(54) Title: BOVINE HERPESVIRUS VACCINE

(57) Abstract: The present invention relates to mutant Bovine Herpesvirus 4 genomes, mutant Bovine Herpesvirus 4 viruses, to mutant Bovine Herpesvirus 4 genomes and viruses carrying a heterologous DNA sequence, cells transfected with mutant Bovine Herpesvirus 4 genomes, cells infected with mutant Bovine Herpesvirus 4 viruses, vaccines and carrier vaccines comprising such mutant Bovine Herpesvirus 4 viruses, methods for the preparation of such mutant Bovine Herpesvirus 4 genomes and such mutant Bovine Herpesvirus 4 viruses and to diagnostic test kits.

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Bovine herpesvirus vaccine

The present invention relates to mutant Bovine Herpesvirus 4 genomes, mutant Bovine
Herpesvirus 4 viruses, to mutant Bovine Herpesvirus 4 genomes and viruses carrying a
5   heterologous DNA sequence, cells transfected with mutant Bovine Herpesvirus 4 genomes, cells
infected with mutant Bovine Herpesvirus 4 viruses, vaccines and carrier vaccines comprising
such mutant Bovine Herpesvirus 4 viruses, methods for the preparation of such mutant Bovine
Herpesvirus 4 genomes and such mutant Bovine Herpesvirus 4 viruses and to diagnostic test kits.

10   Bovine Herpesvirus 4 (BoHV-4) is a member of the subfamily *gammaherpesvirinae*, genus
rhadinovirus, that is distributed worldwide amongst i.a. ruminants. Isolates have been recovered
from cattle, but also from American bison and African buffalo. Isolates have been found also in
sheep. Sporadic isolations were reported in lions, cats and owl monkeys.

15   The virus has been isolated from both healthy and from sick animals. However, although
sporadically a possible role for BoHV-4 in respiratory disease has been reported, by far most
isolates of the BoHV-4 group fail to induce clinical disease in both cattle and in laboratory
animals. Therefore, the pathogenic role of BoHV-4 remains unclear.

20   BoHV-4, although being a member of the subfamily *gammaherpesvirinae*, differs from other
members of this subfamily in that, unlike most other *gammaherpesvirinae*, it replicates in a
variety of primary cultures and cell lines of bovine and various other animal species.
In addition, there is no evidence for oncogenicity or growth transformation by BoHV-4.
Moreover, BoHV-4, like all herpesviruses is capable of accommodating significant amounts of
25   foreign genetic material.
Finally, as mentioned above, apparently BoHV-4 does not seem to induce clinical disease.
These characteristics make BoHV-4 attractive for use as a vector virus.
Such use has been suggested i.a. by Donofrio, G. et al., (J. virol. Methods 101: 49-61 (2002),
Donofrio, G. et al., (Microb. Infect. 8: 898-904 (2006)), Gillet, L. et al., (J. Gen. Virol. 86: 907-
30   917 (2005)).

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A recent example of such use is given by Donofrio, G. et al., (Clinical and Vaccine Immunology 13: 1246-1254 (2006)). In this paper, it is described how BoHV-4 is used for the expression of Glycoprotein D of Bovine Herpesvirus 1, an alphaherpesvirus that is known to be a major pathogen in cattle, that causes significant losses worldwide. BoHV-1 infection is known to cause

5   infectious bovine rhinothracheitis, infectious pustular vulvovaginitis, balanoposthitis, abortion and generalized systemic infection. BoHV-1 is also known to play an important role in the bovine respiratory disease complex commonly referred to as shipping fever. This work is one of the first demonstrations of the use of BoHV-4 as a vector for vaccine purposes and provides a firm basis for BoHV-1-vaccination of cattle by using BoHV-4 as a vector.

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There is however one serious drawback:

Gammaherpesviruses like all *Herpesviridae* exhibit two distinct phases in their life cycle; lytic replication characterised by a transcription program where immediate-early (IE), early (E) and late (L) genes are expressed successively; and latency, characterised by the maintenance of

15   the viral genome as a non-integrated episome and the expression of a limited number of viral genes and micro RNAs (Roizman & Pellett, 2007; Weck *et al.*, 1999; Sarid *et al.*, 1998; Cai *et al.*, 2005). Upon reactivation, latency turns into a lytic replication again and re excretion of infectious particles by latently infected subjects.

