ATTENUATED PESTIVIRUS

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention relates to the field of animal health and in particular to attenuated pestiviruses such as classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV) or border disease virus (BDV).

BACKGROUND INFORMATION

Pestiviruses are causative agents of economically important diseases of animals in many countries worldwide. Presently known virus isolates have been grouped into four different species which together form one genus within the family *Flaviviridae*.

I /II Bovine viral diarrhea virus (BVDV) type 1 (BVDV-1) and type 2 (BVDV-2) cause bovine viral diarrhea (BVD) and mucosal disease (MD) in cattle (Baker, 1987; Moennig and Plagemann, 1992; Thiel et al., 1996). The division of BVDV into 2 species is based on significant differences at the level of genomic sequences (summarized in Heinz et al., 2000) which are also obvious from limited cross neutralizing antibody reactions (Ridpath et al. 1994).

III Classical swine fever virus (CSFV), formerly named hog cholera virus, is responsible for classical swine fever (CSF) or hog cholera (HC) (Moennig and Plagemann, 1992; Thiel et al., 1996).

IV Border disease virus (BDV) is typically found in sheep and causes border disease (BD). After intrauterine infection of lambs with BDV persistently infected lambs can be born that are weak and show different abnormalities among which the 'hairy shaker' syndrome is best known (Moennig and Plagemann, 1992; Thiel et al., 1996).

Pestiviruses are small enveloped viruses with a single stranded RNA genome of positive polarity lacking both 5' cap and 3' poly(A) sequences. The viral genome codes for a polyprotein of about 4000 amino acids giving rise to final cleavage products by co- and posttranslational processing involving cellular and viral proteases. The viral proteins are arranged in the polyprotein in the order NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-

NS5A-NS5B-COOH (Lindenbach and Rice, 2001). Protein C (= core- or capsidprotein) and the glycoproteins E^{rns}, E1 and E2 represent structural components of the pestivirus virion as demonstrated for CSFV (Thiel et al., 1991). This also holds true for BVDV. E2 and to a lesser extent E^{rns} were found to be targets for antibody neutralization (Donis et al., 1988; Paton et al., 1992; van Rijn et al., 1993; Weiland et al., 1990,1992). E^{rns} lacks a typical membrane anchor and is secreted in considerable amounts from the infected cells; this protein has been reported to exhibit RNase activity (Hulst et al., 1994; Schneider et al., 1993; Windisch et al., 1996). The function of this enzymatic activity for the viral life cycle is presently unknown. The enzymatic activity depends on the presence of two stretches of amino acids conserved between the pestivirus E^{rns} and different known RNases of plant and fungal origin. Both of these conserved sequences contain a histidine residue (Schneider et al., 1993). Inactivation of the RNase activity residing within the E^{rns} results in an attenuated apathogenic pestivirus which is capable to be used as a modified live vaccine (WO 99/64604).

The pestivirus glycoprotein E^{rns} is expressed on the surface of virions and in infected cells as a disulfide-linked homodimer. The most C-terminal cysteine residue forms the intermolecular disulfide bond between two E^{rns} monomers, resulting in the E^{rns} homodimer (Schneider et al., 1993). Recently it has been reported for CSFV that a substitution of the most C-terminal cysteine against serine results in a viable CSFV, which, however, lacks the abilitiy to form any E^{rns} homodimers (van Gennip et al., 2005).

N^{pro} represents the first protein encoded by the long open reading frame in the pestivirus RNA. N^{pro} represents a nonstructural protein that has protease activity and cleaves itself of the nascent polyprotein (Stark et al., 1993; Wiskerchen et al., 1991) presumably already during translation. N^{pro} is a cysteine protease (Rümenapf et al., 1998) that is not essential for virus replication (Tratschin et al., 1998). Recently, it was shown that N^{pro} somehow interferes with the cellular antiviral defense so that it can be hypothesized to modulate the immune system within an infected host (Rüggli et al., 2003). Mayer and coworkers presented indications for an attenuation of CSFV in consequence of a deletion of the N^{pro} gene (Mayer et al., 2004).

Present BVDV vaccines for the prevention and treatment of BVDV infections still have drawbacks (Oirschot et al. 1999). Vaccines against the classical BVDV-1 provide only partial protection from BVDV-2 infection, and vaccinated dams may produce calves that are

persistently infected with virulent BVDV-2 (Bolin et al., 1991, Ridpath et al., 1994). This problem is probably due to the great antigenic diversity between type 1 and type 2 strains which is most pronounced in the glycoprotein E2, the major antigen for virus neutralization (Tijssen et al., 1996). Most monoclonal antibodies against type 1 strains fail to bind to type 2 viruses (Ridpath et al., 1994).

Vaccines comprising attenuated or killed viruses or viral proteins expressed in heterologous expression systems have been generated for CSFV and BVDV and are presently used. Conventional BVDV life vaccines are typically generated by cell culture passages resulting in viruses with attenuated virulence in the target species. The structural basis of the attenuation of BVDV used as life vaccines is not known. Therefore it is not possible to assess the molecular stability of the attenuation process. These vaccines, although attenuated, are most often associated with safety problems regarding use in breeding animals. The vaccine viruses may cross the placenta of pregnant animals, e.g. cows and lead to clinical manifestations in the fetus and/or the induction of persistently infected calves. The international patent application WO2005/111201 provides a new generation of a modified live pestivirus vaccine, which comprises a multiple modified pestivirus, having at least one mutation in the coding sequence for glycoprotein E^{rns} and at least another mutation in the coding sequence for N^{pro}, wherein said mutation in the coding sequence for glycoprotein E^{rns} leads to inactivation of RNase activity residing in E^{rns} and/or said mutation in the coding sequence for N^{pro} leads to inactivation of said N^{pro}.

However, in view of the importance of an effective and safe as well as detectable prophylaxis and treatment of pestiviral infections, there is a strong need for attenuated pestiviruses, such as BVDV, with a high potential for induction of immunity as well as a defined basis of attenuation which can also be distinguished from pathogenic pestiviruses, such as BVDV, as well as compositions and vaccines comprising said attenuated pesitiviruses, such as BVDV.

Therefore, the technical problem underlying the present invention is to provide new attenuated pestiviruses, referably an attenuated BVDV for use as live attenuated vaccines. Such improved attenuated pestivirus, preferably BVDV, should especially (i) not cross the placenta themselves and (ii) induce an immunity that prevents viral transmission across the

placenta and thereby prevents pregnancy problems like abortion of the fetus or birth of persistently calves from infected host animals in the case of BVDV infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Growth curves of viruses EP #82(4), EP #98(1) and TF #283(1).

Fig. 2: average clinical score for group 1 / 2 / 3

Fig. 3: average body temperature of group 1 / 2 / 3

Fig. 4: body temperature of group 1 infected with TF #283 (1)

Fig. 5: body temperature of group 2 infected with EP #82 (4)

Fig. 6: body temperature of group 3 infected with TF #1347 / TF #230 / 3

Fig. 7: WBC counts of group 1

Fig. 8: WBC counts of group 2

Fig. 9: WBC counts of group 3

All subsequent Sequences are depicting the deleted regions with dashes (_d), which are also numbered, whereas the sequences in the sequence listing attached hereto are continuously numbered without the deleted regions or amino acid codons.

SEQ ID NO:1 (CSFV_wt) amino acid sequence of CSFV wildtype

SEQ ID NO:2 (CSFV_d) amino acid sequence of CSFV comprising a deletion of cystein at amino acid position 438 as compared to SEQ ID NO:1

SEQ ID NO:3 (CSFV_S) amino acid sequence of CSFV comprising a cystein/serine substitution at amino acid position 438 as compared to SEQ ID NO:1

SEQ ID NO:4 (BVDV_Ke9_wt) amino acid sequence of BVDV type 1 wildtype

SEQ ID NO:5 (BVDV_Ke9_d) amino acid sequence of BVDV type 1 comprising a deletion of cystein at amino acid position 441 as compared to SEQ ID NO:4

SEQ ID NO:6 (BVDV_Ke9_S) amino acid sequence of BVDV type 1 comprising a cystein/serine substitution at amino acid position 441 as compared to SEQ ID NO:4

SEQ ID NO:7 (BVDV_NY_wt) amino acid sequence of BVDV type 2 wildtype

SEQ ID NO:8 (BVDV_NY_d) amino acid sequence of BVDV type 2 comprising a deletion of cystein at amino acid position 441 as compared to SEQ ID NO:7

SEQ ID NO:9 (BVDV_NY_S) amino acid sequence of BVDV type 2 comprising a cystein/serine substitution at amino acid position 441 as compared to SEQ ID NO:7

SEQ ID NO:10 (BDV_x818_wt) amino acid sequence of BDV wildtype

SEQ ID NO:11 (BDV_x818_d) amino acid sequence of BDV comprising a deletion of cystein at amino acid position 439 as compared to SEQ ID NO:10

SEQ ID NO:12 (BDV_x818_S) amino acid sequence of BDV comprising a cystein/serine substitution at amino acid position 439 as compared to SEQ ID NO:10

BRIEF SUMMARY OF THE INVENTION

It has surprisingly been found that a modification in the coding region of the E^{rns} glycoprotein of a pestivirus, which results in the lack of homodimer formation of the E^{rns} glycoprotein, leads to an attenuated pestivirus. Such attenuated pestiviruses can be used as modified live vaccine for the prophylaxis and/or treatment of pestivirus infections. Hence, one aspect of the present patent application relates to a recombinant attenuated pestivirus, wherein said recombinant attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein. Preferably, that pestivirus is selected from the group consisting of CSFV, BVDV and BDV, including any subtype of any of these pestiviruses.

It is known from the international patent application WO2005/111201 that pestiviruses attenuated by a modification within the E^{rns} glycoprotein as well as in the N^{pro} protein show a higher safety level in terms of the prevention of fetal infections by the vaccine virus. Therefore, according to a further aspect, the present invention also relates to attenuated pestiviruses, wherein said attenuated pestiviruses do not produce a dimeric E^{rns} glycoprotein and having at least a mutation in the N^{pro} protein, wherein said mutation in the N^{pro} protein leads to inactivation of said N^{pro} protein. Preferably, that pestivirus is selected from the group consisting of CSFV, BVDV and BDV, including any subtype of any of these pestiviruses.

