

Rapid Communication  
Pathology



 OPEN ACCESS

Received: Mar 27, 2020

Revised: Jul 23, 2020

Accepted: Sep 21, 2020

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Funding

None.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Do VT, Dao HT, Hahn TW; Data curation: Do VT; Formal analysis: Do VT, Dao HT; Investigation: Do VT, Dao HT; Methodology: Do VT, Dao HT, Hahn TW;

# Generation of a cold-adapted PRRSV with a nucleotide substitution in the ORF5 and numerous mutations in the hypervariable region of NSP2

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## ABSTRACT

A cold-adapted porcine reproductive and respiratory syndrome virus (CA-VR2332) was generated from the modified live virus strain VR2332. CA-VR2332 showed impaired growth when cultured at 37°C with numerous mutations (<sup>S</sup>731<sup>F</sup>, <sup>E</sup>819<sup>D</sup>, <sup>G</sup>975<sup>E</sup>, and <sup>D</sup>1014<sup>N</sup>) in the hypervariable region of the NSP2, in which the mutation <sup>S</sup>731<sup>F</sup> might play a vital role in viral replication at 30°C. Conserved amino acid sequences of the GP5 protein suggests that CA-VR2332 is a promising candidate for producing an effective vaccine against PRRSV infection. Further studies on replication and immunogenicity *in vivo* are required to evaluate the properties of CA-VR2332.

**Keywords:** Porcine reproductive and respiratory syndrome; cold-adapted; GP5; hypervariable region; NSP2

## INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) was first reported in the USA in 1987 and in Europe in 1990 [1]. It is caused by the porcine reproductive and respiratory syndrome virus (PRRSV), which is an RNA virus of the order *Nidovirales*, family *Arteriviridae*, genus *Aterivirus* [1]. PRRSV is approximately 15 kb in size, composed of at least nine overlapping open reading frames (ORFs). ORFs 2–5 encode glycosylated membrane proteins GP2–GP5, ORF6 encodes a nonglycosylated membrane protein, and ORF7 encodes the nucleocapsid protein [2]. ORF1a and ORF1b form polyprotein pp1a and the large polyprotein pp1ab, respectively [3]. Among these proteins, GP5 plays an important role in viral infectivity and induces protective antibodies in the host [2]. Nonstructural protein 2 (NSP2), one of the proteins encoded by ORF1a, is the most variable and is involved in the regulation of viral replication and transcription [4].

PRRS is considered one of the most economically important swine disease, causing annually losses of more than \$660 million in the USA, more than €650,000 in Europe [5], and colossal economic losses to the swine industry in China and Asian countries [6]. Several inactivated vaccines and modified live virus (MLV) vaccines are licensed to combat infection

Project administration: Hahn TW; Supervision: Hahn TW; Validation: Do VT, Hahn TW; Writing - original draft: Do VT; Writing - review & editing: Do VT, Hahn TW.

by PRRSV. Inactivated PRRS vaccines improve reproductive performance, but the immune response is often delayed and is controversial [7]. MLV vaccines increase pig farrowing and weaning rates, and improve growth performance, but their tendency to revert to virulence is a major concern [8]. MLV vaccines can get into the target organs and provoke diseases upon reversion of virulence, but cold-adapted vaccines do not replicate at body temperature and do not induce diseases. Thus, we aimed to address the safety concern of MLV PRRS vaccines by generation of a cold-adapted attenuated strain that would show impaired growth at body temperature (37°C), and would not pose a threat. Numerous studies have been conducted to generate cold-adapted attenuated avian influenza virus vaccines, and these have identified promising candidate vaccines. A live cold-adapted H9N2 influenza virus candidate vaccine conferred protection of chickens from challenges with homologous and heterologous H9N2 viruses [9]. A cold-adapted attenuated porcine epidemic diarrhea virus (PEDV) provided durable lactogenic immunity and conferred passive protection to litters against PEDV infection demonstrating a live vaccine in maternal vaccination strategies [10]. Here, we generated a cold-adapted attenuated PRRSV and analyzed the changes in nucleotide and deduced amino acid sequences of GP5 and in the hypervariable region of NSP2.

