each ‘route of inoculation’, groups were assigned to one of five levels of virus exposure. Pigs within each treatment group were inoculated with a 2-mL suspension of PRRSV isolate ISU-P [27] adjusted to contain approximately $10^1$, $10^2$, $10^3$, $10^4$ or $10^5$ fluorescent foci units (FFU) per millilitre based on the predeterminded titre of stock virus. Two groups served as IM and IN negative controls and were inoculated with medium containing no virus.

Each group of three pigs was housed in one room. Pigs were monitored daily for body temperature, loss of appetite, sneezing, coughing, nasal discharge and dyspnea (i.e., ‘thumping’) over a 21-day period post-inoculation (PI) by the same individual. Sera were collected from individual pigs periodically (-2, 0, 2, 4, 7, 9, 11, 14, 17 and 21 days PI) and stored frozen at −70 °C until tested. Prior to performing assays, samples were randomly ordered and relabelled to avoid test bias. Randomized samples were then assayed for the presence of PRRSV and virus-specific antibody.

2.2. Virus assays

The titre of stock virus used for challenging animals was determined using a modification of a microtitration infectivity assay described elsewhere [27]. Virus titre was determined at 70 h post-inoculation and expressed as the mean number of infected cell foci at a given dilution. Foci of infected cells were detected by staining inoculated cells with PRRSV-specific monoclonal antibody SDOW 17 conjugated with FITC (Dr David Benfield, South Dakota State University, Brookings, SD, USA).

To detect viraemia, virus isolation was attempted on sera collected on days 0, 2, 4 and 7 PI. Porcine alveolar macrophages and the MARC-145 clone of an African Green Monkey kidney cell line [11] were used for virus isolation. Procedures for cell maintenance and virus isolation have been described elsewhere [27]. The presence of PRRSV in cell cultures was confirmed by immunofluorescence microscopy using PRRSV-specific monoclonal antibody (SDOW 17). Animals were considered to be viraemic when the virus was isolated from serum samples using either cell type. The virus at a given titre was considered infectious when one or more of the three pigs per treatment became viraemic. If animals were not viraemic during the first 7 days PI, virus isolation was attempted on samples collected at later days PI.

2.3. Antibody detection

A commercial PRRS ELISA kit (PRRS:HerdCheck®, IDEXX Laboratories, Inc., Westbrook, ME, USA) was used to detect virus-specific antibody in serum samples. The test was performed and sample-to-positive
(S/P) ratios were calculated following the procedures recommended by the manufacturer.

2.4. Polymerase chain reaction

A reverse transcription-nested polymerase chain reaction (RT-nPCR) was performed on samples collected on days 0, 2, 4 and 7 PI to confirm virus isolation results. In brief, viral RNA was extracted from serum using Trizol LS reagent (GIBCO/BRL, Grand Island, NY, USA) by following the procedure recommended by the manufacturer. Immediately after extraction, open reading frame (ORF) 7 of individual viral RNA preparations was reverse-transcribed and amplified using the Titan One-tube RT-PCR System (Boehringer Mannheim, Inc., Indianapolis, IN, USA). For that, 2 μL of extracted RNA were added to a mixture containing a final concentration of 2 mM MgCl₂, 5 mM DTT, 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.9 U Prime RNase inhibitor (5Prime-3Prime, Inc., Boulder, CO, USA) per microlitre, 0.08 μM each RT-PCR primers, and 0.5 μL of Titan One-tube RT-PCR System Enzyme Mix. Total volume per sample was adjusted to 25 μL by the addition of molecular biology grade distilled water free of DNase and RNase. All reagents and mixtures were kept on ice and placed in a thermocycler (PE9600, Perkin-Elmer, Foster City, CA, USA) after brief centrifugation in a microcentrifuge (IEC Micromax RF, International Equipment Company, Needham Heights, MA, USA). Reverse transcription was performed for one cycle at 50 °C for 30 min after which the temperature was raised to 94 °C for 2 min. The amplification of cDNA was performed for 35 cycles by denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extending at 68 °C for 45 s. The time of the final extension step was 7 min at 68 °C. RT-PCR products were then amplified further with a set of nested primers. For nested PCR, 0.5 μL of RT-PCR product was added to a mixture containing a final concentration of 4 mM MgCl₂, 10 mM Tris·HCl, 50 mM KCl, 0.2 mM dNTP, 0.8 μM each nested primer, plus 0.25 μL Tag polymerase (5 U/μL, Boehringer Mannheim, Inc., Indianapolis, IN, USA) per reaction. In addition, to allow for direct gel loading of the PCR product, 5 μL of 1 mM Cresol Red in 60 % sucrose (filter sterilized) was added per reaction. Total volume per sample was adjusted to 25 μL by addition of molecular biology grade distilled water free of DNase and RNase. The nested amplification was performed in a thermocycler for 35 cycles with the same annealing, denaturing, and extension temperatures as above, omitting the initial step at 50 °C for 30 min. Primers used in the RT-PCR and nested PCR were designed as previously reported [6] and were synthesized with Integrated DNA Technologies (Coralville, IA, USA). In each run, known positive and negative controls were included for quality control of the test. After completion of the nested PCR, 10 μL of each amplicon was analysed by agarose (2 % FMC NuSieve 3:1) gel electrophoresis with ethidium bromide. Using 100-bp DNA ladder (GIBCO/BRL, Grand Island, NY, USA) as a reference marker, samples showing 236-bp RT-nPCR product were considered positive for PRRSV RNA.

