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(54) **Title:** PRRS VIRUS VARIANT, EUROPEAN PRRS VIRUS CDNA CLONE, AND USES THEREOF

(57) **Abstract:** The present invention belongs to the field of animal health and provides means to study Porcine Reproductive and Respiratory Syndrome (PRRS), a viral disease affecting swine, and for the development of vaccines, therapeutics and diagnostics for the prophylaxis, treatment and diagnosis of PRRS. In a first consideration, the invention relates to a new PRRS virus variant, and, in a second consideration, to a nucleic acid sequence which comprises the genome of an infectious genotype I (EU) PRRS virus clone. Based on this, new PRRS vaccine candidates with improved properties are provided.



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PRRS virus variant, European PRRS virus cDNA clone, and uses thereof

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention belongs to the field of animal health.

In a first consideration, the invention relates to a new PRRS virus variant. The invention also relates to the use of such PRRS virus to study Porcine Reproductive and Respiratory Syndrome (PRRS), a viral disease affecting swine, and in the development of vaccines, therapeutics and diagnostics for the prophylaxis, treatment and diagnosis of PRRS.

In a second consideration, the invention relates to a nucleic acid sequence which comprises the genome of an infectious genotype I (EU) PRRS virus clone. The invention also relates to the use of the nucleic acid sequence of the infectious genotype I PRRS virus clone to produce attenuated live viruses useful for preventing or treating Porcine Reproductive and Respiratory Syndrome (PRRS) in swine and in the development of vaccines, therapeutics and diagnostics for the prophylaxis, treatment and diagnosis of PRRS.

Combining said both considerations, furthermore novel PRRS viruses with improved properties are provided under a third consideration of the invention.

BACKGROUND INFORMATION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the virus family *Arteriviridae* and belongs, together with the *Coronaviridae*, to the virus order *Nidovirales*. PRRSV is an enveloped virus with a single-stranded, positive-sense RNA genome of about 15 kilobases comprising nine open reading frames (ORFs), namely ORF1a, ORF1ab, ORF2a, ORF 2ab, and ORFs 3 through ORF7. ORFs 1a and 1ab encode large polyproteins that are processed into the viral nonstructural proteins (nsp) by auto- and transcleavages of viral proteases nsp1, nsp2, and nsp4 (Snijder and Meulenberg, 1998). ORF4 encodes a minor glycoprotein (GP4) which is, next to a major glycoprotein (GP5) and two other minor glycoproteins (GP2a and GP3), found in the viral envelope, wherein all of said glycoproteins are important for infectious virus production.

PRRSV is considered one of the economically most important infectious agents in pigs causing late-term reproductive failure in sows and respiratory disease in growing pigs. Often, PRRSV infection is complicated by secondary bacterial infections being attributed to the immunosuppressive nature of the virus. Also, PRRSV viremia lasts for weeks, and virus then still can be detected in lymphoid organs for several months, demonstrating difficulties or failure of the host's immune response to clear the virus (Allende et al., 2000).

There are two distinct viral PRRSV genotypes causing similar clinical symptoms that diverge by about 40 % on nucleotide sequence level, genotype I (EU) and genotype II (US). The North American (US) prototype strain is VR-2332, while the European (EU) prototype strain is Lelystad virus.

However, in a first consideration, as PRRS virus strains have a high biological diversity and evolve rapidly on individual farms (Badaoui *et al. BMC Veterinary Research* 2013, **9**:58), new PRRSV isolates are needed for a better understanding of PRRS, for reproducing said disease in its different forms, for comparative tests, and as platform for the development of new vaccines, medications and diagnostics for the prophylaxis, treatment and diagnosis of PRRS.

In a second consideration, a growing number of infectious cDNA clones of the PRRS virus are becoming available to the scientific community, most of which are based on the US type of the virus. For the EU type, however, only few clones are available. Thus, there is a strong need for new infectious cDNA clones of European (genotype I) PRRS virus, for a better understanding of PRRS, for comparative tests, as platform for the development of new vaccines, medications and diagnostics for the prophylaxis, treatment and diagnosis of PRRS, wherein the use of the cDNA clone results in a high yield of virus production. Thus, for experimental convenience in the PRRS vaccine research an infectious cDNA clone would be needed enabling the production of genotype I PRRS virus in high amounts.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present disclosure. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or

group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

DESCRIPTION OF THE INVENTION

1. First consideration of the present invention

According to a first consideration, which is detailed in this section, the invention is based on the isolation of a new PRRS virus which is surprisingly capable to induce severe clinical signs in boars. Closer analyses of this PRRS virus variant revealed a significant deletion within the ORF4 gene of said virus.

In one aspect, the invention thus relates to a Porcine Reproductive and Respiratory Syndrome (PRRS) virus, wherein said virus is selected from the group consisting of the following (a), (b), (c), (d), (e), and (f):

- (a) a PRRS virus comprising an ORF4 protein which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-12;
- (b) a PRRS virus, preferably a genotype I PRRS virus, comprising an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues in the region located between the first two predicted N-terminal β -sheets, as compared to the ORF4 protein of a wild type genotype I PRRS virus;
- (c) a genotype II PRRS virus, comprising an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues in the region between the first two predicted N-terminal β -sheets, as compared to a wild type genotype II PRRS virus;
- (d) a PRRS virus, preferably a genotype I PRRS virus, comprising an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus;
- (e) a genotype II PRRS virus, comprising an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues between amino acid positions 50 to 67, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the PRRS virus VR2332;
- (f) a combination of any of (a), (b), (c), (d), and (e);

and, in a further aspect, the invention relates, respectively, to a Porcine Reproductive and Respiratory Syndrome (PRRS) virus selected from the group consisting of the following A), B), C), D), E), and F):

- A) a PRRS virus whose genome comprises a nucleic acid molecule which encodes an ORF4 protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1-12;
- B) a PRRS virus, preferably a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues in the region located between the first two predicted N-terminal β -sheets, as compared to the ORF4 protein of a wild type genotype I PRRS virus;
- C) a genotype II PRRS virus whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues in the region between the first two predicted N-terminal β -sheets, as compared to a wild type genotype II PRRS virus;
- D) a PRRS virus, preferably a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus;
- E) a genotype II PRRS virus whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues between amino acid positions 50 to 67, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the PRRS virus VR2332;
- F) a combination of any of A), B), C), D), and E).

Preferably, said PRRS virus, which is also termed "PRRS virus of the present invention" hereinafter, is an isolated PRRS virus.

Within the context of the invention, it is in particular understood that the phrase "amino acid residues in the region" is equivalent to the phrase "amino acid residues located in the region" and, respectively, it is particularly understood that the term "amino acid residues between

amino acid positions” is interchangeable with the term “amino acid residues located in the region between amino acid positions”.

It is further understood that the terms “genotype I” and “genotype II” are equivalent to the terms “genotype 1” and “genotype 2” or to the terms “type 1” and “type 2”, as frequently used in the literature in the context of PRRSV.

According to the first aspect ((a)), the PRRS virus of the present invention is thus a PRRS virus comprising an ORF4 protein which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-12, wherein said ORF4 protein preferably comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 13-24, and wherein said ORF4 protein in an exemplary non-limiting embodiment comprises the amino acid sequence of SEQ ID NO: 31.

Respectively, according to the first aspect ((A)), the PRRS virus of the present invention is a PRRS virus whose genome comprises a nucleic acid molecule which encodes an ORF4 protein comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-12, wherein said ORF4 protein preferably comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 13-24, and wherein said ORF4 protein in an exemplary non-limiting embodiment comprises the amino acid sequence of SEQ ID NO: 31.

According to the second aspect ((b)), the PRRS virus of the present invention is a PRRS virus, in particular a genotype I PRRS virus, comprising an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues in the region between the first two predicted N-terminal β -sheets, as compared to the ORF4 protein of a wild type genotype I PRRS virus, wherein said first two predicted N-terminal β -sheets are preferably the two amino acid sequences set forth in SEQ ID NO:25 and SEQ ID NO:26, or are preferably the two amino acid sequences set forth in SEQ ID NO:29 and SEQ ID NO:30, and wherein in an exemplary non-limiting embodiment said ORF4 protein comprises the amino acid sequence of SEQ ID NO:32.

Respectively, according to the second aspect ((B)), the PRRS virus of the present invention is a PRRS virus, in particular a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues in the region between the first two predicted N-terminal β -sheets, as compared

to the ORF4 protein of a wild type genotype I PRRS virus, wherein said first two predicted N-terminal β -sheets are preferably the two amino acid sequences set forth in SEQ ID NO:25 and SEQ ID NO:26, or are preferably the two amino acid sequences set forth in SEQ ID NO:29 and SEQ ID NO:30, and wherein in an exemplary non-limiting embodiment said ORF4 protein comprises the amino acid sequence of SEQ ID NO:32.

As described herein, for purposes of comparison, the wild type genotype I PRRS virus is preferably the prototype genotype I Lelystad virus. The genome of the Lelystad virus is encoded by the nucleic acid sequence of SEQ ID NO:41.

According to the third aspect ((c)), the PRRS virus of the present invention is a genotype II PRRS virus, comprising an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues in the region between the first two predicted N-terminal β -sheets, as compared to a wild type genotype II PRRS virus, wherein the first two predicted N-terminal β -sheets are preferably the two amino acid sequences set forth in SEQ ID NO: 27 and SEQ ID NO: 28, and wherein said ORF4 protein in an exemplary non-limiting example comprises the amino acid sequence of SEQ ID NO:33.

Respectively, according to the third aspect ((C)), the PRRS virus of the present invention is a genotype II PRRS virus whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues in the region between the first two predicted N-terminal β -sheets, as compared to a wild type genotype II PRRS virus, wherein the first two predicted N-terminal β -sheets are preferably the two amino acid sequences set forth in SEQ ID NO: 27 and SEQ ID NO: 28, and wherein said ORF4 protein in an exemplary non-limiting example comprises the amino acid sequence of SEQ ID NO:33.

As mentioned herein, for purposes of comparison, the wild type genotype II PRRS virus is preferably the prototype genotype II virus VR2332. The genome of the virus VR2332 is encoded by the nucleic acid sequence of SEQ ID NO:42.

In the context of the invention, a deletion of amino acid residues is preferably a deletion of consecutive amino acid residues. Thus, for example, a deletion of 9, 10, 11 or more amino acid residues, as described herein, is preferably a deletion of 9, 10, 11 or more consecutive amino acid residues and, respectively, a deletion of 5, 6, 7 or more amino acid residues, as described herein, is preferably a deletion of 5, 6, 7 or more consecutive amino acid residues.

According to the fourth aspect ((d)), the PRRS virus of the present invention is a PRRS virus, preferably a genotype I PRRS virus, comprising an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues, or preferably a deletion of 11, 12, 13, 14, 15, 16, or 17 amino acid residues, between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus, and wherein in a non-limiting exemplary embodiment an ORF4 protein having a deletion of 11 amino acid residues between amino acid positions 50 to 71 is an ORF4 protein which comprises the amino acid sequence of SEQ ID NO:34.

Respectively, according to the fourth aspect ((D)), the PRRS virus of the present invention is a PRRS virus, preferably a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues, or preferably a deletion of 11, 12, 13, 14, 15, 16, or 17 amino acid residues, between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus, and wherein in a non-limiting exemplary embodiment an ORF4 protein having a deletion of 11 amino acid residues between amino acid positions 50 to 71 is an ORF4 protein which comprises the amino acid sequence of SEQ ID NO:34.

As described herein, the numbering of amino acid positions relating to the Lelystad virus refers to the amino acid sequence of full length ORF4 protein of the Lelystad virus. Hence, the numbering of the amino positions as mentioned in this context is with reference to the ORF4 protein of the Lelystad protein having 183 amino acid residues, including a methionine residue at the (N-terminal) amino acid position 1.

Thus, the phrase "wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus", as used in the context of the present invention, relates to the sequence of ORF4 protein as set forth in SEQ ID NO:43.

According to the fifth aspect ((e)), the PRRS virus of the present invention is a genotype II PRRS virus, comprising an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues, or preferably a deletion of 8, 9, 10, 11 or more amino acid residues, between amino acid positions 50 to 67, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the PRRS virus VR2332, and wherein in a non-limiting exemplary embodiment an ORF4 protein having a deletion of 7 amino acid residues

between amino acid positions 50 to 67 is an ORF4 protein which comprises the amino acid sequence of SEQ ID NO:35.

Respectively, according to the fifth aspect ((E)), the PRRS virus of the present invention is a genotype II PRRS virus whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a deletion of 5, 6, 7 or more amino acid residues, or preferably a deletion of 8, 9, 10, 11 or more amino acid residues, between amino acid positions 50 to 67, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the PRRS virus VR2332, and wherein in a non-limiting exemplary embodiment an ORF4 protein having a deletion of 7 amino acid residues between amino acid positions 50 to 67 is an ORF4 protein which comprises the amino acid sequence of SEQ ID NO:35.

As described herein, the numbering of amino acid positions relating to the PRRS virus VR2332 refers to the amino acid sequence of full length ORF4 protein of the PRRS virus VR2332. Hence, the numbering of the amino positions as mentioned in this context is with reference to the ORF4 protein of the VR2332 virus having 178 amino acid residues, including a methionine residue at the (N-terminal) amino acid position 1.

Thus, the phrase "wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the PRRS virus VR2332 having 178 amino acid residues, as used in the context of the present invention, relates to the sequence of ORF4 protein as set forth in SEQ ID NO:44.

