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(54) Title: INFECTIOUS BOVINE VIRAL DIARRHEA VIRUS CLONE

(57) Abstract: The invention belongs to the field of animal health and in particular Bovine Viral Diarrhea Virus (BVDV). The invention provides infectious BVDV clones and methods to produce said BVDV clones. The invention further relates to methods of attenuating said clones, attenuated BVDV clones and vaccines comprising said attenuated clones.

Infectious bovine viral diarrhea virus clone

Field of the invention

The invention belongs to the field of animal health and in particular Bovine Viral Diarrhea Virus (BVDV). The invention provides infectious BVDV clones and methods to produce said BVDV clones. The invention further relates to methods of attenuating said clones, attenuated BVDV clones and vaccines comprising said attenuated clones.

Background of the invention

Bovine Viral Diarrhea Virus (BVDV) is the causative agent of BVD and mucosal disease in cattle (Baker, 1987; Moennig and Plagemann, 1992; Thiel et al., 1996). Fetal infection during pregnancy can result in the resorption of the fetus, abortions as well as birth of immunotolerant calves which are persistently infected with BVDV. These calves lack or have very low neutralizing antibody titers and are continuously shedding high amounts of virus. Next to acutely infected cattle these calves are the major source for virus spreading and are therefore of prime importance in the epidemiology of this disease. The major economical impact of BVD results from high abortion rates, stillbirths, fetal resorption, mummification, congenital malformations, and birth of weak and undersized calves. For a detailed review of the pathogenesis it is hereby referred to the article of Moennig and Liess of 1995.

Two major antigenic groups of BVDV (type 1 and 2) have been described (Becher et al. 1999) which display limited cross neutralizing antibody reactions (Ridpath et al. 1994). Present vaccines for the prevention and treatment of BVDV infections still have drawbacks (Oirschot et al. 1999). Vaccines against the classical BVDV type 1 provide only partial protection from type 2 infection, and vaccinated dams may produce calves that are persistently infected with virulent BVDV type 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 (Tijssen et al., 1996): most monoclonal antibodies against type 1 strains fail to bind to type 2 viruses (Ridpath et al., 1994).

Killed vaccines (inactivated whole virus) or subunit vaccines (conventionally purified or heterologously expressed purified 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.

Live BVDV vaccines, although attenuated, are most often associated with safety problems. As mentioned above, they cross the placenta of pregnant 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 cattle. 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.

Live vaccines with defined mutations as a basis for attenuation would overcome the disadvantages of the present generation of attenuated vaccines. A further advantage of said attenuating mutations lies in their defined molecular uniqueness which can be used as a distinctive label for the attenuated pestivirus to distinguish it from pestiviruses from the field.

In the art, BVDV of defined genetic identity which closely resemble wild-type viruses are hardly known, in particular not for type 2 BVDV. In the art, there was a long lasting need for methods to generate such BVDV. Therefore, the technical problem underlying this invention was to provide a BVDV, in particular a BVDV type 2, of defined genetic identity.

Figure Legends

FIG. 1. Construction of the infectious cDNA clone. The upper part scetches a BVDV genome (kB) and the encoded polyprotein. The middle part shows the cDNA clones (white), the RT-PCR product (light grey) and the PCR products (dark grey) used for engineering the infectious cDNA clone, and the lower part depicts the ends of the genomic cDNA sequences (underlined) and the sequences added at the 5' and 3' ends for in vitro transcription.

Fig. 2: Growth curves of the recombinant virus XIKE-A and the wild type BVDV isolate VLS#399. MDBK cells were infected with the viruses at an m.o.i of 0.1 and harvested by freezing and thawing at the indicated time points. Titers were determined after infection of new MDBK cells by immunofluorescence staining 72h p.i.

Fig. 3: Growth curves of the recombinant virus XIKE-A and the E^{ms} mutants XIKE-B (H349Δ) and XIKE-C (H300L). MDBK cells were infected with the viruses at an m.o.i of 0.1and harvested by freezing and thawing at the indicated time points. Titers were determined after infection of new MDBK cells by immunofluorescence staining 72h p.i.

Fig. 4: Determination of RNAse activity of the recombinant viruses XIKE-A (wild type sequence), XIKE-B (H349Δ) and XIKE-C (H300L) in comparison with the wild type strain new York '93/C from crude cell extracts of MDBK cells infected with the respective viruses. MDBK cells that were not infected served as a negative control (n.i.). The enzymatic degradation of poly(U) was determined by measuring the OD_{260} as a marker of the release of small RNA fragments into the supernatant.

Fig. 5: Body temperatures of animals infected with New York '93/C (animal #275, #612 and #1610, broken lines) or XIKE-A (animal #615, #377 and #091, solid lines).

Fig. 6: White blood cell (WBC) counts of animals infected with New York '93/C (animals #275, #612 and #1610, broken lines) or XIKE-A (animals #615, #377 and #091, solid lines).

Fig. 7: Body temperatures of animals infected with XIKE-A (animal #387, #388 and #418, broken lines) or XIKE-B (animal #415, #417 and #419, solid lines).

Fig. 8: White blood cell I(WBC) counts of animals infected with XIKE-A (animals #387, #388 and #418, broken lines) or XIKE-B (animals #415, #417 and #419, solid lines).

Disclosure 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 virus" includes a plurality of such BVDV viruses, 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 "BVDV" as used herein refers to all viruses belonging to species BVDV 1 and BVDV 2 in the genus pestivirus within the family Flaviviridae (Becher et al. 1999). 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.

A „clone" is a DNA vector or host cell strain into which such vector has been introduced. Preferably, the DNA vector is a plasmid.

An „infectious clone" is a DNA Vector with the capability to serve as a template for transcription into a RNA that induces the generation of the the virus when introduced into susceptible cells. Preferably the RNA is produced by *in vitro* transcription and introduced into the cells by transfection technologies known to the skilled person.

„BVDV particles" or „viral particles" as used herein relates to BVD viruses generated from „infectious clones" via RNA, that will induce production of said BVDV particles when introduced into susceptible cells.
"Attenuated BVDV particles" or "attenuated viral particles" as used herein relates to BVDV particles attenuated by a method according to the invention *(see infra)*.

„Infectivity" is the capability of a virus or viral particle to induce a certain number of plaques in a plaque test or a certain $TCID_{50}$ score in an endpoint test.

A full-length RNA is a RNA comprising at least 98 % of the sequence of a RNA occurring in a wild-type isolate. A full-length complementary DNA is a DNA comprising a sequence complementary to at least 98 % of a RNA occuring in a wild-type isolate.

As used herein, "calf" relates to a bovine animal of six months of age or less.

Virulence: "Authentical virulence" as used herein means that there is no statistically significant difference between the virulence of infectious BVDV particles according to the invention and wild-type BVDV isolates from which said DNA molecules containing a nucleotide sequence complementary to a BVDV RNA, preferably a type 2 RNA have been derived, for at least one predominant clinical parameter. Examples for such predominant clinical parameters are diarrhea, pyrexia and/or lethality.

Attenuation: "An attenuated BVDV particle" as used herein means that there is a statistically significant difference between the virulence of attenuated BVDV particles according to the invention, said attenuated BVDV particles being attenuated by a

method according to the invention, and wild-type BVDV isolates from which said attenuated BVDV particles have been derived, for the predominant clinical parameters diarrhea, pyrexia and lethality in animals infected with the same dose, preferably $6x10^6TCID_{50}$. Thus, said attenuated BVDV particles do not cause diarrhea, pyrexia and lethality and thus may be used in a vaccine.

"RACE" as used herein means rapid amplification of cDNA ends and is known as such in the art (Frohman et al, Proc. Natl. Acad. Sci USA 1988, 85: 8998-9002).

„Susceptible cell" as used herein is a cell which can be infected with BVD virus or transfected with BVDV RNA, wherein said virus or RNA, when introduced into said susceptible cells, induces the generation of infectious BVDV.

A „fragment" according to the invention is any subunit of a DNA molecule or infectious BVDV clone according to the invention, i.e. any subset, characterized in that it is encoded by a shorter nucleic acid molecule than disclosed which can still be transcribed into RNA.

A „functional variant" of the DNA molecule or infectious BVDV clone according to the invention is a DNA molecule or infectious BVDV clone which possesses a biological activity (either functional or structural) that is substantially similar to the DNA molecule or infectious BVDV clone 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 nucleic acid exchanges, deletions or insertions. Said exchanges, deletions or insertions may account for 10% of the entire sequence. 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.

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 tripletts. Said variant at least partially retains its biological activity, or even exhibits improved biological activity.

A „fusion molecule" may be the DNA molecule or infectious BVDV clone according to the invention fused to e.g. a reporter such as a radiolabel, a chemical molecule such as a fluorescent label or any other molecule known in the art.

As used herein, a „chemical derivative" according to the invention is a DNA molecule or infectious BVDV clone according to the invention chemically modified or containing additional chemical moieties not normally being part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life etc.

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.

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 BVDV. The BVDV clone according to the invention confers active immunity that may be transferred passively via maternal antibodies against the immunogens it contains and sometimes also against antigenically related organisms.

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.