20   Therefore, since BoHV-4 has the property to establish latency and to reactivate, this makes wild type strains inappropriate to develop safe recombinant vectors. Indeed, throughout their live, infected animals could reactivate and contaminate naive animals.

Thus, from a vaccine point of view, for BoHV-4 to be used as a vector, the latency of the virus is considered to be a disadvantage.

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The gammaherpesvirus human Herpesvirus 8 (HHV-8 also named KSHV for Kaposi's sarcoma-associated Herpesvirus) is the prototype of the *rhadinovirus* genus. During latency, HHV-8 expresses three main open reading frames (ORFs): ORF71, ORF72 and ORF73 (Sarid *et al.*,1998 and 1999). ORF71 and ORF72 encode viral homologues of cellular FLIP (v-FLIP)

30   (Thome *et al.*, 1997) and D cyclin (v-CYC) (Li *et al.*, 1997), respectively. ORF71 and ORF72 are expressed together with ORF73 on a polycistronic mRNA.

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ORF73 orthologues encoded by different rhadinoviruses have been studied extensively. These studies revealed that the expression product of ORF73 (pORF73) is a multifunctional protein essential for latency. The multiple functions of the protein rely on several molecular domains (Renne *et al.*, 2001; Fowler et al., 2003; Calderwood *et al.*, 2005). The best-characterised pORF73 is the Latency-Associated Nuclear Antigen 1 (LANA-1) of HHV-8. LANA-1 has been shown to associate with mitotic chromosomes to enable episome tethering during division of latently infected cells and its subsequent partitioning between daughter cells (Ballestas *et al.*, 1999; Barbera *et al.*, 2006; Ye *et al.*, 2004). This crucial role in latency is reinforced by the ability of LANA-1 to modulate cellular pathways implicated in cell growth and survival by targeting transcriptional regulators or co-regulators such as p53, pRB, CBP and sin3A (Friborg *et al.*, 1999; Krithivas *et al.*, 2000; Radkov *et al.*, 2000; Lim *et al.*, 2001). Moreover, LANA-1 prevents reactivation from latency by downregulating the expression of ORF50 that encodes the replication and transcriptional activator (RTA) (Lan *et al.*, 2004, 2005; Lu *et al.*, 2006).

BoHV-4 behaves different in many respects, as summarised above, when compared to other herpesviruses, even within the subfamily *gammaherpesvirinae*. Nevertheless, if any ORF73-orthologue exists in BoHV-4, it could then be possible to find out if such ORF73-orthologue, if existent at all, in BoHV-4 would play a comparable role in latency.

However, the only possibly ORF73-like orthologue gene found in BoHV-4 was considered to be a pseudo gene, for the following reasons:

1) The ORF73 orthologue region of BoHV-4 is the site of multiple recombinations (Dewals B., et al., J. Gen. Virol. 87: 1509-1519 (2006)). This is a characteristic of a pseudo gene, but certainly not of a multi-functional gene that is highly depending on full conservation of its sequence.

2) ORF73 orthologue length variations are observed between various BoHV-4 strains. E.g. a short duplication is observed in the M40 strain. Again this is fully in line with the characteristics of a pseudo gene, but it is highly unusual for a functional, let alone a multi-functional gene.

3) ORF73 of HHV-8 encodes a large and multifunctional protein, that has been shown a) to associate with mitotic chromosomes to enable episome tethering during division of latently

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infected cells and its subsequent partitioning between daughter cells, b) to modulate cellular pathways implicated in cell growth and survival by targeting transcriptional regulators or co-regulators such as p53, pRB, CBP and sin3A, and c) to prevent reactivation from latency by downregulating the expression of ORF50 that encodes the replication and transcriptional

5   activator.

Within the rhadinovirus genus, BoHV-4 appeared to encode by far the shortest ORF73 orthologue with a size equivalent to only 22% of ORF73 of HHV-8 (LANA-1) (Fig. 1). BoHV-4 ORF73 consists of only 253 amino acids. Only the 138 N-terminal and the 102 C-terminal amino acid residues exhibit some homology to LANA-1 N- and C-terminal regions, respectively. All

10   other regions are simply missing. Consequently, it was not to be expected that the extremely shortened ORF73 orthologue found in BoHV-4, lacking 78% of the essential structures of LANA-1 including the P-rich domain, the D/E-rich domain, the Q/E-rich domain and the L-zipper, could act as a multifunctional protein having the characteristics of LANA-1.