## MATERIALS AND METHODS

### Generation of a cold-adapted PRRSV

To generate a cold-adapted PRRSV, MARC-145 cells were serially passaged at gradually reducing incubating temperatures from 37°C to 27°C using Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Ireland) containing 10% fetal bovine serum (FBS; Gibco), and 1% antibiotic-antimycotic (Gibco). As a result, MARC-145 cells could adapt and grow well at 30°C. A cold-adapted PRRSV was developed by inoculation of an attenuated modified live PRRSV vaccine strain VR2332 (MLV VR2332; Ingelvac PRRS; Boehringer Ingelheim, USA), on MARC-145 cells in DMEM containing 5% FBS and 1% antibiotic-antimycotic at temperatures ranging from 37°C to 30°C. MLV VR2332 was incubated on MARC-145 cells at 37°C until > 80% of the cells showed CPE, and harvested by three cycles of freezing and thawing. In the same manner, the virus was passaged at temperatures of 35°C, 33°C, 31°C, and 30°C.

### Plaque assay

Plaque assays were performed to determine plaque-forming units (PFUs) of cold-adapted VR2332 (CA-VR2332) at 30°C or 37°C separately as described by Kim et al. [11] with modifications. Briefly, MARC-145 cells were seeded in a 24-well plate at a density of  $1 \times 10^5$  cells/well one day before the assay. Cells were infected with serial 10-fold dilutions of virus (starting with the original virus at an equal multiplicity of infection of 1) for 1 h at 30°C or 37°C. After removing the inoculum, the cells were then overlaid with DMEM containing 0.4% SeaPlaque Agarose (Lonza, Switzerland) and 5% FBS, followed by incubation at 30°C or 37°C, 5% CO<sub>2</sub> for 5–7 days. The plaques were visualized by fixation with 8% formaldehyde and stained with 1% crystal violet [11].

### Sequence alignment

Ten individual CA clones were selected and passaged at 30°C an additional 10 times. Three of the 10 clones showing higher viral titers were selected for genome analysis. The full-length ORF5 and the hypervariable region of NSP2 for nucleotides 2158–3177 and the deduced amino acids 720–1059 (based on the full genome sequence of the MLV VR2332) were sequenced in both directions and compared using the ClustalX 2.0 program (<http://www.clustal.org>).

## RESULTS

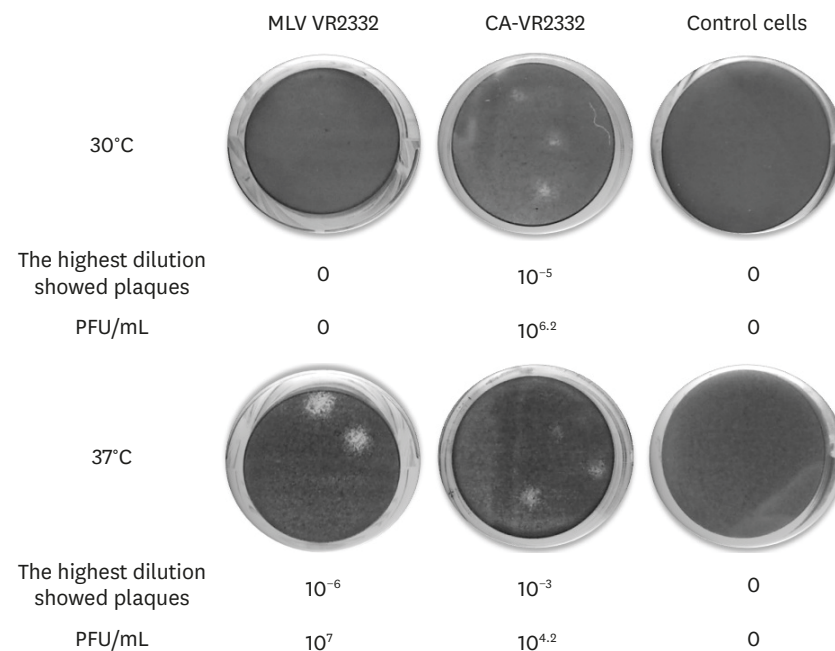
### Generation of CA-VR2332

The MLV VR2332 was inoculated on MARC-145 cells at gradually reducing incubating temperature from 37°C to 30°C. CA-VR2332 clones were generated after 10 passages at 30°C and passaged 10 times more on MARC-145 cells at 30°C to produce a high viral titer of  $10^{6.5}$  TCID<sub>50</sub>/mL.