Disclosure of the invention

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

The long lasting need in the art has been overcome for a live BVDV (bovine viral diarrhea virus) of defined sequence and specificity correlated to virulence which can be used to generate specific attenuated BVDV for use e.g. in a vaccine. The inventors for the first time provided a method to generate infectious clones and infectious BVDV particles derived thereof of defined genetic identity which at the same time have the pathogenicity closely resembling the wild-type virus. Furthermore, the inventors for the first time disclosed an infectious type 2 clone and infectious type 2 BVDV particles

derived thereof. Thirdly, having invented live infectious BVDV particles of defined sequence, the inventors also invented a method to generate attenuated BVDV particles with genetic identity which may be attenuated by modification at only one defined genetic marker site. The invention allows to generate a causal link between genome modification and attenuation, which is essential in order to understand the functional mechanism of the attenuation and therefore helpful to assess the quality in use as a vaccine.

In a first aspect, the invention relates to a DNA molecule containing a nucleotide sequence complementary to a BVDV RNA, wherein said RNA, when introduced into susceptible host cells, induces the generation of infectious BVDV particles

a) with the capability to induce viraemia and leukopenia in calves for a period of at least one day and at least one of the following clinical symptoms of the group comprising diarrhea and/or pyrexia lasting at least one day when infected with a dose of $6x10^6TCID_{50}$.
b) with authentical virulence as defined *supra* as compared to a wild-type BVDV isolate from which such DNA molecule has been derived; and/or
c) which are, when BVDV naive calves are infected at a dose of $6x10^6TCID_{50}$ with such particles, lethal for at least 30 % of such calves within a period of 21days; and/or
d) with a virulence of not less than 90 % of BVDV particles comprising an RNA with a sequence complementary to SEQ ID NO. 1; and/or
e) comprising a sequence complementary to SEQ ID NO. 1.

Said dose of $6x10^6TCID_{50}$ of step a) is preferably administered as $2x10^6$ i.m. (gluteal muscle), $2x10^6$ intranaseally, and $2x10^6$ subcutaneously (over scapula) to obtain a total dose of $6x10^6$. Said clinical symptoms of step a) preferably should be observed in at least two thirds of all infected animals. Said leukopenia of step a) preferably shall be at least a 35% reduction below baseline on at least two consecutive days, wherein "baseline" relates to the average values of all animals 10 days before infection. Diarrhea is a typical symptom of infection with BVDV.

Preferably, in a DNA molecule as described *supra* the pyrexia of step a) is at least 40 °C.

In a second aspect, the invention relates to an infectious BVDV clone,

capable of serving as a template for transcription into an RNA, wherein said RNA, when introduced into susceptible host cells, induces the generation of infectious BVDV particles

f) with the capability to induce viraemia and leukopenia in calves for a period of at least one day and at least one of the following clinical symptoms of the group comprising diarrhea and/or pyrexia lasting at least one day when infected with a dose of $6x10^6TCID_{50}$; and/or

g) with authentical virulence as compared to a wild-type BVDV isolate from which such DNA molecule has been derived; and/or

h) which are, when BVDV naive calves aged from 3 to 6 months are infected at a dose of $6x10^6TCID_{50}$ with such particles, lethal for at least 30 % of such calves within a period of 21days after infection; and/or

i) with a virulence of not less than 90 % of BVDV particles comprising an RNA with a sequence complementary to SEQ ID NO. 1; and/or

j) comprising a sequence complementary to SEQ ID NO. 1.

Said dose of $6x10^6TCID_{50}$ of step f) is preferably administered as $2x10^6$ i.m. (gluteal muscle), $2x10^6$ intranaseally, and $2x10^6$ subcutaneously (over scapula) to obtain a total dose of $6x10^6$. Said clinical symptoms of step a) preferably should be observed in at least two thirds of all infected animals. Said leukopenia of step f) preferably shall be at least a 35% reduction below baseline on at least two consecutive days, wherein "baseline" relates to the average values of all animals 10 days before infection.

Said infectious BVDV clone preferably is a type 1 or type 2 clone.

As it is important that said infectious BVDV clone is of authentical virulence, the virus that serves as the origin for constructing such clone is preferably obtained directly from a field isolate or retransferred to animals and subsequently reisolated from the animal with the strongest clinical symptoms and subsequently passaged no more than twice in

cell culture, preferably once or not at all. The example (example 1) exemplifies this.
The example demonstrates the cDNA-cloning of virus NY93/C which is, after several cell culture passages, retransferred into a bovine animal, isolated and used for RNA preparation and cDNA cloning after not more than two cell culture passages of the isolated virus.

In a third aspect, the invention provides a BVDV particle generated by transcription using the DNA molecule or the BVDV clone according to the invention, the transfection of suitable cells or cell lines with said RNA and the collection of the resulting BVDV particles produced by said cells. Yet another embodiment is a BVDV particle generated by cloning the DNA molecule or the BVDV clone into the genome of a suitable DNA virus, such DNA viruses being known to the artisan, followed by infection of suitable cells resulting in generation of BVDV particles produced by said cells. Preferably also, the DNA or infectious clone may be transfected into suitable cells which then produce the RNA as disclosed for classical swine fever virus (CSFV) by van Gennip et. al. (1999) for cells which stably express T7 Polymerase. Also preferably the DNA or infectious clone may be expressed under control of an eukaryotic promotor in eukaryotic cells leading to the generation of infectious BVDV particles being able to be secreted from the cell (as exemplified by V. Racaniello and D. Baltimore for poliovirus (1981)).
In a fourth aspect, there is provided an infectious BVDV type 2 clone.
In a fifth aspect, there is provided an infectious BVDV type 2 clone, capable of serving as a template for transcription into an RNA, wherein said RNA, when introduced into susceptible host cells, induces the generation of infectious BVDV particles

k) with the capability to induce viraemia and leukopenia in calves for a period of at least 1 day and at least one of the following clinical symptoms of the group comprising diarrhea and/or pyrexia lasting at least one day when infected with a dose of $6x10^6 TCID_{50}$; and/or

l) withauthentical virulence as compared to a wild-type BVDV isolate from which such DNA molecule has been derived; and/or

m) which are, when BVDV naive calves aged from 3 to 6 months are infected at a dose of $6x10^6TCID_{50}$ with such particles, lethal for at least 30 % of such calves within a period of 21 days after infection; and/or

n) with a virulence of not less than 90 % of BVDV particles comprising an RNA with a sequence complementary to SEQ ID NO. 1; and/or

o) comprising a sequence complementary to SEQ ID NO. 1.

Disclosed herein is a BVDV type 2 clone obtainable by a method characterized by the following steps:

aaa) a wild-type BVDV type 2 strain is isolated;

bbb) said wild-type BVDV type 2 strain is passaged in cell-culture;

ccc) said cell culture-passaged BVDV type 2 strain is used to infect bovine animals and a BVDV strain is re-isolated from the most severely infected animal;

ddd) said re-isolated BVDV type 2 strain is passaged no more than twice, preferably once, in cell culture;

eee) said re-isolated BVDV type 2 strain is reverse-transcribed and cloned resulting in a full-length cDNA clone, preferably the 5' and 3' ends are cloned using the RACE-technology.

Said infectious DNA clone may then be transcribed into RNA under appropriate conditions, said RNA is introduced into appropriate cells or cell lines and the resulting BVDV type 2 particle is collected.

Such a clone is exemplified in the non-limiting example 1 and characterized by the cDNA sequence SEQ ID NO. 1. Thus, a sixth aspect relates to an infectious BVDV type 2 clone according to the invention as characterized by the DNA sequence of SEQ ID NO. 1 or a fragment, functional variant, variant based on the degenerative nucleic acid code, fusion molecule or a chemical derivative thereof. A non-limiting example is provided in example 1.

A seventh aspect further relates to a BVDV type 2 particle generated by *in vitro* transcription of the BVDV clone according to the invention into RNA, the transfection of suitable cells or cell lines with said RNA and the collection of the resulting BVDV particles produced by said cells. Preferably also, the DNA or infectious clone may be transfected into suitable cells which then produce the RNA as disclosed for classical swine fever virus (CSFV) by van Gennip et. al. (1999) for cells

which stably express T7 Polymerase. Also preferably the DNA or infectious clone according to the disclosed embodiments may be expressed under control of an eukaryotic promotor in eukaryotic cells leading to the generation of infectious BVDV particles being able to be secreted from the cell (as exemplified by V. Racaniello and D. Baltimore for poliovirus (1981)).

In an eighth aspect of the invention, there is provided a DNA molecule containing a nucleotide sequence complementary to a full-length BVDV type 2 RNA. Preferably, said DNA molecule is characterized by the sequence SEQ ID NO. 1. Thus, the DNA molecule can be characterized by SEQ ID No. 1 or a fragment, functional variant, variant based on the degenerative nucleic acid code, fusion molecule or a chemical derivative thereof. A non-limiting example is provided in example 1.

Most preferably, the DNA molecule according to the disclosed embodiments, consists of the sequence as characterized by SEQ ID No. 1.

A ninth aspect further relates to a RNA molecule complementary to the DNA molecule according to the invention as described *supra*, or to the BVDV clone according to the invention as described *supra*.

A tenth aspect relates to a RNA molecule obtainable by transcription of the DNA molecule according to the invention as described *supra*, or the BVDV clone according to the invention as described supra.

In an eleventh aspect, there is provided a method for the production of an infectious BVDV clone from a wild-type BVDV isolate, said infectious BVDV clone being complementary to a RNA having authentical virulence as compared to said wild-type isolate, comprising the steps of

p) isolating viral particles from an infected animal;

q) passaging not more than twice on suitable cell culture cells;

r) preparing RNA from the viral particles;

s) generating full-length complementary DNA after reverse transcription of the RNA; wherein the reverse transcription includes a step at elevated temperatures sufficient to break or reduce secondary structures of the RNA, and the use of a thermostable enzyme for this step, said enzyme being active at these elevated temperatures;

l) incorporating the complementary DNA (cDNA) into a plasmid vector or into a DNA virus capable of directing the transcription of BVDV cDNA into RNA upon infection of suitable cells.