Any of the attenuated pestiviruses mentioned above are suitable vaccine candidates for developing a modified live vaccine for the prophylaxis and/or treatment of pestivirus infections. Thus, according to a further aspect, the present invention relates to an immunogenic composition comprising an attenuated pestivirus, wherein said attenuated

pestivirus does not produce a dimeric E^{rns} glycoprotein. Preferably, that pestivirus has at least a further mutation in the N^{pro} protein, wherein said mutation in the N^{pro} protein leads to inactivation of said N^{pro} protein. Preferred pestiviruses are selected from the group consisting of CSFV, BVDV and BDV, including any subtype of any of these pestiviruses.

According to a further aspect, the present invention also relates to a method for attenuating a pestivirus, comprising modifications in the E^{rns} glycoprotein of said pestivirus in such that said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein. Preferably, that pestivirus is selected from the group consisting of CSFV, BVDV and BDV, including any subtype of any of these pestiviruses.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS OF TERMS USED IN THE DESCRIPTION:

Before the embodiments of the present invention it must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a BVDV" includes a plurality of such BVDV, reference to the "cell" is a reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies as reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The term “pestivirus” as used herein refers to all members of the genus *Pestivirus*, including BVDV, CSFV and BDV, within the family *Flaviviridae*.

The term “CSFV” as used herein refers to all viruses belonging to species of classical swine fever virus (CSFV) in the genus *Pestivirus* within the family *Flaviviridae*.

The term "BVDV" as used herein refers to all viruses belonging to species bovine viral diarrhea virus (BVDV) type 1 (BVDV-1) and BVDV type 2 (BVDV-2) in the genus *Pestivirus* within the family *Flaviviridae* (Heinz et al., 2000). The more classical BVDV type 1 strains and the more recently recognized BVDV type 2 strains display some limited but distinctive differences in nucleotide and amino acid sequences.

The term "N^{pro}" as understood herein relates to the first protein encoded by the viral open reading frame and cleaves itself from the rest of the synthesized polyprotein (Stark, et al., J. Virol. 67:7088-7093 (1993); Wiskerchen, et al., Virol. 65:4508-4514 (1991)). Said term, depending on the context, may also relate to the remaining "N^{pro}" amino acids after mutation of the encoding nucleotide sequence or to the coding nucleotide sequence for said protein itself. "Protease activity residing in N^{pro}" relates to the polypeptide cleavage activity of said "N^{pro}".

"E^{rns}" as used herein relates to the glycoprotein E^{rns} which represents a structural component of the pestivirus virion (Thiel et al., 1991). E^{rns} lacks a typical membrane anchor and is secreted in considerable amounts from the infected cells; this protein has been reported to exhibit RNase activity (Hulst et al., 1994; Schneider et al., 1993; Windisch et al., 1996). It should be noted that the term glycoprotein E0 is often used synonymously to glycoprotein E^{rns} in publications. Said term, depending on the context, may also relate to the mutated "E^{rns}" protein after mutation of the encoding nucleotide sequence or to the coding nucleotide sequence for said protein itself. "RNase activity residing in glycoprotein E^{rns}" relates to the RNA cleavage activity of said glycoprotein, i.e. the ability of the glycoprotein E^{rns} to hydrolyze RNA. The term "inactivation of the RNase activity residing in said glycoprotein" refers to the inability or reduced capability of a modified glycoprotein E^{rns} to hydrolyze RNA as compared to the unmodified wild type of said glycoprotein E^{rns}.

Attenuation: "An attenuated pestivirus or BVDV particle" as used herein means that there is a statistically significant difference between the virulence of attenuated pestivirus or BVDV particles of the present invention, wherein said attenuated viral particles being attenuated by a method described herein, and wild-type pestivirus or BVDV isolates from which said attenuated pestivirus or BVDV particles have been derived, for the predominant clinical

parameters, in case of BVDV for diarrhea, pyrexia and lethality in animals infected with the same dose, preferably $6x10^6TCID_{50}$. Thus, said attenuated BVDV particles do not cause diarrhea, pyrexia and lethality and thus may be used in a vaccine.

Inactivation of E^{rns} as used herein means RNase activity not significantly above the level measured for noninfected control cells in an RNase assay as described in Meyers et al., 1999. "Not significantly above the level measured for noninfected control cells in an RNase assay as described in Meyers et al., 1999, means for example, that the RNase activity is less than 150% compared to the noninfected control cells.

Inactivation of N^{pro} as used herein means the prevention or considerable reduction of the probable immunemodulating activity of N^{pro} by mutation. In a preferred embodiment this mutation prevents or considerably reduces the interference of N^{pro} with the induction of an interferon response by the infected cells as described by Rüggli et al., (2003). In this case, the inactivation of N^{pro} would allow the cell to mount a normal interferon response.

The "dimeric E^{rns} glycoproteins" means a homodimer of two momomers of E^{rns} glycoproteins. It should be noted that the two monomers of the E^{rns} glycoprotein which build the homodimer may comprise a certain level of sequence diversity within their amino acid sequence. In this context, "a certain level of sequence diversity" shall mean that the monomers forming the homodimer show at least 80%, preferably 90%, more preferably 95%, even more preferably 98% sequence homology in respect to the amino acid sequence of the E^{rns} gene region.

The term "non-dimeric E^{rns} glycoprotein" shall mean, but is not limted to an E^{rns} glycoprotein that is not capable to form a detectable amount of homodimer with a second E^{rns} glycoprotein. Preferably, the term "non-dimeric E^{rns} glycoprotein" shall mean, but is not limted to an E^{rns} glycoprotein that is not capable to any homodimer with a second E^{rns} glycoprotein

"Processing signal" as used herein relates to a substance that ensures the generation of a functional N-terminal of the C protein of the pestivirus, preferably of BVDV, in particular a substance selected from the group of ubiquitin, LC3, SUMO-1, NEDD8, GATE-16 and GABA(A)RAP. Also proteases selected from the group of Intein, picornavirus 3C, caridovirus 2A, and p15 of rabbit hemorrhagic disease virus are understood as "processing

signals" as used herein. Similarly, 2A proteins of aphtoviruses or related sequences that promote the expression of two separate proteins by translational discontinuity are included int eh term "Processing signal". Any other similar processing signal known to the skilled person that ensures the generation of a functional N-terminal of the C protein shall also be comprised in the term "processing signal".

"Protein C" or "C protein" or "C-protein" as used herein relates to a structural component of the pestivirus virion (Thiel et al., 1991). "Protein C" is the capsid or core protein of pestiviruses. Said term, depending on the context, may also relate to the "Protein C" with one or several amino acids exchanges resulting from mutation of the encoding nucleotide sequence.

A „fragment" according to the invention is any subunit of a polynucleotide molecule according to the invention, i.e. any subset. For DNA, said fragment is characterized in that it is shorter than the DNA covering the full length viral genome.

A „functional variant" of the nucleotide molecule according to the invention is a nucleotide molecule which possesses a biological activity (either functional or structural) that is substantially similar to the nucleotide molecule according to the invention. The term „functional variant" also includes „a fragment", „a functional variant", „variant based on the degenerative nucleic acid code" or „chemical derivative". Such a „functional variant" e.g. may carry one or several nucleotide exchanges, deletions or insertions. Said functional variant at least partially retains its biological activity, e.g. function as an infectious clone or a vaccine strain, or even exhibits improved biological activity. "Possess a biological activity that is substantially similar" means with respect to the pestiviruses provided herewith, for example, that said pestivirus is attenuated in a manner described herein and result in a non-pathogenic virus suitable for the production of live attenuated virus, which loss ability to pass the placenta but mediates an immune response after vaccination.

A „variant based on the degenerative nature of the genetic code" is a variant resulting from the fact that a certain amino acid may be encoded by several different nucleotide triplets. Said variant at least partially retains its biological activity, or even exhibits improved biological activity.

A molecule is „substantially similar" to another molecule if both molecules have substantially similar nucleotide sequences or biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein if the nucleotide sequence is not identical, and two molecules which have a similar nucleotide sequence are considered variants as that term is used herein even if their biological activity is not identical.

A mutation as used herein relates to modifications in the nucleic acid molecules encoding the proteins / amino acids according to the invention. Said mutations relate to, but are not limited to, substitutions (replacement of one or several nucleotides/base pairs), deletions (removal of one or several nucleotides/base pairs), and/or insertions (addition of one or several nucleotides/base pairs). As used herein, mutation may be a single mutation or several mutations, therefore, often the term "mutation(s)" is used and relates to both a single mutation and several mutations. Said mutations include, but are not limited to point mutations (single nucleotide mutations) or larger mutations wherein e.g. parts of the encoding nucleic acid molecules are deleted, substituted and/or additional coding nucleic acid is inserted. Said mutations may result in a modified expressed polypeptide due to the change in the coding sequence. Such modified polypeptides are desired, as set out in the disclosure of the invention as set out below.

