VACCINE COMPRISING AN 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 bovine viral diarrhea virus (BVDV).

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). Exchange of each of these residues against lysine in the E^{rns} protein of a CSFV vaccine strain resulted in the destruction of RNase activity (Hulst et al., 1998). Introduction of these mutations into the genome of the CSFV vaccine strain did not influence viral viability or growth properties but led to a virus exhibiting a cytopathogenic phenotype (Hulst et al., 1998). Similarly, Meyers et al. showed that an RNase negative variant of the virulent CSFV strain Alfort/Tübingen was fully viable. However, the respective virus mutant showed no cytopathogenic phenotype (Meyers et al., 1999).

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. Killed

vaccines (inactivated whole virus) or subunit vaccines (conventionally purified or heterologously expressed viral proteins) are most often inferior to live vaccines in their efficacy to produce a full protective immune response even in the presence of adjuvants.

The structural basis of the attenuation of BVDV used as life vaccines is not known. These vaccines, although attenuated, are most often associated with safety problems. 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. Therefore, they cannot be applied to breeding herds that contain pregnant cows. Pregnant cows have to be kept separate from vaccinated cattle to protect fetuses and must not be vaccinated themselves. Furthermore, revertants of attenuated live BVDV pose a serious threat to animals. For conventionally derived attenuated viruses wherein the attenuation is achieved by conventional multiple passaging, the molecular origin as well as the genetic stability of the attenuation remains unknown and reversion to the virulent wild-type is unpredictable.

Because of the importance of an effective and safe as well as detectable prophylaxis and treatment of pestiviral infections, there is a strong need for improved 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 improved attenuated pestivirus, 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 infected host such calves in the case of BVDV infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 Serum neutralisation against NY93/C (BVDV type II)

Fig. 2 Serum neutralisation assay against KE9 (BVDV type I)

Fig. 3 Serum neutralisation assay against NY93/C (BVDV type II)

All subsequent Sequences are the show the deleted regions indicated with dashes (-), 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	XIKE-A-cDNA sequence
SEQ ID NO:2	XIKE-A-NdN-cDNA sequence
SEQ ID NO:3	XIKE-B-cDNA sequence
SEQ ID NO:4	XIKE-B-NdN-cDNA
SEQ ID NO:5	XIKE-A amino acid sequence
SEQ ID NO:6	XIKE-A-NdN amino acid sequence
SEQ ID NO:7	XIKE-B amino acid sequence
SEQ ID NO:8	XIKE-B-NdN amino acid sequence
SEQ ID NO:9	XIKE-C-NdN amino acid sequence
SEQ ID NO:10	XIKE-C-NdN-cDNA sequence
SEQ ID NO:11	XIKE-C-cDNA sequence
SEQ ID NO:12	XIKE-C amino acid sequence

BRIEF SUMMARY OF THE INVENTION

The present invention relates to attenuated pestivivirus, preferably to attenuated BVDV, wherein at least one mutation is in the coding sequence for glycoprotein E^{rns} and at least another mutation in the coding sequence for N^{pro} which preferably leads to combined inactivation of the RNase activity residing in glycoprotein E^{rns} in addition to the inactivation of the (hypothesized) immunemodulating activity residing in N^{pro}. The invention also relates to methods for attenuating pestivirus in such that the attenuation results in an attenuated pestivirus, preferably in an attenuated BVDV, as described above. The present invention furthermore relates to nucleic acids molecules encoding said attenuated pestiviruses, preferably encoding attenuated BVDV, compositions and vaccines comprising the attenuated pestivirus, preferably BVDV as disclosed herein.

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 $6x10^6 TCID_{50}$. 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.

"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. 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 an 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.

It has surprisingly been found that pestiviruses, in particular BVDV can be more effectively attenuated by introducing at least one mutation in the coding sequence for glycoprotein E^{rns} and at least another mutation in the coding sequence for N^{pro} which preferably leads to combined inactivation of the RNase activity residing in glycoprotein E^{rns} in addition to the inactivation of the immunomodulating activity residing in N^{pro}. An immunomodulating effect in one aspect is indicated but not limited to the indicated function for one pestivirus in an exemplary manner by Rüggli et al. (2003).

A pestivirus, in particular BVDV attenuated in accordance with the present invention may be advantageously used in vaccines. Said attenuated pestivirus, in particular said attenuated BVDV now provide live vaccines of high immunogenicity. Surprisingly, the pestivirus, in particular the BVDV according to the invention furthermore are safe for use in pregnant animals as they do not cross the placenta. This is exemplified in a non-limiting manner for BVDV in example 3.

Furthermore, live vaccines with defined mutations as a basis for attenuation will allow to avoid the disadvantages of the present generation of vaccines, e.g. the risk of reversion to an mor pathogenic strain. A further advantage of said attenuating mutations lies in their molecular uniqueness which allows to use them as distinctive labels for an attenuated pestivirus, in particular BVDV and to distinguish them from pestivirus, in particular BVDV from the field. Therefore, in one aspect the present invention provides an attenuated pestivirus, in particular an attenuated BVDV 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}. Preferably, in such attenuated pestivirus, preferably in such attenuated BVDV said mutation in the coding sequence for glycoprotein E^{rns} leads to inactivation of the RNase activity residing in E^{rns} and/or said mutation in the coding sequence for N^{pro} leads to inactivation of said N^{pro}. Said inactivation may take place by any mutation known to the person skilled in the art of the E^{rns}- and the N^{pro}-coding sequence, wherein the mutations are any mutation as defined in the "definitions" section, such as deletions, insertion mutations and/or substitution mutations. Most preferably, the mutation(s) are deletions, as the likelihood for revertation to the wild type is the lowest for deletions.

It has been shown that the glycoprotein E^{rns} forms a disulfide-bonded homodimer of about 97 kD, wherein each monomer consists of 227 amino acids corresponding to the amino acids 268 to 494 of the CSFV polyprotein as described by Rümenapf et al. (1993). The genome sequence of the Alfort/Tübingen strain of CSFV is available in the GenBank/EMBL data library under accession number J04358; alternatively, the amino acid sequence for the BVDV strain CP7 can be accessed in the GenBank/EMBL data library (accession number U63479); in the BVDV CP7 polyprotein, the E^{rns} protein corresponds to residues 271 to 497. Two regions of amino acids are highly conserved in glycoprotein E^{rns} as well as in some plant and fungal RNase-active proteins (Schneider et al., 1993). These two regions are of particular importance to the RNase enzymatic activity. The first region consists of the region at the amino acids at position 295 to 307 (298 to 310 for BVDV strain cp7) and the second region consists of the amino acids at position 338 to 357 (341 to 360 for BVDV strain cp7) of said viral polyprotein as exemplified for the Alfort strain of CSFV in Meyers et al., 1999 (numbering according to the published deduced amino acid sequence of CSFV strain Alfort/Tübingen (Meyers et al., 1989). The amino acids of particular importance to the RNase activity as mentioned above are by no means limited to the exact position as defined for the Alfort/Tübingen strain of CSFV but are simply used in an exemplary manner to point out the preferred amino acids being at that position or corresponding to that position in other strains such as found in BVDV, BDV and pestiviruses in general since they are highly conserved. For pestiviruses other than the CSFV Alfort/Tübingen strain the numbering of the positions of the preferred amino acids can be different but an expert in the field of the molecular biology of pestiviruses will easily identify these preferred amino acids by the high degree of conservation of this amino acid sequence and the position of these motifs in the sequence context. In one particular non-limiting example, the position of CSFV Alfort/Tübingen 346 is identical to position 349 of BVDV strain cp7.

As a consequence, the present invention preferably relates to a BVDV according to the invention, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} are located in the encoding nucleotide sequence corresponding to amino acids at position 298 to 310 and/or position 341 to 360. Preferably, such mutations are (amino acids are given in the one letter symbols; the amino acid before the position number indicates the amino acid to be substituted, the amino acid after the position number the substituting amino acid (del indicates deletion): for example, H300L means histidine 300 was substituted by leucine:

Suitable modification of the glycoprotein E^{rns} 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 without mutations in the N^{pro}region. Suitable mutants of the pestiviral glycoprotein E^{rns} are provided, for example, by WO 99/64604, which is incorporated herein at its whole. It should be noted, however, that according to the present invention, at least one additional mutation in the N^{pro} region, as disclosed in further detail below, must be present.

It was particularly found that deletion or substitution of the histidine residue at position 346 (CSFV) or 349 (BVDV) leads to effective inactivation of E^{rns} and therefore leads to particularly useful pestiviral live vaccines. The present invention demonstrates that pestiviruses are viable and code for an E^{rns} protein without RNase activity when the histidine residue at position 346 of the viral polyprotein (numbering according to the published sequence of CSFV Alfort/Tübingen (Meyers et al., 1989)), or at position 349 (numbering according to the published sequence of BVDV CP7 (Meyers et al., 1996b)) if said pestivirus is BVDV, which represents one of the conserved putative active site residues of the E^{rns} RNase, is deleted. Thus, preferably, the invention also relates to a BVDV according to the invention, wherein said mutation in the coding sequence for glycoprotein E^{rns} is a deletion or substitution of the histidine residue at position 349. Even more specifically, 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 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. Thus, preferably, the invention also relates to a pestivirus, in particular to BVDV having at least one mutation in the coding sequence of the N^{pro} protein and the glycoprotein E^{rns}, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} are located in the nucleotide sequence coding for the conserved E^{rns} sequence SLHGIWPEKIC and/or RHEWNKHGWCNW. Preferably, the mutation is located in only one of said sequences. Thus the invention also relates to a pestivirus, in particular to BVDV having at least one mutation in the coding sequence of the N^{pro} protein and the glycoprotein E^{rns}, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} are located in the nucleotide sequence coding for the conserved E^{rns} sequence SLHGIWPEKIC or RHEWNKHGWCNW. Preferably, such mutations concern two different amino acids, i.e. are double mutations. Thus, said mutations may be 1 to 3 nucleotide mutations in two different tripletts encoding two amino acids. Thus, the invention also relates to a pestivirus, in particular to BVDV having at least one mutation in the coding sequence of the N^{pro} protein and the glycoprotein E^{rns}, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} are two mutations located in the nucleotide sequence coding for the conserved E^{rns} sequence SLHGIWPEKIC and/or RHEWNKHGWCNW. Preferably, such mutations concern a single amino acid. Thus, said mutation may be 1 to 3 nucleotide mutations in one triplett encoding one amino acid. Thus, the invention also relates to a pestivirus, in particular to BVDV having at least one mutation in the coding sequence of the N^{pro} protein and the glycoprotein E^{rns}, wherein a single mutation is located in the conserved E^{rns} sequence SLHGIWPEKIC or RHEWNKHGWCNW.