4) By far most other known ORF73 orthologues are not much smaller in size than LANA-

15   1, or even bigger. Only one relatively small ORF73 orthologue has been described: For murine herpesvirus-68, an ORF73 orthologue has been found. This ORF73 is however still 123% of the size of the BoHV-4 orthologue, and most strikingly, the percentage of amino acid identity is practically negligible: only 25%.

20   Surprisingly it was found now, that this alleged pseudo gene for unknown reasons plays a role that, with regard to its function in latency, appears to be comparable to that of ORF73 in LANA-1. This gene will for reasons of convenience be further referred to as an ORF73 orthologue.

25   Moreover, it was surprisingly found that deletion of BoHV-4 ORF73 did not affect the capacity of the virus to replicate *in vitro*, while it prevented latent infection *in vivo*. All together, the results of the present invention show that despite its extremely small size and the striking lack of homology to any known ORF73-orthologue, BoHV-4 ORF73 is a functional homologue of larger rhadinovirus orthologues. The sequence of BoHV-4 ORF73 is available from

30   Genbank/EMBL/DDBJ accession numbers AY847342-AY847348.

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Thus, it is one of the merits of the present invention that it provides a BoHV-4 virus from which the ORF73 gene is deleted, thus providing a vector with all advantages of BoHV-4 vector viruses without having the disadvantage of causing latent infection.

5   Thus, a first embodiment of the present invention relates to a mutant BoHV-4 genome that has as a characteristic that it does not encode a functional ORF73 protein.

A functional ORF73 protein is a protein that is still capable of causing latency.

10   A second embodiment of the present invention relates to a mutant BoHV-4 virus that it is not capable of making a functional ORF73 protein.

Preferably, the mutant BoHV-4 virus is not capable of making a functional ORF73 protein because it has a mutation in the ORF73 gene.

15   Therefore, a preferred form of this embodiment relates to mutant BoHV-4 virus according to the invention that has as a characteristic that it has a mutation in the ORF73 gene.

A mutation can be an insertion, a deletion or a frame shift mutation. The easiest way of making a mutation is by means of inserting a stretch of nucleotides or by deleting a part or the whole of the

20   ORF73 coding region.
Therefore, a more preferred form of this embodiment relates to mutant BoHV-4 virus wherein said mutation is a deletion or an insertion.

It is known that several genes play a role in the virulence of herpesviruses in general, and in

25   BoHV-4 too. The deletion of genes encoding such virulence factors improves the safety of the virus, because it behaves less virulent than the wild-type virus. Therefore, especially when using the mutant BoHV-4 virus according to the invention as a vaccine or a carrier vaccine, it may be desirable to delete one or more of such virulence factors. Such deletions are for the purpose of this patent herewith defined as second deletions.

30   Thus, an even more preferred form of this embodiment relates to mutant BoHV-4 virus according to the invention that comprises a second deletion.

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Preferred second deletions are deletions in the ORF71-gene encoding a v-FLIP, the ORF16-gene encoding a v-Bcl-2 homologue, the Bo10 gene encoding glycoprotein gp80 or the ORF21-gene encoding thymidine kinase.

Therefore, a most preferred embodiment of the mutant BoHV-4 virus according to the invention

5   relates to such viruses having a second deletion in the ORF71-gene encoding a v-FLIP, the ORF16-gene encoding a v-Bcl-2 homologue, the Bo10 gene encoding glycoprotein gp80 or the ORF21-gene encoding thymidine kinase.

Although the mutant BoHV-4 virus according to the invention can be used for vaccine purposes

10   as such, merely in order to prevent susceptible animals, such as ruminants from BoHV-4 infection, it is most efficiently used as carrier virus for heterologous DNA sequences. In that case, the advantageous characteristics of the mutant BoHV-4 virus according to the invention would be fully used and in addition the virus would e.g. gain additional immunizing or adjuvating properties.

15   Thus, a third embodiment of the present invention relates to a mutant BoHV-4 virus according to the invention, that comprises a heterologous DNA sequence.