The term "vaccine" as used herein refers to a pharmaceutical composition comprising at least one immunologically active component that induces an immunological response in an animal and possibly but not necessarily one or more additional components that enhance the immunological activity of said active component. A vaccine may additionally comprise further components typical to pharmaceutical compostions. The immunologically active component of a vaccine may comprise complete virus particles in either their original form or as attenuated particles in a so called modified live vaccine (MLV) or particles inactivated by appropriate methods in a so called killed vaccine (KV). In another form the immunologically active component of a vaccine may comprise appropriate elements of said organisms (subunit vaccines) whereby these elements are generated either by destroying the whole particle or the growth cultures containing such particles and optionally subsequent purification steps yielding the desired structure(s), or by synthetic processes including an appropriate manipulation by use of a suitable system based on, for example, bacteria, insects, mammalian

or other species plus optionally subsequent isolation and purification procedures, or by induction of said synthetic processes in the animal needing a vaccine by direct incorporation of genetic material using suitable pharmaceutical compositions (polynucleotide vaccination). A vaccine may comprise one or simultaneously more than one of the elements described above. The term "vaccine" as understood herein is a vaccine for veterinary use comprising antigenic substances and is administered for the purpose of inducing a specific and active immunity against a disease provoked by a pestivirus infection, preferably by a BVDV infection. The attenuated pestivirus, in particular the attenuated BVDV as described herein, confer active immunity that may be transferred passively via maternal antibodies against the immunogens it contains and sometimes also against antigenically related organisms. A vaccine of the invention refers to a vaccine as defined above, wherein one immunologically active component is a BVDV or of pestiviral origin or derived from a nucleotide sequence that is more than 70% homologous to any known pestivirus sequence (sense or antisense).

The term "live vaccine" refers to a vaccine comprising a living, in particular, a living viral active component.

Additional components to enhance the immune response are constituents commonly referred to as "adjuvants", like e.g. aluminiumhydroxide, mineral or other oils or ancillary molecules added to the vaccine or generated by the body after the respective induction by such additional components, like but not restricted to interferons, interleukins or growth factors.

A "pharmaceutical composition" essentially consists of one or more ingredients capable of modifying physiological e.g. immunological functions of the organism it is administered to, or of organisms living in or on the organism. The term includes, but is not restricted to, antibiotics or antiparasitics, as well as other constituents commonly used to achieve certain other objectives like, but not limited to, processing traits, sterility, stability, feasibility to administer the composition via enteral or parenteral routes such as oral, intranasal, intravenous, intramuscular, subcutaneous, intradermal or other suitable route, tolerance after administration, controlled release properties. One non-limiting example of such a pharmaceutical composition, solely given for demonstration purposes, could be prepared as follows: Cell culture supernatant of an infected cell culture is mixed with a stabilizer (e.g. spermidine and/or BSA (bovine serum albumin)) and the mixture is subsequently lyophilized

or dehydrated by other methods. Prior to vaccination, said mixture is then rehydrated in aqueous (e.g. saline, PBS (phosphate buffered saline)) or non-aqueous solutions (e.g. oil emulsion, aluminum-based adjuvant).

DISCLOSURE OF THE INVENTION

The solution to the above technical problem is achieved by the description and the embodiments characterized in the claims.

As mentioned above, at least for the CSFV it has been shown that the last C-terminal cystein of the E^{rns} glycoprotein is involved in the homodimer formation of two monomers of E^{rns} glycoprotein. A substitution of this last C-terminal cystein against serine (cystein/serine substitution) results in a pestivirus that is not longer capable to form homodimers of the E^{rns} glycoprotein (van Gennip et al., 2005). Now it has been surprisingly found that a pestivirus lacking the ability to form homodimers of the E^{rns} glycoprotein is also apathogenic for its host. Consequentely, pestiviruses lacking the ability to form homodimers of the E^{rns} glycoprotein are well attenuated and suitable candidates for a modified live vaccine for the prophylaxis and/or treatment of animals against a pestivirus infection (see example section for more details). Hence, one aspect of the present patent application relates to an attenuated pestivirus, wherein said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein. Preferably, that pestivirus is selected from the group consisting of CSFV, BVDV and BDV, including any subtype of any of these pestiviruses.

According to the further aspect, the pestivirus lacking the ability to form homodimers of the E^{rns} glycoprotein can be established by recombinant bioengineering techniques. For instances, a deletion or substitution at least of the cystein at amino acid position 438 of the CSFV pestivirus (according to SEQ ID NO:1 as shown in an exemplarily manner) results in such a recombinant attenuated CSFV pestivirus lacking the ability to form E^{rns} homodimers, because such E^{rns} glycoprotein is not longer able to form intermolecular disulfide bonds between to E^{rns} monomers. With regard to the BVDV pestivirus (type 1 and/or 2), a deletion or substitution of at least the cystein at amino acid position 441 of the BVDV pestivirus (according to SEQ ID NO:4 (BVDV-1) or SEQ ID NO:7 (BVDV type 2) as shown in an exemplarily manner) results in such a recombinant attenuated BVDV pestivirus lacking the ability to form E^{rns} homodimers. In respect to BDV, a deletion or substitution of at least the

cystein at amino acid position 439 of the BDV pestivirus (according to SEQ ID NO:10 as shown in an exemplarily manner) results in such a recombinant attenuated BDV pestivirus lacking the ability to form E^{rns} homodimers. Preferred substitutions are cystein/serine substitutions. However, any other substitutions of the last C-terminal cystein within the E^{rns} glycoprotein (e.g. Cys438 of CSFV, Cys441 of BVDV type 1 or 2, Cys439 of BDV) are also within the meaning of the present invention.

The key element of the present invention is to provide a pestivirus that is not longer able to form homodimers of the E^{rns} glycoprotein. Homodimer formation can be prevented by a deletion or substitution of at least the last C-terminal cystein within the E^{rns} glycoprotein as decribed above, because any such pestiviruses are not longer capable to form intermolecular disulfide bonds between two E^{rns} glycoprotein monomers. Beside the deletion or substitution of the last C-terminal cystein of the E^{rns} glycoprotein, homodimerisation between two monomers of the E^{rns} glycoprotein can also be inhibited by a modification of the amino acid environment of the last C-terminal cystein of the E^{rns} glycoprotein, if this modification leads, for instance, to a change of the charge and/or conformation of the direct environment of the last C-terminal cystein in a manner that the intermolecular disulfide bonds can not be formed any more. For instance, an insertion, deletion or substitution of prolin close to that last C-terminal cystein may change the conformation of the E^{rns} glycoprotein in such that the last C-terminal cystein of the E^{rns} glycoprotein is not longer exposed in a manner that it can form intermolecular disulfide bonds with a second E^{rns} glycoprotein. Moreover, modifications of the cystein environment with result in a strong positive or negative polarity of such environment (e.g by insertion of or substitution by positive charged amino acids such as argnin, lysine and/or histidine; by insertion of or substitution by negative charged amino acids such as aspratate or glutamate) could also inhibit the formation of intermolecular disulfide bonds between two E^{rns} glycoproteins. Thus, the present invention does not only relate to recombinant attenuated pestiviruses, wherein the last C-terminal cystein of such E^{rns} glycoprotein is deleted or substituted by a non-cystein residue, it also relates to any modified attenuated pestivirus, wherein the formation of homodimers of the E^{rns} glycoprotein of said pestivirus is (in general) inhibited by a deletion, insertion or modification. Preferably, such modification is introduced closely to the last C-terminal cystein of the E^{rns} glycoprotein, preferably such modification affects the amino acids between the amino acid positions 410 to 470, preferably 420 to 460. A skilled person in the art is able to modify the coding sequence

of a pestivirus in such that the pestivirus may not longer form any E^{rns} glycoprotein dimers by routine work. The publication of van Gennip et al., (2005) teaches a person skilled in the art how he can estimate dimerization of the E^{rns} glycoprotein.

Thus, one aspect of the present invention relates to a recombinant attenuated pestivirus, wherein said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein.

According to further aspect, the carboxy-terminus of the E^{rns} glycoprotein of said attenuated pestivirus is modified by a deletion, insertion or substitution.

According to further aspect, at least the last C-terminal Cystein-residue of the E^{rns} glyoprotein of said attenuated pestivirus is deleted or substituted by non-Cys amino acid residue.

According to further aspect, at least the most last Cystein-residue in the E^{rns} glycoprotein of said attenuated pestivirus is deleted.

According to further aspect, said attenuated pestivirus selected from the group consisting of CSFV, BVDV type 1 and/or 2, and BDV.

According to further aspect, said attenuated pestivirus is a CSF pestivirus.

According to a further aspect, at least the Cystein-residue at amino acid position 438 according to SEQ ID NO: 1 of the E^{rns} glyoprotein of said attenuated CSFV is deleted or substituted by a non Cystein-residue.

According to further aspect, said attenunated pestivirus is a BVD type 1 and/or 2 pestivirus.

According to a further aspect, at least the Cystein-residue at amino acid position 441 according to SEQ ID NO: 4 (BVDV type 1) or SEQ ID NO:7 (BVDV type 2) of the E^{rns} glyoprotein of said attenuated BVDV is deleted or substituted by a non Cystein-residue.

According to further aspect, said attenunated pestivirus is a BDV pestivirus.

According to a further aspect, at least the Cystein-residue at amino acid position 439 according to SEQ ID NO: 10 of the E^{rns} glyoprotein of said attenuated BDV is deleted or substituted by a non Cystein-residue.

In WO 99/64604 it is described that the inactivation of the RNAse activity residing within the E^{rns} also results in an attenuated apathogenic pestivirus, which is capable to be used as a modified live vaccine. According to a further aspect of the present invention, both modifications can be combined which would result in an attenuated pestivirus, wherein the RNAse activity residing within the E^{rns} is inactivated by a deletion, insertion or substitution and which is not capable to form any E^{rns} dimers. Suitable modifications of the glycoprotein E^{rns} which result in RNase negative E^{rns} glycoproteins are for example, the single substitutions/deletions: S298G, H300K, H300L, H300R, H300del, W303G, P304del, E305A, C308G, R343G, E345del, W346G, K348A, H349K, H349L, H349del, H349Q, H349SV (mutation H349S and insertion of V), K348R, W351P, W351G, W351L, W351K, W351H; the double substitutions/deletions: H300L/H349L, K348del/H349del, H349del/G350del, E345del/H349del, W303G/E305A, H300K/H349K, H300K/H349L and the triple deletions: L299del/H300del/G300del, K348del/H349del/G350del. Numbering is according to the published amino acid sequence of BVDV CP7 for all the mutants listed above (the given numbers minus 3 would correspond to the equivalent residues of the CSFV Alfort/Tübingen amino acid sequence). All the above-listed mutants were at least tested as respective CSFV or BVDV mutants. Suitable mutants of the pestiviral glycoprotein E^{rns} are provided, for example, by WO 99/64604, which is incorporated herein at its whole.