According to the sixth aspect ((f)), the PRRS virus of the present invention is a combination of any of the aspects (a), (b), (c), (d), and (e), as described herein, preferably a combination of any of the aspects (a), (b), and (d) or a combination of any of the aspects (a), (c), and (e). Within this context it is in particular understood that the phrase "combination of any of (a), (b), (c), (d), and (e)" and "combination of any of the aspects (a), (b), (c), (d), and (e)", respectively, means a PRRS virus having a combination of the features of any PRRS viruses of (a), (b), (c), (d), and (e), as described herein, wherein a combination of the features of any of the PRRS viruses of the aspects (a), (b) and/or (c) or a combination of the features of any of the PRRS viruses of the aspects (a), (c), and (e) is in particular preferred.

Respectively, according to the sixth aspect ((F)), the PRRS virus of the present invention is a combination of any of the aspects (A), (B), (C), (D), and (E), as described herein, preferably

a combination of any of the aspects (A), (B), and (D) or a combination of any of the aspects (A), (C), and (E). Within this context it is in particular understood that the phrase "combination of any of (A), (B), (C), (D), and (E)" and "combination of any of the aspects (A), (B), (C), (D), and (E)", respectively, means a PRRS virus having a combination of the features of any PRRS viruses of (A), (B), (C), (D), and (E), as described herein, wherein a combination of the features of any of the PRRS viruses of the aspects (A), (B) and/or (D) or a combination of the features of any of the PRRS viruses of the aspects (A), (C), and (E) is in particular preferred.

The PRRS virus of the present invention preferably comprises

- an ORF4 protein which comprises or consists of an amino acid sequence having a least 84.5% preferably at least 90%, more preferably at least 95%, still more preferably at least 97%, and in particular preferably at least 99% sequence identity with the amino acid sequence of SEQ ID NO:36, or
- an ORF4 protein which comprises or consists of an amino acid sequence encoded by a nucleic acid sequence having a least 83.5% preferably at least 90%, more preferably at least 95%, still more preferably at least 97%, and in particular preferably at least 99% sequence identity with the nucleic acid sequence of SEQ ID NO:37, wherein said PRRS virus is preferably a genotype I PRRS virus,

and wherein said PRRS virus is in particular a genotype I PRRS virus.

As used herein, it is in particular understood that the term "sequence identity with the amino acid sequence of SEQ ID NO: 36" is equivalent to the term "sequence identity with the amino acid sequence of SEQ ID NO: 36 over the length of SEQ ID NO: 36" or to the term "sequence identity with the amino acid sequence of SEQ ID NO: 36 over the whole length of SEQ ID NO: 36", respectively.

Further, as used herein, it is particularly understood that the term "sequence identity with the nucleic acid sequence of SEQ ID NO: 37" is equivalent to the term "sequence identity with the nucleic acid sequence of SEQ ID NO: 37 over the length of SEQ ID NO: 37" or to the term "sequence identity with the nucleic acid sequence of SEQ ID NO: 37 over the whole length of SEQ ID NO: 37", respectively.

Sequence identity in the context of the first consideration of the invention is understood as being based on progressive alignment (Feng, D. F. and Doolittle, R. F. (1987). *Progressive sequence alignment as a prerequisite to correct phylogenetic trees*. J. Mol. Evol., 25(4):351–

360, herein incorporated by reference). This method is based on combining sequences into alignments, which can in turn be combined with other sequences or alignments to form larger alignments. The procedure is repeated until all the input sequences have been joined in a single multiple alignment. For purposes of the present invention, percent sequence identity is determined with the software CLC MAIN WORKBENCH 4.1.1 (CLC BIO).

In one exemplary and non-limiting embodiment the PRRS virus of the present invention is a genotype I PRRS whose genome comprises an RNA molecule encoded by a nucleic acid molecule having at least 84.5%, preferably at least 90%, more preferably at least 95%, still more preferably at least 97%, and in particular preferably at least 99% sequence identity with the nucleic acid sequence of SEQ ID NO: 38.

As used herein, it is in particular understood that the term "sequence identity with the nucleic acid sequence of SEQ ID NO: 38" is equivalent to the term "sequence identity with the nucleic acid sequence of SEQ ID NO: 38 over the length of SEQ ID NO: 38" or to the term "sequence identity with the nucleic acid sequence of SEQ ID NO: 38 over the whole length of SEQ ID NO: 38", respectively.

According to another preferred aspect, the PRRS virus of the present invention is able to induce reproductive symptoms in pregnant sows and/or respiratory symptoms in piglets.

According to further preferred aspect, the PRRS virus of the present invention is able to induce respiratory symptoms in boars.

Thus, the PRRS virus of the present invention is preferably an infectious PRRS virus.

The term "infectious PRRS virus" according to the invention is particularly understood as a PRRS virus which infects swine, causing the associated disease, Porcine reproductive and respiratory syndrome (PRRS).

Said infection of swine by the PRRS virus of the present invention in particular includes attachment of the virus to a host cell, entry of the virus into the cell, disassembly of the virion, replication and transcription of the viral genome, expression of viral proteins and assembly and release of new infectious viral particles.

In another aspect, the invention further relates to a PRRS virus, preferably the PRRS virus of the present invention, genetically modified to contain therein exogenous RNA, wherein the

exogenous RNA is inserted into the ORF4 gene of said virus, and wherein the exogenous RNA is preferably inserted

- a) into the region of the ORF4 gene of said virus encoding the amino acid sequence selected from the group consisting of SEQ ID NOs: 1-12 or 13-24;
- b) into the region of the ORF4 gene of said virus encoding the region located between the first two predicted N-terminal β -sheets, as compared to the ORF4 protein of a wild type genotype I PRRS virus;
- c) into the region of the ORF4 gene of said virus encoding the region located between the first two predicted N-terminal β -sheets, as compared to the ORF4 protein of a wild type genotype II PRRS virus;
- d) into the region of the ORF4 gene of said virus encoding the region located between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus; or
- e) into the region of the ORF4 gene of said virus encoding the region located between amino acid positions 50 to 67, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the PRRS virus VR2332.

As used herein, the term "exogenous RNA" or "exogenous nucleic acid sequence" in particular refers to a nucleic acid sequence that was introduced into the genome of a PRRS virus from an external source, such as from a recombinant sequence. Examples of such external source comprise PRRSV derived sequences as well as non PRRSV derived sequences. More particular, the introduction of the exogenous nucleic acid sequence results in a genome or a gene, respectively, having a non-naturally occurring portion. As used herein, the term "exogenous RNA" thus in particular refers to a nucleotide sequence, which is not naturally found in the PRRS virus genome. Such non-naturally occurring portion or not naturally found sequence, respectively, can also be the result of the insertion of one naturally occurring nucleotide sequence into another naturally occurring nucleotide sequence.

The exogenous RNA, as described herein, in particular encodes an expression product selected from the group consisting of an epitope of interest, a biological response modulator, a growth factor, a recognition sequence, and a fusion protein, and wherein said epitope of interest is preferably an epitope of interest from an antigen or a veterinary pathogen or toxin.

In one preferred embodiment, said epitope of interest is a peptide encoded by the ORF5 gene of PRRS virus, wherein said peptide encoded by the ORF5 gene of PRRS virus in particular comprises or consists of at least 4 consecutive amino acid residues of the

sequence set forth in SEQ ID NO: 39 or, more particular, said peptide encoded by the ORF5 gene of PRRS virus comprises or consists of the amino acid sequence of SEQ ID NO:39.

In another preferred embodiment, said epitope of interest is the ectodomain of the ORF4 protein (GP4) of a different PRRS virus strain, wherein said ectodomain of GP4 of a different PRRS virus strain in particular comprises or consists of at least 4 consecutive amino acid residues of the sequence set forth in SEQ ID NO:40 or, more particular, said ectodomain of GP4 of a different PRRS virus strain comprises or consists of the amino acid sequence of SEQ ID NO:40.

The invention further provides the PRRS virus genetically modified to contain therein exogenous RNA, as described herein, for use as a medicament.

The present invention also provides the PRRS virus described herein for use as a challenge virus, in particular if said PRRS virus inherently induces a vaccinating effect when administered to an animal.

The present invention additionally provides the use of the PRRS virus of the present invention as a challenge virus, in particular if said PRRS virus does not induce a vaccinating effect when administered to an animal.

The term "animal", as mentioned herein, is in particular directed to swine, more particular to a pig, preferably a domestic pig.

Preferably, the PRRS virus is to be administered, or is administered, respectively, via the intranasal, intramuscular, oral, or intrauterine route to an animal.

Also, the present invention provides the use of the PRRS virus described herein as a detection marker, preferably for the differentiation between infected and vaccinated animals (DIVA).

According to a further aspect, the invention also relates to a DNA molecule which encodes the PRRS virus described herein, wherein said DNA molecule is preferably an isolated DNA molecule and/or wherein said DNA molecule preferably comprises a nucleic acid molecule having at least 84.5%, preferably at least 90%, more preferably at least 95%, still more

preferably at least 97%, and in particular preferably at least 99% sequence identity with the nucleic acid sequence of SEQ ID NO: 38.

The present invention further provides a DNA construct comprising the DNA molecule described herein, wherein said DNA construct is in particular a DNA vector such as a plasmid. DNA vectors or plasmids into which the DNA molecule of the present invention can be inserted will be recognized by those of ordinary skill in the art. The DNA construct, as described herein, is preferably an isolated DNA construct. As used herein, the term "comprising the DNA molecule" is in particular understood to be equivalent to the term "comprising the sequence of the DNA molecule".

Further, the present invention provides a RNA transcript of the DNA construct described herein, wherein said RNA transcript is preferably an isolated RNA transcript.

The present invention also provides a cell transfected with the DNA construct described herein, wherein said cell is preferably an isolated cell.

Further, the present invention provides a cell transfected with the RNA transcript mentioned herein, wherein said cell is preferably an isolated cell.

The term "cells" or "cell", as mentioned herein, is preferably directed to mammalian cells, in particular porcine or simian cells, such as MA-104 cells or MARC-145 cells or Vero cells, more preferably it is understood that the term "cells" or "cell" is directed to the host cells of PRRS virus, namely to porcine macrophages. Hence, a cell, as mentioned herein, is preferably selected from the group consisting of porcine cell, simian cell, MA-104 cell, MARC-145 cell, Vero cell and porcine macrophage.

In a further aspect, the invention provides a method for producing the PRRS virus described herein, wherein the method comprises the step of transfecting a cell with the DNA construct described herein and optionally harvesting the virus from the cell and/or the medium.

In another aspect, the invention provides a method for producing the PRRS virus described herein, wherein the method comprises the step of transfecting a host cell with the RNA transcript described herein and optionally harvesting the virus from the cell and/or the medium.

Production of the nucleic acid/DNA molecules described herein, is within the skill in the art and can be carried out according to recombinant techniques described, among other places, in Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel, et al., 2003, Current Protocols In Molecular Biology, Greene Publishing Associates & Wiley Interscience, NY; Innis et al. (eds), 1995, PCR Strategies, Academic Press, Inc., San Diego; and Erlich (ed), 1994, PCR Technology, Oxford University Press, New York, all of which are incorporated herein by reference.

2. Second consideration of the present invention

According to a second consideration, which is detailed in this section, the invention provides, in one aspect, a nucleic acid molecule which encodes a genotype I PRRS virus and which is capable of producing live virus when transfected into cells, wherein said molecule comprises

- a first nucleic acid sequence having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:45,
- a second nucleic acid sequence flanking the 5' end of the first nucleic acid sequence and having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:46,
- a third nucleic acid sequence flanking the 3' end of the first nucleic acid sequence and having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:47, and
- a polyadenine nucleotide sequence flanking the 3' end of the third nucleic acid sequence.

Preferably,

- said first nucleic acid sequence has at least 96%, preferably at least 97%, more preferably at least 98%, still more preferably at least 99%, and in particular preferably 100% sequence identity with the nucleic acid sequence of SEQ ID NO:45; and/or
- said second nucleic acid sequence having at least 96%, preferably at least 97%, more preferably at least 98%, still more preferably at least 99%, and in particular

preferably 100% sequence identity with the nucleic acid sequence of SEQ ID NO:46; and/or

- said third nucleic acid sequence having at least 96%, preferably at least 97%, more preferably at least 98%, still more preferably at least 99%, and in particular preferably 100% sequence identity with the nucleic acid sequence of SEQ ID NO:47; and/or
- said polyadenine nucleotide sequence is composed of n adenine nucleotides, wherein n is any integer between 1 and 51, and wherein n is preferably 12, 13 or 14.

The nucleic acid molecule of the present invention is preferably a DNA molecule. Preferably, said nucleic acid molecule is an isolated nucleic acid molecule.

Within the context of the present invention it is in particular understood that the term "polyadenine nucleotide sequence" is equivalent to the term "polyadenylic acid sequence" or "poly (A) tail", respectively. The term "adenine nucleotide(s)", as described herein, is in particular understood to be equivalent to the term "deoxyadenylate(s)".

The phrase "nucleotide sequence flanking the 5' end of" as described herein is in particular equivalent to the phrase "nucleotide sequence covalently linked with the 5' end of" or, respectively, with the phrase "nucleotide sequence, wherein the 3' terminal nucleotide thereof is covalently linked with the 5' terminal nucleotide of", and wherein it is particularly understood that said two terminal nucleotides are linked covalently between the phosphate group attached to the 5' carbon of the pentose and the 3' carbon atom of the adjacent pentose.

The phrase "nucleotide sequence flanking the 3' end of" as described herein is in particular equivalent to the phrase "nucleotide sequence covalently linked with the 3' end of" or, respectively, to the phrase "nucleotide sequence, wherein the 5' terminal nucleotide thereof is covalently linked with the 3' terminal nucleotide of", and wherein it is particularly understood that said two terminal nucleotides are linked covalently between the 3' carbon atom of the pentose and the phosphate group attached to the 5' carbon of the adjacent pentose.

It is further particularly understood that the phrase "having 100% sequence identity with the nucleic acid sequence of", as used herein, is equivalent to the phrase "being identical to the the nucleic acid sequence of" or "consisting of the nucleic acid sequence of", respectively.