As mentioned above, the attenuated pestiviruses provided by the present invention, having at least on mutation in the coding sequence of the glycoprotein E^{rns} and in the coding sequence of the the N^{Pro} protein, wherein said mutation preferably result in inactivation of the RNase activity residing in the glycoprotein E^{RNS} and of the immunomodulating activity residing in N^{pro}. 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) 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:

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

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;

[**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:

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

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:

$$M[C\text{-term}].$$

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:

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

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:

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

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:

$$MELF\text{-}[C\text{-}term^*],$$

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:

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

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).

Also more preferably, said BVDV according to the invention as described *supra* is a BVDV type 1 BVDV. Most preferably, said BVDV according to the invention as described *supra* is a BVDV type 2 BVDV. BVDV-1 and BVDV-2 are differentiated according to features of their genomic sequences (Heinz et al., 2000 and references therein). BVDV-1 as disclosed herein may be used in the manufacture of a composition for use in the prevention and/or treatment of BVDV type 1 infections in breeding stocks of cattle, in pregnant cows and in the induction of fetal protection against BVDV type 1 infection is pregnant cows. Surprisingly, a BVDV-2 as disclosed herein may be used in the manufacture of a composition for use in the prevention and/or treatment of BVDV type 1 infections in breeding stocks of cattle. In particular, the invention relates to the use of a BVDV type 2 according to the invention in the manufacture of a composition for use in the prevention and/or treatment of BVDV type 1 infections in pregnant cows. Preferably, the BVDV type 2 according to the invention may be used in the manufacture of a composition for use in the induction of fetal protection against BVDV type 1 infections in pregnant cows. Surprisingly also, a BVDV-1 as disclosed herein may be used in the manufacture of a composition for use in the prevention and/or treatment of BVDV type 2 infections in breeding stocks of cattle. In particular, the invention relates to the use of a BVDV type 1 according to the invention in the manufacture of a composition for use in the prevention and/or treatment of BVDV type 2 infections in pregnant cows. Preferably, the BVDV type 1 according to the invention may be used in the manufacture of a composition for use in the induction of fetal protection against BVDV type 2 infections in pregnant cows. Most preferred is the use of BVDV type 1 and type 2 in combination for the manufacture of a composition for use in the prevention and/or treatment of BVDV type 1 and or type 2 infections in breeding stocks of cattle, in pregnant cows and in the induction of fetal protection against BVDV type 1 and/or type 2 infections is pregnant cows.

Most preferably, the wild type BVDV according to the invention which is to be mutated as disclosed herein corresponds to amino acid sequence SEQ ID No. 5 (termed XIKE A) or is a functional variant thereof. Most preferably also, the BVDV according to the invention has a N^{pro} mutation according to the invention and corresponds to amino acid sequence SEQ ID No. 6 (termed XIKE-A-NdN) or is a functional variant thereof. Preferably, such a functional variant is

at least 65% homologous to the amino acid sequence disclosed herein. On the amino acid level, homologies are very roughly: BVDV-1/-BVDV-1: 93%; BVDV-1/-BVDV-2: 84%; BVDV-2/-BVDV-2: 98%. Therefore, more preferable, such a functional variant is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90% homologous to the amino acid sequence disclosed herein. More preferably also, such functional variant is at least 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% homologous to the amino acid sequence disclosed herein. Most preferably, such functional variant is at least 99% or 99.9% homologous to the amino acid sequence disclosed herein.

Most preferably also, the BVDV according to the invention has a E^{rns} mutation according to the invention which has a deletion of the codon coding for histidine 349, and corresponds to amino acid sequence SEQ ID No. 7 (termed XIKE-B) or is a functional variant thereof. Most preferably also, the BVDV according to the invention has both a E^{rns} mutation and a N^{pro} mutation according to the invention, wherein the codon coding for histidine 349 of E^{rns} is deleted and also the complete N^{pro} coding region is deleted, except for codons 1 to 4, thus amino acids MELF of N^{pro} remain. Said mutant corresponds to amino acid sequence SEQ ID No. 8 (termed XIKE-B-NdN) or is a functional variant thereof. Preferably, such a functional variant is at least 65% homologous to the amino acid sequence disclosed herein. More preferable, such a functional variant is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90% homologous to the amino acid sequence disclosed herein. More preferably also, such functional variant is at least 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% homologous to the amino acid sequence disclosed herein. Most preferably, such functional variant is at least 99% or 99.9% homologous to the amino acid sequence disclosed herein.

Most preferably also, the BVDV according to the invention has a E^{rns} mutation according to the invention which has a substitution of the codon coding for histidine 300 by the codon coding for leucine and corresponds to amino acid sequence SEQ ID No. 9 (termed XIKE-C) or is a functional variant thereof. Most preferably also, the BVDV according to the invention has both a E^{rns} mutation and a N^{pro} mutation according to the invention, wherein the codon coding for histidine 300 is substituted by the codon coding for leucine and also the complete N^{pro} coding region is deleted, except for codons 1 to 4, thus amino acids MELF of N^{pro} remain. Said mutant corresponds to amino acid sequence SEQ ID No. 10 (termed XIKE-C NdN) or is a functional variant thereof. Preferably, such a functional variant is at least 65% homologous to the amino

acid sequence disclosed herein. More preferable, such a functional variant is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90% homologous to the amino acid sequence disclosed herein. More preferably also, such functional variant is at least 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% homologous to the amino acid sequence disclosed herein. Most preferably, such functional variant is at least 99% or 99.9% homologous to the amino acid sequence disclosed herein.

Another important embodiment of the invention described herein is a composition 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 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 a composition according to the invention, the pestivirus, in particular BVDV may be solved in:

Pestivirus (preferably BVDV)	*$10^2 - 10^8$ TCID50*
*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 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).

Preferably, the composition according to the invention induces an immunological response in an animal. More preferred, the 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 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 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 pharmaceutical 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 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, having at least one mutation in the coding sequence of N^{pro} and at least one in the coding sequence of glycoprotein E^{rns}, wherein said mutations result in an combined inactivation of the RNase activity residing in glycoprotein E^{rns} and in the inactivation of the immunomodulating activity residing in N^{pro}. 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]
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.

Most preferably, the wild type BVDV according to the invention which is to be mutated as disclosed herein is encoded by the nucleic acid sequence SEQ ID No. 1 (termed XIKE A) or a functional variant thereof. Most preferably also, the BVDV according to the invention has a N^{pro} mutation according to the invention and is encoded by nucleic acid sequence SEQ ID No. 2 (termed XIKE-A-NdN) or a functional variant thereof. Preferably, such a functional variant is at least 65% homologous to the nucleic acid sequence disclosed herein. On the nucleic acid level, homologies are very roughly: BVDV-1/-BVDV-1: 80%; BVDV-1/-BVDV-2: 70%; BVDV-2/-BVDV-2: 96%. Therefore, more preferable, such a functional variant is at least 70%, 71%, 72%,

73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90% homologous to the nucleic acid sequence disclosed herein. More preferably also, such functional variant is at least 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% homologous to the nucleic acid sequence disclosed herein. Most preferably, such functional variant is at least 99% or 99.9% homologous to the nucleic acid sequence disclosed herein.

Most preferably also, the BVDV according to the invention has a E^{rns} mutation according to the invention which has a deletion of codon H349 and is encoded by nucleic acid sequence SEQ ID No. 7 (termed XIKE-B) or by a functional variant thereof. Most preferably also, the BVDV according to the invention has both a E^{rns} mutation and a N^{pro} mutation according to the invention, wherein the codon coding for histidine 349 of E^{rns} is deleted and also the complete N^{pro} coding region is deleted, except for codons 1 to 4, thus amino acids MELF of N^{pro} remain. Said mutant is encoded by nucleic acid sequence SEQ ID No. 8 (termed XIKE-B-NdN) or by a functional variant thereof. Preferably, such a functional variant is at least 65% homologous to the nucleic acid sequence disclosed herein. More preferable, such a functional variant is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90% homologous to the nucleic acid sequence disclosed herein. More preferably also, such functional variant is at least 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% homologous to the nucleic acid sequence disclosed herein. Most preferably, such functional variant is at least 99% or 99.9% homologous to the nucleic acid sequence disclosed herein.

Most preferably also, the BVDV according to the invention has a E^{rns} mutation according to the invention which is a substitution of codon "H300" by a leucine codon, and is encoded by nucleic acid sequence SEQ ID No. 11 (termed XIKE-C) or a functional variant thereof. Most preferably also, the BVDV according to the invention has both a E^{rns} mutation and a N^{pro} mutation according to the invention, wherein the codon coding for histidine 300 is substituted by the codon coding for leucine and also the complete N^{pro} coding region is deleted, except for codons 1 to 4, thus amino acids MELF of N^{pro} remain. Said mutant is encoded by nucleic acid sequence SEQ ID No. 12 (termed XIKE-C-NdN) or by a functional variant thereof. Preferably, such a functional variant is at least 65% homologous to the nucleic acid sequence disclosed herein. More preferable, such a functional variant is at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89% or 90% homologous to the nucleic acid sequence disclosed herein. More preferably also, such functional variant is at least 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% homologous to the nucleic acid sequence

disclosed herein. Most preferably, such functional variant is at least 99% or 99.9% homologous to the nucleic acid sequence disclosed herein.