The putative active site of the RNase is represented by the conserved E^{rns} sequences SLHGIWPEKICTG and/or LQRHEWNKHGWCNWFHIEPW (sequence of the BVDV-2 New York'93 protein given here in an exemplary manner; minor changes can possibly be found in other pestivirus sequences but the identity of the motif will always be obvious for an expert in the field. As an example, the corresponding amino acid sequences of BVDV-1 CP7 would be SLHGIWPEKICTG and/or LQRHEWNKHGWCNWYNIEPW and that of CSFV Alfort/Tübingen SLHGIWPEKICKG and/or LQRHEWNKHGWCNWYNIDPW). Thus, preferably, the invention further relates to a BVDV according to the invention, wherein said RNase negative mutation(s) in the coding sequence for glycoprotein E^{rns} are located in the nucleotide sequence coding for the conserved E^{rns} sequence SLHGIWPEKICTG and/or

LQRHEWNKHGWCNWFHIEPW. These sequences are representing the putative active site of the RNase. The sequences SLHGIWPEKIC and RHEWNKHGWCNW of the putative E^{rns} active site are even more conserved across pestiviruses.

It is known from the international patent application WO2005/111201 that pestiviruses attenuated by a modification within the E^{rns} glycoprotein as well as in the N^{pro} protein show a higher safety level in terms of the prevention of fetal infections. Therefore, according to a further aspect, the present invention also relates to attenuated pestiviruses, wherein said attenuated pestiviruses do not produce a dimeric E^{RNS} glycoprotein and having at least a mutation in the N^{pro} protein, wherein said mutation in the N^{pro} protein leads to inactivation of said N^{pro} protein. Preferably, that pestivirus is selected from the group consisting of CSFV, BVDV and BDV, including any subtype of any of these pestiviruses.

Inactivation of the N^{pro} is achieved in pestiviruses, in particular BVDV of the specified formula described more in detail below, wherein between 0 and all amino acids of N^{pro} are present; ubiquitin or LC3 or another sequence serving as processing signal (e.g. SUMO-1, NEDD8, GATE-16,GABA(A)RAP, or proteases like e.g. Intein, picornavirus 3C, caridovirus 2A, or p15 of rabbit hemorrhagic disease virus, or sequences like aphtovirus 2A that lead to discontinuous translation) is present or absent. In case a processing signal is present, the coding sequence of the processing signal is inserted at or close to the C-terminal end of the (remaining part of the) N^{pro}-protein. Only in the case that a processing signal is present, any number of amino acids coding for N^{pro} (=N^{pro} amino acids) may be present. In case no processing signal sequence is inserted, a maximum of 12 amino acids, preferably aminoterminal amino acids, of N^{pro} may be present, the remaining amino acids have to be deleted. Furthermore, other than the E^{rns} mutations as disclosed above (at least one of which has to be present in the pestivirus, in particular in BVDV according to the invention), the remaining sequences of the pestivirus, in particular BVDV may remain unchanged, i.e. are not mutated, or may also have mutations close to the N-terminal end of the C-protein. A number of more specific embodiments as disclosed below exemplify this.

Thus, the invention relates to a pestivirus, in particular to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_x$-$[PS]_y$-[C-term]

and wherein:

[N^{pro}] relates to the N^{pro} portion of said polyprotein, wherein "**x**" represents the number of amino acids of the N^{pro} present in the polyprotein;

[PS] relates to a processing signal selected from: ubiquitin, LC3, SUMO-1, NEDD8, GATE-16 or GABA(A)RAP) or proteases like e.g. Intein, picornavirus 3C, caridovirus 2A, or p15 of rabbit hemorrhagic disease virus or any processing signal known to the skilled person that ensures the generation of a functional N-terminal of the C-protein. "Y" may be = 0, which means that no processing signal is present (= PS is absent), or "Y" may be = 1, which means that a processing signal is present (= PS present).

[C-term] relates to the complete pestivirus, in particular the complete BVDV polyprotein except for N^{pro}, but including the capsid (C)-protein and any other protein present in the pestivirus polyprotein, in particular in the BVDV polyprotein including the carboxyterminal NS5B. Preferably, the glycoprotein E^{rns} in said [C-term] is mutated, in such that the RNase activity residing in the glycoprotein E^{rns} is inactivated. The term "any other protein present in the pestivirus polyprotein /BVDV polyprotein" relates to E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B and NS5A, wherin glycoprotein E^{rns} is mutated, preferably as disclosed herein (see above), in such that the RNase activity residing in the glycoprotein E^{rns} is inactivated. Preferably, the pestivirus, in particular the BVDV according to the invention has a C-protein which is not mutated except for the amino acid at position 2 which is changed from D to N. Therefore, [C-term*] is the same as [C-term] but with a mutation at position 2 of the C-protein (N instead of D);

if "**y**" is = 0 (means no [PS] present) then"x" is 0 to 12, (means no N^{pro} specific amino acid or 1 to 12 amino acids of N^{pro}, preferably of the N-terminus of N^{pro}, are present);

if "**y**" is = 1 (means [PS] is present) then "x" is 0 to 168; (means no N^{pro} specific amino acid or 1 to all 168 amino acids of N^{pro}, preferably of the N-terminus of N^{pro}, are present).

Also more preferably, the invention relates to a pestivirus, in particular to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_1$-$[PS]_0$-[C-term]

and wherein the definitions are as defined above.

A specific example thereof is disclosed below, wherein the N-terminal methionine is followed by the C-protein and any other protein present in the polyprotein including the carboxyterminal NS5B. Hence, most preferably, the invention relates to a pestivirus, in particular BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

M[C-term].

and wherein the definitions are as defined above.

Also more preferably, the invention relates to a pestivirus, in particular to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_3$-$[PS]_0$-[C-term]

and wherein the definitions are as defined above.

A specific example of BVDV is disclosed below, wherein the N-terminal methionine is followed by the N^{pro} sequence EL and the C-protein and any other protein present in the polyprotein including the carboxyterminal NS5B. Hence, most preferably, the invention relates to a BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

MEL-[C-term]

and wherein the definitions are as defined above.

Also more preferably, the invention relates to a pestivirus, in particular to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_4$-$[PS]_0$-[C-term]

and wherein the definitions are as defined above.

A specific example of BVDV is disclosed below, wherein the N-terminal methionine is followed by the N^{pro} sequence ELF and the C-protein and any other protein present in the polyprotein including the carboxyterminal NS5B. Hence, most preferably, the invention relates to a BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

MELF-[C-term].

and wherein the definitions are as defined above.

Also more preferably, the invention relates to pestivirus, in particular to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_6$-$[PS]_0$-[C-term]

and wherein the definitions are as defined above.

A specific example of BVDV is disclosed below, wherein the N-terminal methionine is followed by the N^{pro} sequence ELFSN and the C-protein and any other protein present in the polyprotein including the carboxyterminal NS5B. Hence, most preferably, the invention relates to a BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

MELFSN-[C-term].

and wherein the definitions are as defined above.

Also more preferably, the invention relates to a pestivirus, in particular to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_4$-$[PS]_0$-[C-term*]

and wherein the definitions are as defined above except for the fact that the aminoterminal part of the C-protein is changed.

A specific example of BVDV is disclosed below, wherein the N-terminal methionine is followed by the N^{pro} sequence ELF and in the C-protein sequence, the amino acid at position 2 is changed from D to N. Therefore, the aminoterminal C-protein sequence is SNEGSK... instead of SDEGSK. Hence, most preferably, the invention relates to a BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

MELF-[C-term*],

wherein in the C-protein the amino acid at position 2 is changed from D to N, and

wherein the definitions are as defined above.

Also more preferably, the invention relates to a pestivirus, in particular BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_x$-$[PS]_1$-[C-term],

wherein the definitions are as defined as above,

and wherein PS is any of the PS disclosed above, preferably selected from the group of ubiquitin or LC3.

A specific example of BVDV is disclosed below, wherein the N-terminal methionine is followed by any 21 or 28 N^{pro} amino acids, ubiquitin or LC3 and the C-protein. Hence most preferably, the invention relates to a BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$[N^{pro}]_{22}$-$[PS]_1$-[C-term], wherein preferably, the PS is ubiquitin or LC3 or

$[N^{pro}]_{29}$-$[PS]_1$-[C-term], wherein preferably, the PS is ubiquitin or LC3.

Ubiquitin is a well known highly conserved cellular protein of 76 amino acids. Among other functions, ubiquitin is a key player in protein catabolism since conjugation with ubiquitin can mark a protein for degradation via the proteasome. Ubiquitin conjugated with or fused to

other proteins via the carboxyterminal glycin can be cleaved off by cellular ubiquitin-specific proteases. Thus, fusion of a protein to the carboxyterminus of ubiquitin will usually result in defined proteolytic cleavage of the fusion protein into its components when expressed within a cell.

LC3 (light chain 3 of microtubule associated proteins) represents a cellular protein of 125 amino acids that serves a variety of functions (length given for bovine LC3). Recently, a fundamental role of the protein in autophagy has been defined. During this process, LC3 is activated by carboxyterminal cleavage. Thereby, a new carboxyterminus is generated that consists of glycine. LC3 is then conjugated via the carboxyterminal glycine to phosphatidylethanolamine present in the membranes of autophagic vesicles. Because of this process, a protein fused to the carboxyterminus of LC3 will be cleaved off by a cellular protease at a defined position.