Said viral particles preferably are isolated during viremia (step k)). The full length complementary DNA (cDNA) of step m) preferably may be generated by assembling overlapping partial cDNA fragments (see also example 1).

Another preferred embodiment relates to a method for the production of an infectious BVDV clone from a wild-type BVDV isolate, said infectious BVDV clone being complementary to a RNA having authentical virulence as compared to said wild-type isolate, comprising the steps of

ppp) isolating RNA from cells of an infected animal during viraemia or optionally after killing of said animal from its organ(s);

qqq) generating full-length complementary BVDV DNA which preferably is assembled from DNA fragments after reverse transcription of the RNA; wherein the reverse transcription includes a step at elevated temperatures sufficient to break or reduce secondary structures of the RNA, and the use of a thermostable enzyme for this step, said enzyme being active at these elevated temperatures;

rrr) incorporating the complementary DNA (cDNA) into a plasmid vector or into a DNA virus capable of directing the transcription of BVDV cDNA into RNA upon infection of suitable cells.

Suitable cells for cell culture are Madin-Darby bovine kidney (MDBK) cells, RD (bovine testicular) cells or bovine Turbinat (BT) cells. Further suitable cells are known to the person skilled in the art.

The infectious clone produced by the method may be a type 1clone or preferably a type 2 clone.

In a twelfth aspect, there is provided a method for the production of an infectious BVDV clone from a wild-type BVDV isolate, said infectious BVDV clone being complementary to a RNA having a virulence of not less than 90 % of said wild-type isolate, comprising the steps of

u) isolating viral particles from an infected animal;
for example passaging not more than twice is suitable cell culture cells; preferably one or not at all;

v) preparing RNA from the viral particles;

w) generating full-length complementary DNA after reverse transcription of the RNA; wherein the reverse transcription includes a step at elevated temperatures sufficient to break or reduce secondary structures of the RNA, and the use of a thermostable enzyme for this step, said enzyme being active at these elevated temperatures;

x) incorporating the complementary DNA (cDNA) into a plasmid vector or into a DNA virus capable of directing the transcription of BVDV cDNA into RNA upon infection of suitable cells.

Said viral particles preferably are isolated during viremia (step u)). The full length complementary DNA (cDNA) of step x) preferably may be generated by assembling overlapping partial cDNA fragments (see also example 1).

There was a particular difficulty in the art to clone the 5' and 3' region of an infectious BVDV. The inventors developed an inventive method to obtain authentical 5' and 3' regions. Surprisingly, this was possible by applying the RACE-technology. However, only the modification by the inventors of this technique led to the surprising and unexpected generation of BVDV clones of authentic virulence.

Disclosed herein is a method, wherein the 5' end of the RNA is generated using RACE. Surprisingly, only by applying the RACE technology in conjunction with a thermostable polymerase it was possible to dissolve the secondary structure of the genome successfully.

Standard molecular biology methods are known to the skilled person and can also be found e.g. in Sambrook et al.(1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York and Bertram, S. and Gassen, H.G. Gentechnische Methoden, G. Fischer Verlag, Stuttgart, New York, 1991).

Preferably, the RACE is carried out with a thermostable polymerase allowing reaction temperatures of at least 48°C, preferably 50-55°C, preferably also 56-60°C.

Having invented live infectious BVDV particles of defined sequence, the inventors also invented a method to generate attenuated BVDV particles with a defined genetic identity which preferably are attenuated at only one defined genetic marker site. This surprisingly allows the simple determination of revertants or the successful attenuation as only the presence of the genetic marker site needs to be determined by molecular biology methods known to the artisan. XIKE-B and XIKE-C of example 1 are non-limiting examples for such attenuated BVDV particles of defined sequence.

In a thirteenth aspect, there is provided a method of BVD virus attenuation by introducing one or more mutations into the DNA molecule according to the invention as described *supra* or the infectious BVDV clone as described *supra*, wherein said mutation or mutations lead to or increase an attenuated phenotype of the recovered BVD virus.

In a fourteenth aspect, there is provided a method of attenuation of a BVDV strain, comprising the steps of

y) introducing one or more mutations into the DNA molecule according to the invention as described *supra*, or into the infectious BVDV clone according to the invention as described *supra*;

z) introducing the mutated DNA into susceptible host cells wherein said DNA is transcribed into RNA or introducing an RNA transcribed from said DNA into said cells; and

aa)collecting viral particles produced by these cells;

wherein said mutation or mutations results in attenuation.

The mutation or mutations can be a nucleotide substitution, deletion, insertion, addition, or combination thereof.

According to the disclosed embodiments, "mutation" means the replacement of a nucleotide by another (e.g. C for a T) a so-called "substitution" or any other mutation such as "deletion" or "insertion". "Deletion" means the removal of one or several nucleotides or amino acids.

As these infectious BVDV clones according to the disclosed embodiments can be viruses of authentic virulence closely resembling wild-type viruses and at the same time having a defined genotype, said virus can be used as a positive control in animal experiments. Said

infectious clones are excellent tools for generating specifically attenuated BVDV clones to be used for e. g. vaccination. In one embodiment, there is provided BVDV clones wherein the RNase activity residing in glycoprotein E^{RNS} is inactivated. Preferably, said RNAse activity is inactivated by deletion and/or other mutation such as substitution. Preferably, said deletions and/or other mutations are located at the amino acids at position 295 to 307 and/or position 338 to 357.

Thus, a more preferred embodiment is a method of attenuation according to the invention, wherein the mutation or mutations is in the glycoprotein E^{ms} and causes impaired or loss of function of the mutated protein.

A more preferred embodiment is a method of attenuation according to the invention, wherein the mutation consists of

bb) deletion of all or part of the glycoprotein E^{ms}; and/or

cc) deletion or substitution of histidine at position 300 of SEQ ID NO. 1; and/or

dd) deletion or substitution of histidine at position 349 of SEQ ID NO. 1.

Most preferably, yet another important embodiment is a method for the attenuation of BVDV, comprising the mutation of a BVDV clone at histidine positions 300 and/or 349 wherein the coding triplett is deleted or substituted.

Yet another important embodiment is a method for the attenuation of BVDV according to the disclosed embodiments, wherein the codon for histidine 300 is substituted by a codon for leucine.

Yet another important embodiment is a method for the attenuation of BVDV according to the disclosed embodiments, wherein the codon for histidine 349 is deleted.

In a fifteenth aspect, there is provided an attenuated BVDV clone or BVDV strain obtainable by a method according to the invention.

In a sixteenth aspect, there is provided a vaccine comprising an attenuated BVDV clone or strain according to the invention, optionally in combination with a pharmaceutically acceptable carrier or excipient.

In a seventeenth aspect, there is provided a use of a attenuated BVDV clone or strain according to the invention in the manufacture of a vaccine for the prophylaxis and treatment of BVDV infections.

Preferably, a vaccine of the disclosed embodiments refers to a vaccine as defined above, wherein one immunologically active component is a live BVDV, wherein the RNase activity in its protein E^{RNS} is inactivated. The term "live vaccine" refers to a vaccine comprising a particle capable of replication, in particular, a replication active viral component.

Preferably, a vaccine according to the disclosed embodiments comprises an attenuated BVD virus type 1 according to the disclosed embodiments combined with an attenuated BVD virus type 2 according to the disclosed embodiments or any other antigenetic group and a pharmaceutically acceptable carrier or excipient. Said vaccine may be administered as a combined vaccine. Most preferably, said attenuated BVD virus type 1 according to the disclosed embodiments may be administered first followed by an administration of an attenuated BVD virus type 2 according to the invention three to four weeks later.

Preferably, a vaccine according to the disclosed embodiments comprises an attenuated BVD virus type 1 according to the disclosed embodiments wherein the RNase activity in its protein E^{RNS} is inactivated, combined with an attenuated BVD virus type 2 according to the disclosed embodiments wherein the RNase activity in its protein E^{RNS} is inactivated, or any other antigenetic group wherein the RNase activity in its protein E^{RNS} is inactivated, and a pharmaceutically acceptable carrier or excipient. Said vaccine may be administered as a combined vaccine. Most preferably, said attenuated BVD virus type 1 as described *supra* may be administered first followed by an administration of an attenuated BVD virus type 2 as described supra three to four weeks later.

Also disclosed herein is a method of treating a BVDV-infected bovine animal with an attenuated BVDV as described supra, wherein the said attenuated BVDV or the vaccine composition as disclosed supra is administered to the bovine animal in need thereof at a suitable dosis as known to the skilled person and the reduction of BVDV symptoms such as viremia and leukopenia and/or pyrexia and/or diarrhea is monitored. Said treatment preferably may be repeated.

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

MATERIALS AND METHODS

Cells and viruses. MDBK cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS; tested for the absence of pestivirus and antibodies against pestiviruses) and nonessential amino acids.

Bovine viral diarrhea strain New York '93 (field isolate VLS#399) was kindly provided by E. J. Dubovi (New York State College of Veterinary Medicine, Cornell University, Ithaca). The virus underwent one animal passage and was termed „New York '93/C" thereafter.