Another important aspect of the invention is a method for attenuating a pestivirus, characterized in that at least one mutation in the coding sequence for glycoprotein E^{rns} and at least another mutation in the coding sequence for N^{pro} is generated in a pestivirus genome. According to a preferred embodiment, said pestivirus is BVDV.

According to a more preferred embodiment, 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} 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.

Regarding the method for attenuating a BVDV according to the invention, said preferred methods comprisses the steps:

a) reverse transcription of a wild-type BVDV 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} 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, wherein the said pestivirus or said composition 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, wherein the said BVDV or said composition 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

BVDV XIKE-B: foetopathogenicity assessment in pregnant heifers

BVDV XIKE-B, an RNase negative mutant of the highly pathogenic BVDV type 2 isolate NewYork'93/C was recovered from the infectious cDNA clone pKANE40B and showed wild type-like (wt-like) growth characteristics in tissue culture. In animal experiments the mutant virus was found to be considerably attenuated so that it represented a promising candidate for development of a live attenuated vaccine virus (Meyer et al., 2002). To test whether this attenuated virus is still able to cross the placenta and infect the fetus, pregnant heifers were infected with XIKE-B. As a control wild type BVDV recovered from cDNA clone pKANE40A was used The respective virus named XIKE-A expresses an active E^{rns} RNase in the infected cell.

The study aimed to assess the safety of XIKE-A and XIKE-B in pregnant animals.

EXPERIMENTAL DESIGN

Ten pregnant heifers were selected from a BVDV negative herd. The following groups of 5 heifers were included in the trial:

	No.	Inoculation	Virus
Group 1:	5	One i. n. administration, 3 ml in each nostril	XIKE-A
Group 2:	5	One i. n. administration, 3 ml in each nostril	XIKE-B

Heifers were moved to the experimental facilities 8 days before inoculations. Pregnancy status was confirmed after transport into the experimental facility. Heifers were between days 60 and 90 of gestation on the day of inoculation. Inoculation took place for all animals at one point of time.

Heifers were monitored for the presence of clinical signs of BVDV infection including abortions during the observation period. Blood samples were collected from the animals for serology, antigen detection and white blood cells were counted. The experiment was terminated 9 weeks after infection. Non-aborted cows were slaughtered, the uterus examined and collected. Foetal organ samples were collected during routine necropsy and examined for BVDV infection.

The presence of fetal infection was the main evaluation parameter, composed from the number of BVDV-related cow mortality, the number of BVDV-related abortions and the number of BVDV positive fetuses at termination. In addition to the main parameter, clinical signs characteristic for BVDV infection, viraemia and white blood cell counts in cows and rectal temperature after challenge were evaluated.

Animals

Heifers were purchased from a farm free of BVDV.

Only animals, which met the following inclusion criteria, were used.

Inclusion criteria

- Free of BVD antibodies; each individual will be tested in the serum antibody test prior to transport and at the initiation of the study (at the animal test facility).

- Free of BVDV; plasma and/or buffy-coat preparation from each individual will be tested by a suitable test.
- Clinically healthy at the initiation of the study judged upon physical examination. The health examination of the animals was accomplished in accordance with the current, generally accepted veterinary practice.

- Pregnancy confirmed by physical examination before inoculation. Pregnancy was between 60 - 90 days at the time of inoculation, proven by insemination records.

Test Strain A

Description:	XIKE A, life virus BVDV strain
Composition:	Experimental material comprising of cell culture supernatant of low passaged XIKE-A
BVD components:	BVDV type II strain: XIKE-A
Supplied by:	Dr. Gregor Meyers, "Bundesforschungsanstalt für Viruskrankheiten der Tiere" (BFAV), Paul-Ehrlich-Straße 28, 72076 Tübingen, Germany
Applied BVD virus dose:	Type 1 strain: 10^5 $TCID_{50}$/6ml (TCID = **T**issue **C**ulture **I**nfective **D**ose)
Applied vaccine volume:	3 ml per nostril
Application route:	Intranasal
Preparation of dosage form:	The inoculum was sent in a pre-diluted frozen form in a 50 ml vial on dry ice and was to be stored at – 70 °C before inoculation. Immediately before inoculation of Group 1 heifers, the material was thawed avoiding local temperatures above 37 °C. After no ice was visible in the fluid, material was gently stirred and immediately used for inoculation of the animals.
Unused inoculum:	The volume of the unused material was be measured and split on two aliquots before immediate freezing in dry ice or liquid nitrogen and stored for re-titration purposes. Virus and contaminated plastic or glassware were incubated with an appropriate volume of an 8 – 10 % formaldehyde solution for at least 24 hours at room temperature before discarding in order to inactivate viruses.

Test Strain B

Description:	XIKE B, life virus BVDV strain
Composition:	Experimental material comprising of cell culture supernatant of low passaged XIKE B
BVD components:	BVDV type II strain: XIKE-B
Supplied by:	Dr. Gregor Meyers, "Bundesforschungsanstalt für Viruskrankheiten der Tiere" (BFAV), Paul-Ehrlich-Straße 28, 72076 Tübingen, Germany
Applied BVD virus dose:	Type 1 strain: 10^5 $TCID_{50}$/6ml (TCID = **T**issue **C**ulture **I**nfective **D**ose)
Applied vaccine volume:	3 ml per nostril
Application route:	Intranasal
Preparation of dosage form:	The inoculum was sent in a pre-diluted frozen form in a 50 ml vial on dry ice and was stored at –70 °C before inoculation. Immediately before inoculation of Group 2 heifers, the material was thawed avoiding local temperatures above 37 °C. After no ice was visible in the fluid, material was gently stirred and immediately used for inoculation of the animals.
Unused vaccine:	The volume of the unused material was measured and split on two aliquots before immediate freezing in dry ice or liquid nitrogen and stored for re-titration purposes. Virus and contaminated plastic or glassware was incubated with an appropriate volume of an 8 – 10 % formaldehyde solution for at least 24 hours at room temperature before discarding in order to inactivate viruses.

Pregnancy Control

Pregnancy was confirmed immediately before inoculation.

Inoculation of Heifers

The inoculation is Day 0 of the experiment.

In each nostril, 3 ml of the test material was administered intranasally by syringe without needle. Each time a new sterile syringe was taken. Administration was performed during the aspiration phase in order to minimize loss of fluid via expiration of material.

Post-Inoculation Observations

Collection and examination of blood samples

Blood was collected following standard, aseptic procedures (disinfecting the bleeding site). A new sterile syringe and needle was used for each animal.

Blood collection to prepare serum

At least 10 ml blood was collected from the heifers immediately before inoculation, then weekly after infection and at the termination of the study. Serum was stored at –20 °C until required.

Blood collection for leukocyte counts and buffy coat preparations

For leukocyte counting, 3 ml blood was transferred immediately after collection to suitable sterile vessels (Venoject, Terumo Europe N.V., Leuven, Belgium), pre-filled with 0.06 ml EDTA (0.235 MOL/L).

For buffy coat preparations, at least 15 ml blood was transferred immediately after collection to suitable sterile vessels, pre-filled with 0.1 ml Heparin solution (Na-heparin for inj., 5 000 IU/ml lot. A7B163A exp. date: 11/2000: Gedeon Richter RT, Budapest, Hungary) yielding at least 20 IU Heparin per ml blood in the blood sample. The content was carefully mixed thereafter.

For preparation of buffy coats and leukocyte counting, blood was collected from the heifers

- on every day, between Day 0 and Day 14 after infection;
- on every second day, between Day 15 and Day 40, or until all animals were negative for virus isolation for three consecutive sampling time points.

Preparation of serum

Blood was allowed to clot at room temperature, and separated by centrifugation. Each serum sample was divided into two aliquots of at least 2 ml each. One set of aliquots was assayed for BVDV specific antibodies by ELISA. The rest of the sera was frozen and stored at –20 °C until required.

Leukocyte counts

Leukocyte counts was determined with a coulter-counter semi-automated electronic device (Diatron Minicell-16, Messtechnik GmbH, Wien, Austria) with a claimed accuracy of 0.1 x 10^9 / l, 100 / µl. The instrument was used (calibration and leukocyte-counts) according to the manufacturer's recommendations.

Preparation of buffy coats

Heparin blood samples was transported to the laboratory as soon as possible. Buffy coat preparation procedure, following a standard laboratory procedure was performed under aseptic conditions (sterile pipettes, handling, clean bench etc.).

The obtained buffy coats were re-suspended in a small volume (2 ml) of RPMI 1640 and frozen at –70 °C in two aliquots of 0,5 ml. The residual 1ml buffy coats was immediately used for determination of blood cell associated BVDV by co-cultivation in a permissive cell culture.

BVD serum antibody ELISA-test

Each serum sample was tested for the presence of BVDV-antibodies using a suitable and validated ELISA test (Svanovir™ BVDV antibody test Cat# 10-2200-10). Test was validated and performed according to the manufacturer's recommendations. Positive samples were diluted according to the $\log_2$ scale to determine BVDV antibody titers.

BVD antigen assay(s)

Each buffy coat sample was assayed for the presence of BVDV by co-cultivation of the freshly prepared buffy-coats with susceptible cells or a cell-line. No freezing was allowed before co-cultivation.

Plasma was collected and provided to Man-Gene from each sample.

Clinical observations

Observation of heifers

Animals were examined daily from Day 0 - 42 post inoculation for the presence of clinical symptoms by a sufficiently trained veterinarian.