In a particular preferred aspect, the nucleic acid molecule of the present invention comprises a nucleic acid sequence having at least 99% sequence identity with the nucleic acid sequence of SEQ ID NO:48, or wherein said nucleic acid molecule comprises or consists of a RNA copy of a nucleic acid sequence having at least 99% sequence identity with the nucleic acid sequence of SEQ ID NO:48.

The term "cells" or "cell", as mentioned herein, is preferably directed to mammalian cells, in particular porcine or simian cells, such as MA-104 cells or MARC-145 cells or Vero cells, more preferably it is understood that the term "cells" or "cell" is directed to the host cells of PRRS virus, namely to porcine macrophages. Hence, a cell, as mentioned herein, is preferably selected from the group consisting of porcine cell, simian cell, MA-104 cell, MARC-145 cell, Vero cell and porcine macrophage.

The term "live virus" according to the invention is particularly understood as a PRRS virus having the ability of infecting an appropriate subject (as opposed to an inactivated (killed) virus) and/or whose infectivity is similar or identical to a native virus. In particular, a live virus can infect its native host cells.

Said infection of host cells by the PRRS virus produced by the nucleic acid molecule of the present invention in particular includes attachment of the virus to a host cell, entry of the virus into the cell, disassembly of the virion, replication and transcription of the viral genome, expression of viral proteins and assembly and release of new infectious viral particles. Said infection of host cells by the PRRS virus produced by the nucleic acid molecule of the present invention further preferably includes the transcription of the cDNA sequence, in particular in BHK cells, to yield a functional RNA molecule, transfection of cultured cells, preferably porcine cell, simian cell, MA-104 cell, MARC-145 cell, Vero cell and porcine macrophage, with said RNA molecule, generation of live virions by viral replication in said cultured cells, isolation of such virions and infection of host cells.

In particular, the nucleic acid molecule of the present invention preferably encodes an attenuated genotype I PRRS virus or, respectively, the nucleic acid molecule of the present invention is capable of producing live attenuated virus when transfected into cells.

More particular the nucleic acid molecule of the present invention encodes a genotype I PRRS virus which is not able to induce a severe Porcine Reproductive and Respiratory Syndrome (PRRS) in swine or, respectively, the nucleic acid molecule of the present invention is capable of producing live virus when transfected into cells, wherein said live virus is not able to induce a severe, wild-type virus-like Porcine Reproductive and Respiratory Syndrome (PRRS) in swine as caused by virulent field PRRS viruses.

In one particular embodiment, the nucleic acid molecule of the present invention encodes a genotype I PRRS virus which is able to reach titers of at least 5×10^5 to 1×10^6 tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 24 hours post infection of MA104 cells, wherein said MA104 cells are preferably infected with said virus at an MOI (multiplicity of infection) of 0.001 to 0.1.

Particularly, the nucleic acid molecule of the present invention encodes a genotype I PRRS virus which is able to reach titers of 5×10^6 to 1×10^7 or more tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 48 hours post infection of MA104 cells, wherein said MA104 cells are preferably infected with said virus at an MOI (multiplicity of infection) of 0.001 to 0.1.

Thus, the nucleic acid molecule of the present invention preferably encodes a genotype I PRRS virus which is able to

- reach titers of at least 5×10^5 to 1×10^6 tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 24 hours and/or
- reach titers of at least 5×10^6 to 1×10^7 tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 48 hours post infection of MA104 cells

at an MOI (multiplicity of infection) of 0.001 to 0.1,

in particular at an MOI of 0.001 or 0.01 or 0.1.

In the context of the PRRS virus as described herein, it is understood that the term “genotype I” is equivalent to the terms “genotype 1” or “type 1” or “European (EU)” as frequently used in the literature in the context of PRRSV.

In another preferred embodiment, the nucleic acid molecule of the present invention comprises a nucleic acid sequence having at least 99.1% or 99.2%, preferably at least 99.3% or 99.4%, more preferably at least 99.5% or 99.6%, still more preferably at least 99.8% or 99.9%, and in particular preferably at least 99.95% sequence identity with the nucleic acid sequence set forth in SEQ ID NO:48.

Sequence identity in the context of the second consideration of the invention is understood as being based on pairwise determined similarity between nucleotide sequences. The determination of percent identity between two sequences is preferably accomplished using a mathematical algorithm, in particular the well-known Smith-Waterman algorithm (Smith and Waterman, M. S. (1981) J Mol Biol, 147(1):195-197). For purposes of the present invention, percent sequence identity of a nucleotide sequence is determined using the Smith-Waterman

homology search algorithm using a gap open penalty of 25 and a gap extension penalty of 5. The Smith-Waterman homology search algorithm is taught in Smith and Waterman (1981) Adv. Appl. Math 2:482-489, herein incorporated by reference. Such a determination of sequence identity can be performed using, for example, the DeCypher Hardware Accelerator from TimeLogic Version G, or the sequence identity is determined with the software CLC MAIN WORKBENCH 4.1.1 (CLC BIO).

As used herein, it is in particular understood that the term "having at least X% sequence identity with the nucleic acid sequence of SEQ ID NO:Y" (or, alternatively, the term "having at least X% sequence identity with the nucleic acid sequence set forth in SEQ ID NO:Y") is equivalent to the term "having at least X% sequence identity with the nucleic acid sequence of SEQ ID NO:Y over the length of SEQ ID NO:Y" or to the term "having at least X% sequence identity with the nucleic acid sequence of SEQ ID NO:Y over the whole length of SEQ ID NO:Y", respectively. In this context, "X" is any number from 95 to 100, in particular any integer selected from 95 to 99, such that "X% sequence identity" represents any of the percent sequence identities mentioned herein. Respectively, "Y" in this context is any integer selected from 1 to 6, such that "SEQ ID NO:Y" represents any of the SEQ ID NOs mentioned herein.

In a particular preferred embodiment, the nucleic acid molecule of the present invention comprises the nucleic acid sequence of SEQ ID NO:48.

In another preferred embodiment, the nucleic acid molecule of the present invention encodes a genotype I PRRS virus which is not able to induce Porcine Reproductive and Respiratory Syndrome (PRRS) in swine or, respectively, the nucleic acid molecule of the present invention is capable of producing live virus when transfected into cells, wherein said infectious virus is not able to induce Porcine Reproductive and Respiratory Syndrome (PRRS) in swine.

As used herein, the term "is not able to induce Porcine Reproductive and Respiratory Syndrome (PRRS)" in particular refers to a reduction of the clinical signs of PRRS or of signs associated with PRRSV infection, respectively, such as lung lesions in piglets, reproductive failure in pregnant sows, and/or prolonged PRRSV viremia, when compared to a wild-type PRRS virus. In one aspect, the genotype I PRRS virus which is not able to induce PRRS in swine is thus a virus showing one or more reduced clinical signs when administered to swine, in comparison with a wild type PRRS virus administered to swine. The term "wild type PRRS virus", as mentioned herein, in particular relates to a wild type genotype I PRRS virus.

The present invention further provides a DNA construct comprising the nucleic acid molecule according to the invention, wherein said DNA construct is in particular a DNA vector such as a plasmid. DNA vectors or plasmids into which the nucleotide molecule of the present invention can be inserted will be recognized by those of ordinary skill in the art. The DNA construct, as described herein, is preferably an isolated DNA construct. As used herein, the term “comprising the nucleic acid molecule” or “comprising a DNA molecule”, respectively, is in particular understood to be equivalent to the term “comprising the sequence of the nucleic acid molecule” or “comprising the sequence of a DNA molecule”, respectively.

Further, the present invention provides a RNA transcript of the DNA construct described herein, wherein said RNA transcript is preferably an isolated RNA transcript.

The present invention also provides a cell transfected with the DNA construct described herein, wherein said cell is preferably an isolated cell.

Thus, the present invention also provides genotype I PRRS virus produced by the aforementioned cell, wherein said genotype I PRRS virus is preferably an isolated genotype I PRRS virus.

Further, the present invention provides a cell transfected with the RNA transcript mentioned herein, wherein said cell is preferably an isolated cell.

Hence, the present invention also provides genotype I PRRS virus produced by the aforementioned cell, wherein said genotype I PRRS virus is preferably an isolated genotype I PRRS virus.

The present invention further provides a genotype I PRRS virus whose genome comprises the nucleic acid molecule of the present invention or whose genome comprises an RNA molecule encoded by a nucleic acid molecule of the present invention, wherein said genotype I PRRS virus is preferably an isolated genotype I PRRS virus.

In another aspect, the present invention provides a method for producing a genotype I PRRS virus, said method comprising transfecting a cell with the DNA construct described herein.

Moreover, the present invention provides a method for producing a genotype I PRRS virus, said method comprising transfecting a cell with the RNA transcript mentioned herein.

In yet another aspect, the present invention provides a composition, said composition comprising the nucleic acid molecule according to the invention suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.

Production of the nucleic acid molecules described herein is within the skill in the art and can be carried out according to recombinant techniques described, among other places, in Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Ausubel, et al., 2003, Current Protocols In Molecular Biology, Greene Publishing Associates & Wiley Interscience, NY; Innis et al. (eds), 1995, PCR Strategies, Academic Press, Inc., San Diego; and Erlich (ed), 1994, PCR Technology, Oxford University Press, New York, all of which are incorporated herein by reference.

In still another aspect, the invention further relates to the use of the nucleic acid molecule according to the invention or of the DNA construct described herein for producing an attenuated genotype I PRRS virus, wherein one or more mutations are introduced into the nucleic acid molecule or into the DNA construct.

The invention also provides a method of producing an attenuated genotype I PRRS virus comprising the step of introducing one or more mutations into the nucleic acid molecule according to the invention or into the DNA construct described herein.

Preferably, the one or more mutations described herein are introduced into the first nucleic acid sequence having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:45.

The term “attenuated PRRS virus”, as described herein, is in particular directed to a PRRS virus which is attenuated *in vitro* and/or *in vivo*, more particular in susceptible cell lines and/or the host.

The term “host”, as used herein, is in particular directed to animals infectable with PRRS virus, in particular swine, more particular pigs, such as domestic pigs.

As mentioned herein, “attenuated” particularly relates to a reduced virulence of a pathogen, in particular of a wild type PRRS virus, wherein “virulence” is understood to be the degree of pathogenicity, and wherein “pathogenicity” is directed to the ability of the pathogen to induce clinical signs in the host or the offspring of the host, such as reproductive failure.

The term “wild type PRRS virus” or “wild type PRRSV”, respectively, as used herein, is in particular directed to an infectious pathogenic PRRS virus, which is particularly capable of causing PRRS in swine. In one particular preferred embodiment, the term “wild type PRRS virus” is directed to a PRRS virus whose genome comprises a RNA sequence or consists of a RNA polynucleotide, wherein said RNA sequence or RNA polynucleotide is a RNA copy of SEQ ID NO:41 (corresponding to Lelystad virus complete genome).

Preferably, the one or more mutations, as described herein, comprise or consist of one or more point mutations and/or one or more genomic deletions and/or one or more insertions.

Also, the invention provides an attenuated genotype I PRRS virus whose genome comprises an RNA molecule encoded by a nucleic acid molecule according to the invention but wherein said first nucleic acid sequence having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:45 contains one or more mutations that attenuate the encoded PRRS virus and/or that disable the encoded PRRS virus to suppress the interferon type I production and secretion by a cell infected by said virus, and wherein said attenuated genotype I PRRS virus is preferably an isolated attenuated genotype I PRRS virus.

The invention further provides the use of the attenuated genotype I PRRS virus described herein for the preparation of a medicament, in particular of a vaccine or vaccine composition, for preventing an animal from clinical signs of a PRRSV infection, such as by reducing the clinical signs of a PRRSV infection, e.g. reducing the duration of PRRSV viremia.

The term “preventing” or “reducing”, respectively, as used herein, means, but is not limited to, a process which includes the administration of a PRRSV antigen, namely of the attenuated genotype I PRRS virus described herein, to an animal, wherein said PRRSV antigen, when administered to said animal elicits or is able to elicit an immune response in said animal against PRRSV. Altogether, such treatment results in reduction of the clinical signs of PRRS or of signs associated with PRRSV infection, respectively. More specifically, the term “preventing, as used herein, means generally a process of prophylaxis in which an animal is exposed to the immunogenic composition of the present invention prior to the induction or onset of the disease process (PRRS).

Herein, “reducing the clinical signs of a PRRSV infection” means, but is not limited to, reducing the number of infected subjects in a group, reducing or eliminating the number of subjects exhibiting clinical signs of infection, or reducing the severity of any clinical signs that are present in the subjects, in comparison to wild type PRRS virus infection. For example, it should refer to any reduction of pathogen load, pathogen shedding, reduction in pathogen

transmission, or reduction of any clinical sign typical of PRRSV infection, in particular of reproductive failure and/or induction of lung lesions. Preferably these clinical signs are reduced in subjects receiving the attenuated genotype I PRRS virus of the present invention by at least 10% in comparison to subjects not receiving the composition and may become infected. More preferably, clinical signs are reduced in subjects receiving the composition of the present invention by at least 20%, preferably by at least 30%, more preferably by at least 40%, even more preferably by at least 50%, even more preferably by at least 60%, even more preferably by at least 70%, even more preferably by at least 80%, even more preferably by at least 90%, and most preferably by 100%.

The term “subject”, as mentioned herein, in particular relates to an animal.

The term “animal”, as mentioned herein, is in particular directed to swine, more particular to a pig, preferably a domestic pig.

The term “reducing the duration of PRRSV viremia” means, but is not limited to, the reduction of the duration of PRRS virus entering the bloodstream of an animal by at least one day in comparison to subjects not receiving the composition and become infected by a wild type PRRSV.

The term “viremia” refers to the presence of PRRSV in the blood of infected animals as reflected by e.g. the detection of PRRSV RNA copies in blood serum.