2.5. Statistical analysis

The effects of dose of virus and route of exposure on the onset of clinical signs or severity of clinical signs were evaluated. Because of the censored nature of the onset of clinical sign data, i.e. the possibility for onset ('time-to-clinical signs') to occur after the termination of the 21-day study period, we made the assumption of lognormally distributed errors and fitted the model:

\[
\text{onset} = \beta_0 + \beta_1 \text{[dose]} + \beta_2 \text{[route]} + \text{error}
\]

using the LIFEREG procedure of the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA). Exploratory data analysis indicated that the lognormality assumption for the errors was appropriate for these data. The parameters in the model were estimated by maximum likelihood. The significance of each parameter was then tested using a \( \chi^2 \) test of the null hypothesis that the parameter is equal to zero. Each parameter was considered to be strongly associated with onset when the \( P \) value for the test was less than 0.05.
3. RESULTS

Negative control pigs remained normal in appearance and behaviour throughout the monitoring period. Virus isolation and ELISA results provided evidence that the negative controls remained free of PRRSV infection throughout the study. In contrast, all inoculated pigs developed transient fever (≥ 40 °C) and anorexia at some time during the study. Infrequent diarrhoea, sneezing, mucous nasal discharge, mild coughing and dyspnea were observed in some of the inoculated animals. Time of onset of respiratory clinical signs was highly associated with challenge dose \((P < 0.01)\). Nasal discharge \((P = 0.0001)\), sneezing \((P = 0.0001)\) and dyspnea \((P = 0.0026)\) appeared earlier in animals exposed to a higher dose of PRRSV, regardless of route of challenge \((figure 1)\). However, no dose-dependent response or route-dependent response was observed with respect to the severity of clinical signs.

Virus isolation results are summarized in table I. Regardless of challenge dose of PRRSV, all inoculated pigs, except two pigs inoculated IN with PRRSV at the lowest dose \((10^1 \text{ FFU/mL})\), became viraemic by day 4 PI. One of the two pigs became viraemic by day 7 PI and the other by day 14 PI. This was considered to be the result of transmission of PRRSV from pen mates. PCR results on sera were in agreement with virus isolation results on the same samples.

All pigs in the IM challenge group seroconverted to PRRSV between days 9 and 11 PI. In contrast, pigs in the IN challenge group seroconverted between days 11 and 21 PI \(table II\). No statistically significant difference in the onset or level of the humoral immune response to PRRSV was observed among different treatments \(figure 2\). However, IM exposure to PRRSV appeared to induce a more uniform antibody response than IN inoculation.

4. DISCUSSION

The minimum infectious dose (MID) for a number of human viral pathogens has been found to vary considerably, ranging from about 1 virus to \(10^4\) particles \([7, 24, 25]\). The MID has been

<table>
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<tr>
<th>Challenge route</th>
<th>Dose ((\text{log}_{10}))</th>
<th>Days post-inoculation</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intranasal</td>
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<td>0*</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
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<td>0</td>
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<td>3</td>
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<td></td>
</tr>
<tr>
<td>Intramuscular</td>
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<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
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<td>0</td>
<td>3</td>
<td>3</td>
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</tr>
</tbody>
</table>

* Number of pigs with detectable viraemia at a given day post-inoculation. Three pigs per group.
Figure 1. Appearance of respiratory clinical signs (top panel: nasal discharge; middle panel: sneezing; bottom panel: dyspnea) in pigs exposed to PRRSV at different levels and by different routes. Dots are mean days of first observation of each clinical sign. Solid lines indicate standard error of mean (SEM). Dotted lines are regression lines.
studied for a limited number of viruses infectious for animals and, as in the case of viral pathogens of humans, varies widely. For example, the MID of encephalomyocarditis (EMC) virus in pigs was reported to be $10^{7.8} \text{TCID}_50/\text{mL}$ by the oral route [28]. In other species, the MID of lactate dehydrogenase elevating virus (LDV) of mice has been estimated to be $10^{3.3-10^5.3}$ virions of $50\%$ infectious dose by exposure via mucosal surfaces [1]. In a study of five species (Takydromus tachydomoides, Eumeces latiscutatus, E. barbouri, E. marginatus oshimensis and Gekko japonicus), the MID of Japanese B encephalitis virus for lizards was reported to be $10^3 50\%$ mouse lethal dose by intraperitoneal injection of the virus [15].