All clinical signs were recorded and described by its nature, consistence/touch, severity (mild, medium or severe) location, size of the area affected, and they will be scored according to agreed and standard definitions. Special attention was paid to respiratory signs (respiration, its rate; nasal or ocular discharge; conjunctivitis, sneezing, coughing, etc.) and diarrhea.

Rectal temperatures

Rectal temperatures were measured daily in each heifer, at the same hour of the day (preferably in the morning) from 5 days prior to the inoculation till 21 days post infection.

Daily measurement of rectal temperature was continued until each animal had rectal temperatures below or equal to 39 °C for at least 3 consecutive days.

Detection of interrupted pregnancy

Pregnancy was confirmed and suspicion for abortion or resorption of the fetus was established by rectal examination. A trained veterinarian examined all animals at inoculation, 1 and 2 months post-inoculation. The examination was carried out according to the generally accepted veterinary practice.

Heifers were examined daily for any sign of abortion until termination of the study (8 - 12 weeks post-challenge).

Termination of the Study

The study was terminated by slaughtering the heifers and extracting the fetuses. Fetuses and fetal material were transferred into closed transport containers marked with the number of the cow and the date/time. Containers were transported to a selected necropsy room.

Necropsy of the heifers was not required. Necropsy will be performed on fetuses, findings recorded and a panel of samples collected as described below.

Post-Mortem Examination

A detailed necropsy of the experimental animals was done in each case of death.

Post-mortem examinations were carried out by an experienced veterinary surgeon and the data were recorded on appropriate data sheets. Further laboratory tests were performed according to the clinical signs and lesions observed. If the diagnosis of the necropsy referred to a disease caused by microbial agent the diagnosis was verified by an appropriate test, specific for the agent.

Each tissue sample was collected in at least 2 separate, labeled containers and snap-frozen in liquid nitrogen. Samples were stored at –70 °C until required.

Aborted fetuses and study termination

At least the following tissue samples were collected from the fetuses:

- exudate from the peritoneal cavity or thorax, if present,
- mesenteric lymph nodes,
- spleen,
- thymus,
- cerebellum,
- kidney,
- bone marrow from the sternum,
- sample from the placenta, if available.

Dead or sacrificed heifers

At least the following tissue samples were collected:

- blood for buffy coat, if available,
- blood for serum, if available,
- Peyer's patches,
- mesenteric lymph nodes,
- spleen,
- kidney,
- uterus, including a sample from the placenta, if available.

Storage and Transport of Samples

Samples:	Storage:
Serum	–20 °C
Buffy coat	–70 °C
Virus	–70 °C
Tissue from heifers	–70 °C
Tissue from fetuses	–70 °C

Samples were sent for laboratory analysis as required by the sponsor. The choice of samples and the timing of transport were agreed with the study monitor or the project manager. As a matter of

general principle, samples coming from aborted material or from new-born calves were investigated as soon as possible.

RESULTS

Mortality

Heifer No. 626 (Group 1) died on Day 13 PI (post inoculation). The following table summarises the observed clinical signs and lesions revealed during necropsy:

Heifer	In-life observations	Post mortem findings
No. 626	• signs of disease from 7 DPI • lachrymation, nasal discharge on 7 - 12 DPI • loss of appetite from 8 - 12 DPI • diarrhoea on 11 - 12 DPI • elevated respiratory rate on 9 - 10 and 12 DPI • coughing on 9 DPI • abnormal breathing on 12 DPI	• dehydration • haemorrhages on the serous membranes • hyperaemia of the Peyer's patches • oedema of the lung

These clinical and gross-pathological findings are consistent with BVDV induced lesions, therefore it may be concluded that the reason of death was the BVDV infection.

Abortions after Infection

One heifer had clinical abortion in each group. Heifer No. 615 (Group 1) aborted on Day 38 PI, Heifer No. 469 (Group 2) aborted on Day 39 PI. Both foetuses showed the signs of autolysis, and they were estimated to die at least 3 - 7 days before the abortion (around 32 - 35 DPI).

In Group 1, no foetus was found in Heifer No. 526 during the slaughter examination at termination. Gross-pathology of the uterus revealed the followings:

- the right uterine horn was slightly enlarged,
- the remains of placenta with progressed autolysis was retained in the lumen.

The findings on the uterus of Heifer No. 526 is consistent with a "silent" abortion, most likely due to the BVD infection.

Clinical Observation of Heifers

A summary of the clinical observation data and duration of clinical signs in the groups are presented below.

Clinical signs and the days post inoculation (DPI) when they were observed

Group 1 (XIKE-A)

Clinical sign	Animal ID				
	526	598	615	618	626*
	DPI				
Loss of appetite	8 - 13	8 - 18	8 - 18	8 - 16	8 - 12
Lachrymation	7 - 10	7- 8, 10-12	8 - 10	8 - 10	7 - 8
Conjunctivitis	9 - 10	9 - 12	9 - 11	9 - 11	-
Nasal discharge	7 - 13	7-9, 11-12	8 -13	8 - 12	7 - 12
Oral erosion	-	-	-	-	-
Oral haemorrhage	-	-	-	-	-
Diarrhoea	-	11 - 16	10 - 15	11 - 15	11 - 12
Coughing	9	10, 15	9	8 - 9, 13	9
Abnormal breathing	-	11 - 14	12 - 14	12 - 14	12
Elevated respiratory rate	-	10 - 13	9 - 13	8 - 13	9 - 10, 12
Hoof erosion	-	-	-	-	-

*Heifer No. 626 died on Day 13 PI

Group 2 (XIKE-B)

Clinical sign	Animal ID				
	469	588	565	608	619
Loss of appetite	-	-	-	-	-
Lachrymation	-	-	-	-	-
Conjunctivitis	-	-	-	-	-
Nasal discharge	-	-	-	-	-
Oral erosion	-	-	-	-	-
Oral haemorrhage	-	-	-	-	-
Diarrhoea	-	-	-	-	-
Coughing	-	-	-	-	-
Abnormal breathing	-	-	-	-	-
Elevated respiratory rate	-	-	-	-	-
Hoof erosion	-	-	-	-	-

All Group 1 animals infected with XIKE-A exhibited a broad spectrum of clinical signs. Respiratory signs appeared first accompanied by loss of appetite, and a few days later heifers developed diarrhoea with the exception of Heifer No. 526. One heifer died and another one aborted (see before) after infection. All these signs are consistent with the symptoms expected after infection with a virulent BVDV strain.

All Group 2 animals infected with XIKE-B were free of clinical signs. At the same time, one heifer had abortion during the observation period.

Rectal Temperatures

No abnormal temperature changes were detected before the infection of the animals.

In Group 2, all temperature values remained within the physiological range from Day 0 to Day 21 after infection.

All Group 1 animals showed elevated rectal temperature after infection that were detected between Days 7 - 11 PI.

Findings at Study Termination

At study termination, foetuses were examined at slaughter.

No foetus was recovered from Heifer No. 526 (see section 10.2 "Abortions after Infection").

The following findings were observed at the necropsy of the foetuses:

Animal No.	Findings	Conclusion
Group 1		
598	Ascites, general oedema, autolysis	Died at least 2 weeks earlier
618	Ascites, general oedema, autolysis	Died at least 3 weeks earlier
Group 2		
565	Ascites, general oedema, liver degeneration	Foetus considered non-viable
588	Normal	-
608	Normal, perirenal oedema	-
619	General autolysis	Died 3 - 6 weeks earlier

The findings suggest that 2 Group 1 animals (Heifers No. 598 and No. 618) and one Group 2 animal (Heifer No. 619) died several weeks before extraction, and so they can be considered abortions.

Abortions Modified by Post-Mortem Findings

After the post-mortem examination it was not clear why some of the heifers had not had abortion. Dead foetuses should be considered as abortions, therefore the clinical picture was modified after the termination of the study as follows:

Group 1

Animal No.	Conclusion
526	BVD abortion (uterus with placenta post-mortem)
598	BVD abortion (foetus post-mortem)
615	Clinical BVD abortion
618	BVD abortion (foetus post-mortem)
626	Died due to BVD

Group 2

Animal No.	Conclusion
469	Clinical BVD abortion
565	Expected BVD abortion; non-viable foetus
588	Normal
608	Normal
619	BVD abortion (foetus post-mortem)

Examination of Blood Samples

Leukocyte count

WBC counting was interrupted on Day 26 PI, as all animals became negative for virus isolation for this time point.

0 DPI values were considered as individual baseline for comparison.

In Group 2, the leukocyte counts never went to 40 % or more below the baseline value until the end of the observation period (26 DPI).

In Group 1, one animal (Heifer No. 598) had WBC count below the 40 % baseline for one day.

Serology

None of the selected animals had BVDV specific antibody in their sera before the infection.

After infection, all surviving Group 1 heifers developed BVDV specific antibodies detected from 3 weeks PI and lasted until the end of the observation period in all study animals. In Group 2, 4 out of the 5 heifers had BVDV specific antibodies detected from 4 weeks PI. Measurable antibody response lasted only in 3 animals until the end of the observation period. Titres were lower in Group 2 than in Group 1.

Virus Detection by Co-Cultivation

Buffy coats

BVDV was detected in both groups. The duration of virus detection is summarised below. All samples were co-cultivated immediately after collection, i. e. without freezing.

Animal No.	DPI when BVDV was detected
Group 1	
615	5 - 12
526	5 - 9
626	5 - 12
618	5 - 11, 14
598	5 - 11, 13
Group 2	
565	7 - 9
588	8
608	6 - 9
469	8
619	5 - 11

Tissue samples

The presence of BVD virus in the dead heifer and the foetuses is summarised below:

Heifer:

Animal No.		BVDV in tissue samples
Group 1		
626		Present[#]

Foetuses:

Animal No.	BVDV in tissue samples
Group 1	
615	Not present[#]
526	NT
626	Present[#]
618	Not present
598	Present

Group 2	
565	Present
588	Not present
608	Present
469	Not present#
619	Not present

NT = Not tested

Samples were co-cultivated immediately after collection (i. e. without freezing), except "#" marked ones, from which only frozen samples were available.