Also, the invention relates to a vaccine composition comprising the attenuated genotype I PRRS virus described herein suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.

The one or more pharmaceutically acceptable carriers or excipients, as mentioned herein, are preferably selected from the group consisting of solvents, dispersion media, adjuvants, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, and adsorption delaying agents.

In a preferred aspect, the immunogenic composition of the invention comprises an amount of 10^1 to 10^7 viral particles of the attenuated genotype I PRRS virus described herein per dose, preferably 10^3 to 10^6 particles per dose, more preferably 10^4 to 10^6 particles per dose.

In another preferred aspect, the immunogenic composition of the invention comprises an amount of the PRRS virus according to the invention which is equivalent to a virus titer of at least about 10^3 TCID₅₀/mL per dose, preferably between 10^3 to 10^5 TCID₅₀/mL per dose

As used herein, the term "vaccine composition" in particular refers to a composition that will elicit a protective immune response in an animal that has been exposed to the composition. An immune response may include induction of antibodies and/or induction of a T-cell response.

Usually, an "immune response" includes but is not limited to one or more of the following effects: the production or activation of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or a protective immunological (memory) response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction in number or severity of, or lack of one or more of the clinical signs associated with the infection of the pathogen, in the delay of onset of viremia, in a reduced viral persistence, in a reduction of the overall viral load and/or in a reduction of viral excretion.

Thus, an "immune response" in particular means but is not limited to the development in a subset of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest.

Further, the invention relates to the vaccine composition of the invention for use in a method for preventing an animal from clinical signs of a PRRSV infection, such as by reducing the clinical signs of a PRRSV infection, e.g. reducing the duration of PRRSV viremia.

Moreover, the invention provides a method for preventing an animal from clinical signs of a PRRSV infection, such as by reducing the clinical signs of a PRRSV infection, e.g. reducing the duration of PRRSV viremia, wherein said method comprises the step of administering the vaccine of the invention to an animal in need thereof.

EMBODIMENTS according to the second consideration of the present invention

The following clauses are also described herein:

1. A nucleic acid molecule which encodes a genotype I PRRS virus and which is capable of producing live virus when transfected into cells, wherein said molecule comprises
 - a first nucleic acid sequence having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:45,

- a second nucleic acid sequence flanking the 5' end of the first nucleic acid sequence and having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:46,
- a third nucleic acid sequence flanking the 3' end of the first nucleic acid sequence and having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:47, and

a polyadenine nucleotide sequence flanking the 3' end of the third nucleic acid sequence.

2. The nucleic acid molecule of clause 1, wherein

- said first nucleic acid sequence having at least 96%, preferably at least 97%, more preferably at least 98%, still more preferably at least 99%, and in particular preferably 100% sequence identity with the nucleic acid sequence of SEQ ID NO:45; and/or
- said second nucleic acid sequence having at least 96%, preferably at least 97%, more preferably at least 98%, still more preferably at least 99%, and in particular preferably 100% sequence identity with the nucleic acid sequence of SEQ ID NO:46; and/or
- said third nucleic acid sequence having at least 96%, preferably at least 97%, more preferably at least 98%, still more preferably at least 99%, and in particular preferably 100% sequence identity with the nucleic acid sequence of SEQ ID NO:47; and/or
- said polyadenine nucleotide sequence is composed of n adenine nucleotides, wherein n is any integer between 1 and 51, and wherein n is preferably 12, 13 or 14.

3. The nucleic acid molecule of clause 1 or 2, wherein said virus is attenuated and/or wherein said virus is able to induce a protective immune response against respiratory and/or reproductive signs of disease after infection with Porcine Reproductive and Respiratory Syndrome (PRRS) virus in swine.

4. The nucleic acid molecule of any one of clauses 1 to 3, wherein said virus is able to reach titers of at least 5×10^5 to 1×10^6 tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 24 hours post infection of MA104 cells, preferably at an MOI (multiplicity of infection) of 0.001 to 0.1.

5. The nucleic acid molecule of any one of clauses 1 to 4, wherein said virus is able to reach titers of at least 5×10^6 to 1×10^7 tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 48 hours post infection of MA104 cells, preferably at an MOI (multiplicity of infection) of 0.001 to 0.1.
6. The nucleic acid molecule of any one of clauses 1 to 5, wherein said molecule comprises a nucleic acid sequence having at least 91% or 92%, preferably at least 93% or 94%, more preferably at least 95% or 96%, still more preferably at least 98% or 99%, and in particular preferably at least 99% sequence identity with the nucleic acid sequence of SEQ ID NO:48.
7. The nucleic acid molecule of any one of clauses 1 to 6, wherein said molecule comprises a nucleic acid sequence having at least 99.1% or 99.2%, preferably at least 99.3% or 99.4%, more preferably at least 99.5% or 99.6%, still more preferably at least 99.8% or 99.9%, and in particular preferably at least 99.95% sequence identity with the nucleic acid sequence of SEQ ID NO:48.
8. The nucleic acid molecule of any one of clauses 1 to 7, wherein said molecule comprises the nucleic acid sequence of SEQ ID NO:48.
9. The nucleic acid molecule of any one of clauses 1 to 8, wherein said virus is not able to induce a severe, Porcine Reproductive and Respiratory Syndrome (PRRS) in swine as caused by virulent field PRRS viruses.
10. The nucleic acid molecule of any one of clauses 1 to 9, wherein said molecule is a DNA molecule.
11. A DNA construct comprising a DNA molecule according to clause 10.
12. An RNA transcript of the DNA construct of clause 11.
13. A cell transfected with the DNA construct of clause 11.
14. A cell transfected with the RNA transcript of clause 12.
15. A genotype I PRRS virus produced by the cell of clause 13.
16. A genotype I PRRS virus produced by the cell of clause 14.
17. A genotype I PRRS virus whose genome comprises a nucleic acid molecule according to any one of clauses 1 to 9 or whose genome comprises an RNA

molecule encoded by a nucleic acid molecule according to any one of clauses 1 to 10.

18. A method for producing a genotype I PRRS virus comprising transfecting a cell with the DNA construct of clause 11.
19. A method for producing a genotype I PRRS virus comprising transfecting a host cell with the RNA transcript of clause 12.
20. A composition comprising a nucleic acid molecule of any one of clauses 1 to 10 suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.
21. Use of the nucleic acid molecule of any one of clauses 1 to 10 or of the DNA construct of clause 11 for producing an attenuated genotype I PRRS virus, wherein one or more mutations are introduced into the nucleic acid molecule or into the DNA construct.
22. Method of producing an attenuated genotype I PRRS virus comprising the step of introducing one or more mutations into the nucleic acid molecule of any one of clauses 1 to 10 or into the DNA construct of clause 11.
23. An attenuated genotype I PRRS virus whose genome comprises an RNA molecule encoded by a nucleic acid molecule according to any one of clauses 1 to 10 but wherein said first nucleic acid sequence having at least 95% sequence identity with the nucleic acid sequence of SEQ ID NO:45 contains one or more mutations that disable the encoded PRRS virus to suppress the interferon type I production and secretion by a cell infected by said virus.
24. Use of the attenuated genotype I PRRS virus of any one of clauses 21 to 23 for the preparation of a medicament for preventing an animal from clinical signs of a PRRSV infection.
25. A vaccine composition comprising the attenuated genotype I PRRS virus of any one of clauses 21 to 23 suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.
26. The vaccine composition of clause 25 for use in a method for preventing an animal from clinical signs of a PRRSV infection.

27. Method for preventing an animal from clinical signs of a PRRSV infection comprising the step of administering the vaccine composition of clause 26 to an animal in need thereof.

3. Third consideration of the present invention

According to a third consideration, which is detailed in this section, the invention is based on the finding that the first consideration of the present invention can be combined with the second consideration of the present invention. Thus, the third consideration of the present invention relates to a combination of (1) the aspects and embodiments of the first consideration of the present invention and (2) the aspects and embodiments of the second consideration of the present invention. Hence, it is understood that all possible features and definitions, in particular the features and definitions relating to a genotype I PRRS virus, of the first consideration of the present invention can be arbitrarily combined with all features and definitions of the second consideration of the present invention.

In one aspect, the nucleic acid molecule according to the second consideration of the present invention thus encodes the Porcine Reproductive and Respiratory Syndrome (PRRS) virus according to the first consideration of the present invention, as recited in any one of the claims 36 to 42.

In another aspect, respectively, the Porcine Reproductive and Respiratory Syndrome (PRRS) virus according to the first consideration of the present invention is thus encoded by the nucleic acid molecule according to the second consideration of the present invention, as recited in claims 56 or 57.

Hence, the combination of all possible aspects of the first consideration of the present invention with all possible aspects of the second consideration of the present invention is in particular also reflected by said claims and the claims depending thereon.

The invention is directed, furthermore, to a genotype I PRRS virus, in particular the aforementioned PRRS virus, whose genome is encoded by a nucleic acid molecule which encodes a genotype I PRRS virus and which is capable of producing live virus when transfected into cells, wherein said molecule comprises a nucleic acid sequence having at least 91% or 92%, preferably at least 93% or 94%, more preferably at least 95% or 96%, still more preferably at least 98% or 99%, and in particular preferably at least 99% or 100%

sequence identity with the nucleic acid sequence of SEQ ID NO:48, but wherein said nucleic acid sequence contains a mutation resulting in the production of said virus comprising an ORF4 protein having a deletion of 9, 10, 11 or more amino acid residues between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus.

The invention also concerns a genotype I PRRS virus, whose genome is encoded by a nucleic acid molecule which encodes a genotype I PRRS virus and which is capable of producing live virus when transfected into cells, wherein said molecule comprises a nucleic acid sequence having at least 91% or 92%, preferably at least 93% or 94%, more preferably at least 95% or 96%, still more preferably at least 98% or 99%, and in particular preferably at least 99% or 100% sequence identity with the nucleic acid sequence of the nucleic acid sequence of SEQ ID NO:48, but wherein said nucleic acid sequence contains a mutation resulting in the production of said virus comprising an ORF4 protein having a deletion of 11, 12, 13, 14, 15, 16, or 17 amino acid residues between amino acid positions 50 to 71, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus.

The invention moreover contemplates a genotype I PRRS virus, whose genome is encoded by a nucleic acid molecule which encodes a genotype I PRRS virus and which is capable of producing live virus when transfected into cells, wherein said molecule comprises a nucleic acid sequence having at least 91% or 92%, preferably at least 93% or 94%, more preferably at least 95% or 96%, still more preferably at least 98% or 99%, and in particular preferably at least 99% or 100% sequence identity with the nucleic acid sequence of the nucleic acid sequence of SEQ ID NO:48, but wherein said nucleic acid sequence contains a mutation resulting in the production of said virus comprising an ORF4 protein having a deletion of 13 amino acid residues between amino acid positions 56 to 70 or between amino acid positions 57 to 69, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus.

The mutation, as referred to herein, is preferably a deletion.

Preferably, the PRRS virus of the invention is genetically modified to contain therein exogenous RNA, wherein the exogenous RNA is inserted into the orf4 gene of said virus, and wherein the exogenous RNA is in particular inserted into the region of the orf4 gene of said virus encoding the region located between amino acid positions 50 to 71, wherein the

numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus.

In another preferred aspect, the exogenous RNA is inserted into the orf4 gene of the virus and replaces the nucleotide sequence encoding the amino acid residues deleted within the context of the invention.

According to a further preferred aspect, the exogenous RNA encodes an expression product selected from the group consisting of an epitope of interest, a biological response modulator, a growth factor, a recognition sequence, a fusion protein, wherein the epitope of interest is preferably an epitope of interest from an antigen or a veterinary pathogen or toxin.

In particular, the epitope of interest is a peptide encoded by the orf5 gene of PRRS virus or is an amino acid sequence encoded by the orf5 gene of PRRS virus, wherein said peptide or amino acid sequence encoded by the orf5 gene of PRRS virus preferably comprises or consists of the amino acid sequence of SEQ ID NO:39 or SEQ ID NO:50 or preferably comprises or consists of at least 4 consecutive amino acid residues of the sequence set forth in SEQ ID NO: 39 or SEQ ID NO:50, or preferably comprises or consists of the amino acid sequence of SEQ ID NO:51 or SEQ ID NO:52.

According to a another preferred aspect, the exogenous RNA encodes an amino acid sequence selected from the group consisting of SEQ ID NOs: 53-55.

In a particular preferred aspect, the invention provides, as a non limiting example, a genotype I PRRS virus, whose genome is encoded by a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of any one of SEQ ID NOs:56-59.

The PRRS virus of any one of claims 58 to 73, wherein said virus is an isolated virus and/or wherein said mutation is a deletion.

The PRRS virus, as mentioned to herein, is preferably an isolated virus and/or a non-naturally occurring virus.

The invention is directed, furthermore, to a genotype I PRRS virus, wherein said virus comprises an ORF4 protein having a proline residue at amino acid position 56 and/or having a glutamine residue at amino acid position 66, wherein the numbering of the amino acid

positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus, and wherein the amino acid sequence of ORF4 protein of the Lelystad virus is the sequence set forth in SEQ ID NO:43.

The invention also concerns a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a proline residue at amino acid position 56 and/or having a glutamine residue at amino acid position 66, wherein the numbering of the amino acid positions refers to the amino acid sequence of ORF4 protein of the Lelystad virus, and wherein the genome of said virus is preferably encoded by a nucleic acid molecule, wherein said molecule comprises a nucleic acid sequence having at least 91% or 92%, preferably at least 93% or 94%, more preferably at least 95% or 96%, still more preferably at least 98% or 99%, and in particular preferably at least 99% sequence identity with the nucleic acid sequence of the nucleic acid sequence of SEQ ID NO:45 or SEQ ID NO:48.