Infection of cells, immunofluorescence assay and virus peroxidase assay. Since pestiviruses are highly associated with their host cells, lysates of infected cells were used for reinfection of culture cells. Lysates were prepared by freezing and thawing cells 3 to 5 days after infection and were stored at –70°C. Unless indicated otherwise in the text, a multiplicity of infection (m.o.i.) of 0,1 was used for infection of culture cells.

For immunofluorescence and peroxidase assays, the infected cells were fixed with ice-cold acetone:methanol (1:1) for 15 min at –20°C, air dried and rehydrated with phosphate buffered saline (PBS). Cells were then incubated with a mixture of anti-BVDV monospecific antibodies directed against E2 (Weiland et al. 1989). After three washes with PBS, a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Dianova, Hamburg, Germany) was used for detecting bound antibodies in the immunofluorescence assays. For peroxidase assays, peroxidase-conjugated goat anti-mouse antibody (Dianova) was used as second antibody. After incubation for one hour at room temperature, cells were washed three times with PBS. Bound antibodies were detected with a solution composed of 50 mM sodium acetate buffer pH 5.0, 1 µM aminoethylcarbazole and 0,1 % H_2O_2.

Northern (RNA) hybridization. RNA was prepared 48 hours after infection by cesium density gradient centrifugation as described before (Rümenapf et al. 1989). Gel electrophoresis, radioactive labelling of the probe, hybridization, and posthybridization washes were done as described before (Rümenapf et al. 1989). A radioactively labelled

PCR product (nucleotides 4301 to 5302) from strain New York 93/C was used as a probe.

PCR and RT-PCR. PCR was carried out either with *Tfl*-Polymerase (Promega, Mannheim, Germany) or with *Taq*-Polymerase (Appligene, Heidelberg, Germany) following the manufacturer's recommendations and using ca. 50-100 ng of DNA template and 25 pmol of each primer. The sequences of the primers used for amplification of the 5' end of the genome were upstream, $T_{25}V$ primer (Display Systems Biotech, Copenhagen, Denmark); and downstream, CM79: CTCCATGTGCCATGTACAGCAGAG for the first round and CM86: CTCGTCCACATGGCATCTCGAGAC for the nested PCR. The primers used for amplification of the 3' end of the genome were upstream, CM46: GCACTGGTGTCACTCTGTTG for the first round and CM80: GAGAAGGCTGAGGGTGATGCTGATG for the nested PCR and downstream, nls-: GACTTTCCGCTTCTTTTTAGG. Reverse transcription PCR (RT-PCR) was was done with the Titan™ One Tube RT-PCR System (Boehringer Mannheim, Germany), using 2 µg of total RNA as a template and following the manufacturer's instructions. The primers for amplification of the E^{rns} coding region were upstream, CM28: GGAGAGAATATCACCCAGTG; and downstream, CM21: CTCCACTCCGCAGTATGGACTTGC.

The amplified RT-PCR products were purified by preparative agarose gel electrophoresis and elution with the Nucleotrap kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer.

Phosphorylation and ligation of DNA-oligonucleotides to the 3' ends of RNA. For ligation of a DNA primer to the 3' end of the virus genome, the primer was phosphorylated. 10 µg of the oligonucleotide nls+: CCTAAAAAGAAGCGGAAAGTC were incubated with 5 units of T4 polynucleotide kinase (New England Biolabs, Schwalbach, Germany) in 30 µl kinase-mix (2 mM ATP, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 25 µg/ml bovine serum albumin) for 40 min at 37°C. The primer was passed through a sephadex G-15 spin column (Sambrook et al. 1989) and further purified by phenol/chloroform extraction and ethanol precipitation.

Ligation was carried out using 5 µg of total RNA prepared from infected culture cells and 150 pmol of the phosphorylated oligonucleotide with 20 units of T4-RNA-Ligase

(New England Biolabs, Schwalbach, Germany) in 50 µl of ligase-mix (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 40% polyethylene glycol and 50 units of RNA guard (Amersham, Freiburg, Germany)) for 16 hours at 17°C. The product was purified by phenol/chloroform extraction and ethanol precipitation.

Synthesis and tailing of single-stranded DNA. Single-stranded (-) DNA from the 5' end of the virus genome was generated with displayThermo-RT reverse transcriptase (Display Systems Biotech, Copenhagen, Denmark) using 2 µg of total RNA from infected cells and 100 pmol of primer CM79 (see „PCR and RT-PCR"), and following the manufacturer's instructions (reaction: 65 °C for 10 min, 42°C for 40 min, 65°C for 15 min). The DNA was purified by two sequential phenol/chloroform extractions and ethanol precipitations with ¼ vol of 10 M ammonium acetate (Schaefer 1995).

A poly-dA tail was added to the first cDNA strand with Terminal deoxynucleotidyl Transferase (TdT) (Roche Molecular Biochemicals, Mannheim, Germany) using 50% of the „first strand" product, 50 units terminal transferase, 6,25 µM dATP and 1,5 mM $CoCl_2$ in 50 µl of TdT buffer as recommended by the manufacturer. After incubation at 37°C for 30 min, the product was purified by phenol/chloroform extraction and ethanol precipitation.

Construction of a cDNA library and nucleotide sequencing. Synthesis of cDNA, cloning and library screening were generally carried out as described previously (Meyers et al. 1991). cDNA synthesis was primed with oligos BVD13, BVD14 and BVD15 (Meyers et al. 1991) as well as with B22.1R (GTTGACATGGCATTTTTCGTG), B12.1R (CCTCTTATACGTTCTCACAACG), BVD33 (GCATCCATCATXCCRTG$^{A}_{TG}$AT), N7-3-7 (CAAATCTCTGATCAGTTGTTCCAC), B23-RII (TTGCACACGGCAGGTCC), and B-3' (GTCCCCCGGGGGCTGTTAAGGGTTTTCCTAGTCCA). The probe used for screening the library was the *XhoI/AatII* insert of a cDNA clone from BVDV strain cp7 (GenBank accession no. U63479, Meyers et al. 1996b); hybridisation was carried out at 52°C.

Exonuclease III and nuclease S1 were used to establish deletion libraries of cDNA clones (Henikoff 1987). Nucleotide sequencing of double-stranded DNA was carried out with the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Weiterstadt, Germany). As a rule, both DNA strands of the cDNA clones were

sequenced; overlaps between independent cDNA clones were sequenced on at least two clones. In total, about 47.000 nucleotides were analyzed which equals an overall coverage of ~ 3.8 for the entire genome. Sequence analysis and alignments were done with Genetics Computer Group software (Devereux et al. 1984).

Construction of the full-length cDNA clone. Restriction, cloning and other standard procedures were generally carried out as described elsewhere (Sambrook et al. 1989). Restriction and modifying enzymes were obtained from New England Biolabs (Schwalbach, Germany), Pharmacia (Freiburg, Germany), GibcoBRL (Eggenstein, Germany), and Boehringer Mannheim (Germany).

Five cDNA clones from the library were used for construction of the full length cDNA clone: plasmid C3/8 (nucleotides 35 to 2411), plasmid C5/11 (nucleotides 22 to 2400) plasmid 8/11 (nucleotides 3400 to 7814), plasmid 13/27 (nucleotides 4783 to 9910) and plasmid C4/24 (nucleotides 8658 to 12322). A fragment „RT-E2" reaching from nucleotide position 2144 to position 4447 was obtained by RT-PCR with primers CM29 (GATGTAGACACATGCGACAAGAACC) and CM51 (GCTTCCACTCTTATGCCTTG), using total RNA from MDBK cells infected with field isolate VLS#399 as a template. In the following description, plasmid restriction sites flanking the viral cDNA inserts are underlined.

First, clone C3/8 was cut with *Aat*II and *Hind*III, and the cDNA insert was transferred to pACYC177 cut with the same enzymes. The resulting plasmid was named pKANE5. RT-PCR product „RT-E2„ was inserted into the *Nde*I / *Hind*III sites of this plasmid after restriction with the same enzymes; the resulting plasmid was pKANE8. Then, the *Aat*II fragment from clone C5/11 was transferred into the *Aat*II site of pKANE8, yielding plasmid pKANE14.

The 5' end of the recombinant cDNA clone was generated by PCR with primers CM87 (GCTCTAGACGGCCGTAATACGACTCACTATAGGTATACGAGATTAGCTAAAGAACT CGTATATGGATTGGACGTCAAC) that introduces a T7 promoter sequence upstream of the first cDNA nucleotide, and CM79 (see „PCR and RT-PCR"); plasmid C5/11 was used as the PCR template. The PCR product was ligated into the *Xba*I and *Bsr*GI sites of cDNA clone C5/9, resulting in plasmid pKANE22. Later it was found that oligo CM87 contained a false nucleotide, and pKANE22 was repaired by PCR with oligos CM88 (GACGGCCGTAATACGACTCACTATAGTATACG) and CM79. The PCR product was

treated with *E. coli* DNA-Polymerase I (Klenow fragment) to produce blunt ends and then restricted with *Bsr*GI. It was cloned into the *Spe*I^{blunt} / *Bsr*GI sites of pKANE22, resulting in plasmid pKANE22A.