Summary of BVD related clinical and laboratory data

Group 1

Animal No.	Conclusion	BVD
526	BVD abortion (uterus with placenta post-mortem)	NT (no sample found)
598	BVD abortion (foetus post-mortem)	+ (foetus)*
615	Clinical BVD abortion	- (foetus)*
618	BVD abortion (foetus post-mortem)	- (foetus)*
626	Died due to BVD	+ (foetus) / + (heifer)

NT: not tested

Group 2

Animal No.	Conclusion	BVD
469	Clinical BVD abortion	- (foetus)*
565	Expected BVD abortion; non-viable foetus	+ (foetus)
588	Normal	- (foetus)
608	Normal	+ (foetus)
619	BVD abortion (foetus post-mortem)	- (foetus)*

* Foetuses were autolysed at the time of sampling

Conclusion:

The study aimed to assess the safety of XIKE-A and XIKE-B in pregnant animals.

Ten pregnant heifers were selected from a BVDV negative herd. Two groups of 5 heifers were included in the trial: one was inoculated with XIKE-A the other with XIKE-B virus strain. Heifers were between days 60 and 90 of gestation on the day of inoculation.

Heifers were monitored for the presence of clinical signs of BVDV infection including abortions during the observation period. Blood samples were collected from the animals for serology, antigen detection and white blood cells were counted. The experiment was terminated 9 weeks after infection. Non-aborted cows were slaughtered, the uterus examined and collected. Foetal organ samples were collected during routine necropsy and examined for BVDV infection.

The presence of fetal infection was the main evaluation parameter, composed from the number of BVDV-related cow mortality, the number of BVDV-related abortions and the number of BVD positive fetuses at termination. In addition to the main parameter, clinical signs characteristic for BVDV infection, viraemia and white blood cell count in cows and rectal temperature after challenge were evaluated.

The XIKE-B virus proved to be less pathogenic than XIKE-A, nevertheless BVD related abortion and infection of the foetus was observed in the XIKE-B group, too. Therfore it can be concluded that the inactivation of the E^{rns} RNase does not prevent fetal infection.

EXAMPLE 2

BVDV XIKE-A-NdN: foetopathogenicity assessment in pregnant heifers

The N^{pro} gene has been shown to be nonessential for growth of CSFV in tissue culture (Tratschin et al., 1998). Even though a proof for BVDV attenuation in consequence of N^{pro} deletion is still missing, a role of this protein in the interaction between virus and host seemed to be possible and was actually indicated by recent experiments for CSFV (Mayer et al., 2004; Rüggli et al., 2003). We therefore wanted to investigate, whether the deletion of the major part of the N^{pro} coding sequence leads to a virus that no longer infects the fetus in pregnant heifers. The N^{pro} gene except for the 5' terminal 4 codons was deleted from the full length cDNA clone pKANE40A according to standard procedures. The resulting mutant full length clone was used as template for in vitro transcription and the resulting cRNA was transfected into MDBK cells as described (Meyer et al., 2002). The recovered virus was amplified in tissue culture and then used in the animal experiment described below. BVDV XIKE-B served as a control since it was shown before that it is able to cross the placenta (EXAMPLE 1).

OBJECTIVE(S)/PURPOSE OF THE STUDY

The study aims to assess the safety of a live attenuated BVDV with a genomic deletion of most of the N^{pro} coding region in pregnant animals.

Material and Methods applied are described in Example 1

STUDY DESIGN

Eight pregnant heifers were assigned at random to two groups. They were treated and observed according to the following schedule:

	Group1	Group 2
N	5	3
Treatments	XIKE-A-NdN	XIKE-B / control
Route	Intramuscular	
Vaccination time	between days 60 and 90 of pregnancy (day 0 of the study)	
Observations Post-vaccination (in life)	• Clinical signs • Serum at days 0, 14, 28, 42 and at termination • WBC at day 0 and then daily for 14 days • Buffy coat at day 0 and then daily for 14 days	
Post-mortem (day 60)	• Gross-pathology • Organ panel for virus isolation	

Type of study:	open controlled clinical study
Experimental unit:	Individual animal
Method of blinding:	Partial blinding. No detailed procedures for blinding and access to treatment schedule were applied. The observing veterinarian at the study location and the pathologist were not be aware of the treatment; they only received a protocol extract relevant to their tasks. Vaccination was performed by the investigator or his assignee. Samples for virus isolation were coded by the investigator until all results are available.

RESULTS

All heifers were healthy and pregnant at study start. All animals proved to be free of BVDV and BVDV antibodies before the initiation of the.

Preparation and Control of the Virus used for the Infection

Samples were collected throughout the dilution steps and assayed on the day of preparation, i.e. without freezing by co-cultivation on suitable tissue culture. The results of virus titration are shown in the following table.

Sample ID	Virus strain	Dilution / description	Log_{10} titre/ml
VT1a	XIKE-A/NdN (S)	1:2 (at 4 °C)	4.4
VT1b		#2a on ice without opening	4.0
VT1c		Return of #2b	2.8
VT2a	XIKE-B	1:2.2 (at 4 °C)	2.3
VT2b		#3a on ice without opening	2.8
VT2c		Return of #3b	Negative

Clinical Symptoms of BVDV Infection

The table below gives a summary about the animals that had clinical signs during the observation period.

Clinical signs and the days post inoculation (DPI) when they were observed

Group 1 (XIKE-A NdN) Group 2 (XIKE-B)

Clinical sign	Animal ID	Animal ID	
	1583	1438	1585
Loss of appetite	8	-	10
Lachrymation	-	-	-
Conjunctivitis	-	-	-
Nasal discharge	-	-	-
Oral erosion	-	-	-
Oral haemorrhage	-	-	-
Diarrhoea	-	-	-
Coughing	-	12	10-13
Abnormal breathing	-	-	-
Elevated respiratory rate	-	-	-
Hoof erosion	-	-	-

Only mild and transient clinical signs were observed in some of the animals in each group. In Group 1, one out of the 5 heifers had loss of appetite on day 8 PI. In Group 2, two out of the 3 animals had clinical signs. Both heifers experienced coughing around day 21 PI that was accompanied with loss of appetite in one of the animals.

Rectal Temperatures

No abnormal temperature changes were detected before the inoculation of the animals. The few

cases of elevated temperatures measured after the inoculation are summarised in the table below.

Group	Animal ID	Temperature (°C)	PI day
1	1583	39.9	8
	1621	39.0	5
2	1438	39.0	2
	1585	40.8	9

One animal had slightly elevated temperature in each group, and also one animal had fever in each group. Fever was detected on day 8 or 9 PI. Temperature values always returned to normal value on the following day.

Leukocyte Counts

Some leukopenia was observed in all groups between PI days 3 – 8. The number of animals with at least 40 % reduction in white blood cell count was the following:

Group	Number of animals having leukopenia / total
1	3/5 (60%)
2	1/3 (33%)

Serology (BVDV antibodies)

In compliance with the study protocol, all heifers were free of BVDV antibodies before vaccination. In Group 1 (inoculated with XIKE-A NdN) and Group 2 (inoculated with XIKE-B), complete seroconversion was detected only at study termination (2 months after inoculation).

BVD virus isolation from buffy coats

No viremia was detected

BVD virus isolation from foetal tissue samples

	Group 1	Group 2
N	5	3
Treatments	XIKE-A-NdN	XIKE-B / control
Route	Intramuscular	Intramuscular
Number of fetuses in which fetal transmission was detected:	4 out of 5 foetuses infected	2 out of 3 foetuses infected

Conclusion of the virus used for treatment has the potential to be transmitted over the placenta:	Fetal transmission for XIKE-A-NdN observed	Fetal transmission for XIKE – B observed

Conclusion

The N^{pro} deletion resulted in a considerable attenuation of the BVDV in comparison to the parental virus XIKE-A that was shown to be highly pathogenic (Meyer et al., 2002). However, the N^{pro} deletion alone is not preventing transmission of a NY93 based virus recombinant to the foetus after inoculation of pregnant cows.

EXAMPLE 3

BVDV XIKE-B-NdN: foetopathogenicity assessment in pregnant heifers

To be able to test the potential of a combination of RNase inactivation and N^{pro} deletion with regard to BVDV attenuation and fetal transmission, different BVDV-2 mutants with deletions within the N^{pro} coding region were established based on the infectious cDNA clone pKANE40B, the RNase negative mutant of pKANE40A with a deletion of codon 349. The recovered viruses were analyzed with regard to presence of the desired mutations, the absence of second site mutations in the regions flanking the introduced changes and their growth characteristics in tissue culture. XIKE-B-NdN (V-pK88C), a variant containing a deletion of the complete N^{pro} coding region except for codons 1 to 4 in addition to the RNase inactivating deletion of codon 349 was chosen for an animal experiment since it combined the desired mutations with acceptable growth characteristics. The aim of the study was to assess the safety of a live attenuated BVDV isolate in pregnant animals.

Five BVDV-negative, pregnant heifers were inoculated intranasally with an infective dose of 10^5 TCID50/animal XIKE-B-NdN (back titration data are depicted in Table 3.1). Clinical data were recorded daily. Blood samples were collected for white blood cell counting, for buffy-coat preparation and serology. After termination of the study foetal tissues were collected for virus isolation.

MATERIAL AND METHODS:

As detailed for example 1:

RESULTS

No clinical data were observed (data not shown). Leukocyte counts remained virtually unchanged except for a significant decrease by approx. 40% below the baseline value (day0) in heifer no- 1015 on a single day (day 6 p.i.) (data not shown).