Preferably, such a heterologous DNA sequence encodes an immunogenic protein of another virus or microorganism that is pathogenic to animals that are sensitive to BoHV-4 infection, such as

20   ruminants. The advantage of such a mutant BoHV-4 virus would be that by administrating such virus to such animals, e.g. ruminants, one would be able to protect against BoHV-4, and against that other pathogenic virus or microorganism. And even if there would be no interest in obtaining protection against BoHV-4 infection, such a virus would have all the advantages of a very safe live carrier virus: it is a very safe life BoHV-4 vaccine that at the same time mimics a live

25   vaccine against that other (e.g. ruminant) pathogenic virus or microorganism. Merely as an example: a mutant BoHV-4 virus according to the invention would be a very attractive BoHV-1 gD carrier alternative instead of the BoHV-4 strain as used by Donofrio G., et al., in Clinical and Vaccine Immunology 13: 1246-1254, (2006).

Also, preferably such heterologous DNA sequence encode a cytokine. Cytokines comprise i.a.

30   the interleukins, interferons and tumor necrosis factors. Several cytokines, e.g. interferons are known to play an important role as immune modulators in mammals, e.g. ruminants. Thus it may be advantageous to include a gene encoding this kind of molecule into said virus.

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Thus, a preferred form of this embodiment relates to mutant BoHV-4 virus according to the
invention comprising a heterologous DNA sequence encoding an immunogenic protein of
another virus or microorganism pathogenic to animals that are sensitive to BoHV-4 infection, e.g.
ruminants, and/or a cytokine.

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The most common and/or important (i.a. ruminant) pathogenic microorganisms and viruses are
formed by the group consisting of Bovine Herpesvirus, Bovine Viral Diarrhoea virus,
Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella
haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria* sp., *Babesia* sp., *Trypanosoma*

10   *species, Anaplasma* sp*., Neospora caninum, Staphylococcus aureus, Streptococcus agalactiae,*
Mycoplasma, *E. coli, Enterobacter, Klebsiella, Citrobacter* and *Streptococcus dysgalactiae*.
Therefore, a more preferred form of this embodiment relates to mutant BoHV-4 virus according
to the invention wherein that virus or micro-organism is selected from the group consisting of
Bovine Herpesvirus, Bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine

15   Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory
Syncytial Virus, *Theileria* sp., *Babesia* sp., *Trypanosoma species, Anaplasma* sp*., Neospora
caninum, Staphylococcus aureus, Streptococcus agalactiae,* Mycoplasma, *E. coli, Enterobacter,
Klebsiella, Citrobacter* and *Streptococcus dysgalactiae.*

20   As said above, mutant BoHV-4 virus according to the invention is not capable of making a
functional ORF73 protein. This can i.a. be obtained by making an insertion in the gene. It would
therefore be most convenient to insert the heterologous DNA sequence directly into the ORF73
gene. That would avoid the necessity to find a suitable location in the viral genome to introduce
the heterologous DNA sequence and would at the same time make the gene encoding ORF73

25   nonfunctional.

Also from a safety point of view, it is very beneficial to insert the heterologous DNA sequence
directly into ORF73: if a recombination occurs between a wild type strain and the vaccine, the
recombinant that acquires the heterologous DNA sequence will also acquire the genetic defect of

30   the vaccine and thus not be able to persist as a latent virus.

Thus a most preferred form of this embodiment relates to mutant BoHV-4 virus according to the invention, wherein the heterologous DNA sequence is inserted in the ORF73 gene.

The heterologous DNA sequence encoding the antigen or cytokine is preferably placed under the
5   control of a functional promoter. A functional promoter is a promoter that is recognized in ruminant cells.

A large number of suitable promoters is known in the art, that are recognized for their very efficient level of expression. Such promoters are e.g. the Pseudorabies gX-promoter, the Pseudorabies TK-
10   promoter, the Adenovirus Major Late promoter, the Retroviral Long Terminal Repeat, the SV40 Early and Late promoters, the Mouse Cytomegalovirus immediate early (MCMViel) promoter, the Mouse Cytomegalovirus early (MCMVe1) promoter, the Human Cytomegalovirus immediate early (HCMViel) promoter, and the BHV-gE promoter. All these promoters have been described and are known in the art for many years. Merely as an example in Donofrio G., et al. describe the use of the
15   Human Cytomegalovirus enhancer-promoter for the expression of gD of BoHV-1. (Donofrio G., et al., Clinical and Vaccine Immunology 13: 1246-1254, (2006))

As extensively motivated above, the mutant BoHV-4 viruses according to the invention are very suitable as vaccine viruses or carrier vaccine viruses.
20   Therefore, again another embodiment of the present invention relates to vaccines for combating BoHV-4 infection in animals susceptible for BoHV-4 such as ruminants, wherein such vaccines comprise a mutant BoHV-4 genome according to the invention or a mutant BoHV-4 virus according to the invention, and a pharmaceutically acceptable carrier.