Such a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a proline residue at amino acid position 56, is in an exemplary non-limiting aspect a PRRS virus, whose genome is encoded by a nucleic acid molecule comprising the nucleic acid sequence SEQ ID NO:58.

In another exemplary non-limiting aspect, such a genotype I PRRS virus, whose genome comprises a nucleic acid molecule which encodes an ORF4 protein having a glutamine residue at amino acid position 66, is a PRRS virus, whose genome is encoded by a nucleic acid molecule comprising the nucleic acid sequence SEQ ID NO:57.

The PRRS virus of the invention is preferably for use as a medicament or for use in the prophylaxis or treatment of Porcine Reproductive and Respiratory Syndrome, in particular in swine, and wherein optionally said virus is to be administered, or is administered, respectively, via the intranasal, intramuscular, oral, or intrauterine route to an animal, in particular to a pig.

A medicament as referred to throughout this disclosure is preferably a vaccine.

According to another aspect, the PRRS virus of the invention is preferably used as a detection marker, preferably for the differentiation between infected and vaccinated animals (DIVA).

In still a further aspect, the invention relates to a DNA molecule which encodes the PRRS virus of the invention, and wherein said DNA molecule preferably comprises a nucleic acid molecule having a sequence selected from the group consisting of SEQ ID NOs:56-58.

In yet a further aspect, the invention relates to a preferably isolated DNA construct comprising said DNA molecule and to a preferably isolated RNA transcript thereof.

According to still another aspect the invention also relates to a preferably isolated cell transfected with said DNA construct or said RNA transcript.

The invention furthermore relates to a method for producing the PRRS virus of the invention, wherein said method comprises the step of transfecting a cell with said DNA construct or comprises the step of transfecting a host cell with said RNA transcript.

In conclusion, the knowledge of having the possibility to insert a deletion to the extent according to the present invention into the sequence coding for the ectodomain of GP4 of a PRRS virus, such as a genotype I PRRSV, now provides a number of beneficial uses:

- The virus based on this knowledge can be used as a challenge isolate for parenteral, oral, intranasal, intrauterine infection and for infection by means of sperm in PRRSV positive and PRRSV naive and/or PRRSV sensitive species.
- The invention provides deletion markers for serological differentiation or for sequence differentiation (DIVA concept), of each conceivable PRRSV strain, to PRRSV strains of the genotype II, regardless of whether deletions are already present at the respective site or not.
- Further, deletion markers are provided for serological differentiation also in connection or in combination with other epitopes. For example, PRRS viruses without deletion could be distinguished serologically from PRRS viruses with a complete or partial deletion of these epitopes (e.g. Lelystad GP4 aa60-aa71: AAQEKISFGKS as included in SEQ ID NO:43) by using antibodies directed against this epitope. For instance, two PRRS viruses having a deletion in this region/domain could be differentiated from each other in conjunction with other epitopes.

The invention further provides an insertion region/domain for the introduction of „foreign RNA instead of the viral RNA at the position, where the deletion according to the invention is located (ectodomain of GP4).

The insertion can be done for various purposes and for every conceivable PRRSV strain, also for PRRSV strains of the genotype II which already have a small deletion in this region.

The insertion of the foreign sequence can take place e.g. in the PRRSV genotype I strain BI EU described herein and replace the sequence coding for the ectodomain of GP4 of said strain with the amino acid (aa) sequence aa54-aa70 (QSHRASTAQGTTPLRRS (SEQ ID NO:40)) or with shortened or mutagenized derivatives thereof.

Moreover, for the improvement of the immune response it is also possible to insert one or more sequential T- oder B-cell epitopes

a) from other gene/genomic regions of PRRSV, e.g. from (i) the region coding for the glycoprotein 5 (aa) of the PRRSV genotype I strain BI EU described herein, e.g. sequences coding for the amino acids (aa) aa36-aa52 (SSHLQLIYNLTICELNG (SEQ ID NO:39)) or for shortened or for mutagenized derivatives thereof, also with suitable linker(s), for instance with the aa motif GSS; accordingly also from other PRRSV isolates, e.g. from the PRRSV genotype I prototype isolate Lelystad or, accordingly, from other genotypes of PRRSV, such as e.g. from the PRRSV genotype II prototype isolate VR2332;

b) from other pathogens, e.g. from another swine pathogen, for establishing an or enhancing the immune response against said pathogen(s);

c) from non-PRRSV-specific T- oder B-cell epitopes as a genetic or serological positive marker, also in combination with a);

d) from immuno-enhancers different from a), e.g. cytokines, as for instance interleukins, also in combination with b).

For the improvement of the immune response it is also possible to insert one or more sequential T- oder B-cell epitopes for the reduction of the pathogenicity of the virus.

EXAMPLES

Example 1

a) Isolation of PRRSV

PRRSV was isolated from blood samples (bS-720789) previously tested positive in a PRRSV EU-type detection PCR. Isolation of virus was performed on MA104 cells. After propagation of the isolated EU-type PRRSV on MA104 cells, a virus stock for full genome sequencing was prepared by ultracentrifugation on a sucrose cushion, followed by RNase and DNase treatment. Finally, viral RNA was extracted from the virus stock and submitted for full genome sequencing (Roche 454 platform). The genome sequence obtained (14 854 nucleotides) was compared to the EU-type reference genome sequence of strain Lelystad, revealing a deletion of 33 nucleotides in ORF4.

b) Infection

Infection of boars with the virus of a) produces severe clinical signs of PRRS.

Example 2

a) Generation and characterization of a novel EU type PRRSV infectious cDNA clone

This example describes the generation and characterization of a novel EU type PRRSV infectious cDNA clone which is designated "BI EU" in the following. BI EU is based on but not identical to an attenuated EU type PRRSV strain and is 89% identical on nucleotide level to the EU prototype strain Lelystad virus or 87% identical to the PRRSV cDNA insert of the EU type PRRSV infectious cDNA clone LoN94-13 (WO 2013017568 A1) respectively. The cDNA sequence of BI EU is provided in SEQ ID NO:48.

Live virus was recovered from cDNA clone BI EU after transfecting synthetic capped transcripts into BHK21 cells and subsequent transfer of cell culture supernatant from transfected cells onto PRRSV-susceptible MA104 cells. A strong cytopathic effect (CPE) was detectable within 3 to 4 days post transfer of cell culture supernatant from transfected BHK21

cells to MA104 cells (Figure 1 A). After staining the cells with the PRRSV capsid protein-specific monoclonal antibody SDOW17 (Rural Technologies), a strong signal was detectable in the CPE positive MA104 cells (Figure 1 B) but not in cells which received supernatants of mock transfected BHK21 cells (not shown).

To test growth of the BI EU cDNA clone-derived virus, MA104 cells were infected with the recovered virus using a multiplicity of infection (MOI) of 0.001, 0.01 or 0.1, respectively. Supernatants of infected cells were collected at 0, 24, 48, 72 and 96 hours post infection and virus titers were determined by serial virus dilutions on 96-well plates containing MA104 cells. The resulting growth curve for virus recovered from BI EU is shown in **Figure 2**.

Independent of the MOI used for infection of MA104 cells, the virus BI EU reached titers of 5×10^5 to 1×10^6 tissue culture infectious dose 50 (TCID₅₀) per milliliter (ml) within 24 hours post infection. Titers peaked around 48 hours post infection with 1×10^6 to 1×10^7 TCID₅₀/ml, demonstrating highly efficient replication of the BI EU virus on MA104 cells.

This finding allows to use BI EU as a platform for PRRSV vaccine research, e.g., as one of many applications, to investigate the PRRSV interplay with host immune responses to viral infection.

b) Use of the novel EU type PRRSV infectious cDNA clone in PRRS vaccine research

The specific immune response to PRRSV infection is characterized by delayed induction of neutralizing antibodies (Lopez and Osorio, 2004) and short cell-mediated immune response (Xiao et al., 2004). It is commonly accepted that these effects can in part be attributed, along with presentation of decoy epitopes (Ostrowski et al., 2002; Ansari et al., 2006) and glycan shielding of viral envelope proteins (Ansari et al., 2006), to the viral inhibition of the host's innate immune system. It has been demonstrated that PRRSV infection does not or only weakly or delayedly induce production of type I interferon (IFN), (interferon- α and interferon- β ; (Miller et al., 2004)) or type II IFN, (interferon- γ ; (Meier et al., 2003)) in susceptible cell lines (swine pulmonary alveolar macrophages, monkey kidney cells MARC-145) and/or pigs (Buddaert et al., 1998).

IFNs play an important role in establishing an effective adaptive immune response against viral infections, and many viruses therefore have developed strategies to counteract onset of the host's innate immune system (Haller and Weber, 2009). In the interest to identify the anticipated PRRSV IFN antagonist(s), extensive screening analyses based on cell lines

stably expressing genes of interest or on cells transfected with protein-expressing plasmids have identified several PRRSV nonstructural proteins (nsps) including nsp1 (see below), nsp2 (Beura et al., 2010; Li et al., 2010), nsp4 (Beura et al., 2010), and nsp11 (Beura et al., 2010; Shi et al., 2011a) to be involved in blocking the induction of type I IFN.

nsp1 is located at the N-terminus of the PRRSV ORF1a-derived polyprotein 1a and is processed into two multifunctional subunits, nsp1 α and nsp1 β , each of which contains a papain-like cysteine protease (PCP) domain essential for self-release from the viral polyprotein (den Boon et al., 1995; Chen et al., 2010). nsp1 α contains an N-terminal zinc-finger domain and the PCP α protease domain, while nsp1 β contains PCP β . For both nsp1 subunits, nsp1 α and nsp1 β , the three-dimensional crystal structure has been resolved (Sun et al., 2009; Xue et al., 2010). According to these analyses, nsp1 β consists of an N-terminal domain (NTD), a linker domain (LKD), the PCP domain (PCP beta), and a C-terminal extension (CTE); (Xue et al., 2010). C-terminal, nsp1 β -mediated cleavage of nsp1 from nsp2 occurs at site WYG/AGR for PRRSV US strains (Kroese et al., 2008) or is predicted at site WYG/AAG for PRRSV EU strains (Chen et al., 2010), while nsp1 α /nsp1 β cleavage occurs at site ECAM/AxVYD for PRRSV US strains or is predicted at site EEAH/SxVYR for PRRSV EU strains (Chen et al., 2010).

Several studies demonstrated to the mechanistic detail that PRRSV nsp1 and/or its autocleavage-derived subunits nsp1 α and/or nsp1 β inhibit type I IFN production by interfering with IFN transcription (Song et al., 2010; Kim et al., 2010; Chen et al., 2010; Beura et al., 2010). In addition, it has been demonstrated that nsp1 β interferes with the cellular response to interferon (interferon signaling); (Chen et al., 2010). Moreover, it was demonstrated that PRRSV infection inhibits IFN- α and/or IFN- β production in PRRSV infected cells *in vitro* (Kim et al., 2010; Beura et al., 2010), the subcellular localization of nsp1 (subunits) was determined (Song et al., 2010; Chen et al., 2010), and mechanistic aspects of type I IFN inhibition that were obtained by others from single protein expression experiments were confirmed in cells infected with PRRSV (Shi et al., 2010). Finally, a nsp1 mutagenesis study based on nsp1 protein expression investigated effects on viral IFN inhibition (Shi et al., 2011b).

Previously viable PRRSV (EU) strains have been generated (as described in WO 2013017570 A1) that contained mutations (deletions) in the nsp1 β gene that induced type I IFN (IFN- β) production in susceptible cells (MARC145) and that are sensitive to type I IFN (IFN- β).

To test whether such and also different IFN inducing virus mutants could get generated based on the novel infectious clone BI EU, a set of viruses harboring deletions in the nsp1 β gene was designed. More precisely, these deletions were located in the N-terminal domain (NTD) of nsp1 β which has been shown to be required for homodimerization of the protein (Xue et al., 2010). **Figure 3** shows an nsp1 β aminoacid sequence alignment of several US and EU type PRRSV strains. Indicated are aminoacids predicted to form strands (blue) or alpha helices (red) formation.

Ten nsp1 β deletion mutants were generated on the basis of the infectious cDNA clone BI EU. Deletions included aminoacids that were predictedly not involved in beta strand or alpha helix formation and that were (partially) conserved within all EU type PRRSV strains analyzed in the alignment (framed in red in **Figure 3**).

The deletions introduced in the nsp1 β gene are visualized in the aminoacid sequence alignment shown in **Figure 4**. The BI EU-nsp1 β deletion mutants are designated BI EU-nsp1 β -delALEV, BI EU-nsp1 β -delEV, BI EU-nsp1 β -delLEVL, BI EU-nsp1 β -delLE, BI EU-nsp1 β -delDD, BI EU-nsp1 β -delSDDS, BI EU-nsp1 β -delHH, BI EU-nsp1 β -delGRSR, BI EU-nsp1 β -delRSR and BI EU-nsp1 β -delSDGRSR, respectively.

To test viability of the nsp1 β deletion mutants, synthetic transcripts of BI EU cDNAs harbouring the respective deletion were transfected into BHK21 cells. After transfer of cell culture supernatant from transfected cells onto PRRSV-susceptible MA104 cells, cytopathic effects (CPE) and nucleocapsid-specific immunofluorescence staining indicating PRRSV mutant viability were detectable for nine of the ten nsp1 β deletion mutants generated (not shown). These findings demonstrated that, with the exception of BI EU-nsp1 β -delLEVL, all nsp1 β deletion mutants were viable. To further analyze whether the nsp1 β deletion mutants could be grown to high titers on IFN-competent MA104 cells, growth curves were performed essentially as described above for the BI EU virus. Briefly, MA104 cells were infected with one of the nine nsp1 β deletion mutants or the virus BI EU as control. Cell culture supernatants were harvested at 0, 24, 48, 72 and 93 hours post infection and titrated on MA104 cells on 96-well plates. Viral titers were calculated based on CPE-positive wells. **Figure 5** shows the result of two independent experiments and demonstrates that BI EU-nsp1 β deletion mutants can be grown on MA104 cells as efficiently as the parent BI EU virus. Peak titers of 5×10^6 to 1×10^7 TCID₅₀/ml were observed at 48 hours post infection.