Also more preferably, the invention relates to a pestivirus, preferably to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula selected from the group of :

$[N^{pro}]_2$-$[PS]_y$-[C-term] and preferably ME-$[PS]_y$-[C-term];

$[N^{pro}]_5$-$[PS]_y$-[C-term] and preferably MELFS-$[PS]_y$-[C-term];

$[N^{pro}]_7$-$[PS]_y$-[C-term] and preferably MELFSNE-$[PS]_y$-[C-term];

$[N^{pro}]_8$-$[PS]_y$-[C-term] and preferably MELFSNEL-$[PS]_y$-[C-term];

$[N^{pro}]_9$-$[PS]_y$-[C-term] and preferably MELFSNELL-$[PS]_y$-[C-term];

$[N^{pro}]_{10}$-$[PS]_y$-[C-term] and preferably MELFSNELLY-$[PS]_y$-[C-term];

$[N^{pro}]_{11}$-$[PS]_y$-[C-term] and preferably MELFSNELLYK-$[PS]_y$-[C-term]; and

$[N^{pro}]_{12}$-$[PS]_y$-[C-term] and preferably MELFSNELLYKT-$[PS]_y$-[C-term]

and wherein the definitions are as defined as above. The preferably disclosed embodiments refers to BVDV.

Most preferably, y is 0 (no PS present).

According to a further aspect, the invention relates to a pestivirus, preferably to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula selected from the group of :

M-[PS]$_0$-[C-term];

MEL-[PS]$_0$-[C-term];

MELF-[PS]$_0$-[C-term];

MELFS-[PS]$_0$-[C-term];

MELFSN-[PS]$_0$-[C-term];

MELFSNE-[PS]$_0$-[C-term];

MELFSNEL-[PS]$_0$-[C-term];

MELFSNELL-[PS]$_0$-[C-term];

MELFSNELLY-[PS]$_0$-[C-term];

MELFSNELLYK-[PS]$_0$-[C-term];

MELFSNELLYKT-[PS]$_0$-[C-term]

According to a further aspect, the invention relates to a pestivirus, preferably to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula selected from the group of :

MELI-[PS]$_0$-[C-term];

MELIS-[PS]$_0$-[C-term];

MELISN-[PS]$_0$-[C-term];

MELISNE-[PS]$_0$-[C-term];

MELISNEL-[PS]$_0$-[C-term];

MELISNELL-[PS]$_0$-[C-term];

MELISNELLY-[PS]$_0$-[C-term];

MELISNELLYK-[PS]$_0$-[C-term];

MELISNELLYKT-[PS]$_0$-[C-term];

According to a further aspect, the invention relates to a pestivirus, preferably to BVDV according to the invention, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula selected from the group of :

MELIT-[PS]$_0$-[C-term];

MELITN-[PS]$_0$-[C-term];

MELITNE-[PS]$_0$-[C-term];

MELITNEL-[PS]$_0$-[C-term];

MELITNELL-[PS]$_0$-[C-term];

MELITNELLY-[PS]$_0$-[C-term];

MELITNELLYK-[PS]$_0$-[C-term];

MELITNELLYKT-[PS]$_0$-[C-term];

Another important aspect of the invention described herein are immunogenic compositions comprising a pestivirus, in particular a BVDV according to the invention, and a solution. The skilled person knows additional components which may be comprised in said composition (see also Remington's Pharmaceutical Sciences. (1990). 18th ed. Mack Publ., Easton). The expert may use known injectable, physiologically acceptable sterile solutions. For preparing a ready-to-use solution for parenteral injection or infusion, aqueous isotonic solutions, such as e.g. saline or corresponding plasma protein solutions are readily available. The pharmaceutical compositions may be present as lyophylisates or dry preparations, which can be reconstituted with a known injectable solution directly before use under sterile conditions, e.g. as a kit of parts.

The final preparation of the immunogenic compositions of the present invention are prepared for e.g. injection by mixing said pestivirus, preferably BVDV according to the invention with a sterile physiologically acceptable solution, that may be supplemented with known carrier substances or/and additives (e.g. serum albumin, dextrose, sodium bisulfite, EDTA). Said solution may be based on a physiologically acceptable solvent, e.g. an aqueous solution between pH 7 and 8. The pH may be stabilised by a pharmaceutically acceptable buffer. The solution may also contain further stabilising agents like a detergent like Tween 20, serum albumin such as BSA (bovine serum albumin), ascorbic acid, and/or spermidine. The composition may also comprise adjuvants, e.g. aluminiumhydroxide, mineral or other oils or ancillary molecules added to the vaccine or generated by the body after the respective induction by such additional components, like but not restricted to interferons, interleukins or growth factors.

For example, in an immunogenic composition according to the invention, the pestivirus, in particular BVDV may be solved in:

Pestivirus (preferably BVDV)	*$10^2 - 10^8$ $TCID_{50}$*
*SGS**	*25 % v/v*
Cell culture medium	*qsp 1 dose*

** SGS:*	*Composition per 2 ml*
Sucrose	*75 mg*
Gelatine	*20 mg*
Potassium hydroxide	*0.274 mg*
L- glutamic acid	*0.72 mg*
Potassium dihydrogen phosphate	*0.516 mg*
Dipotassium phosphate	*1.254 mg*
Water for injection	*qsp 2 ml*

If the immunogenic composition is first lyophilized or dehydrated by other methods, then, prior to vaccination, said composition is rehydrated in aqueous (e.g. saline, PBS (phosphate buffered saline)) or non-aqueous solutions (e.g. oil emulsion (mineral oil, or vegetable/metabolizable oil based/single or double emulsion based), aluminum-based, carbomer based adjuvant).

The immunogenic composition according to the invention is capable to induce an immunological response in an animal. More preferred, the immunogenic composition according to the invention is a vaccine. A vaccine as understood herein comprises a pestivirus, in particular BVDV according to the invention and is defined above (section "definitions")

Most preferred, the immunogenic composition according to the invention further comprises a pharmaceutically acceptable carrier or excipient. Several carriers or excipients are disclosed above. The composition may comprise, if aimed at injections or infusion, substances for preparing isotonic solutions, preservatives such as p-hydroxybenzoates, stabilizers, such as alkalisalts of ethylendiamintetracetic acid, possibly also containing emulsifying and/ or dispersing.

The immunogenic composition according to the invention may be applied intradermally, intratracheally, or intravaginally. The composition preferably may be applied intramuscularly or intranasally. In an animal body, it can prove advantageous to apply the immunogenic compositions as described above via an intravenous or by direct injection into target tissues. For systemic application, the intravenous, intravascular, intramuscular, intranasal, intraarterial, intraperitoneal, oral, or intrathecal routes are preferred. A more local application can be effected subcutaneously, intradermally, intracutaneously, intracardially, intralobally, intramedullarly, intrapulmonarily or directly in or near the tissue to be treated (connective-, bone-, muscle-, nerve-, epithelial tissue). Depending on the desired duration and effectiveness of the treatment, the immunogenic compositions according to the invention may be administered once or several times, also intermittently, for instance on a daily basis for several days, weeks or months and in different dosages.

The invention also relates to the use of a pestivirus, in particular BVDV according to the invention in the manufacture of a vaccine for the prophylaxis and treatment of pestiviral infections, in particular of BVDV infections.

Another important part of the invention is a polynucleotide molecule comprsing the nucleic acid coding for a pestivirus, in particular for a BVDV according to the invention, or a fragment, functional variant, variant based on the degenerative nucleic acid code, fusion molecule or a chemical derivative thereof. Preferably, said polynucleotide molecule is DNA. Also preferably, said polynucleotide molecule is RNA. In a more preferred embodiment, said polynucleotide molecule also comprises the nucleotide sequence of a functional 5'- and/or 3'-non-translated region of a pestivirus, in particular of BVDV.

There are several nucleotide sequences known in the art, which represents the basis for the production of a polynucleotide molecule coding for a pestivirus attenuated according to the present invention. Examples of nuclecic acid sequences of wild-type sequences of several members of pestiviruses are listed below:

Border disease virus	
Strain BD31	NCBI GenBank Accession No. [U70263]
Strain X818	NCBI GenBank Accession No. [AF037405]
Bovine viral diarrhea virus 1	
Strain NADL	NCBI GenBank Accession No. [M31182]
Strain Osloss	NCBI GenBank Accession No. [M96687]
Strain SD-1	NCBI GenBank Accession No. [M96751]
Strain CP7	NCBI GenBank Accession No. [U63479]
Bovine viral diarrhea virus 2	
Strain 890	NCBI GenBank Accession No. [U18059]
Strain C413	NCBI GenBank Accession No. [AF002227]
Strain New York'93	NCBI GenBank Accession No. [AF502399]
Classical swine fever virus	
Strain Alfort/187	NCBI GenBank Accession No. [X87939]
Strain Alfort-Tübingen	NCBI GenBank Accession No. [J04358]
Strain Brescia	NCBI GenBank Accession No. [M31768]
Strain C strain	NCBI GenBank Accession No. [Z46258]

The mutations/modifications according to the invention relating to the coding sequence of N^{pro} and E^{rns} are described above more in detail. Having this information, a person skilled in the art is able to realize the manufacture of any polynucleotide/polynucleic acid coding for a pestivirus according to the present invention. Furthermore, this person is able to manufacture an attenuated pestivirus according to the invention. Molecular method for introducing a mutation into a polynucleotide sequence, cloning and amplification of said mutated polynucleotide are for example provided by Sambrook et 1989 or Ausubel et al. 1994.

Another important aspect of the invention is a method for attenuating a pestivirus which results in a pestivirus according to the invention, comprising modifying the E^{rns} glycoprotein of said pestivirus in such that said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein. According to further aspect, the carboxy-terminus of the E^{rns} glycoprotein of said pestivirus is modified by a deletion, insertion or substitution. According to further aspect, at least the last C-terminal Cystein-residue of the E^{rns} glyoprotein of said pestivirus is deleted

or substituted by a non-Cys amino acid residue. According to further aspect, at least the last C-ternimal Cystein-residue in the E^{rns} glycoprotein of said pestivirus is deleted. According to further aspect, said pestivirus selected from the group consisting of CSFV, BVDV type 1 and/or 2, and BDV. According to further aspect, said attenunated pestivirus is a CSF pestivirus. According to a further aspect, at least the Cystein-residue at amino acid position 438 according to SEQ ID NO: 1 of the E^{rns} glyoprotein of said CSFV pestivirus is deleted or is substituted by a non Cystein-residue. According to further aspect, said pestivirus is a BVD type 1 and/or 2 pestivirus. According to a further aspect, at least the Cystein-residue at amino acid position 441 according to SEQ ID NO: 4 (BVDV type 1) or SEQ ID NO:7 (BVDV type 2) of the E^{rns} glyoprotein of said BVDV is deleted or substituted by a non Cystein-residue. According to further aspect, said pestivirus is a BD pestivirus. According to a further aspect, at least the Cystein-residue at amino acid position 439 according to SEQ ID NO: 10 of the E^{rns} glyoprotein of said BDV is deleted or is substituted by a non Cystein-residue.