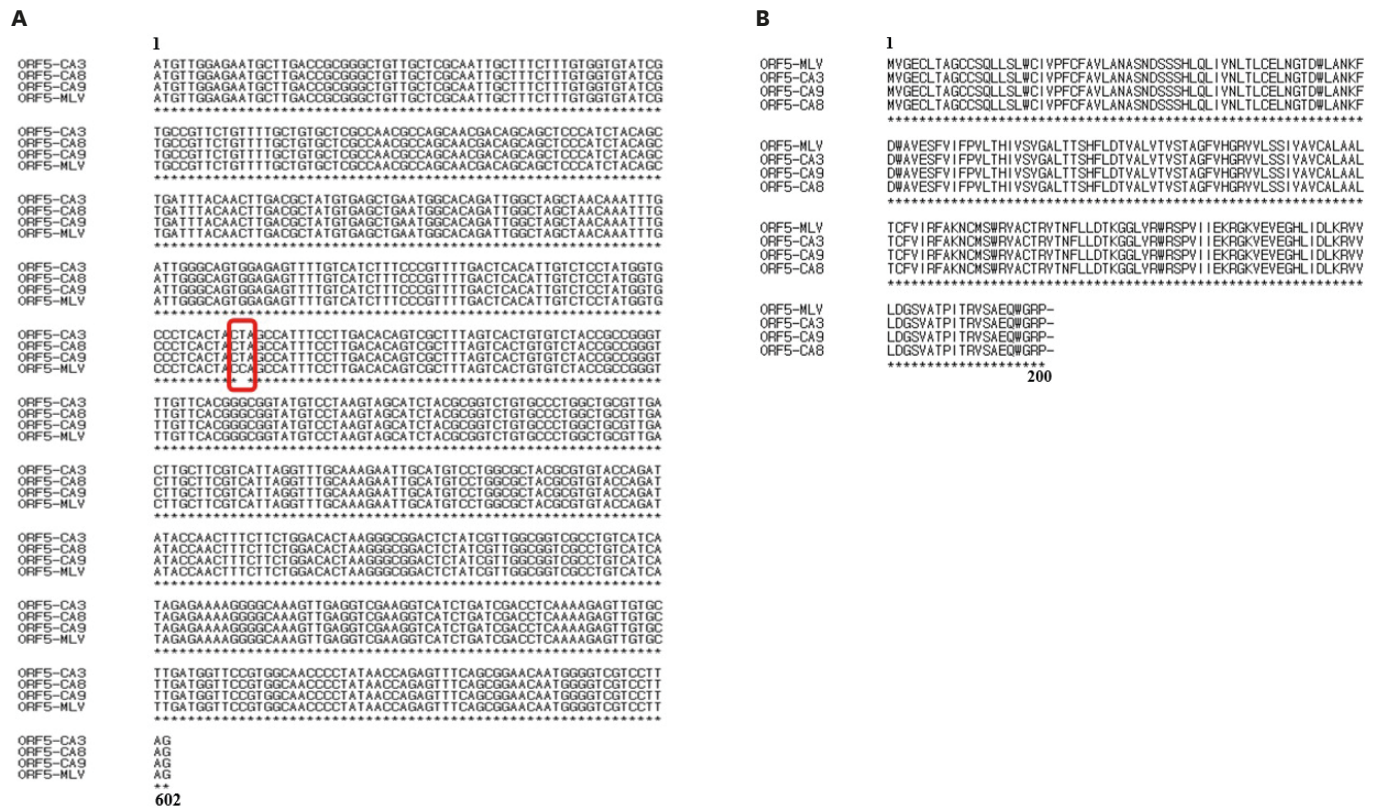
CA-VR2332 reached  $10^{6.2}$  PFU/mL at 30°C but reduced forming PFU to  $10^{4.2}$  PFU/mL at 37°C. No PFUs were developed by the MLV VR2332 and control cells at 30°C. In contrast, the MLV VR2332 resulted in  $10^7$  PFU/mL and formed bigger plaques at 37°C (**Fig. 1**). CA-VR2332 showed a 100-fold reduction in PFUs when incubated at 37°C compared with that at 30°C. Thus, CA-VR2332 showed impaired growth when cultured at normal body temperature (37°C), suggesting the successful generation of CA-VR2332.