It was next analyzed whether the deletions introduced into the nsp1 β gene would indeed abolish the IFN antagonistic activity of the nsp1 β protein. Therefore IFN- β levels in 100 μ l samples collected at 0, 24, 48, 72 and 93 hours post infection throughout the growth curve

experiment described above were measured using a commercial ELISA specific for human IFN- β (Invitrogen). According to the manufacturer, this ELISA can also be applied for the detection of non-human primate IFN- β and worked well for samples from MA104 cells which are epithelial Green Monkey kidney cells (see Figure 6). For quantification of the obtained results, a calibration curve was included using a positive control of the ELISA manufacturer.

IFN- β levels measured in the supernatants of MA104 cells infected with one of the nine viable nsp1 β deletion mutants or with the parent BI EU virus and obtained from two independent experiments are shown in **Figure 6**.

As expected, parental BI EU efficiently blocked the secretion of IFN- β throughout the course of infection which is attributed to functional viral IFN antagonist(s). No or only little amounts of IFN- β were detectable in the cell culture supernatant at 0, 24 and 48 hours post infection with the various BI EU-nsp1 β deletion mutants. At later timepoints however, some mutants were unable to inhibit the expression of IFN- β in infected MA104 cells, indicating a defect in the nsp1 β IFN antagonistic activity. Interestingly, this defect varied significantly between the nine BI EU-nsp1 β deletion mutants analyzed. While most of the mutants induced IFN- β levels below 50 international units (IU) per 100 μ l cell culture supernatant, the mutant BI EU-nsp1 β -delALEV was completely unable to antagonize the expression of IFN- β in infected MA104 cells. The amounts of IFN- β measured at 72 and 93 hours post infection even exceeded the limit of the ELISA test which is set at ~200 IU per 100 μ l. This result clearly demonstrated that the IFN antagonistic activity of the nsp1 β protein can be abolished by deleting the aminoacids A₃₀LEV₃₃ in the BI EU infectious cDNA clone.

Taken together, a novel EU type PRRSV infectious cDNA clone was generated that can be efficiently grown to titers of 1×10^7 TCID₅₀/ml in Green Monkey kidney MA104 cells. Based on this clone, nine viable BI EU-nsp1 β mutants were generated which harboured deletions in the NTD of nsp1 β which has been shown to be required for homodimerization of the protein (Xue et al., 2010). These mutants could all be grown to high titers on MA104 cells. Mutants BI EU-nsp1 β -delALEV, BI EU-nsp1 β -delEV, BI EU-nsp1 β -delLE, BI EU-nsp1 β -delSDDS, BI EU-nsp1 β -delGRSR, BI EU-nsp1 β -delRSR and BI EU-nsp1 β -delSDGRSR all induced the secretion of IFN- β at late timepoints of infection which is in strict contrast to the parent BI EU virus. Out of these seven mutants, the four mutants BI EU-nsp1 β -delALEV, BI EU-nsp1 β -delEV, BI EU-nsp1 β -delLE, and BI EU-nsp1 β -delSDDS represent a new class of mutants that has not previously been described in WO 2013017570 A1. In particular, infection with the mutant BI EU-nsp1 β -delALEV induced extremely high amounts of IFN- β in MA104 cells

which leads to the conclusion that this virus is severely impaired in blocking the induction of IFN type I.

This finding has strong implications for PRRSV vaccine development since it can be assumed that the immune response of the natural host against PRRSV can be significantly enhanced by introducing deletions, e.g. by deleting aminoacids A₃₀LEV₃₃ in the nsp1 β protein of genotype I PRRSV strains.

The nsp1 β deletion mutants described therein, either alone or in combination with other attenuating mutations, represent promising candidates for live attenuated PRRSV vaccines.

Example 3

a) Introducing a deletion within the ORF4 protein of the EU type PRRSV infectious cDNA clone BI EU

It was tested whether a deletion, as described according to the first consideration of the present invention, could be introduced into the ORF4 gene of any PRRS virus strain without negatively affecting viral replication. Therefore, a deletion was introduced into the genomic region coding for the ectodomain of the ORF4 protein between amino acid positions 50 to 71 of the EU type PRRSV infectious cDNA clone BI EU (comprising the sequence of SEQ ID NO:48). The deletion within the ORF4 protein of BI EU included amino acids 57-69 (as encoded by SEQ ID NO:49).

To test viability of the ORF4 deletion mutant, a synthetic transcript of BI EU cDNA harboring the deletion was transfected into BHK21 cells. After transfer of cell culture supernatants from transfected cells onto PRRSV-susceptible MA104 cells, a cytopathic effect (CPE) was detectable within 3 to 4 days post transfer of cell culture supernatants from transfected BHK21 cells to MA104 cells. After staining the cells with the PRRSV capsid protein-specific monoclonal antibody SDOW17 (Rural Technologies), a strong signal was detectable in the CPE positive MA104 cells but not in cells which received supernatants of mock transfected BHK21 cells (not shown). These findings demonstrated that the BI EU-ORF4 deletion mutant was viable. The recovered mutant virus is designated as BI EU-GP5-36-46-ctr (compare example b) in the following.

To further analyze whether BI EU-GP5-36-46-ctr could be grown to high titers on MA104 cells, growth kinetics were performed. Therefore, MA104 cells were infected with the recovered virus and with the parental BI EU virus as control using a multiplicity of infection

(MOI) of 0.01. Supernatants of infected cells were collected at 0, 24, 48, 72 and 96 hours post infection and virus titers were determined by serial virus dilutions on 96-well plates containing MA104 cells. **Figure 7** shows the result of three independent experiments and demonstrates that BI EU-GP5-36-46-ctr can be grown on MA104 cells as efficiently as the parental BI EU virus. Peak titers of $\sim 1 \times 10^7$ TCID₅₀/ml were observed for both viruses at 48 hours post infection.

Taken together, deletion of amino acids 57-69 within the ORF4 protein does not negatively influence growth of BI EU, indicating that sequence variations within this region are well tolerated by PRRSV *in vitro*. Concluding from these results, the region located between amino acid positions 50 to 71 of the BI EU ORF4 protein might also be used as insertion site for exogenous sequences.

b) Use of the ORF4 protein deletion site for inserting exogenous RNA: Insertion of the PRRSV ORF5 protein neutralizing epitope sequence into the ORF4 gene of the infectious cDNA clone BI EU

This example describes the insertion of an exogenous RNA into the region located between amino acid positions 50 to 71 of the BI EU ORF4 protein. The exogenous RNA in this example codes for the neutralizing epitope located within the ORF5 protein of PRRS virus (Ostrowski, M. et al.) and consists of amino acids 1-11 of SEQ ID NO:39. This sequence (SEQ ID NO:51) was chosen to be inserted into the ectodomain of the ORF4 protein in order to increase accessibility of the ORF5 neutralizing epitope in a potential vaccine candidate allowing improved immune responses in vaccinated animals.

For generating the recombinant virus, the exogenous sequence was introduced into the ORF4 deletion site described in example a) and replaced amino acids 57-69 of the BI EU ORF4 protein by amino acids 1-11 of SEQ ID NO: 39 (representing amino acids 36-46 within the ORF5 protein of type 2 PRRSV strains) flanked by a G-G linker. The insertion resulted in a final sequence of Gly₅₇-Ser-Ser-His-Leu-Gln-Leu-Ile-Tyr-Asn-Leu-Thr-Gly₆₉ (SEQ ID NO:53) within the ORF4 protein of BI EU. The recombinant virus harboring the insertion is designated as BI EU-GP5-36-46 (comprising the sequence of SEQ ID NO:56) in the following.

In order to test whether BI EU-GP5-36-46 could be recovered, a synthetic transcript of BI EU cDNA harboring the mutation was transfected into BHK21 cells. The recombinant virus could be rescued by the same method as described above. A cytopathic effect (CPE) was observable within 3 to 4 days post transfer of cell culture supernatants from transfected BHK21 cells to PRRSV susceptible MA104 cells. Furthermore, PRRSV capsid protein-

specific staining was detectable in CPE positive MA104 cells but not in cells which received supernatants of mock transfected BHK21 cells (not shown).

Growth kinetics were performed in order to test whether the recombinant virus could be grown to high titers. Therefore, MA104 cells were infected with BI EU-GP5-36-46 and with the parental BI EU virus as control using a MOI of 0.01. Supernatants of infected cells were collected at 0, 24, 48, 72 and 96 hours post infection and virus titers were determined by serial virus dilutions on 96-well plates containing MA104 cells. The result of three independent experiments is depicted in **Figure 7**. At 48 hours post infection the virus mutant BI EU-GP5-36-46 reached the same peak titer of $\sim 1 \times 10^7$ TCID₅₀/ml as the parental BI EU virus showing that the inserted sequence within the ORF4 protein does not negatively influence high titer virus growth.

Further experiments on MA104 cells revealed that the exogenous RNA sequence was stably maintained over multiple passages. Sequence analyses demonstrated stability of the insert over all passages analyzed. Interestingly a single nucleotide mutation of adenine to thymine, resulting in an amino acid exchange of His to Pro at position 56, upstream of the insertion site was detectable after passage 1 in independent experiments. Therefore, this additional mutation was inserted into BI EU-GP5-36-46 by reverse genetics. For generating this recombinant virus, an exogenous sequence was introduced into the ORF4 deletion site described in example a) and replaced amino acids 56-69 of the BI EU ORF4 protein by amino acids 1-11 of SEQ ID NO: 39 (representing amino acids 36-46 within the ORF5 protein of type 2 PRRSV strains) N-terminally flanked by the amino acid sequence PG and C-terminally flanked by a G-linker. The insertion resulted in a final sequence of Pro₅₆-Gly-Ser-Ser-His-Leu-Gln-Leu-Ile-Tyr-Asn-Leu-Thr-Gly₆₉ (SEQ ID NO:55) within the ORF4 protein of BI EU. The resulting recombinant virus is designated as BI EU-GP5-36-46-AtoC (comprising the sequence of SEQ ID NO:58) in the following. Growth kinetics depicted in **Figure 7** demonstrated that BI EU-GP5-36-46-AtoC could be grown to similar titers as BI EU-GP5-36-46 and BI EU wild type, respectively.

To test whether the ORF5-derived sequences in the ectodomain-encoding region of ORF4 in BI EU-GP5-36-46-AtoC would render the mutant virus more sensitive to serum neutralization, serum neutralization tests (SNTs) were performed. It was postulated that

increased accessibility of the inserted ORF5-derived neutralizing epitope located in the ORF4 protein ectodomain would result in enhanced sensitivity of the recombinant virus to the action of neutralizing antibodies as compared to the parental virus BI EU.

For the SNTs sera taken from six sows at 48 days post vaccination with BI EU wild type virus were serially diluted and mixed either with BI EU-GP5-36-46-AtoC or with wild type BI EU.

After incubation for one hour at 37°C and 5% CO₂, MA104 cells were added to the samples. Serum titers were determined four days later based on CPE induced by non-neutralized virus. Sera taken from the same animals previous to vaccination served as negative controls (not shown). Mean values and standard deviations of two independent experiments are depicted in **Figure 8**.

It could be demonstrated that BI EU-GP5-36-46-AtoC was consistently more sensitive to *in vitro* neutralization when compared to BI EU wild type virus despite variations that were observable between the six animals analyzed. Serum titers measured for BI EU-GP5-36-46-AtoC were 3 to 15 fold higher than the titers determined for the parental virus BI EU (**Figure 8**). Data obtained from a different experiment further suggested that serum titers for BI EU-GP5-36-46-AtoC might be even more increasable by mutating the N-glycosylation site (amino acid N₉ of SEQ ID NO: 39) present in the ORF5-derived sequence from Asn₉ to Gln₉ (SEQ ID NO: 50 and 52) as N-glycosylation naturally shields the ORF5 neutralizing epitope ((Ansari et al., 2006) and data not shown). In summary, the findings depicted in **Figure 8** strongly indicated that the ORF5-derived neutralizing epitope inserted into the ORF4 protein ectodomain is highly accessible in the recombinant virus BI EU-GP5-36-46-AtoC making the latter a promising vaccine candidate. The demonstrated higher sensitivity to sera containing PRRSV-specific neutralizing antibodies should allow faster clearance and increased safety of the vaccine virus.

Also, it can be expected that PRRSV-specific neutralizing antibodies will be induced to higher levels and at earlier time points in piglets or sows that were vaccinated with BI EU-GP5-36-46-AtoC when compared to animals that were vaccinated with the parental virus BI EU. Early induction of neutralizing antibodies after vaccination should result in faster clearance and therefore less shedding of the vaccine virus (increased safety) and in a more efficient immune response after natural infection with PRRSV (increased efficacy).

The recombinant virus BI EU-GP5-36-46-AtoC therefore represents a promising candidate for a live attenuated PRRSV vaccine with improved safety and efficacy.

LIST OF FIGURES

Figure 1: A. Infectious virus recovered from the BI EU cDNA clone induced a strong CPE on MA104 cells as shown by bright field microscopy. B. PRRSV capsid protein-specific immunofluorescence (IF) staining of BI EU-infected MA104 cells.

Figure 2: Growth of virus recovered from the infectious cDNA clone BI EU on MA104 cells.

Figure 3: nsp1 β N-terminal domain (NTD) amino acid sequence alignment of several US (type II, top) and EU (type I, bottom) PRRSV strains. The NTD aminoacid sequence of BI EU is given at the very bottom. Amino acids R22, PR24, E32, SFP and H52 are indicated above the alignment and have been shown to be crucial for nsp1 β homodimerization (Xue et al., 2010). Target regions for nsp1 β mutagenesis are framed in red. The SDGRSR motif corresponds to the region described in WO 2013017570 A1 using PRRSV EU cDNA clone LoN94-13.