Analysis of buffy coat preparations:

Approximately 10^6 leukocytes were cultured in duplicates with MDBK-cells in 24-well tissue culture plates for 5 days. Samples were freeze-thawed twice. One hundred microliter aliquots of thawed samples were inoculated onto freshly seeded 24-well tissue culture plates and tested for virus by indirect immuno-fluorescence staining (mAb Code 4, directed against a conserved epitope in nonstructural protein NS3). No BVDV could be isolated from the buffy coat preparations of animals # 921, 1013, 1015, 1055 and 1075 (Table 3.2) whereas positive controls clearly showed the correct conduction of the test.

b) Post-mortem examination of foetal tissues

After termination of the study the following foetal tissues were collected for virus isolation: spleen, kidney, thymus, sternum, cerebellum, placenta, intestine and abdominal fluid. Briefly, tissue suspensions were made in a mortar using sterile sea sand and ice-cold PBS without Ca^{2+} and Mg^{2+}. Mortars were rinsed with 1 ml ice-cold PBS without Ca^{2+} and Mg^{2+} and suspensions were centrifuged for 10 min. at 2000 x g (4°C). The supernatant was first passed through a disposable 0.45 μm filter holder, followed by a second filter passage (0.2 μm pore size). Virus isolation was carried out in duplicates (400 μl foetal tissue suspension or 100 μl foetal abdominal fluid) on a monolayer of MDBK-cells in a 24 wells tissue culture plate (37°C, 7% CO2). Tissue samples were controlled daily for cytopathic effects or bacterial contamination, and after an incubation time of 5 days plates were frozen and thawed twice. 100 μl of samples were passaged to freshly seeded MDBK-cells. Virus was detected by indirect immuno-fluorescence staining (mAb Code 4). No BVDV could be detected in the tissue samples or foetal abdominal fluid (Table 3.3).

c) Serological Findings

Serum neutralization titres were determined before inoculation, 1 month post-inoculation and at termination of the study. Sera from all animals were tested in triplicates for neutralizing antibodies against NY93/C, and the endpoint dilution was read by indirect immunofluorescence staining. Results were expressed as the endpoint dilution, which neutralized approximately 100

$TCID_{50}$ and calculated by the method of Kaerber. No definite data could be obtained for day 0, and 1 and 2 weeks post infection as the sera were toxic for MBDK-cells in dilutions up to 1:16 and no neutralization could be detected at higher dilutions. Starting with the third week post vaccination all animals developed neutralising antibodies against the homologous BVDV-2 virus NY'93/C lasting till the end of the experiment (Table 3.4 and Fig. 1).

d) Conclusions

The data obtained during the animal study clearly show that BVDV XIKE-B-NdN represents a highly attenuated virus. In contrast to wild type virus or the single mutants XIKE-B or XIKE-A-NdN that show foetal transmission in pregnant heifers at high rates the double mutant did not cross the placenta. BVDV XIKE-B-NdN as well as similar double mutants are extremely suitable for the use in a live attenuated vaccine.

Study No.: B01 BIVI020 and B01 BIVI022

Table 3.1: Back Titration of Viruses

Sample ID	Virus Strain	Dilution	Titre
1a	XIKE-B-NdN	concentrated virus	$10^{5,44}$ $TCID_{50}$/ ml
1		1:4	$10^{4,86}$ $TCID_{50}$/ ml
6		residues of infection (#1)	$10^{4,27}$ $TCID_{50}$/ ml

Table 3.2
Detection of Viremia

Animal ID No.	*Days after vaccination*															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
0921	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1. isolation
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2. isolation
1013	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1. isolation
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2. isolation
1015	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1. isolation
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2. isolation
1055	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1. isolation
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2. isolation
1075	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1. isolation
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2. isolation
Date	13.12 2001	14.12 2001	15.12 2001	16.12 2001	17.12 2001	18.12 2001	19.12 2001	20.12 2001	21.12 2001	22.12 2001	23.12 2001	24.12 2001	25.12 2001	26.12 2001	27.12 2001	

- sample negative

Table 3.3
Analysis of foetus tissue samples for the presence of BVDV

Animal No.	Abdominal fluid	Thoracic Fluid	Mesenteric lymph nodes	Spleen	Kidney	Thymus	Bone marrow (sternum)	Cerebellum	Placenta	Intestine	Date of tissue collection	Isolation
0921	-- --	NC	NC	-- --	-- --	-- --	-- --	-- --	-- --	-- --	12.02.02	1. isolation 2. isolation
1013	-- --	NC	NC	-- --	-- --	-- --	-- --	-- --	-- --	-- --	12.02.02	1. isolation 2. isolation
1015	-- --	NC	NC	-- --	-- --	-- --	-- --	-- --	-- --	-- --	12.02.02	1. isolation 2. isolation
1055	-- --	NC	NC	-- --	-- --	-- --	-- --	-- --	-- --	-- --	12.02.02	1. isolation 2. isolation
1075	-- --	NC	NC	-- --	-- --	-- --	-- --	-- --	-- --	-- --	12.02.02	1. isolation 2. isolation

NC = Not collected

- = sample negative

Table 3.4: B01 BIVI022 / BVDV XIKE-B-NdN; foetal protection study
Serum Neutralization Assay

Animal ID No.	From the heifers									
	at selection	during ACC	1 wPV	2 wPV	3 wPV	4 wPV	5 wPV	6 wPV	7 wPV	8 wPV
0921		*	*	*	1:40[(2)]	1:161[(1)]	1:256[(1)]	1:323[(1)]	1:128[(1)]	1:256[(1)]
1013		*	*	*	1:3[(2)]	NA	1:161[(1)]	1:323[(1)]	1:406[(1)]	1:256[(1)]
1015		*	*	*	1:64[(2)]	1:161[(1)]	1:256[(1)]	1:323[(1)]	1:406[(1)]	1:323[(1)]
1055		*	*	*	1:32[(2)]	1:40[(2)]	1:256[(1)]	1:323[(1)]	1:406[(1)]	1:406[(1)]
1075		*	*	*	NA	1:128[(2)]	1:102[(1)]	1:203[(1)]	1:161[(1)]	1:406[(1)]
Date		06.12.2001	20.12.2001	27.12.2001	03.01.2002	10.01.2002	17.01.2002	24.01.2002	31.01.2002	07.02.2002

(1) SNT against 1456 Nase (= NY93/C) $10^{2,03}$ $TCID_{50}$/ 50µl * Serum toxic for MBDK-cells in dilutions up to 1:16 ⇒ no data available

(2) SNT against 1456 Nase (= NY93/C) $10^{1,57}$ $TCID_{50}$/ 50µl NA data not available

The Serum Neutralisation Assay against NY93/C is illustrated in Fig. 1.

Efficacy and crossprotection study

Two possible problems have to be faced with regard to vaccination with attenuated virus mutants BVDV XIKE-B or BVDV XIKE-B-NdN. First, there is a general problem concerning cross protection between BVDV-1 and BVDV-2. At least vaccination with inactivated BVDV-1 vaccines did not prevent the transmission of BVDV-2 to the foetus in pregnant animals. Since protection against foetal infection represents the major aim of anti BVDV vaccination, such vaccines cannot be regarded to induce a protective immunity on a broad range. The question therefore was, whether vaccination with live attenuated BVDV-2 can prevent virus transmission to the foetus. Second, the reduced growth rates of BVDV XIKE-B-NdN might result in only a low level of protection not able to prevent transplacental infection of the foetus in pregnant heifers. To address these problems, an animal study was started. The animals (2 groups of 10 animals each) were vaccinated either with BVDV XIKE-B or XIKE-B-NdN (intended dosage: 1ml of supernatant with 10^5 $TCID_{50}$ of virus; backtitration is shown in Tab. 3.5). None of the animals showed significant clinical signs after the vaccination except for one animal of the nonvaccinated control group with mild coughing for one day. Rectal temperature values were below 39 °C except for one animal of the nonvaccinated control group that hat 39.1°C for one day. Buffy coat samples prepared after vaccination were analysed for the presence of virus as described above. The experiments showed that only 5 of the 20 animals contained virus in the blood for 1 or 2 days at 4 to 8 days post infection (Tab. 3.6).

Table 3.5: Back Titration of Viruses used for vaccination

Sample ID	Virus Strain	Dilution	Titre
1a	XIKE-B-NdN	concentrated virus	$10^{5,44}$ $TCID_{50}$/ ml
1		1:4	$10^{4,86}$ $TCID_{50}$/ ml
6		residues of infection (#1)	$10^{4,27}$ $TCID_{50}$/ ml
3	XIKE-B	1:11	$10^{5,76}$ $TCID_{50}$/ ml
4		1:110	$10^{4,92}$ $TCID_{50}$/ ml
5		residues of infection (#4)	$10^{4,27}$ $TCID_{50}$/ ml

Study No. / Id.: B01 BIVI020 / BVDV Tü XIKE-B/XIKE-B-NdN; foetal protection study
Table 3.6: Inoculation with white blood cell (buffy coat) preparations collected after vaccination

Animal ID No.	*Days after vaccination*															
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
1134(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1141(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1142(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	+Ø	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1145(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1149(1)	ØØ	ØØ	ØØ	ØØ	+Ø	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1151(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1152(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1156(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1158(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1160(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
Date	13.12 2001	14.12 2001	15.12 2001	16.12 2001	17.12 2001	18.12 2001	19.12 2001	20.12 2001	21.12 2001	22.12 2001	23.12 2001	24.12 2001	25.12 2001	26.12 2001	27.12 2001	

Animal ID No.	*Days after vaccination*															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1197(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1200(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1206(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1210(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1212(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1214(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1216(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1217(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1218(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1225(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
Date	13.12	14.12	15.12	16.12	17.12	18.12	19.12	20.12	21.12	22.12	23.12	24.12	25.12	26.12	27.12	
	2001	2001	2001	2001	2001	2001	2001	2001	2001	2001	2001	2001	2001	2001	2001	

Immunofluorescence staining: Code 4

- □ sample negative
- \+ sample positive
- B bacterial contamination in well

Code of animal numbers:

(1) vaccination with BVDV XIKE-B (RNase mutant)

(2) vaccination with BVDV XIKE-B-NdN (RNase and N^{pro} double mutant)

Four weeks after vaccination, insemination of the animals was carried out. Challenge infections were performed 60 to 90 days later using either a BVDV-1 strain (BVDV KE-9, heterologous challenge, animals vaccinated with XIKE-B) or a heterologous BVDV-2 strain (BVDV KE-13, homologous challenge, animals vaccinated with XIKE-B-NdN) (intended dosage: 10^5 TCID50 in 6ml; backtitration is shown in Tab. 3.7). From each group of vaccinated animals 5 pregnant heifers were randomly selected for the challenge infection. Animals vaccinated with BVDV XIKE-B were challenged with the BVDV-1 strain KE-9, whereas heifers vaccinated with BVDV XIKE-B/NdN were challenged with BVDV-2 KE-13. In addition, 2 nonvaccinated control animals were infected with each of the challenge viruses.