25   The pharmaceutically acceptable carrier can be as simple as water or a buffer. The pharmaceutically acceptable carrier may also comprise stabilizers. It can also comprise an adjuvant, or it can in itself be an adjuvant.

The vaccine can be safely stored for long periods of time when it is freeze-dried. Therefore, the
30   vaccine according to the invention is preferably in a freeze-dried form.

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Still another embodiment relates to mutant BoHV-4 carrier vaccines that comprise a BoHV-4 virus according to the invention comprising a heterologous DNA and a pharmaceutically acceptable carrier.

5   Again another embodiment of the invention relates to a mutant BoHV-4 genome according to the invention or mutant BoHV-4 virus according to the invention for use in a vaccine.

Still another embodiment of the invention relates to the use of a mutant BoHV-4 genome according to the invention or a mutant BoHV-4 virus according to the invention for the

10   manufacture of a vaccine for the protection of susceptible animals such as ruminants against BoHV-4 infection.

And again another embodiment of the invention relates to the use of a mutant BoHV-4 genome according to the invention or a mutant BoHV-4 virus according to the invention for the

15   manufacture of a BoHV-4 carrier vaccine.

As follows i.a. from the Examples, it is possible to make the necessary mutant BoHV-4 genome in vitro. If a cell is transfected with this genome, this leads to the generation of progeny virus. Thus, another embodiment of the present invention relates to a cell comprising a mutant BoHV-4

20   genome according to the invention.

The progeny virus in itself is fully infective, and therefore, that progeny virus can be propagated on susceptible cells, such as e.g. MDBK cells.
Therefore, another embodiment of the present invention relates to a cell comprising a mutant

25   BoHV-4 virus according to the invention.

For administration to animals, the BoHV-4 mutant according to the present invention can be given inter alia intranasally, intradermally, subcutaneously or intramuscularly. The routes and methods for vaccination can be the same as for other live attenuated BHV-viruses, more

30   specifically BoHV-4 vaccines.

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The useful effective amount to be administered will vary depending on the age, weight, mode of administration and type of pathogen against which vaccination is sought.

For most applications, a very suitable dosage range is between about $10^{3.0}$ - $10^{7.0}$ pfu/animal.

Another embodiment of the present invention relates to methods for the production of a mutant BoHV-4 genome according to the invention or a mutant BoHV-4 virus according to the invention, wherein that method comprises the step of mutating the ORF73 gene so that it no longer encodes a functional ORF73 protein.

An interesting effect of the fact that mutant BoHV-4 according to the invention can carry a heterologous gene, is that it can thus be used as a marker vaccine. A marker vaccine allows to discriminate between field viruses and vaccine viruses on the basis of differences in antibody panels raised against wild-type BoHV-4 and mutant BoHV-4 virus according to the invention when carrying an additional gene.

Sera from mammals infected with a mutant BoHV-4 strain according to the invention that carries a heterologous gene will react with immunogenic BoHV-4-proteins and with an immunogenic protein encoded by a heterologous gene inserted in mutant BoHV-4 according to the invention. Sera from mammals infected with an BoHV-4 field strain however will only react with immunogenic BoHV-4-proteins. Tests based upon purified protein encoded by the heterologous gene directly show the presence or absence of antibodies against the protein encoded by the heterologous gene. Therefore, mutant BoHV-4 viruses according to the present invention that carry a heterologous gene turn out to be very suitable marker vaccines, i.e. vaccines that can be serologically discriminated from a field strain. They can be discriminated by using specific diagnostic tests.

Therefore still another embodiment of the invention relates to diagnostic test kits for differentiating BoHV-4 field-virus infected mammals from mammals vaccinated with a vaccine comprising mutant BoHV-4 according to the invention and carrying a heterologous gene. Such tests comprise the testing of serum for the absence or presence of antibodies against the protein encoded by the heterologous gene.