According to a further aspect the present invention provides a method for attenuating a pestivirus, characterized in that the glycoprotein E^{rns} is modified in such that the RNAse activity residing within the E^{rns} is inactivated by a deletion, insertion or substitution, and in such that said pestivirus is not capable to form any E^{rns} dimer. According to a preferred embodiment, said pestivirus is selected from the group consisting of CSFV, BVDV, and BDV, including any subtypes thereof.

According to a further aspect, the present invention provides a method for attenuating a pestivirus, characterized in that glycoprotein E^{rns} is modified by a deletion, insertion or substitution in such that said pestivirus is not capable to form any E^{rns} dimer and by modifying the N^{pro} protein by a deletion, insertion or substitution in such that the N^{pro} protein is inactivated..

According to a further aspect, said method comprises the steps:

a) reverse transcription of a wild-type pestivirus nucleotide sequence into a cDNA;
b) cloning said cDNA;
c) introducing mutations selected from the group of deletions, insertion mutations and/or substitution mutations into said cDNA, wherein said mutations are located in the coding

sequence encoding glycoprotein E^{rns}, or the coding sequence encoding glycoprotein E^{rns} and the protease N^{pro},

d) incorporating the cDNA into a plasmid or into a DNA virus capable of directing the transcription of pestivirus cDNA into RNA in vitro or upon infection of suitable cells.

Yet another important embodiment of the invention is a method of treatment of disease caused by a pestivirus, wherein a pestivirus according to the invention or a composition according to the invention is administered to an animal in need thereof at a suitable dosis as known to the skilled person and the reduction of symptoms of said pestivirus infection.

Yet another important embodiment of the invention is a method of treatment of disease caused by BVDV, wherein a BVDV according to the invention or a composition according to the invention is administered to an animal in need thereof at a suitable dosis as known to the skilled person and the reduction of symptoms of BVDV infection such as viremia and leukopenia and/or pyrexia and/or diarrhea is monitored.

EXAMPLES

The following examples serve to further illustrate the present invention; but the same should not be construed as limiting the scope of the invention disclosed herein.

EXAMPLE 1

The objective of the study TV#26 was to define the outcome of clinical signs after infection of pigs with a CSFV Alfort/Tübingen mutant exhibiting a deletion of the cysteine codon at position 438 in the polypeptide (171 in the E^{rns} sequence). This mutation prevents E^{rns} dimerization (EP #82(4)). As controls an RNase negative variant of CSFV Alfort/Tübingen with mutation H297K and wt virus were used.

In this study, 12 animals with weight of about 20 kg were split in three groups of four animals each. The CSFV mutants (first group: TF #283(1) / second group: EP #82(4)) and CSFV wild type virus (third group: EP #98(1)) were applied via the intramuscular and intranasal route on

day 0 (0 dpi). Each animal received 10^6 KID_{50} virus in 1,5 ml DMEM. After infection, animals were monitored for 22 days and rectal temperatures were recorded daily or every second day. A clinical score was determined according to Mittelholzer with modifications.

a) Viruses for the animal study

1. TF #283 (1): CSFV Alfort/Tübingen, RNase negative
2. EP#82(4): CSFV Alfort/Tübingen, incompetent for E^{rns} dimerization
3. EP#98(1): CSFV Alfort/Tübingen, wild type virus.

The mutants show only a slightly reduced growth rate between 24 to 70 h p.i.in cell culture compared to the parental strain. It will have to be tested in further experiments whether this difference is significant (Fig. 1).

The CSFV mutants (first group: TF #283(1) / second group: EP #82 (4)) and CSFV wild type virus (EP #98(1)) were applied intramuscularly and intranasally on day 0 (0 dpi). Each animal received 10^6 KID_{50} virus in 1.5 ml DMEM. Two-thirds of the suspension were applied intranasally (0.5 ml per nostril). For better i.m. application the last 0.5 ml of virus was filled up to 2 ml with DMEM and were injected in the muscle brachiocephalicus.

For titre confirmation of challenge virus 1 ml virus suspension was collected after different dilution and transport steps:

a) original virus (stock solution)

b) first dilution step with DMEM as diluent to obtain a virus concentration of $10^{5,824}$ KID_{50} / ml (10^6 KID_{50} /1.5 ml) directly to -70 °C

c) This sample was taken along "on ice" with the inoculum and handled the same way. After returning from the stable this sample was frozen at -70 °C immediately.

d) second dilution step for i.m. application (see above)

After returning from the stable this sample was frozen at -70 °C immediately.

Titre confirmation has not beenfinished yet.

	TF #283(1) 4. pas (14.12.07)		**EP #82(4)** 7. pas (10.01.08)		**EP #98(1)** 1. pas (14.12.07)	
	expected	determined	expected	determined	expected	determined
original virus	10^6		$10^{6,25}$		$10^{6,125}$	
b	$10^{5,8}$		$10^{5,8}$		$10^{5,8}$	
c	$10^{5,8}$		$10^{5,8}$		$10^{5,8}$	

d	$10^{5,22}$		$10^{5,22}$		$10^{5,22}$	

Tab. 1: Titer of challenge virus preparations
b = directly to -70°C after first dilution step (for intranasal application)
c = return on ice
d = second dilution step (for i.m. application)

b) Operating schedule

Blood was taken from the jugular vein for BC isolation, leukocyte counting, plasma isolation, FACS analysis and serum preparation according to table 3. After infection, animals were monitored for 22 days and rectal temperatures were recorded daily or, in the late stage of the experiment, every second day.

	whole blood for BC isolation + leukocyte count+ plasma	**whole blood for** FACS analysis	**Serum** (SNT)
-1 dpi	X	X	X
0 dpi	Infektion		
2 dpi	X		
5 dpi	X		
8 dpi	X	X	
9 dpi = term (group 3)	X (group 3)	X (group 3)	X (group 3)
12 dpi	X (group 1+2)	X (group 1+2)	
15 dpi	X (group 1+2)	X (group 1+2)	
22 dpi = term (group 1+2) 27.03.2008	X (group 1+2)	X (group 1+2)	X (group 1+2)

Tab. 2: Operating schedule for blood collection

c) Animals and observation during the experiment

Twelve animals with an average weight of 20 kg arrived at the institute on March 28, 2008. The pigs were divided into three different groups of 4 animals. After six days of acclimatisation the animals were infected with the mutant and wild type viruses.

	group 1	2	3
virus	**TF #283(1)**	**EP #82 (4)**	**EP #98(1)**
anim al-ID	841	847	574
	842	848	576

	843	849	593
	844	850	598

Tab. 3: allocation of the animals and challenge viruses to the 3 groups

Pigs were observed daily for general health status. The animals of group #3 challenged with the wild type virus had to be killed prematurely on day 9 p.i. because of considerable signs of CSF. In groups infected with the RNase negative mutant and the dimerization incompetent mutant all animals showed signs of disease typical for CSF. In comparison with the wild type infected animals (group 3) the severity of symptoms in group 1 and 2 were significantly less distinctive.

A clinical score according to Mittelholzer (with the following modifications: defecation: soft feces, normal amount = 0; Reduced amount of feces, dry / thin feces = 1; only small amount of dry, fibrin-covered feces, or diarrhea = 2; no feces, mucus in rectum, or watery or bloody diarrhea = 3) was recorded to evaluate the pathological signs.

		group 1				group 2				group 3			
		841	842	843	844	847	848	849	850	574	576	593	598
days post infection	-1	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0
	4	**1**	**1**	**1**	**1**	**1**	0	**1**	0	**1**	**1**	**1**	**1**
	5	**2**	**2**	**3**	**2**	**5**	**3**	**3**	**3**	**3**	**3**	**3**	**4**
	6	0	0	0	0	**1**	0	**4**	0	**3**	**3**	**3**	**6**
	7	**1**	**1**	**1**	0	0	0	**2**	0	**5**	**4**	**7**	**7**
	8	0	0	**1**	0	0	0	0	0	**9**	**8**	**9**	**8**
	9	0	0	0	0	0	0	0	0	**11**	**10**	**13**	**10**
	10	0	0	0	0	**1**	0	0	0				
	11	0	0	0	0	0	0	0	0				
	12	0	0	0	0	**1**	0	0	0				
	13	0	0	0	0	0	0	0	0				
	14	0	0	0	0	0	0	0	0				
	15	0	0	0	0	0	0	0	0				
	16	0	0	0	0	**1**	0	0	0				
	17	0	0	0	0	0	0	0	0				
	18	0	0	0	0	0	0	0	0				
	19	0	0	0	0	0	0	0	0				
	20	0	0	0	0	0	0	0	0				
	21	0	0	0	0	0	0	0	0				
	22	0	0	0	0	0	0	0	0				

Tab. 4: clinical score for group 1 / 2 / 3 according to Mittelholzer
Animal was euthanised

Rectal temperatures were recorded on -3 and -2 dpi and daily or every second day from 0 dpi up to 20 dpi. In group 1 and 2 every animal reached the critical temperature of 40 °C on 4 / 5 or 6 days post infection. The temperature recovered in all animals of these groups to normal values till 10 dpi or 11 dpi. In the wild type virus infected group body temperature increased from day 4 post infection and no descent was detectable until euthanasia.

d) Analysis of buffy coat preparation

Detection of viremia through virus isolation in Buffy coat preparations has not been finished yet.

	group 1				group 2				group 3			
	841	842	843	844	847	848	849	850	574	576	593	598
-1 dpi												
2 dpi												
5 dpi												
8 dpi												
9 dpi												
12 dpi												
15 dpi												
22 dpi												

Tab. 5: buffy coat preparation
Animal was euthanised

e) White blood cell counts

WBC counts were determined in a haemocytometer, "Neubauer chamber", by standard laboratory procedure. For all animals a reduction of WBC was detectable during the study. The surviving animals showed an increase to almost normal values until study termination.

		group 1				group 2				group 3			
		841	842	843	844	847	848	849	850	574	576	593	596
days post infection	- 1	11972	17527	11706	12722	19694	19694	19361	15944	20441	18371	20666	11361
	2	9896	9842	12027	9457	18194	15472	17333	9611	14555	16248	22055	12500
	5	6664	7194	7861	10111	9694	9051	13111	11944	12472	15055	10055	8030
	8	13321	12399	8852	11130	14609	14542	16828	7583	5190	7612	10611	6430

	9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3429	7555	7194	5171
	12	11138	13729	12611	13527	20277	15083	25611	10833				
	15	12527	13861	18083	13722	22194	14906	27887	11194				
	22	11080	11885	13634	8850	24392	14049	30146	11366				

Tab. 6: WBC counts
n.d. = not determined
Animal was euthanised

f) Serum neutralisation assay

Determination of antibody titers has not been finished yet.