Study No. / Id.: B01 BIVI020 / BVDV Tü XIKE-B-NdN; foetal protection study

Table 3.7: Back titration of challenge viruses

Virus Strain	Sample ID	Titre ($TCID_{50}$ / ml)	Mean Titre ($TCID_{50}$ / ml)
KE 9	1	$10^{4,44}$	$10^{4,94}$
		$10^{5,10}$	
	2	$10^{4,69}$*	$10^{4,44}$
	3	**	
KE13	1	$10^{4,69}$	$10^{4,76}$
		$10^{4,82}$	
	2	$10^{4,57}$	$10^{4,63}$
		$10^{4,69}$	
	3***	$10^{3,5}$	$10^{3,5}$

Sample 1: stock of inoculate
Sample2: stock of inoculate returned from the stable
Sample3: excess inoculate
* Second inoculation of KE9, sample 2 wasn't interpretable because of cell death.
** KE9, sample 3 wasn't interpretable because of cell death or bacterial contamination.
*** First inoculation of KE13, sample 3 wasn't interpretable because of bacterial contamination.

The vaccinated animals did not show viremia or clinical symptoms upon challenge infection. The challenge was successful as all non-vaccinated controls were BVDV positive (Tab. 3.8). Only mild signs of disease were observed in the control groups. The white blood cell counts were nearly normal (not shown).

Study No. / Id.: B01 BIVI020 / BVDV Tü XIKE-B/XIKE-B-NdN; foetal protection study
Table 3.8: Inoculation with white blood cell (buffy coat) preparations collected after challenge

Animal ID No.	*Days after challenge*																
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	Isolation
1104(3)	ØØ	ØØ	ØØ	Ø+	++	++	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	+Ø	++	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1108(3)	ØØ	ØØ	ØØ	ØØ	++	+Ø	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ	ØØ	ØØ	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	2. isolation
1126(3)	ØØ	ØØ	++	+Ø	++	Ø+	ØØ	Ø+	Ø+	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
	ØØ							ØØ									2. isolation
1145(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ		ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
							ØØ										2. isolation
1151(2)	ØØ	ØØ	ØØ	ØØ		ØØ	ØØ	ØØ		ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
								ØØ	ØØ								2. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ					3. isolation
1152(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ		1. isolation
																ØØ	2. isolation
														ØØ	ØØ	ØØ	3. isolation
1156(2)	ØØ	ØØ	ØØ	ØØ	ØØ		ØØ	ØØ			ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation
									ØØ								2. isolation
	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ					3. isolation
Date	04.22	04.25	04.27	04.29	05.01	05.03	05.05	05.07	05.09	05.11	05.13	05.15	05.17	05.19	05.21	05.23	
	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	

Animal ID No.	Days after challenge																
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	
1197(1)	ØØ ØØ	ØØ ØØ	ØØ ØØ	ØØ ØØ	 ØØ	ØØ ØØ	ØØ ØØ	ØØ ØØ	ØØ ØØ	ØØ ØØ	ØØ ØØ	ØØ ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation 2. isolation 3. isolation
1200(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation 2. isolation
1214(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ ØØ	ØØ	1. isolation 2. isolation 3. isolation
1216(2)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ ØØ	ØØ	ØØ	ØØ ØØ ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation 2. isolation 3. isolation
1217(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ ØØ	ØØ	1. isolation 2. isolation 3. isolation
1218(1)	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	1. isolation 2. isolation
1249(3)	ØØ	ØØ	ØØ	ØØ	++ ++	ØØ +Ø	ØØ ØØ	ØØ ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ ØØ	ØØ	1. isolation 2. isolation
Date	04.22 2002	04.25 2002	04.27 2002	04.29 2002	05.01 2002	05.03 2002	05.05 2002	05.07 2002	05.09 2002	05.11 2002	05.13 2002	05.15 2002	05.17 2002	05.19 2002	05.21 2002	05.23 2002	

Immunofluorescence staining: Code 4

□ sample negative
\+ sample positive
B bacterial contamination in well

Code of animal numbers:

(1) **vaccination with BVDV XIKE-B (RNase mutant)**
(2) **vaccination with BVDV XIKE-B-NdN (RNase and N^{pro} double mutant)**
(3) **nonvaccinated controls**

Serum neutralization titers were determined before inoculation, 1 month post-inoculation, before challenge, 1 month after challenge and at termination of the study. Sera from all animals were tested in triplicates for neutralizing antibodies against KE9 and NY93/C (1456Nase), and the endpoint dilution was read by indirect immunofluorescence staining. Results were expressed as the endpoint dilution, which neutralized approximately 100 $TCID_{50}$ and calculated by the method of Kaerber. At some of the higher antibody titres, the used endpoint dilution was not high enough. Against KE9, only animals vaccinated with XIKE-B developed low antibody titres starting about week 4. At challenge, all animals had antibody titres, which increased considerably starting around week 4 post challenge. XIKE-B vaccinated animals had higher antibody titres then those vaccinated with XIKE-B-NdN vaccinated. All animals developed about the same neutralization titre against NY93/C four weeks post vaccination, with marginally lower titres in XIKE-B-NdN vaccinated animals. After challenge all animals had high antibody titres. Fig. 2 shows the serum neutralization assay against KE9 (BVDV-1) and Fig. 3 shows the serum neutralization assay against NY93/C (BVDV-2).

Analysis of tissue samples obtained after termination of the study from the foetuses revealed that the material obtained from the vaccinated animals gave negative results whereas transmission had occurred in all 4 control animals (Tab. 3.9). Thus, it is clear that the established BVDV-2 mutants are well suited as efficient cross protective vaccine viruses .

Study No. / Id.: B01 BIVI020 / BVDV Tü XIKE-B/XIKE-B-NdN; foetal protection study

Table 3.9: Analysis of foetus tissue samples for the presence of BVDV

Animal No.	Abdominal Fluid	Thoracic fluid	Mesenteric lymph nodes	Small intestine	Spleen	Thymus	Kidney	Bone marrow (sternum)	Cerebellum	Placenta	Date
1214(1)	NA	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	17.06.02
1126(3)	++	***	++	++	++	++	++	++	++	++	17.06.02
1249(3)	++	NA	++	++	++	++	++	++	++	Ø+	17.06.02
1218*(1)	NA	NA	NA	NA	NA	NA	NA	NA	NA	ØØ**	17.06.02
1197(1)	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	17.06.02
1217(1)	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	17.06.02
1200(1)	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	17.06.02

Animal No.	Abdominal Fluid	Thoracic fluid	Mesenteric lymph nodes	Small intestine	Spleen	Thymus	Kidney	Bone marrow (sternum)	Cerebellum	Placenta	Date
1145[(2)]	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	21.06.02
1108[(3)]	+Ø	NA	++	++	++	++	++	++	++	ØØ	21.06.02
1156[(2)]	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	21.06.02
1104[(3)]	++	NA	++	++	++	++	++	++	++	++	21.06.02
1216[(2)]	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	21.06.02
1151[(2)]	NA	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	21.06.02
1152[(2)]	ØØ	NA	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	ØØ	21.06.02

NA = not available
*No foetus was found in the uterus of heifer #1218
**Endometrium (also collected for histology)
*** Sample was not sent to BFA Tübingen

Code of animal numbers:

(1) **vaccination with BVDV XIKE-B (RNase mutant)**
(2) **vaccination with BVDV XIKE-B-NdN (RNase and N^{pro} double mutant)**
(3) **nonvaccinated controls**

Conclusion

The challenge was successful as all non-vaccinated controls were BVDV viraemic and foetuses of all non-vaccinated controls were BVDV positive.

Both isolates gave full protection under the present test and assay conditions. Isolate XIKE-B, with the single genetic marker was shown to cross-protect against type 1 BVDV challenge in terms of BVD viraemia and transmission to the foetus after challenge. Isolate XIKE-B-NdN with the double genetic marker was able to fully protect against a heterologue type 2 BVDV challenge strain in terms of BVD viraemia and transmission to the foetus after challenge.

1. Isolate XIKE-B (type 2 isolate) was shown to cross-protect against type 1 BVDV challenge in terms of BVD viraemia and transmission to the foetus after challenge under the present test and assay conditions (n=4).
2. Isolate XIKE-B-NdN (type 2 isolate) fully protected against a heterologues type 2 BVDV challenge strain in terms of BVD viraemia and transmission to the foetus after challenge under the present test and assay conditions (n=5).

EXAMPLE 4

Establishment of N^{pro} mutants

Further analyses of BVDV-2 mutants with N^{pro} deletions. Different mutants with deletions in the N^{pro}-coding region of the genome were established. Initially, only true deletions or a deletion accompanied by a point mutation were introduced.