Figure 4: Amino acid sequence alignment of BI EU-nsp1 β deletion mutants.

Figure 5: Growth of BI EU-nsp1 β deletion mutants on IFN-competent MA104 cells.

Figure 6: IFN- β levels measured at different timepoints in the cell culture supernatant of MA104 cells infected with the BI EU-nsp1 β deletion mutants or with parent BI EU virus.

Figure 7: Growth kinetics of recombinant BI EU viruses harboring deletions or insertions within the ORF4 protein.

Figure 8: Serum neutralization tests for the recombinant virus BI EU-GP5-36-46-AtoC and the parental virus BI EU.

In the sequence listing:

SEQ ID NOs:1-24 correspond to sequences of the ectodomain of PRRSV ORF4 protein with a deletion;

SEQ ID NO:25 and SEQ ID NO:26 correspond to sequences of the first two predicted N-terminal β -sheets of PRRSV (genotype I) ORF4 protein;

SEQ ID NO:27 and SEQ ID NO:28 correspond to sequences of the first two predicted N-terminal β -sheets of PRRSV (genotype II) ORF4 protein;

SEQ ID NO:29 and SEQ ID NO:30 correspond to sequences of the first two predicted N-terminal β -sheets of PRRSV (genotype I) ORF4 protein;

SEQ ID NO:31 and SEQ ID NO:32 correspond to sequences of the first two predicted N-terminal β -sheets of PRRSV (genotype II) ORF4 protein;

SEQ ID NO:32 corresponds to a (partial) sequence of a PRRSV (genotype I) ORF4 protein having a deletion of 11 amino acid residues in the region between the first two predicted N-terminal β -sheets;

SEQ ID NO:33 corresponds to a (partial) sequence of a PRRSV (genotype II) ORF4 protein having a deletion of 7 amino acid residues in the region between the first two predicted N-terminal β -sheets;

SEQ ID NO:34 corresponds to the sequence of the ectodomain of a PRRSV (genotype I) ORF4 protein having a deletion of 11 amino acid residues;

SEQ ID NO:35 corresponds to the sequence of the ectodomain of a PRRSV (genotype II) ORF4 protein having a deletion of 7 amino acid residues;

SEQ ID NO:36 corresponds to the sequence of a PRRSV (genotype I) ORF4 protein having a deletion of 11 amino acid residues (and including the sequence of SEQ ID NO:34, respectively);

SEQ ID NO:37 corresponds to a nucleotide sequence encoding the sequence of SEQ ID NO:36;

SEQ ID NO:38 corresponds to a nucleotide sequence encoding a genotype I PRRSV whose genome comprises a nucleic acid molecule which codes for the sequence of SEQ ID NO:36;

SEQ ID NO:39 corresponds to the sequence of a peptide encoded by the ORF5 gene of PRRS virus;

SEQ ID NO:40 corresponds to the sequence of a peptide encoded by the ORF5 gene of PRRS virus;

SEQ ID NO:41 corresponds to Lelystad virus complete genome;

SEQ ID NO:42 corresponds to VR2332 virus complete genome;

SEQ ID NO:43 corresponds to the sequence of ORF4 protein of the Lelystad virus;

SEQ ID NO:44 corresponds to the sequence of ORF4 protein of the VR2332 virus;

SEQ ID NO:45 corresponds to a first nucleic acid sequence as described herein;

SEQ ID NO:46 corresponds to a second nucleic acid sequence as described herein, which flanks the 5' end of the first nucleic acid sequence;

SEQ ID NO:47 corresponds to a third nucleic acid sequence as described herein, which flanks the 3' end of the first nucleic acid sequence;

SEQ ID NO:48 corresponds to BI EU complete viral cDNA insert;

SEQ ID NO:49 corresponds to the sequence of SEQ ID NO:48 with a deletion, thereby encoding an ORF4 protein having a deletion of 13aa (aa 57-69);

SEQ ID NO:50 corresponds to the sequence of SEQ ID NO:39 with the substitution N->Q at position 9;

SEQ ID NO:51 corresponds to the sequence of aa 1-11 of SEQ ID NO:39;

SEQ ID NO:52 corresponds to the sequence of SEQ ID NO:51 with the substitution N->Q at position 9;

SEQ ID NO:53 corresponds to the sequence of SEQ ID NO:51 with a Gly-Gly linker;

SEQ ID NO:54 corresponds to the sequence of SEQ ID NO:52 with a Gly-Gly linker;

SEQ ID NO:55 corresponds to the sequence of SEQ ID NO:53 with an N-terminal proline residue;

SEQ ID NO:56 corresponds to the sequence of SEQ ID NO:49 with an insert, thereby encoding the sequence of SEQ ID NO:53;

SEQ ID NO:57 corresponds to the sequence of SEQ ID NO:49 with an insert, thereby encoding the sequence of SEQ ID NO:54;

SEQ ID NO:58 corresponds to the sequence of SEQ ID NO:48 with a deletion, thereby encoding an ORF4 protein having a deletion of 14aa (aa 56-69), wherein an insert coding for the sequence of SEQ ID NO: 55 is included.

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Claims:

1. A nucleic acid molecule which encodes a genotype I PRRS virus and which is capable of producing live virus when transfected into cells, wherein said molecule comprises a nucleic acid sequence having at least 99.6% sequence identity with the nucleic acid sequence of SEQ ID NO:48 over the whole length of SEQ ID NO:48, or wherein said molecule comprises the nucleic acid sequence of SEQ ID NO:48.
2. The nucleic acid molecule of claim 1, wherein the nucleic acid sequence has at least 99.8% sequence identity with the nucleic acid sequence of SEQ ID NO:48 over the whole length of SEQ ID NO:48.
3. The nucleic acid molecule of claim 2, wherein the nucleic acid sequence has at least 99.9% sequence identity with the nucleic acid sequence of SEQ ID NO:48 over the whole length of SEQ ID NO:48.
4. The nucleic acid molecule of claim 3, wherein the nucleic acid sequence has at least 99.95% sequence identity with the nucleic acid sequence of SEQ ID NO:48 over the whole length of SEQ ID NO:48.
5. The nucleic acid molecule of any one of claims 1 to 4, wherein said virus is attenuated.
6. The nucleic acid molecule of any one of claims 1 to 5, wherein said molecule is a DNA molecule.
7. A DNA construct comprising a DNA molecule according to claim 6.
8. An RNA transcript of the DNA construct of claim 7.
9. A cell transfected with the DNA construct of claim 7 or with the RNA transcript of claim 8.
10. A genotype I PRRS virus produced by the cell of claim 9.
11. A genotype I PRRS virus whose genome comprises a nucleic acid molecule according to any one of claims 1 to 5 or whose genome comprises an RNA molecule encoded by

a nucleic acid molecule according to any one of claims 1 to 6 or whose genome is encoded by the nucleic acid molecule of any one of claims 1 to 6.

12. A method for producing a genotype I PRRS virus comprising transfecting a cell with the DNA construct of claim 7 or comprising transfecting a host cell with the RNA transcript of claim 8.
13. A composition comprising a nucleic acid molecule of any one of claims 1 to 5 suspended in a suitable amount of a pharmaceutically acceptable diluent or excipient.
14. Use of the PRRS virus according to claim 10 or claim 11 for manufacture of a medicament for preventing or treating Porcine Reproductive and Respiratory Syndrome.
15. Use of the PRRS virus according to claim 10 or claim 11 for prophylaxis or treatment of Porcine Reproductive and Respiratory Syndrome.
16. The use of claim 14 or claim 15, wherein the virus is formulated to be administered to swine.
17. The use of any one of claims 14 to 16, wherein the virus is formulated to be administered via the intranasal, intramuscular, oral, or intrauterine route to an animal.
18. A method of preventing or treating Porcine Reproductive and Respiratory Syndrome in an animal comprising administering the PRRS virus according to claim 10 or claim 11.
19. The method of claim 18, wherein the animal is swine.
20. The method of claim 18 or claim 19, wherein the virus is administered via the intranasal, intramuscular, oral, or intrauterine route to the animal.

Figure 1

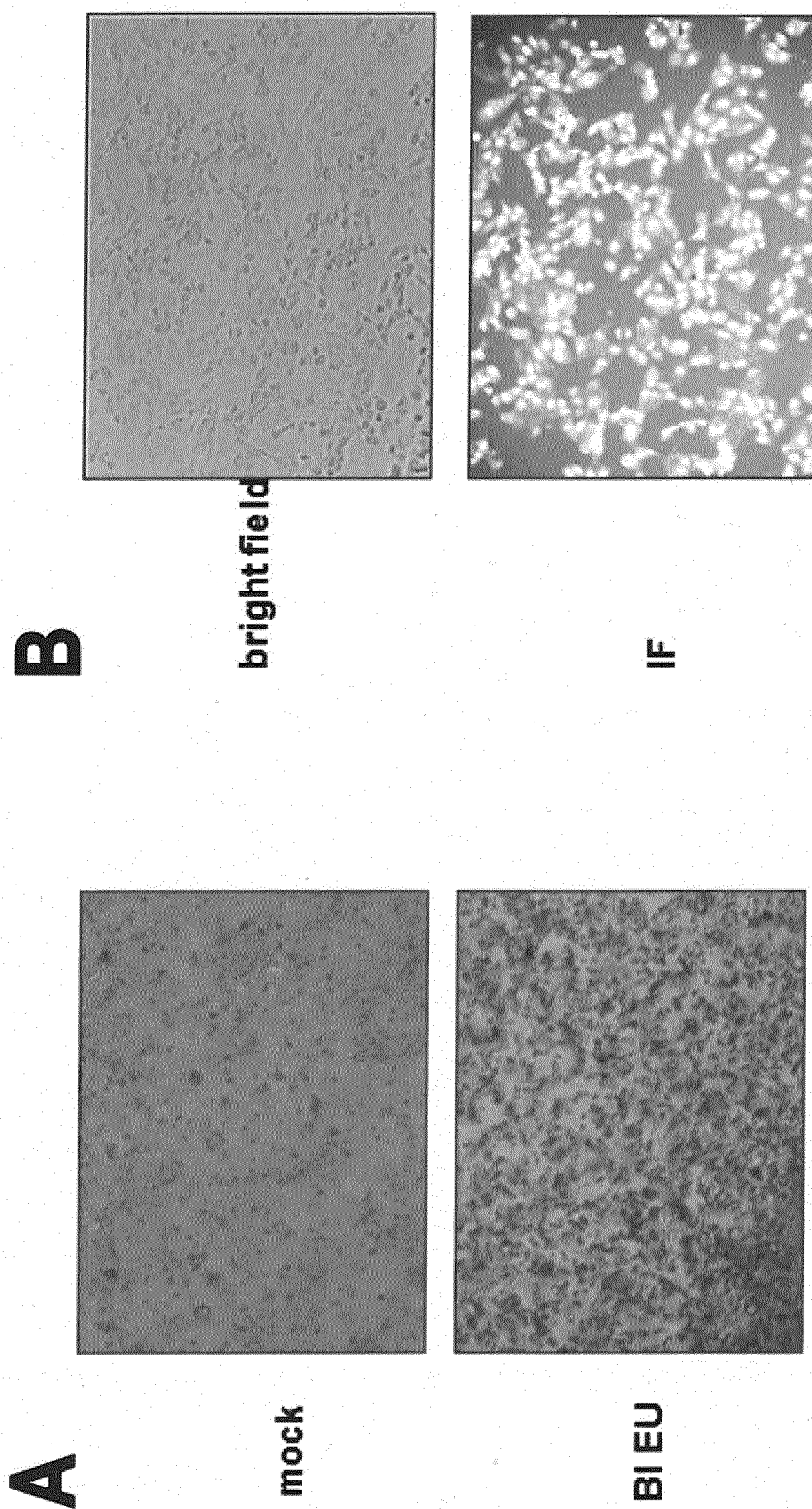


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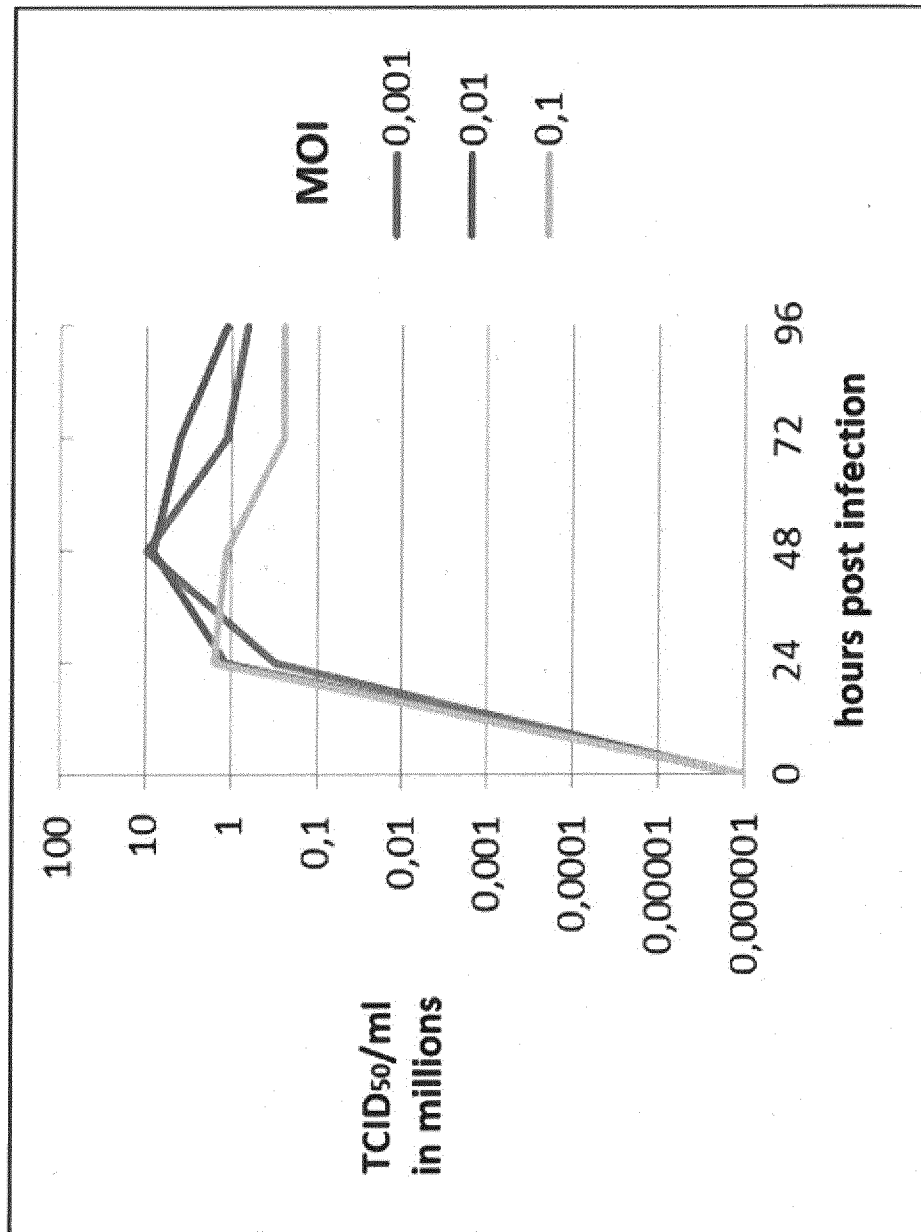