### Sequence comparison of MLV VR2332 and CA-VR2332

To investigate how cold adaptation caused the change in sequences of ORF5 encoding GP5 and the hypervariable region of NSP2, 10 cold-adapted clones were harvested and propagated on MARC-145 cells at 30°C individually for an additional 10 passages. Three CA-VR2332 clones (CA3, CA8, and CA9) with high viral titers at passage 30 were selected for sequencing and comparison with the MLV VR2332. As shown in **Fig. 2A and B**, cold adaptation caused one nucleotide substitution <sup>C251T</sup>, but no change in the deduced amino acid sequences of GP5 of all three CA-VR2332 clones was seen. Notably, sequence alignment of the hypervariable region of NSP2 showed that all three clones contained the same nucleotide and amino acid substitutions. Changes in nucleotide sequences were observed at five positions (**Fig. 3A**), in which one mutation did not cause substitution of amino acid 725. Four substitutions of amino acids occurred at residues <sup>S731F</sup>, <sup>E819D</sup>, <sup>G975E</sup>, and <sup>D1014N</sup> (**Fig. 3B**).



**Fig. 1.** Plaque-forming units of the MLV VR2332 and CA-VR2332 at 30 °C or 37 °C. PFU, plaque-forming unit.



**Fig. 2.** Nucleotide sequence alignment of ORF5 (A), and the deduced amino acid sequence comparison of GP5 (B) of the MLV VR2332 and CA-VR2332 clones. Nucleotide substitutions are shown in the red box. No substitutions in the amino acid sequences of GP5 were observed.

## DISCUSSION

The ORF5 encodes GP5, which is the main structural protein of PRRSV. GP5 has high immunogenicity and induces neutralizing antibodies in the host [2]. An et al. [12] generated an attenuated PRRSV strain HuN4-F112 by passaging a virulent strain on MARC-145 cells 112 times at 37°C, and compared the GP5 sequences of the virulent and attenuated strains. An amino acid substitution (<sup>Q196</sup>R) was detected at passage 20 and an additional amino acid change (<sup>N34</sup>P) occurred at passage 65. No further change in GP5 sequence was observed until passage 112. In the present study, sequencing showed one nucleotide change, but it did not cause any change in the deduced amino acid sequences of GP5 compared with the MLV VR2332 (**Fig. 2B**). Thus, cold adaptation did not appear to affect the amino acid sequence of GP5.

ORF1a and ORF1b encode a large polypeptide containing cleavage products involving viral transcription and replication, in which NSP1, 2, 3, 4, and 5 are encoded by ORF1a [13]. NSP2 is the most variable nonstructural protein, primarily in its middle region, sharing 32% amino acid sequences among subtypes [13]. Faaberg et al. [14] indicated that amino acids 727–813 of NSP2 were important for PRRSV replication *in vivo*. Deletion of these amino acids in PRRSV produced a significant decrease in the enlargement of lymph nodes in infected pigs compared with full-length PRRSV VR2332. In the present study, four substitutions of amino acids occurred at residues <sup>S731</sup>F, <sup>E819</sup>D, <sup>G975</sup>E, and <sup>D1014</sup>N (**Fig. 3B**), with mutation <sup>S731</sup>F in an important sequence (amino acids 727–813), suggesting that this mutation could play a vital role in replication of the MLV VR2332 at 30°C.