The insert of cDNA clone 8/11 was cut with *Xho*I and *Bam*HI and cloned into pACYC177 cut with the same enzymes; the resulting plasmid was named pKANE6. The *Avr*II / *Bam*HI fragment of cDNA clone 13/27 was transferred to pKANE6, yielding plasmid pKANE15. Then, the *Eco*RV / *Mfe*I fragment from pKANE14 was inserted into pKANE15 digested with the same enzymes. The resulting plasmid was pKANE21. pKANE21 was digested with *Sac*II and *Eco*RV, and a corresponding fragment from pKANE14 was cloned into these sites, leading to plasmid pKANE24. Then the *Sac*II/*Sac*II fragment from pKANE 22A was cloned into pKANE24 cut with the same enzyme. The resulting plasmid was pKANE28AII.

The 3' end of the genome was generated by PCR with primers B2-11500 (CCTAACCATGATATATGCCTTCTG) and CM81 (CGGAATTCGCCCGGGCTGTTAGAGGTCTTCCCTAGT) which adds an *Srf*I site to the 3' end of the genome. The PCR product was cut with *Bam*HI and *Eco*RI and cloned into pACYC177, resulting in plasmid pKANE17. Then, the *Sac*I/*Kpn*2I fragment of cDNA clone C4/24 was transferred to pKANE17; the plasmid was called pKANE20. The *Stu*I / *Eco*RI fragment was excised from pKANE20 and cloned into plasmid pKANE21 which was digested with *Eco*RI and partially digested with *Stu*I. The resulting plasmid was pKANE23. Finally, the *Xba*I / *Psh*AI fragment from pKANE28AII was inserted into plasmid pKANE23 cut with the same enzymes, leading to the full-length cDNA clone pKANE40.

Site-directed mutagenesis. All mutants were generated by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) following the manufacturer's instructions. The plasmid used for introducing mutations into the region coding for E^{rns} was C5/9, a clone obtained from the initial cDNA library (nucleotides 50 to 2411). Oligonucleotides for generating mutant H"346"Δ were CM126 (GAGTGGAATAAAGGTTGGTGTAAC) and CM127 (GTTACACCAACCTTTATTCCACTC), oligos for mutant H"297"L were CM128 (AACAGGAGTCTATTAGGAATTTGGCCA) and CM129

(TGGCCAAATTCCTAATAGACTCCTGTT). The presence of the desired mutations and the absence of second site mutations were verified by nucleotide sequencing.

***in vitro* transcription and RNA transfection**

Transcription of RNA and transfection of MDBK cells were done essentially as described before (Meyers et al. 1996a). Briefly, 2 ug of the respective cDNA construct was linearized with SrfI and purified by phenol extraction and ethanol precipitation. Transcription with T7 RNA polymerase (NEB, Schwalbach, Germany) was carried out in a total volume of 50ul transcription mix (40mM Tris-HCl, pH 7.5; 6mM $MgCl_2$; 2mM spermidine; 10mM NaCl; 0.5 mM of each ATP, GTP, CTP and UTP; 10 mM dithiothreitol; 100ug/ml of bovine serum albumine) with 50 units of T7 RNA polymerase in the presence of 15 units RNAguard (Pharmacia, Freiburg, Germany). After incubation at 37°C for 1h the reaction mixture was passed through a Sephadex G-50 spun column and further purified by phenol extraction and ethanol precipitation.

If not specified otherwise, transfection was done with a suspension of ca. $3x10^6$ MDBK cells and about 0.5 µg of *in vitro* transcribed RNA bound to DEAE-dextran (Pharmacia, Freiburg, Germany). The RNA/DEAE-dextran complex was established by mixing RNA dissolved in 100 µl HBSS (5 g of Hepes, 8 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO4.2H_2O$ and 1 g of dextrose per Liter; pH 7.05) with 100 µl DEAE-dextran (1mg/ml in HBSS) and incubation for 30 minutes on ice. Pelleted cells were washed once with DMEM without FCS, centrifuged and then resuspended in the RNA/DEAE-dextran mixture. After 30 minutes incubation at 37°C, 20 µl dimethyl sulfoxide was added and the mixture incubated for 2 minutes at room temperature. After addition of 2 ml HBSS, cells were pelleted and washed once with HBSS and once with medium without FCS. Cells were resuspended in DMEM with FCS and seeded in a 10.0-cm-diameter dish. 48h to 72h post transfection cells were split and seeded as appropriate for subsequent analyses.

Electroporation was used for determination of the specific infectivity of RNA. $3x10^6$ MDBK cells in 0.5 ml of phosphate buffered saline (PBS) without magnesium and calcium were mixed with appropriate amounts of RNA and transferred into a 2 mm electroporation cuvette. Electroporation was done with one pulse of 960 µF, 180 Volt in a Hoefer PG 200 Progenefor II. Afterwards, the cells were seeded in 3.5 cm dishes and analyzed by immunofluorescence about 20 h later.

Determination of RNAse activity. MDBK cells were infected with the recombinant viruses and grown for 48 hours. Cells infected with the wild type virus served as a positive control, and uninfected cells were used as a negative control. Cell preparation and measurement of RNAse activity were carried out as described before (Meyers et al. 1999) with the exception that incubation of the probes at 37°C was 30 min instead of 1 hour because longer incubation resulted in considerable background activity in MDBK cells.

Animal experiments. Two animal experiments were carried out to test the recombinant viruses. In the first experiment, two groups of 3 flecked cattle female animals (8 to 10 months old) were inoculated intranasally with $10^5 TCID_{50}$ per animal. In the second experiment, 6 male Holstein and Holstein-cross calves (7 to 10 weeks old) were infected intranasally with $5x10^5 TCID_{50}$ per animal. In the challenge experiment, animals were inoculated with $5x10^6 TCID_{50}$. All animals were tested free of BVDV specific antigen and antibody prior to infection. The different groups were housed in separate isolation units. Clinical parameters were recorded daily as indicated in the results section. Blood was taken from the vena jugularis externa at the time points indicated in the results section and was stabilized with Heparin (ca. 35 I.U./ml) unless it was used for the production of serum.

In order to determine the presence of virus in the blood, buffy coats were prepared from all blood samples. 5 ml ice cold lysis buffer were added to an aliquot of heparin stabilized blood (containing ca. 10^7 leucocytes) and incubated on ice for 10 min, followed by centrifugation The pellet was washed once with lysis buffer and twice with PBS without Ca^{2+} and Mg^{2+} before it was resuspended in 2 ml PBS. MDBK cells seeded in 24-well plates were inoculated with 200 µl of the buffy coat preparations and incubated for 5 days. Viral antigen was detected by immunofluorescence microscopy with the BVDV E2 mAb mix (see above).

The presence of virus-neutralizing antibodies was tested in serum samples that had been inactivated by incubation at 56°C for 30 min. The sera were diluted in steps of 1:2 on 96 well microtitre plates and inoculated with a suspension of strain New York '93/C /100 $TCID_{50}$ per well) for 1 hour at 37°C. $10^{1.75}$ MDBK cells were added to each well and incubated for 5 days. Infection was analysed by immunofluorescence, calculated

by the method of Kaerber (Mayr et al. 1974) and expressed as the 50 % endpoint dilution which neutralized approx. 100 $TCID_{50}$.

To detect virus in nasal discharge, nasal swabs were taken at the time points indicated in the results section, diluted in 2 ml of transport buffer (PBS supplemented with 5 % FCS, 100 I.U./ ml penicillin G, 0.1 mg / ml streptomycin and 2.5 µg / ml amphotericin B) and passed through a 0.2 µm filter. MDBK cells were inoculated in 24 well plates with 100 µl of these preparations and analysed by indirect immunofluorescence microscopy after 5 days.

RESULTS

Genome analysis. The strain NY'93/C is the second BVDV type 2 genome that has been fully sequenced. Northern blot analysis showed that, contrary to strain 890 (Ridpath and Bolin 1995), the genome of NY'93/C contains no large insertions or deletions (data not shown). Nucleotide sequence analysis revealed that the genome is 12332 nucleotides long and contains one open reading frame encoding a polyprotein of 3913 amino acids.

The 5' untranslated region (position 1 to 385) was determined by RACE technology and was found to be identical with the New York '93 sequence published by Topliff and Kelling (1998) except for position 21. In contrast to other known type 2 genomes (Ridpath 1995; Topliff and Kelling 1998), strain NY'93/C has adenine at this position instead of thymin.

Construction and analysis of an infectious cDNA clone for NY'93/C. Although a number of infectious cDNA clones have been established for CSFV and BVDV type 1 (Mendez et al. 1998; Meyers et al. 1996a and 1996b; Moormann et al. 1996; Vassilev et al 1997; Kümmerer and Meyers 2000), this is the first report of an infectious clone from a BVDV type 2 strain. The clone was designed for runoff transcription with T7 RNA polymerase, resulting in a genome-like RNA without any heterologous additions. The full-length clone was constituted from four cDNA plasmids selected from the initial phage library and one RT-PCR product encompassing the region between positions 2265 and 4301. At the 5' end, the sequence of the T7 promoter was added for *in vitro*

transcription, and an *Srf*I site was added to the 3' end for plasmid linearization (Fig. 1). The full-length clone was named pKANE40A.

MDBK cells were transfected with RNA generated from the linearized pKANE40A template by *in vitro* transcription. A runoff transcript from plasmid pKANE28AII which terminates 19 codons upstream of the NS5B coding region served as a negative control. Three days post transfection, BVDV-specific signals were detected after immunofluorescence staining in cells transfected with RNA from pKANE40A but not in the control. The virus generated from the infectious clone pKANE40A was termed XIKE-A. The transfected cells were passaged twice, and the stock of the second passage was used for all further experiments. The virus was analysed by RT-PCR sequencing, taking the nucleotide exchange from C to T at position 1630 as proof of the identity of XIKE-A.