A: $[N^{pro}]_1$-[C-term];

B: $[N^{pro}]_3$-[C-term];

C: $[N^{pro}]_4$-[C-term];

D: $[N^{pro}]_6$-[C-term];

E: $[N^{pro}]_4$-[C-term*]

In the formulas $[N^{pro}]_x$ represents the number of residues of the aminoterminus of N^{pro} that are left in the mutated polyprotein amino acids, [C-term] is the complete polyprotein except for N^{pro} (starting with the C protein and ending with NS5B), and [C-term*] is the same as [C-term] but with a mutation at position 2 of the C protein (N instead of D).

The growth rates of the recovered viruses were considerably lower than those of wild type XIKE-A or the RNase negative mutant XIKE-B. There are two possible explanations for this finding: (i) dependent on the virus strain, sequences of variable length of the N^{pro}-coding region are necessary for efficient translation initiation (Myers et al., 2001; Tautz et al., 1999) and (ii) the fusion of additional sequences to the aminoterminus of the capsid protein interferes with capsid protein function.

To obtain better growing N^{pro} deletion mutants, a second set of mutants was generated with either a bovine ubiquitin gene or a fragment of the bovine LC3-coding sequence replacing the major part of the N^{pro} gene. These constructs allow efficient translation and generate a capsid protein with the correct amino terminus.

$[N^{pro}]_{22}$-[PS]- [C-term]

wherein PS is ubiquitin or LC3, C-term is the complete polyprotein except for N^{pro} (starting with the C protein and ending with NS5B).

The growth rates of these mutants were more similar to what was determined for XIKE-A. It even seemed that the two RNase positive viruses according to the formula $[N^{pro}]_{22}$-[PS]- [C-term] named V-pK87F and V-pK87G showed no significant growth retardation at all, whereas the RNase negative counterpart V-pK88G once again was somewhat hampered in propagation but to a lesser extend than the formerly descirbed mutants.

Further examples of N^{pro} deletion mutants may be:

*ME*SDEGSK...

*MELFS*SDEGSK...

*MELFSNE*SDEGSK...

*MELFSNEL*SDEGSK...

*MELFSNELL*SDEGSK...

*MELFSNELLY*SDEGSK...

*MELFSNELLYK*SDEGSK...

*MELFSNELLYKT*SDEGSK...

MELFSNELLYKT represents the aminoterminal sequence of N^{pro} of the BVDV isolate NewYork93/C.

It may also be possible to use variants of this sequence with one or several mutations. Especially the naturally occurring variations as found in other pestiviruses can be expected to be functional. Therefore, the complete list of the tested or proposed variants with the different parts of the aminoterminal end of N^{pro} can be enlarged by equivalent sets with amino acid exchanges. Below, typical examples of the respective sequences are given for several pestiviruses but the possible variations are not limited to these examples.

BVDV NewYork93/C: *MELFSNELLYKT*

BVDV CP13: *MELISNELLYKT*

BVDV SD1: *MELITNELLYKT*___

CSFV Brescia: *MELNHFELLYKT*

BDV X818: *MELNKFELLYKT*

Thus, these variants for example may include: *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];

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];

These formulas may also have $[PS]_1$, i.e. PS may also be one of the PS as described herein. Sequences belonging to the N^{pro} protein are in italics. Amino acid exchanges with regard to the sequence of BVDV NewYork93/C are in bold.

Further examples can be found e.g. by using the GenBank accession numbers given in Becher et al., 2003, Virology 311, 96-104) or by standard sequence data searches.

A further possibility could be the use of a processing signal (PS) inserted between the (residual) N^{pro} sequence and the aminoterminus of the capsid protein. The PS leads to a cleavage that generates a functional capsid protein. The configuration of such constructs could be as follows:

$[N^{pro}]_{22}$-**PS**[C-term]

PS: Processing signal. Can either be a target for a protease (e.g. ubiquitin, LC3 as defined herein or a protease or an unstable peptide leading to processing at its own carboxyterminus like e.g. intein (Chong et al. 1998 and references therein) or 3C of picornaviruses, 2A of cardioviruses or aphtoviruses, p15 of rabbit hemorrhagic disease virus or the corresponding protease of other caliciviruses (Proter 1993, and references therein; Meyers et al., 2000 and references therein). When using a PS, a large number of different variants are possible since the PS ensures the generation of the correct amino terminus of the capsid protein C. Thus, when using a PS construct, all kinds of deletions or mutations of the N^{pro} sequence are expected to result in viable mutants as long as the reading frame is not shifted or translation stopped by an in frame stop codon. As an example we established a viable CSFV N^{pro} deletion mutant according to the formula

$[N^{pro}]_{29}$-**PS**[C-term]

Especially interesting could be N^{pro} mutations blocking the proteolytic activity of the protein. Rümenapf et al. (1998) have published the identification of the active site residues of the protease for CSFV Alfort Tübingen. The respective amino acids (glutamic acid at position 22, histidine at position 49 and cysteine at postion 69) are conserved for other pestiviruses. Thus, exchanges of any amino acid expect for serine or threonine for the cysteine at position 69 will result in destruction of the protease activity. Similarly, changing the glutamic acid at position 22 will most likely result in inactivation of the protease unless the new amino acid is aspartic acid. Similarly most if not all exchanges at position 49 will lead to an inactive protease).

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.

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 attenuated 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}.

2. The virus according to claim 1, 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}.

3. The virus according to claim 1 or 2, wherein said mutations are selected from the group of deletions, insertion mutations and/or substitution mutations.

4. The the virus according to any one of claims 1 to 3, wherein said mutation(s) are deletions.

5. The virus according to any one of claims 1 to 4, wherein said pestivirus is a Bovine viral diarrhea virus (BVDV).

6. The virus according to claim 5, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} are located in the encoding nucleotide sequence corresponding to amino acids at position 298 to 310 and/or position 341 to 360.

7. The virus according to claim 5 or 6, wherein said mutation in the coding sequence for glycoprotein E^{rns} is a deletion or substitution of the histidine at position 349.

8. The virus according to anyone of claims 1 to 7, wherein said 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.

9. The virus according to any one of claims 1 to 8, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} located in the nucleotide sequence coding for the conserved E^{rns} sequence SLHGIWPEKIC and/or RHEWNKHGWCNW.

10. The virus according to anyone of claims 1 to 9, wherein said mutation(s) in the coding sequence for glycoprotein E^{rns} are two mutations located in the nucleotide sequence coding for the conserved E^{rns} sequence SLHGIWPEKIC and/or RHEWNKHGWCNW.

11. The virus according to anyone of claims 1 to 10, wherein said mutation in the coding sequence for glycoprotein E^{rns} is a single mutation located in the conserved E^{rns} sequence SLHGIWPEKIC or RHEWNKHGWCNW.

12. The virus according to any one of claims 1 to 11, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

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 grpup 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 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).

13. The virus according to claim 12, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

14. The virus according to claim 12, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

and wherein the definitions are as defined in claim 11.

15. The virus according to claim 12, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

16. The virus according to claim 12, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

17. The virus according to to claim 12, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

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

18. The virus according to claim 12, wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by the following formula:

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

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

19. The virus according to claim 12, wherein said said virus is BVDV, and wherein mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by a formula selected from the group consisting of :

$$M\text{-}[PS]_0\text{-}[C\text{-term}];$$

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]

20. The virus according to claim 12, wherein said said virus is BVDV, and wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by a formula selected from the group consisting 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];

21. The virus according to claim 12, wherein said said virus is BVDV, and wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by a formula selected from the group consisting 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];

22. The virus according to claim 12, wherein said said virus is BVDV, and wherein said mutation(s) in the coding sequence for N^{pro} lead to an encoded polyprotein as characterized by a formula selected from the group consisting of :

$[N^{pro}]_x$-$[PS]_0$-MELF-$[PS]_0$-[C-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.

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

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

and wherein PS is ubiquitin or LC3.

24. The virus according to any one of claims 19 to 22, 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, and p15 of rabbit hemorrhagic disease virus.

25. The BVDV according to any one of claims 5 to 24, wherein said BVDV is selected from the group of BVDV type 1 or BVDV type 2.

26. The BVDV according to any one of claims 5 to 25, wherein the BVDV corresponds to SEQ ID No. 8 or a functional variant thereof.

27. A composition comprising the virus according to any one of claims 1 to 26 and a solution.

28. The composition according to claim 27, which induces an immunological response in an animal.

29. The composition according to claim 27or 28, which is a vaccine.

30. The composition according to any one of claims 27 to 29, wherein said composition further comprises a pharmaceutically acceptable carrier or excipient.

31. Use of a virus according to any one of claims 1 to 26 in the manufacture of a vaccine for the prophylaxis and treatment of a pestivirus infection.

32. Use of a BVDV according to any one of claims 5 to 26 in the manufacture of a vaccine for the prophylaxis and treatment of a BVDV infection.

33. A nucleic acid molecule comprising the nucleic acid encoding a live attenuated BVDV according to any one of claims 5 to 26 or a fragment, functional variant, variant based on the degenerative nucleic acid code, fusion molecule or a chemical derivative thereof.

34. The nucleic acid molecule according to claim 33, wherein said nucleotide molecule is DNA.

35. The nucleic acid molecule according to claim 34, wherein said nucleotide molecule is RNA.

36. A method for attenuating a pestivirus, characterized in that at least one mutation in the coding sequence for glycoprotein E^{rns} and at least another mutation in the coding sequence for N^{pro} is generated in a pestivirus.

37. The method according to claim 36, comprising the following 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} 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.

38. The method according to claim 36 or 37, wherein said pestivirus is BVDV.

39. Method of treatment of disease caused by BVDV, wherein a BVDV according to any one of claims 4 to 26 or a composition according to claims 27 to 30, wherein the said BVDV or said composition 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.