Serum neutralisation assays will be performed using SP50 as test virus on day -1 before infection, 9 days post infection for group 3 and 22 dpi for group 1 and 2.

	group 1				group 2				group 3			
	841	842	843	844	847	848	849	850	574	576	593	598
-1 dpi												
9 dpi	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.				
22 dpi												

Tab. 7: Antibody titers against SP50 determined in SNT, n.d.= not determined
The reciprocal values of serum dilutions that neutralise ca. 100 TCID50 of SP50 are given.

g) Conclusion

It is obvious that the deletion of cysteine #438 and thereby the prevention of E^{rns} dimer formation (biochemical data available) leads to attenuation of CSFV. This attenuation can hardly be due to a general growth retardation of the virus mutant since only marginal differences between the growth rates of the mutant and wt viruses were observed. The development and intensity of the clinical scores and fever as well as the degree of the WBC number reduction are very similar between the virus mutant lacking the E^{rns} RNase activity and the variant with the deletion preventing E^{rns} dimer formation. Since the dimerization has been found to be crucial for the biological functions but not enzymatic activity of other RNases, it can be hypothesized that the attenuation of CSFV in consequence of the prevention of E^{rns} dimer formation and the abrogation of the RNase activity rely on the same principles of the virus host interaction, namely the blockage of the E^{rns} RNase effect. Thus, the prevention of dimer formation is most likely equivalent to blocking the RNase activity, even though the virus is able to express an active RNase.

References

Ausubel, F.M. et al., Current Protocols in molecular biology. New York : Greene Publishing Associates and Wiley-Interscience. 1994 (updated)

Baker, J.C. 1987. Bovine viral diarrhea virus: a review. J. Am. Vet. Med.Assoc. 190: 1449-1458.

Becher, P., König, M., Paton, D.J., Thiel, H.J., 1995, Further characterization of border disease virus isolates: evidence for the presence of more than three species within the genus pesivirus. Virology 209 (1), 200-206.

Chong, S., Williams, K.S., Wotkowicz, C., and Xu, M.Q.1998. Modulation of Protein Splicing of the *Saccharomyces cerevisiae* Vacuolar Membrane ATPase Intein. J. Biol. Chem. 273: 10567 - 10577.Donis, R.O., Corapi, W., and Dubovi, E.J. 1988. Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56K to 58K glycoprotein. J. Gen. Virol. 69: 77-86.

Fuerst T.R. et al. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. 83: 8122-8126.

Heinz, F.X., Collett, M.S., Purcell., R.H., Cold, E.A., Howard, C.R., Houghton, M., Moormann, R.J.M., Rice, C.M., and Thiel, H.-J. 2000. Familiy Flaviviridae. PP 859-878. In: Virus Taxonomy (van Regenmortel., H.H.V., Fauquet, C.M., and Bishop, D.H.L, Eds.). Academic Press, San Diego.

Hulst, M.M., Himes, G., Newbigin, E., Moormann, R.J.M. 1994. Glycoprotein E2 of classical swine fever virus: expression in insect cells and identification as a ribonuclease. Virology 200: 558-565.

Hulst, M.M., F.E. Panoto, A. Hooekmann, H.G.P. van Gennip., and Moormann, R.J.M. 1998. Inactivation of the RNase activity of glycoprotein E^{rns} of classical swine fever virus results in a cytopathogenic virus. J. Virol. 72: 151-157.

Kit, M. and S. Kit. 1991. Sensitive glycoprotein gIII blocking ELISA to distinguish between pseudorabies (Aujeszky's disease) –infected and vaccinated pigs. Veterinary Microbiology 28:141-155.

Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. 154:367-392.

König, Matthias, 1994, Virus der klassischen Schweinepest: Untersuchungen zur Pathogenese und zur Induktion einer protektiven Immunantwort. Dissertation, Tierärztliche Hochschule Hannover, Germany.

Lindenbach, B.D., and Rice, C. M. 2001. The pestiviruses. In Fields Virology, eds. Knipe, D.M., & Howley, P.M. (Lippincott-Raven, Philadelphia), pp. 991-1042.Mayer, D., Hofmann, M.A., and Tratschin, J.D. 2004. Attenuation of classical swine fever virus by deletion of the viral N(pro) gene. Vaccine. 22:317-328.

Meyers, G., Rümenapf, T. and Thiel, H.-J. 1989. Molecular cloning and nucleotide sequence of the genome of hog cholera virus.Virology 171: 555-567.

Meyers,G., Saalmüller,A., and Büttner,M. (1999). Mutations abrogating the RNase activity in glycoprotein e(rns) of the pestivirus classical swine fever virus lead to virus attenuation. J Virol **73:** 10224-10235.

Meyers, G., Tautz, N., Becher, P., Thiel, H.-J., & Kümmerer, B.M. 1996b. Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. J. Virol., 70: 8606-8613.

Meyers, G., Thiel, H.-J., and Rümenapf, T. 1996a. Classical swine fever virus: Recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic swine fever virus. J. Virol. 67:7088-709526.

Meyers, G., Wirblich, C., Thiel. H.-J. and Thumfart, J.O. 2000. Rabbit hemorrhagic disease Virus: genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. Virology 276: 349-363.

Moennig, V. and Plagemann, J. 1992. The pestiviruses. Adv. Virus Res. 41: 53-91.

Paton, D.J., Lowings, J.P., Barrett, A.D. 1992. Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. Virology 190: 763-772.

Pellerin, C. et. al. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities, Virology 203, 1994:260-268.

Porter, A.G. (1993). Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. *J. Virol.* **67**, 6917-6921.

Rüggli, N., Tratschin, J.D., Schweizer, M., McCullough, K.C., Hofmann, M.A., Summerfield, A. 2003. Classical swine fever virus interferes with cellular antiviral defense: evidence for a novel function of N(pro). J. Virol. **77**:7645-7654.

Rümenapf, T., Stark, R., Heimann, M., and Thiel, H.-J. 1998. N-terminal protease of pestiviruses: identification of putative catalytic residues by site directed mutagenesis. J. Virol. **72**: 2544-2547.

Rümenapf, T., Unger, G., Strauss, J.H., and Thiel, H.-J. 1993. Processing of the evelope glycoproteins of pestiviruses. J. Virol. 67: 3288-3294.Schneider, R., G. Unger, R. Stark, E. Schneider-Scherzer, and H.-J. Thiel. 1993. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science 261: 1169-1171.

Sambrook, J., Fritsch, E.F. & Maniatis, T.,Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989

Stark, R., Meyers, G., Rümenapf, T., and Thiel, H.-J. (1993): Processing of pestivirus polyprotein: Cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. J. Virol., **67**, 7088-7095.Thiel, H.-J., Plagemann, G.W., & Moennig, V. 1996. The pestiviruses. In Fields Virology, eds. Fields, B.N., Knipe, D.M., & Howley, P.M. (Lippincott-Raven, Philadelphia), pp.1059-1073.

Thiel, H.-J., Stark, R., Weiland, E., Rümenapf, T. & Meyers, G. 1991. Hog cholera virus: molecular composition of virions from a pestivirus. J. Virol. 65: 4705-4712.31.

Tratschin, J.-D., Moser, C., Ruggli, N., and Hofmann, M.A. 1998. Classical swine fever virus leader proteinase Npro is not required for viral replication in cell culture. J. Virol. **72**, 7681-7684.van Rijn, P.A., van Gennip, H.G., de Meijer, E.J., Moormann, R.J. 1993. Epitope mapping of envelope glycoprotein E1 of hog cholera virus strain Brescia. J. Gen. Virol. 74: 2053-2060.

Van Gennip H.G.P., Hesselink, A.T., Moormann, R.J.M., Hulst M. M. (2005). Dimerisation of glycoprotein Erns of classical swine fever virus is not essential for viral replication and infection. Arch. Virology 150, 2271-2286.

Weiland, E., Thiel, H.-J., Hess, G., and Weiland, F. (1989). Development of monoclonal neutralizing antibodies agaist bovine viral diarrhea virus after pretreatment of mice with normal bovine cells and cyclophosphamide. J. Virol. Methods 24: 237-244.

Weiland, E., Stark, R., Haas, B., Rümenapf, T., Meyers, G. and Thiel, H.-J. (1990). Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J. Virology 64, 3563-3569.

Weiland, E., Ahl, R., Stark, R., Weiland, F. and Thiel, H.-J. (1992). A second envelope glycoprotein mediates neutralization of a pestivirus, hog cholera virus. J. Virology 66, 3677-3682.