The specific infectivity of the RNA derived from pKANE40A was determined in comparison to RNA prepared from cells infected with the wild type virus NY'93/C. To this end, the concentration of viral RNA in samples used for transfection of MDBK cells was measured in comparison with defined amounts of the *in vitro* transcribed RNA after Northern blotting and hybridization, using a phosphoimager. MDBK cells were transfected with similar amounts of both RNAs, and plaques were counted three days post transfection. On the average, the infectivity of RNA derived from pKANE40A was 4.32×10^2 pfu/µg, and the wild-type RNA yielded 4×10^2 pfu/µg.

The growth characteristics of the recombinant virus were analysed through a growth curve, using the original field isolate VLS#399 as a control in the same experiment (Fig. 2). MDBK cells were infected with an m.o.i. of 0.1, and samples were taken at seven time points from 2 hours to 96 hours post infection. The growth curve of the recombinant XIKE-A is somewhat smoother than that of VLS#399, but both viruses reach a titre of $10^{6,39}$ after 96 hours. XIKE-A was therefore deemed suitable for further experiments.

Construction and analysis of E^{ms} mutants. Previous experiments with CSFV (Meyers et al. 1999) had shown that the RNAse activity of the glycoprotein E^{ms} is destroyed by substitution of histidine 297 or 346 (the numbers represent the residue positions in CSFV strain Alfort/Tübingen) by leucine or lysin, or by deletion of codon "H346". The

mutant viruses are viable, but clinically attenuated. In BVDV strain NY'93/C, the two histidine residues are located at position 300 and 349, respectively. To test whether the effects of mutations at these positions would be similar to CSFV in a BVDV type 2 genome, two infectious clones were engineered with either a deletion of codon "H349" or a substitution of codon "H300" by leucine. The resulting recombinant virus mutants were named XIKE-B (H349Δ) and XIKE-C (H300L).

Both mutants were stable in MDBK cells for at least five passages as determined by nucleotide sequencing of RT-PCR products encompassing the E^{ms} coding region. The growth characteristics of the two mutant viruses were compared with virus derived from the wild type infectious clone XIKE-A (Fig. 3).

The RNAse activity of XIKE-A, XIKE-B and XIKE-C was determined in crude cell extracts of cells infected with the same m.o.i. of either virus two days post infection. Aliquots of the preparations were tested for their ability to degrade poly(U); cells infected with the wild type strain NY'93/C served as a positive control, and uninfected cells were used as a negative control. After 30 min of incubation, the residual high molecular weight RNA was precipitated, and OD_{260} measurement of the supernatants revealed the presence of small degraded RNA fragments (Meyers et al. 1999). High RNAse activity was found in the NY'93/C and XIKE-A samples whereas the two mutants XIKE-B and XIKE-C were in the same range as the negative control (Fig. 4).

Animal experiment with XIKE-A and NY'93/C. The purpose of the first animal experiment was to compare the virulence and pathogenicity of the recombinant virus XIKE-A derived from the infectious cDNA clone with the wild type strain NY'93/C. Two groups of three animals (8 to 9 months old) were each infected with $10^5 TCID_{50}$ of either XIKE-A (animals #615, #377, #091) or NY'93/C (animals #275, #612, #1610). Each group was housed in a separate isolation unit. Body temperatures and clinical signs were recorded daily; blood samples were taken on days 0, 2 to 16 and 21 p.i. for leukocyte counts and detection of viremia. Sera from all calves were collected for detection of neutralizing antibodies against NY'93/C on days 0, 7, 14, 21, 29 and 35 p.i.. Nasal swabs for virus isolation were taken on day 0, 2 to 16 and 21 p.i.

Virus isolation from buffy coat preparations							Virus isolation from nasal swabs					
Days p.i.	#275	#612	#1610	#615	#377	#091	#275	#612	#1610	#615	#377	#091
-26	--	--	--	--	--	--	--	--	--	--	--	--
0	--	--	--	--	--	--	--	--	--	--	--	--
2	--	--	--	--	--	--	--	--	--	--	--	--
3	++	--	--	-+	++	++	--	--	--	--	--	--
4	-+	++	++	++	++	-+	--	--	--	--	--	--
5	++	++	+-	++	++	++	--	--	--	--	--	--
6	++	++	++	++	++	++	--	--	--	--	--	--
7	++	-+	++	++	++	++	--	--	--	--	--	--
8	++	--	--	++	++	++	--	--	--	--	--	--
9	--	--	--	--	++	--	++	+-	--	++	++	++
10	--	--	--	--	--	--	bac	--	--	+-	bac	+-
11	++	--	--	--	--	--	bac	--	--	--	--	--
12	--	--	--	--	--	--	--	--	--	--	--	--
13	--	--	--	--	--	++	--	--	--	--	--	--
14	--	--	--	--	--	*	--	--	--	--	--	*
15	--	--	--	--	--	*	--	--	--	--	--	*
16	--	--	--	--	--	*	--	--	--	--	--	*
21	--	--	--	--	--	*	--	--	--	--	--	*
total	7	4	4	6	7	7	1	1	0	2	1	2
Ø		5			6,7			0,7			1,7	

Table 1: Virus isolation from buffy coat preparations and nasal swabs of animals infected with New York '93/C or XIKE-A. + virus detected, - no virus detected, bac = bacteria, *animal was euthanized on day 13 p.i.

All animals in both groups developed fever (Fig. 5) and a broad spectrum of clinical signs including respiratory symptoms and gastrointestinal disorders. Animal #091 was killed on day 13 p.i. for welfare reasons. All calves in both groups showed leukopenia starting on day 3 p.i. and persisting for up to day 15 p.i. (Fig. 6). Virus was detected in buffy coat preparations from animals infected with NY'93/C for 5 days, and with XIKE-A for 7 days. Nasal shedding was found for 1 or 2 days (Table 1).

The identity of the viruses was checked by nucleotide sequencing of RT-PCR products from RNA prepared from buffy coat preparations from all animals. The entire E^{ms} coding region (positions 1140 to 1780) was sequenced and found to be identical with the known sequences of NY'93/C or XIKE-A, respectively. Neutralizing antibodies were found in the serum of all calves starting on day 14 p.i. (Table 2).

days p.i.	615	377	091	275	612	1610
-26	<2	<2	<2	<2	<2	<2
0	<2	<2	<2	<2	<2	<2
7	<2	<2	<2	<2	<2	<2
13/14	645	323	406	256	128	40
21	1024	1290	*	1290	512	51
29	2580	4096	*	813	2580	2580
35	3251	3251	*	8192	2580	5161

* animal were euthanized on day 13

Table 2: Neutralizing antibody titres determined in serum samples of all calves after experimental infection with New York '93/C or XIKE-A. Results are expressed as the reciprocal of the serum BVDV-specific neutralizing antibody titers against New York '93/C ($10^{2,07}$ $TCID_{50}$).

The results of this study demonstrated that the recombinant virus XIKE-A is highly similar to the wild type virus NY'93/C with regard to both pathogenicity and an the induction of an immune response in the natural host. It is therefore plausbile to assume that any deviation from this clinical picture that might be observed in a virus mutant generated on the basis of the infectious clone pKANE40A would indeed be caused by the desired mutation.

Animal experiment with XIKE-B and XIKE-A. In the second animal experiment, the clinical and immunological characteristics of the RNAse negative mutant XIKE-B were analysed in comparison with XIKE-A. The H349Δ mutant was given precedence over the H300L mutant to minimize the danger of a genomic reversion to wildtype.

Two groups of three calves (7 to 10 weeks old) each were inoculated with a dose of $5x10^5TCID_{50}$ of either XIKE-A (animals #387, #388, #418) or XIKE-B virus (animals #415, #417, #419). The groups were housed in separate isolation units. Rectal temperatures and clinical symptoms were monitored daily; nasal swabs and blood samples were taken on days -8, 0, 2 to 14, 17 and 21. Serum samples were collected on days 0, 8, 12 /14, 21, 28 and 38/40.

Nine to ten days post infection, the calves infected with XIKE-A developed fever for up to 3 days; in addition animal #387 had fever on day 3 p.i. (Fig. 7) that was accompanied by diarrhea and respiratory symptoms. Calf #388 showed convulsions. The group was euthanized for welfare reasons on day 12 p.i. in a state of marked

depression and anorexia. None of the calves infected with XIKE-B had elevated body temperatures (Fig. 7). Only mild respiratory symptoms were observed for up to 6 days. Leucopenia was found in all animals; however, the decrease of leucocyte numbers was more pronounced in the calves infected with wild type XIKE-A than in the XIKE-B group (Fig. 8).

Virus was found in buffy coat preparations of all animals starting on day 4 p.i.; however, viremia was shorter for the E^{ms} mutant (Ø 4 days) than for the virus with wild type sequence (Ø 8 days). Nasal shedding of virus could be observed for up to 8 days (Ø 4,7) with XIKE-A animals, but for a maximum of 1 day (Ø 0,7) with XIKE-B animals (Table 3).