Parameters which influence the MID include the type and strain of virus, the assay system (e.g. cell type or host organism being infected), and the route of infection [24]. In the case of LDV of mice, the MID has been shown to vary considerably depending on the route of exposure. Cafruny and Hovinen [1] found that the MID by intraperitoneal or tail cartilage injections approached one virus particle, i.e. very similar to PRRSV. In contrast, exposure via mucosal surfaces such as ocular, vaginal or oral routes required $10^{3.3-10^5.3}$ virions, depending on the specific anatomical location [1]. The route dependence of the MID has also been observed with other viruses. For example, at the same exposure dose of virus, pigs were shown to be more susceptible to encephalomyocarditis virus by transdermal, intratracheal or intramuscular routes than by intranasal or garbage exposure [28].

In our study, assuming that 1 FFU represented the focus of infected cells derived from a single cell infected with one virus particle, we found that ten or fewer virions were sufficient to achieve infection by either intramuscular or intranasal routes of exposure. The route of inoculation did not affect the infectious dose except at the lowest IN inoculation dose (10$^1$ FFU/mL) at which level only one of three pigs became infected by exposure to the inoculum. To achieve a $100\%$ infection rate by intranasal exposure, our data suggested that pigs should receive a dose of at least $10^2$ virions per millilitre. In light of the wide differences in the MID of LDV by route

### Table II. Effect of PRRS virus challenge dose on seroconversion of pigs as determined by ELISA.

<table>
<thead>
<tr>
<th>Challenge route</th>
<th>Dose (log$_{10}$)</th>
<th>Days post-inoculation</th>
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<tbody>
<tr>
<td></td>
<td>0 2 4 7 9 11 14 21</td>
<td></td>
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<tr>
<td><strong>Intranasal</strong></td>
<td></td>
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<tr>
<td>1</td>
<td>0* 0 0 0 1 2 3 3 3</td>
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<tr>
<td>2</td>
<td>0 0 0 0 2 3 3 3 3</td>
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<td>3</td>
<td>0 0 0 1 2 2 3 3 3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0 0 0 0 0 2 3 3 3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 0 0 0 0 2 3 3 3</td>
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</tr>
<tr>
<td><strong>Intramuscular</strong></td>
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<td></td>
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<tr>
<td>1</td>
<td>0 0 0 0 1 3 3 3 3</td>
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<td>0 0 0 0 1 3 3 3 3</td>
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<td>3</td>
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<td>4</td>
<td>0 0 0 0 0 3 3 3 3</td>
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<tr>
<td>5</td>
<td>0 0 0 0 0 3 3 3 3</td>
<td></td>
</tr>
</tbody>
</table>

* Number of pigs in which PRRSV-specific antibody was detected at a given day post-inoculation. Three pigs per group.
of exposure, these results were unexpected.

Dose and route have also been reported to influence the host's immune response. Paul and Mengeling [16] observed that the highest immune response to attenuated porcine parvovirus was achieved by administering a high dose (e.g. $10^5$ TCID$_{50}$). These investigators also found that oronasal administration of the virus did not consistently induce an immune response in pigs, whereas IM injection did. Similar observations were also made in chickens with the infectious bronchitis virus (IBV) [23]. Chickens vaccinated with $10^6$ 50% embryo infectious dose (EID$_{50}$) of IBV (strain H-120) developed both IgG and IgA antibody responses to the virus. In contrast, chickens that received a lower dose of the virus ($10^2$ EID$_{50}$) did not develop either IgG or IgA virus-specific antibodies. Furthermore, higher IgG levels were detected after vaccination by the ocular route as compared to vaccination via the drinking water in chickens that received the same dose of vaccine. Our study did not reveal significant differences in the time of onset or degree of serological response to PRRSV infection based on route of inoculation and/or dose of virus. It should be noted that this study was not intended to monitor the immune response and route- and/or dose-dependent differences might be detected if other facets of immunity were monitored.

This study quantified the highly infectious nature of PRRSV; an important fact in understanding the epidemiology, prevention and control of PRRS. However, in addition to intramuscular and intranasal exposure, pigs are known to be susceptible to PRRSV by several other routes including oral [13], intraperitoneal [22], intravenous [21], intrauterine [5] and vaginal [8, 26]. Our study demonstrated that pigs are infected by intranasal or intramuscular exposure to low levels of virus. Additional work is needed to define the minimum infectious dose and subsequent immune response following exposure by other routes.

Figure 2. Antibody response of pigs to PRRSV infection at different levels and by different routes as determined by ELISA. Lines on each graph represent curve fitting, and symbols show distribution of serological responses. Pigs in the same treatment group are shown by the same symbol at a given day post-inoculation: ■: pigs inoculated with $10^1$ FFU/mL; △: pigs inoculated with $10^2$ FFU/mL; ▲: pigs inoculated with $10^3$ FFU/mL; ▲: pigs inoculated with $10^5$ FFU/mL.
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