Cold adaptation of PRRSV causes mutations in NSP2

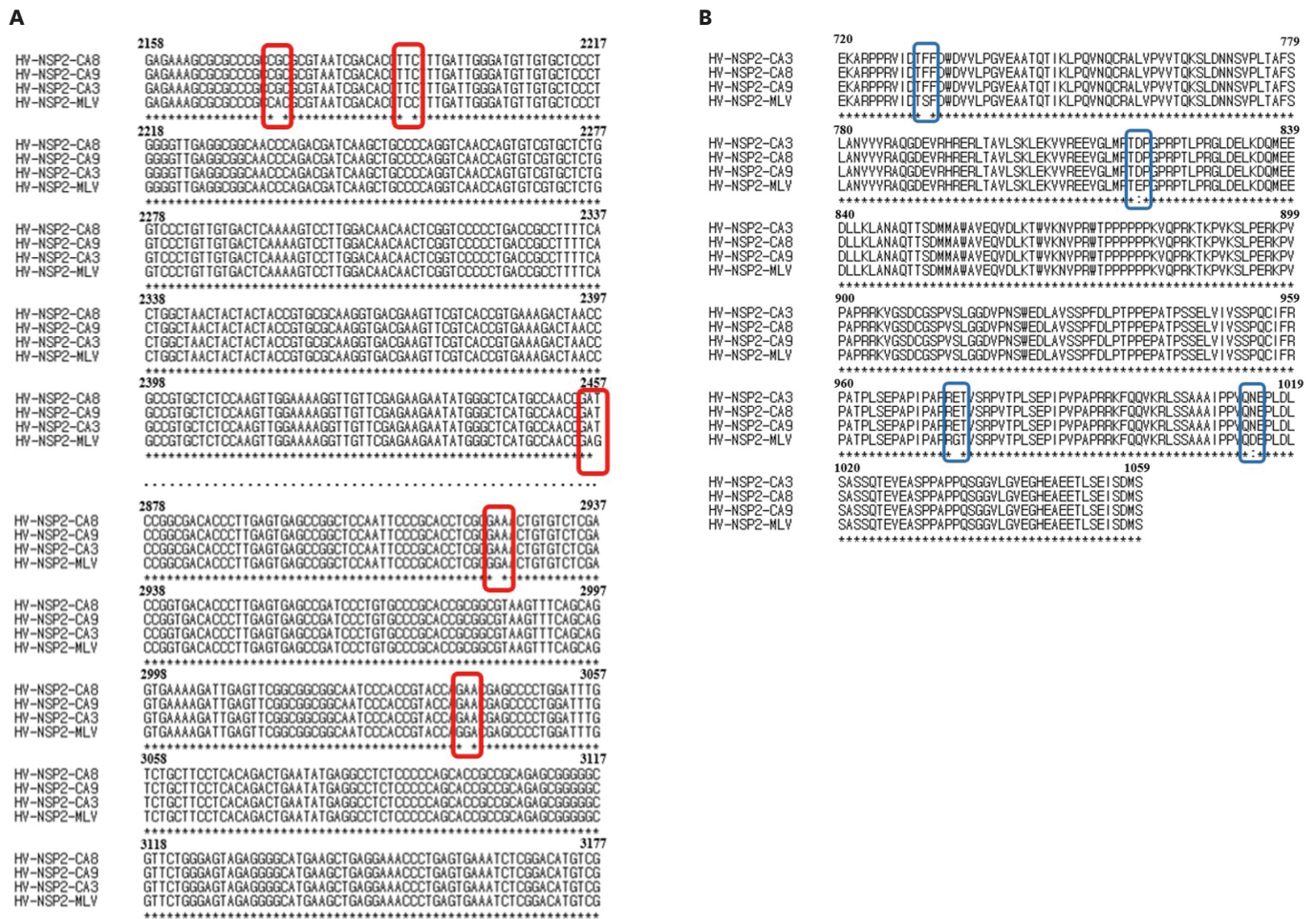


Fig. 3. Nucleotide sequence alignment (A), and the deduced amino acid sequence comparison (B) of the hypervariable region of NSP2 of the MLV VR2332 and CA-VR2332 clones. Nucleotide substitutions are shown in the red boxes, and substitutions of deduced amino acids are shown in the blue boxes.

In a previous study, attenuation of virulent PRRSV strains on MARC-145 cells at 37°C caused eight mutations in NSP2 (<sup>H</sup>401<sup>R</sup>, <sup>T</sup>477<sup>I</sup>, <sup>D</sup>799<sup>N</sup>, <sup>V</sup>894<sup>A</sup>, <sup>S</sup>1009<sup>P</sup>, <sup>N</sup>1090<sup>S</sup>, <sup>C</sup>1166<sup>S</sup>, and <sup>T</sup>1481<sup>A</sup>), in which the mutation <sup>D</sup>799<sup>N</sup> is within a sequence that is important for viral replication [12]. Thus, mutations in NSP2 occurred not only in this important sequence but also in the full-length protein needed for viral replication and attenuation. In our study, progressive cold adaptation caused mutations in this key sequence and in the hypervariable sequence of NSP2. These mutations occurred at the same positions among three of the CA clones. Thus, mutations in this highly variable region of NSP2, especially amino acids 727–813, have significant effects not only in replication and attenuation but also in cold adaptation of PRRSV.

To our knowledge, this is the first study to generate a cold-adapted attenuated PRRSV. We successfully generated CA-VR2332 and the changes in the sequences of nucleotides and amino acids of GP5 protein and the hypervariable region in NSP2 were observed. There is a need for further studies on the replication and immunogenicity of this cold-adapted PRRSV in pigs.

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