	Virus Isolation from buffy coat preparations						Virus Isolation from nasal swabs					
Days p.i.	#415	#417	#419	#387	#388	#418	#415	#417	#419	#387	#388	#418
-8	–	–	–	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–
4	+-	+-	–	++	++	++	–	–	–	–	–	–
5	++	+-	+-	++	++	++	–	–	–	–	–	+-
6	++	+-	++	++	++	++	–	+-	–	–	–	+-
7	++	++	++	++	++	++	–	–	+-	+-	–	+-
8	–	+-	–	++	++	++	–	–	–	–	–	+-
9	–	–	–	++	+-	++	–	–	–	++	++	+-
10	–	–	–	++	+-	+-	–	–	–	+-	+-	++
11	–	–	–	++	+-	++	–	–	–	+-	–	+-
12	–	–	–	–	–	–	–	–	–	–	–	++
13	–	–	–	*	*	*	–	–	–	*	*	*
14	–	–	–	*	*	*	–	–	–	*	*	*
17	–	–	–	*	*	*	–	–	–	*	*	*
21				*	*	*	–	–	–	*	*	*
total	4	5	3	8	8	8	0	1	1	4	2	8
Ø		4			8			0,7			4,7	

Table 3: Virus isolation from buffy coat preparations and nasal swabs of animals infected with the recombinant virus XIKE-A (animals #387, #388 and #418) or the E^{ms} mutant XIKE-B (animals #415, #417 and #419). +virus detected, -no virus detected, *animals were euthanized on day 12 p.i.

Again, nucleotide sequencing of RT-PCR products encompassing the entire E^{ms} coding region was used for virus identification in buffy coat preparations. As expected, isolates from animals #387, #388 and #418 were wild type. A deletion of the "H349" codon was confirmed for animals #415, #417 and #419. Interestingly, an additional point mutation was found in RT-PCR products from two of these animals (#415 and #419): nucleotide position 1246 was changed from guanin to thymin, resulting in the amino acid substitution Q287H. Neutralizing antibodies were first detected on day 12 p.i. in the

serum of the calves infected with XIKE-A, and on day 14 p.i. in the serum of calves infected with the E^{ms} mutant (Table 4).

days p.i.	387	388	418	415	417	419
0	<2	<2	<2	<2	<2	<2
8	<2	<2	<2	<2	<2	<2
12/14	20	8	128	51	203	64
21	*	*	*	512	1024	406
28	*	*	*	2048	1024	4096
38/40	*	*	*	8182	4096	4096

* animals were euthanized on day 12

Table 4: Neutralizing antibody titres determined in serum samples of all calves after experimental infection with XIKE-A (wild type sequence) or XIKE-B (H346?). Results are expressed as the reciprocal of the serum BVDV-specific neutralizing antibody titers against New York '93/C ($10^{1,7}$ $TCID_{50}$).

Example 2

Experimental design

Twelve pregnant heifers were selected from a BVDV negative herd. The following group of 5 / 7 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	NY-93

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 with 2.5×10^4 $TCID_{50}$/ml of the respective virus applied in 6ml tissue culture supernatant.

Heifers were monitored for the presence of clinical signs of BVDV infection including abortions during the observation period. 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.

Results:

Group 1

Animal No.	Conclusion
526	BVD abortion
598	BVD abortion
615	BVD abortion
618	BVD abortion
626	Heifer Died due to BVD

Group 2

Animal No.	Conclusion
184	Heifer Died due to BVD
203	BVD abortion
232	Heifer Died due to BVD
233	Foetus BVD positiv (viremic)
252	BVD abortion
267	Heifer died due to BVD
306	BVD abortion

Example 3:

The study aimed to assess the efficacy of BVDV isolates against foetal infection. Efficacy of the NY93 infectious copy derivative BVDV recombinant (type II) with a deletion of the Rnase function in the E(RNS) protein XIKE-B (H349Δ) is investigated to prevent fetal infection after an heterologous type I challenge.

Between day 60 and 90 is the most sensitive period for fetal exposure to BVDV. Therefore in this trial heifers derived from BVDV-free farm (and confirmed seronegative

for BVDV) have been immunized by a single exposure with XIKE B (i.m.). Thereafter heifers were inseminated and between day 60-90 animals, when animals are supposed to be highly sensitive to BVDV fetal infection, a challenge infection with a wild type field virus was performed. The intranasal route for challenge was chosen as this mimics the normal route of infection in the field best.

Experimental design:

Heifers were selected from a BVDV negative herd. The heifers were tested serologically and virologically negative for BVDV. The following groups of heifers were included in the trial:

Group	Treatment	Challenge	No. of heifers:	
		BVDV	Vaccinated	Challenged
1	None	Type I	NA	2
2	Isolate XIKE-B	Type I	10	4

Group 1 remained untreated in the herd of origin until challenge. Blood samples were collected post-vaccination for buffy coat preparation and serology.

Inseminations started 4 weeks after immunisation for all groups. Group 1 was transported to the experimental facility before challenge.

Heifers were challenged 4 months and 10 days after vaccination. At the day of inoculation, pregnancy status was between day 60 and 90 of pregnancy.

The prevention of foetal infection was the main evaluation parameter.

Sequence of events and time schedule

Immunisation	10 days after transport to the animal facility
Insemination	In a period of 30 days, started approximately 4 weeks after Immunisation
Second transport to the challenge facility	At least 10 days before challenge
Challenge	Between day 60 to 90 of pregnancy
Observations	Continuous for about 2 months
Slaughter of animals and harvest of foetuses for virus isolation testing of fetal organ samples	About 2 months post-challenge

BVDV challenge viruses

The virus is grown in BVDV free medium as appropriate, aliquoted and frozen at –70 °C [±10 C].

Type / designation::	Type I / ncp KE#9
Passage level:	4
Composition:	Isolate obtained from German field
Challenge dose:	10^5 per animal
Applied volume:	6 ml per animal (3ml per nostril)
Inoculation route:	Intranasal

Vaccinations

The vaccination schedule is described in the Experimental Design Section.

Description:	XIKE-B, live virus BVDV strain
Passage number:	10
Virus dose:	10^5 per animal
Applied vaccine volume:	2 ml per animal
Application route:	Intramuscular (i. m.)

Results:

Rectal temperatures

The temperature values were below 39°C in all but one cases, and no unusual fluctuations were seen during the observation period. Heifer N° 1249 (Group 1) had a temperature of 39.1°C on 14 DPI (=days post infection) that returned to normal value on the next day.

Leukocyte Counts

0 DPI values were considered as individual baseline for comparison. No lower limit of leukocyte counts was defined in the study protocol. However, a reduction of leukocyte counts by 40% or more, i.e., values reaching 60% of the baseline value (established on the day of challenge) or lower, was considered biologically significant.

Individual mean leukocyte counts are shown in Table 1 below.

Table 1. Individual mean leukocyte counts

Group 1

ID No.	Days post infection (DPI)															
	0*	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
1249	11,2	12,3	7,6	6,9	6,6	12,8	11,3	10,4	9,6	8,6	11,2	12,2	12,0	11,8	10,4	7,5
1126	10,0	8,3	6,1	6,1	6,5	13,2	14,2	11,0	11,7	8,9	12,6	11,4	9,9	11,9	12,4	8,3

Group 2

1200	13,2	13,1	15,2	14,8	17,3	13,7	16,5	12,8	10,6	11,5	13,3	13,2	13,8	11,2	10,1	11,8
1217	9,4	8,0	9,4	14,3	10,4	11,1	15,0	10,4	8,1	8,2	12,7	10,8	11,8	10,3	12,2	8,0
1197	11,3	11,1	12,5	11,8	8,4	11,7	9,0	6,4	8,5	8,8	9,5	12,2	12,1	9,1	9,8	8,5
1214	8,9	9,4	9,2	10,2	12,8	7,5	10,6	7,7	7,6	11,1	10,7	10,9	8,8	9,1	7,8	10,2

*O day samples were collected on the day before infection

Baseline leukocyte counts were similar in all groups. While both heifers in Group 1 (infected with Type I strain) experienced a biologically significant reduction in leukocyte counts (values highlighted with grey colour) after the challenge (maximum drop noted 4 – 8 DPI), the corresponding vaccinated heifers (Group 2) had no remarkable falls in leukocytes. The only exception was heifer N° 1197 who showed a significant decrease

on a single day, on Day 14 PI. On the very next day, leukocyte count returned to what was considered normal (less than 40 % deviation from baseline).

Virus isolation data

Methods applied for virus isolation investigations are detailed in previous examples.

Virus isolation data from buffy coats (described as day post infection (=DPI) with vaccine candidate (XIKE B):

Group	Animal ID	0 DPI	2 DPI	4 DPI	6 DPI	8 DPI	10 DPI	12 DPI	14 DPI
1	1126	-	-	+	+	+	+	-	+
1	1249	-	-	-	-	+	+	-	-
2	1197	-	-	-	-	-	-	-	-
2	1200	-	-	-	-	-	-	-	-
2	1214	-	-	-	-	-	-	-	-
2	1217	-	-	-	-	-	-	-	-

Group	Animal ID	16 DPI	18 DPI	20 DPI	22 DPI	24 DPI	26 DPI	28 DPI	30 DPI
1	1126	+	-	-	-	-	-	-	-
1	1249	-	-	-	-	-	-	-	-
2	1197	-	-	-	-	-	-	-	-
2	1200	-	-	-	-	-	-	-	-
2	1214	-	-	-	-	-	-	-	-
2	1217	-	-	-	-	-	-	-	-

Virus isolation from fetal organs:

Group	Animal ID	Mesenteric lymph nodes	Small intestine	Spleen	Thymus	kidney	Sternum bone marrow	cerebellum	placenta
1	1126	+	+	+	+	+	+	+	+
1	1249	+	+	+	+	+	+	+	+
2	1197	-	-	-	-	-	-	-	-
2	1200	-	-	-	-	-	-	-	-
2	1214	-	-	-	-	-	-	-	-
2	1217	-	-	-	-	-	-	-	-

All heifers did not show any clinical symptomes typical for BVDV infection after vaccination with XIKE B. After challenge heifers of group 1 had on at least one day viremia, whereas in group 2 on no day after challenge viremia could be detected.