Figure 3

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Figure 4

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Figure 5

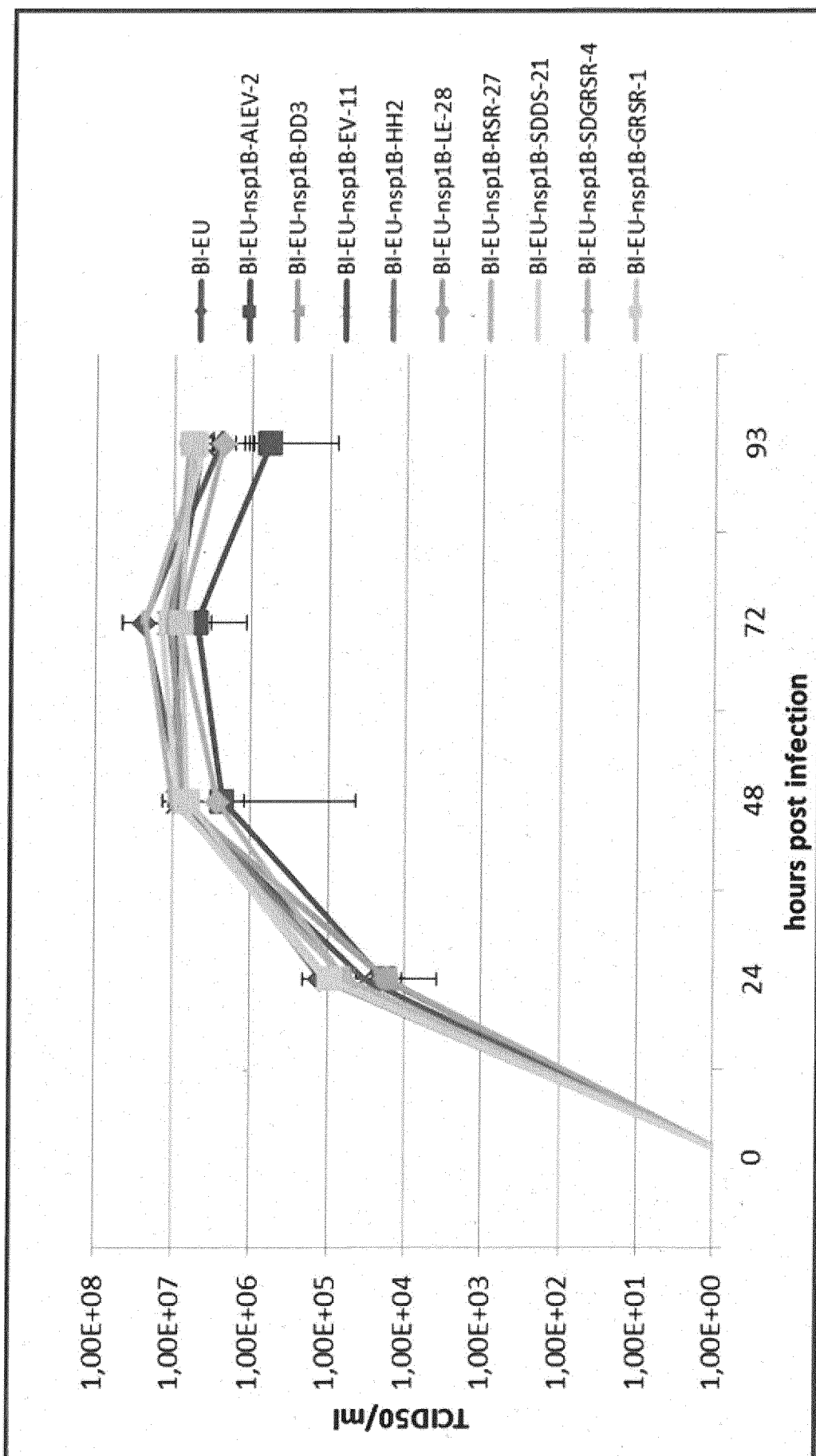


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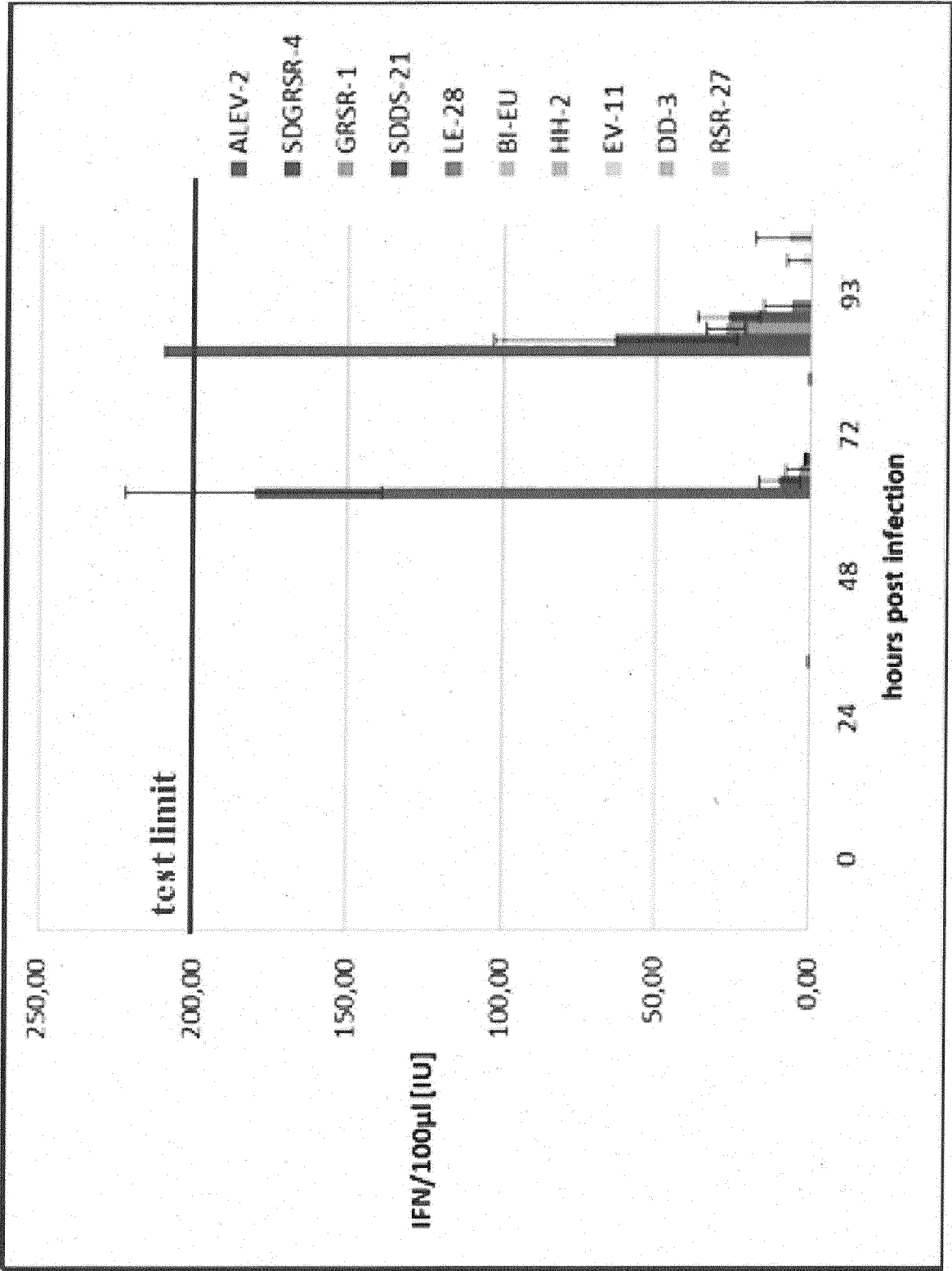


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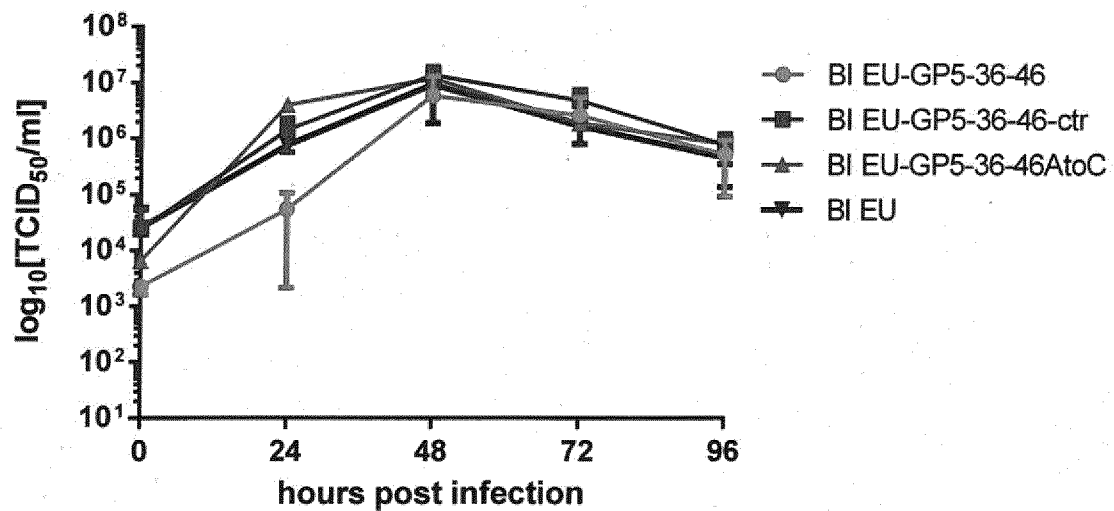
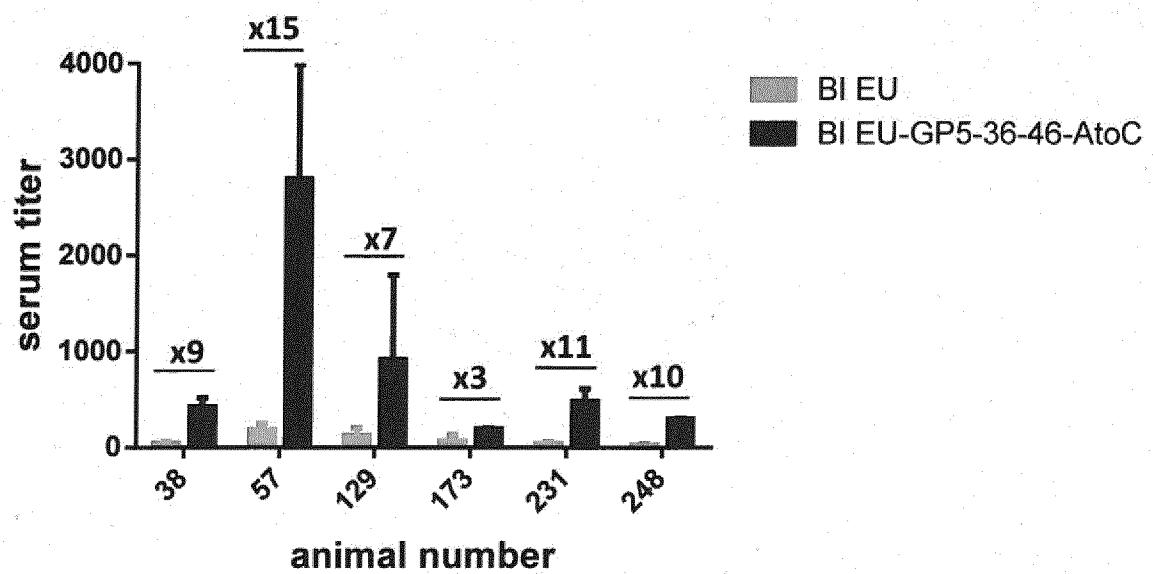


Figure 8



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Lys Ile Glu Cys Leu Gln Ala Pro Gly Thr Arg Ser Gln Cys Arg Glu
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eolf-seql.txt

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eolf-seql.txt

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