Windisch, J.M., Schneider, R., Stark, R., Weiland, E., Meyers, G., and Thiel, H.-J. 1996. RNase of classical swine fever virus: biochemical characterization and inhibition by virus-neutralizing monoclonal antibodies. J. Virol. 70: 352-358

Wiskerchen, M., Belzer, S.K., and Collett, M. S. 1991. Pestivirus gene expression: the first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity J. Virol. 65:4508-4514.

CLAIMS:

1. An immunogenic composition comprising an attenuated pestivirus, wherein said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein.

2. The immunogenic composition according to claim 1, wherein the carboxy-terminus of the E^{rns} glycoprotein of said attenuated pestivirus is modified by a deletion, insertion or substitution.

3. The immunogenic composition according to claim 1 or 2, wherein at least the last Cystein-residue of the E^{rns} glyoprotein of said attenuated pestivirus is deleted or substituted by non-Cys amino acid residue.

4. The immunogenic composition according to claim 3, wherein the last Cystein-residue of the E^{rns} glyoprotein of said attenuated pestivirus is deleted.

5. The immunogenic composition according to any one of claims 1 to 4, wherein said attenuated pestivirus is a Bovine viral diarrhea virus (BVDV), Classical swine fever virus (CSFV) or Border disease virus (BDV).

6. The immunogenic composition according claim 5:
   a. wherein at least the Cystein-residue of the E^{rns} glyoprotein of said attenuated BVDV at amino acid position 441 according to SEQ ID NOs: 4 or 7 is deleted or substituted by a non Cystein-residue;
   b. wherein the Cystein-residue of the E^{rns} glyoprotein of said attenuated CSFV at amino acid position 438 according to SEQ ID NO: 1 is deleted or substituted by a non Cystein-residue;
   c. wherein the Cystein-residue of the E^{rns} glyoprotein of said attenuated BDV at amino acid position 439 according to SEQ ID NO: 10 is deleted or substituted by a non Cystein-residue.

7. The immunogenic composition according to any one claims 1 to 6, wherein said attenuated pestivirus further has at least a mutation in the N^{pro} protein, wherein said mutation in the N^{pro} protein leads to inactivation of said N^{pro} protein.

8. The immunogenic composition according to claim 7, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_x\text{-}[PS]_y\text{-}[C\text{-}term]$$

and wherein:

[N^{pro}] relates to the N^{pro} portion of said polyprotein, wherein "**x**" represents the number of amino acids of the N^{pro} present in the polyprotein; and wherein

[PS] relates to a processing signal selected from the group consisting of: ubiquitin, LC3, SUMO-1, NEDD8, GATE-16 or GABA(A)RAP), Intein, picornavirus 3C, caridovirus 2A, or p15 of rabbit hemorrhagic disease virus; and wherein

"Y" may be = 0, which means that no processing signal is present, or "Y" may be = 1, which means that a processing signal is present; and wherein

[C-term] relates to the complete virus polyprotein except for N^{pro}, but including the capsid (C)-protein, modified E^{RNS} protein and any other protein present in the virus polyprotein including the carboxyterminal NS5B; and wherein

if "**y**" is = 0, then "x" is 0 to 12, (means no N^{pro} specific amino acid or 1 to 12 amino acids of N^{pro} are present); and wherein

if "**y**" is = 1, then "x" is 0 to 168; (means no N^{pro} specific amino acid or 1 to all 168 amino acids of N^{pro} are present).

9. The immunogenic composition according to claim 8, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_4\text{-}[PS]_0\text{-}[C\text{-}term]$$

10. The immunogenic composition according to claim 8, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_4\text{-}[PS]_0\text{-}[C\text{-}term^*],$$

and wherein [C-term]* is = [C-term] wherein in the C-protein the amino acid at position 2 is changed from D to N.

11. The immunogenic composition according to claim 8, wherein said attenuated virus is BVDV, and wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by a formula selected from the group consisting of :

$$[N^{pro}]_x\text{-}[PS]_0\text{-MELF-}[PS]_0\text{-}[C\text{-term}^*];$$

and wherein [C-term]* is = [C-term] wherein in the C-protein the amino acid at position 2 is changed from D to N.

12. The immunogenic composition according to claim 8, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by a formula selected from the group consisting of :

$$[\,N^{pro}]_{22}\text{-}[PS]_1\text{-}[C\text{-term}],$$

and wherein PS is ubiquitin or LC3.

13. The immunogenic composition according to claim 12, wherein the $[PS]_0$ is replaced by $[PS]_1$, and wherein said PS is selected from the group of consisting of: ubiquitin, LC3, SUMO-1, NEDD8, GATE-16, GABA(A)RAP, Intein, picornavirus 3C, caridovirus 2A, aphtovirus 2A and p15 of rabbit hemorrhagic disease virus.

14. The immunogenic composition according to any one of claims 1 to 13, wherein said composition further comprises a pharmaceutically acceptable carrier or excipients.

15. The immunogenic composition according to any one of claims 1 or 14, wherein said composition comprises at least two attenuated pestiviruses of the same genus.

16. The immunogenic composition according to claim 15, wherein said attenuated pestiviruses are different isolates or subtypes of the same genus.

17. The immunogenic composition according to claim 15 or 16, wherein said attenuated pestiviruses are modified at same coding regions in such that none of the attenuated viruses can revert to a pathogenic pestivirus by recombination.

18. The immunogenic compositions according to any one of claims 1 to 17, wherein said immunogenic composition is a vaccine for the prophylaxis of a pestivirus infection.

19. An attenuated pestivirus, wherein said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein and having at least a mutation in the N^{pro} protein, wherein said mutation in the N^{pro} protein leads to inactivation of said N^{pro} protein.

20. The attenuated pestivirus according to claim 19, wherein the carboxy-terminus of the E^{rns} glycoprotein is modified by a deletion, insertion or substitution, and said mutation in the N^{pro} is selected from the group of deletions, insertion mutations and/or substitution mutations.

21. The attenuated pestivirus according to claim 19 or 20, wherein at least the last Cystein-residue in the E^{rns} glyoprotein is deleted or substituted by non-Cys amino acid residue.

22. The attenuated pestivirus according to any one of claims 19 to 21, wherein said attenuated pestivirus is a Bovine viral diarrhea virus (BVDV), Classical swine fever virus (CSFV) or Border disease virus (BDV).

23. The attenuated pestivirus according claim 22:

a. wherein at least the Cystein-residue of the E^{rns} glyoprotein of said attenuated BVDV at amino acid position 441 according to SEQ ID NOs: 4 or 7 is deleted or substituted by a non Cystein-residue;

b. wherein the Cystein-residue of the E^{rns} glyoprotein of said attenuated CSFV at amino acid position 438 according to SEQ ID NO: 1 is deleted or substituted by a non Cystein-residue;

c. wherein the Cystein-residue of the E^{rns} glyoprotein of said attenuated BDV at amino acid position 439 according to SEQ ID NO: 10 is deleted or substituted by a non Cystein-residue.

24. The attenuated virus according to any one of claims 30 to 38, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_x\text{-}[PS]_y\text{-}[C\text{-term}]$$

and wherein:

[N^{pro}] relates to the N^{pro} portion of said polyprotein, wherein "**x**" represents the number of amino acids of the N^{pro} present in the polyprotein; and wherein

[PS] relates to a processing signal selected from the group consisting of: ubiquitin, LC3, SUMO-1, NEDD8, GATE-16 or GABA(A)RAP), Intein, picornavirus 3C, caridovirus 2A, aphtovirus 2A or p15 of rabbit hemorrhagic disease virus; and wherein

"Y" may be = 0, which means that no processing signal is present, or "Y" may be = 1, which means that a processing signal is present; and wherein

[C-term] relates to the complete virus polyprotein except for N^{pro}, but including the region of capsid (C)-protein, modified E^{RNS} protein and any other protein present in the virus polyprotein including the carboxyterminal NS5B; and wherein

if "**y**" is = 0, then "x" is 0 to 12, (means no N^{pro} specific amino acid or 1 to 12 amino acids of N^{pro} are present); and wherein

if "**y**" is = 1, then "x" is 0 to 168; (means no N^{pro} specific amino acid or 1 to all 168 amino acids of N^{pro} are present).

25. The attenuated virus according to claim 24, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_4\text{-}[PS]_0\text{-}[C\text{-term}]$$

26. The attenuated virus according to claim 24, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_4\text{-}[PS]_0\text{-}[C\text{-term}^*],$$

and wherein [C-term]* is = [C-term] wherein in the C-protein the amino acid at position 2 is changed from D to N.

27. The attenuated virus according to claim 24, wherein said mutation(s) in the N^{pro} lead to an encoded polyprotein as characterized by the following formula:

$$[N^{pro}]_x\text{-}[PS]_1\text{-}[C\text{-term}]$$

and wherein PS is selected from the group of ubiquitin or LC3.

28. A recombinant attenuated BVDV, wherein said attenuated BVDV does not produce a dimeric E^{rns} glycoprotein.

29. The recombinant attenuated BVDV according to claim 28, wherein the carboxy-terminus of the E^{rns} glycoprotein of said attenuated BVDV is modified by a deletion, insertion or substitution.

30. The recombinant attenuated BVDV according to claim 27or 28, wherein at least the last Cystein-residue of the E^{rns} glyoprotein of said attenuated BVDV is deleted or substituted by non-Cys amino acid residue.

31. The recombinant attenuated BVDV according to any one of claims 28 to 30, wherein at least the Cystein-residue at amino acid position 441 according to SEQ ID NOs: 4 or 7 is deleted or substituted by a non Cystein-residue.

32. A method for attenuating a pestivirus, comprising modifying the E^{rns} glycoprotein of said pestivirus in such that said attenuated pestivirus does not produce a dimeric E^{rns} glycoprotein.

33. The method according to claim 32, wherein the carboxy-terminus of the E^{rns} glycoprotein of said attenuated pestivirus is modified by a deletion, insertion or substitution.

34. The method according to claim 32 or 33, wherein at least the last Cystein-residue of the E^{rns} glyoprotein of said attenuated pestivirus is deleted or substituted by non-Cys amino acid residue.

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