All fetuses from heifers of group 1 were positive for BVDV (all of the following organs were positiv tested for BVDV by virus isolation (mesenteric lymph nodes; small

intestine, spleen, thymus, kidney, sternum, bone marrow, cerebellum); the fetuses from from heifers of group 2 were all negative (in all tested organs consistently: mesenteric lymph nodes; small intestine, spleen, thymus, kidney, sternum, bone marrow, cerebellum) for BVDV.

Therefore infectious copy derived virus was attenuated successfully and the potential of the use as vaccine virus in order to prevent fetal infection was shown.

The XIKE B virus belongs antigenetically to the BVDV type II viruses and is effective in preventing fetal infection after challenge with an heterologous challenge virus belonging to the BVDV type I antigenic group.

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Claims

1. DNA molecule containing a nucleotide sequence complementary to a BVDV RNA, wherein said RNA, when introduced into susceptible host cells, induces the generation of infectious BVDV particles
   a) with the capability to induce viraemia and leukopenia in calves for a period of at least 1 day and at least one of the following clinical symptoms of the group comprising diarrhea and/or pyrexia lasting at least one day when infected with a dose of $6x10^6TCID_{50}$; and/or
   b) with authentical virulence as compared to a wild-type BVDV isolate from which such DNA molecule has been derived; and/or
   c) which are, when BVDV naive calves are infected at a dose of $6x10^6TCID_{50}$ with such particles, lethal for at least 30 % of such calves within a period of 21 days; and/or
   d) with a virulence of not less than 90 % of BVDV particles comprising an RNA with a sequence complementary to SEQ ID NO. 1; and/or
   e) comprising a sequence complementary to SEQ ID NO. 1.
2. Infectious BVDV clone, capable of serving as a template for transcription into an RNA, wherein said RNA, when introduced into susceptible host cells, induces the generation of infectious BVDV particles
   f) with the capability to induce viraemia and leukopenia in calves for a period of at least 1 day and at least one of the following clinical symptoms of the group comprising diarrhea and/or pyrexia lasting at least one day when infected with a dose of $6x10^6TCID_{50}$; and/or
   g) with authentical virulence as compared to a wild-type BVDV isolate from which such DNA molecule has been derived; and/or
   h) which are, when BVDV naive calves aged from 3 to 6 months are infected at a dose of $6x10^6TCID_{50}$ with such particles, lethal for at least 30 % of such calves within a period of 21 days after infection; and/or
   i) with a virulence of not less than 90 % of BVDV particles comprising an RNA with a sequence complementary to SEQ ID NO. 1; and/or
   j) comprising a sequence complementary to SEQ ID NO. 1.

3. DNA molecule according to claim 1, wherein the pyrexia of step a) is at least 40 °C.

4. BVDV particle generated by transcription using the DNA molecule according to claim 1 or 3 or the BVDV clone according to claim 2, the transfection of suitable cells or cell lines with said RNA and the collection of the resulting BVDV particles produced by said cells.

5. Infectious BVDV type 2 clone.

6. Infectious BVDV type 2 clone, capable of serving as a template for transcription into an RNA, wherein said RNA, when introduced into susceptible host cells, induces the generation of infectious BVDV particles

k) with the capability to induce viraemia and leukopenia in calves for a period of at least 1 day and at least one of the following clinical symptoms of the group comprising diarrhea and/or pyrexia lasting at least one day when infected with a dose of $6x10^6TCID_{50}$; and/or

l) with authentical virulence as compared to a wild-type BVDV isolate from which such DNA molecule has been derived; and/or

m) which are, when BVDV naive calves aged from 3 to 6 months are infected at a dose of $6x10^6TCID_{50}$ with such particles, lethal for at least 30 % of such calves within a period of 21 days after infection; and/or

n) with a virulence of not less than 90 % of BVDV particles comprising an RNA with a sequence complementary to SEQ ID NO. 1; and/or

o) comprising a sequence complementary to SEQ ID NO. 1.

7. Infectious BVDV type 2 clone according to claim 5 or 6, as characterized by the DNA sequence of SEQ ID NO. 1 or a fragment, functional variant, variant based on the degenerative nucleic acid code, fusion molecule or a chemical derivative thereof.

8. BVDV type 2 particle generated by *in vitro* transcription of the BVDV clone according to any one of claims 5 to 7 into RNA, the transfection of suitable cells or cell lines with said RNA and the collection of the resulting BVDV particles produced by said cells.

9. DNA molecule containing a nucleotide sequence complementary to a full-length BVDV type 2 RNA.

10. DNA molecule according to claim 9, characterized by SEQ ID No. 1 or a fragment, functional variant, variant based on the degenerative nucleic acid code, fusion molecule or a chemical derivative thereof.

11. DNA molecule according to claim 9 or 10, consisting of the sequence as characterized by SEQ ID No. 1.

12. RNA molecule complementary to the DNA molecule of claim 1 or claim 3 or any one of claims 9 to 11, or to the BVDV clone of claim 2 or claim 4 or claim 5 to 7.

13. RNA molecule obtainable by transcription of the DNA molecule of claim 1 or claim 3 or any one of claims 9 to 11, or the BVDV clone of claim 2 or claim 4 or claim 5 to 7.

14. Method for the production of an infectious BVDV clone from a wild-type BVDV isolate, said infectious BVDV clone being complementary to a RNA having authentical virulence as compared to said wild-type isolate, comprising the steps of

p) isolating viral particles from an infected animal;

q) passaging not more than twice on suitable cell culture cells;

r) preparing RNA from the viral particles;

s) generating full-length complementary DNA after reverse transcription of the RNA; wherein the reverse transcription includes a step at elevated temperatures sufficient to break or reduce secondary structures of the RNA, and the use of a thermostable enzyme for this step, said enzyme being active at these elevated temperatures;

t) incorporating the complementary DNA (cDNA) into a plasmid vector or into a DNA virus capable of directing the transcription of BVDV cDNA into RNA upon infection of suitable cells.

15. Method for the production of an infectious BVDV clone from a wild-type BVDV isolate, said infectious BVDV clone being complementary to a RNA having a virulence of not less than 90 % of said wild-type isolate, comprising the steps of

u) isolating viral particles from an infected animal;

v) preparing RNA from the viral particles;

w) generating full-length complementary DNA after reverse transcription of the RNA; wherein the reverse transcription includes a step at elevated temperatures sufficient to break or reduce secondary structures of the RNA,

23. Attenuated BVDV clone or BVDV strain obtainable by a method according to any one of claims 18 to 22.

24. Vaccine comprising an attenuated BVDV clone or strain according to claim 23, optionally in combination with a pharmaceutically acceptable carrier or excipient.

25. Use of a attenuated BVDV clone or strain according to claim 23 in the manufacture of a vaccine for the prophylaxis and treatment of BVDV infections.

Sequenzlisting1249.ST25.txt
SEQUENCE LISTING

<110> Boehringer Ingelheim Vetmedica GmbH

<120> Infectious bovine viral diarrhea virus clone

<130> not yet assigned

<150> DE10143813.3
<151> 2001-09-06

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<170> PatentIn version 3.1

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and the use of a thermostable enzyme for this step, said enzyme being active at these elevated temperatures;

x) incorporating the complementary DNA (cDNA) into a plasmid or into a DNA virus capable of directing the transcription of BVDV cDNA into RNA upon infection of suitable cells.

16. The method of claim 14 or 15, wherein the 5' end of the RNA is transcribed using RACE.

17. The method of claim 16, wherein RACE is carried out with a thermostable polymerase allowing reaction temperatures of at least 42°C.

18. Method of BVDV attenuation, or comprising introducing one or more mutations into the DNA molecule of claim 1 or claim 3 or any one of claims 9 to 11, or into the infectious BVDV clone of claim 2 or claim 4 or claim 5 to 7, wherein said mutation or mutations lead to or increase an attenuated phenotype of the recovered BVD virus.

19. Method of attenuation of a BVDV strain, comprising the steps of

y) introducing one or more mutations into the DNA molecule of claim 1 or claim 3 or any one of claims 9 to 11, or into the infectious BVDV clone of claim 2 or claim 4 or claim 5 to 7;

z) introducing the mutated DNA into susceptible host cells wherein said DNA is transcribed into RNA or introducing an RNA transcribed from said DNA into said cells; and

aa) collecting viral particles produced by these cells;

wherein said mutation or mutations results in attenuation.

20. Method of claims 18 or 19, wherein the mutation or mutations is a substitution, deletion, insertion, addition, or combination thereof.

21. Method of any one of claims 18 to 20, wherein the mutation or mutations is in the glycoprotein E^{ms} and causes impaired or loss of function of the mutated protein(s).

22. Method of claim 21, wherein the mutation consists of

bb) deletion of all or part of the glycoprotein E^{ms}; and/or

cc) deletion or substitution of histidine at position 300 of SEQ ID NO. 1; and/or

dd) deletion or substitution of histidine at position 349 of SEQ ID NO. 1.

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