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A diagnostic test kit for the differentiation between animals vaccinated with mutant BoHV-4 strains according to the invention and carrying a heterologous gene, and animals infected with BoHV-4 field strains can comprise e.g. a simple blocking-ELISA test in which a mutant BoHV-4 strain according to the invention and carrying a heterologous gene is coated to the wall of a

5   microtiter-plate. Such a plate is then incubated with serum of the animal to be tested. Subsequently, the plate is incubated with a specific and labeled antiserum against the heterologous protein. If the labeled antiserum reacts with the coated virus, i.e. it is not blocked by the antiserum of the animal to be tested, this shows that that antiserum does not contain antibodies against the heterologous protein. It can then be concluded that the animal was infected

10   by a field strain.

Alternatively, such a diagnostic test kit can comprise an ELISA-test in which possibly purified heterologous protein is coated to the wall of separate wells of an ELISA-plate. Incubation with serum from mammals to be tested, followed by e.g. incubation with a labeled antibody against

15   the heterologous protein can then reveal the presence or absence of antibodies against the heterologous protein in the respective wells. Another example of a diagnostic test system is e.g. the incubation of a Western blot comprising the heterologous protein with serum of mammals to be tested, followed by analysis of the blot.
Detection of specific anti-heterologous protein antibodies indicates that the tested mammal has

20   been vaccinated with a mutant BoHV-4 strain according to the invention and carrying a heterologous gene, whereas the lack of antibody-reaction with the heterologous protein band indicates that the mammal has been infected with a BoHV-4 field strain.

Therefore, in another embodiment the present invention relates to a diagnostic kit for the

25   detection of antibodies against a mutant BoHV-4 strain according to the invention and carrying a heterologous gene, in which the kit comprises purified heterologous protein or a fragment thereof still comprising an antigenic determinant.

Another approach for using mutant BoHV-4 virus according to the invention in marker vaccines

30   is to discriminate between field viruses and vaccine viruses on the basis of differences in reactions in PCR tests between wild-type BoHV-4 and mutant BoHV-4 virus according to the invention. Such a test in a very basic form could comprise a first set of PCR-primers against any

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wild-type BoHV-4 sequence and a second set of PCR-primers against a region of ORF73 that has been deleted from the mutant BoHV-4 virus according to the invention.

If blood or tissue from an animal shows a reaction with both the first and the second primer set, this indicates that the animal was infected with a field virus. If blood or tissue from an animal

5   however only shows a reaction with the first and not with the second primer set, this indicates that the animal was vaccinated with a mutant BoHV-4 virus according to the invention.

Merely to exemplify aspects of the invention, the Examples below will explain (without being restrictive in any way) various aspects of the invention in more detail.

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EXAMPLES

15   **EXAMPLE 1.**

Computational analysis. Protein sequences were analysed with ClustalX sequence alignment program (Thompson *et al.*, 1997).

20   **Cells and Viruses.** Madin-Darby bovine kidney cell line (MDBK; ATCC CCL-22) was cultured in minimum essential medium (MEM, Invitrogen) containing 5% fetal calf serum (FCS, Harlan). Embryonic bovine lung (EBL, German collection of micro-organisms and cell culture DSMZ ACC192), EBL stably expressing the Cre recombinase (EBL-NLS-Cre) (Gillet *et al.*, 2005) and bovine macrophage (BOMAC) (Stabel & Stabel, 1995) cell lines were cultured in

25   Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% FCS. The BoHV-4 V. test strain and the V. test BAC G derived bacterial artificial chromosome (BAC) clone were described elsewhere (Gillet *et al.*, 2005).

RT-PCR, determination of kinetic class of transcription. For the determination of kinetic

30   class of transcription, cycloheximide (CHX, 100µg/ml, Sigma) or phosphonoacetic acid (PAA, 300 µg/ml, Sigma) were added to the culture medium at the time of infection to inhibit *de novo* protein synthesis or viral DNA polymerase, respectively. 24 h post infection (p.i.) of MDBK cells

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(multiplicity of infection (MOI) of 1 plaque forming unit (PFU)/cell), cytoplasmic RNA was extracted using the RNeasy Mini Kit (Qiagen) with on-column DNase I digestion. Reverse transcription and PCR reactions were performed using the SuperScript III One-step RT-PCR System with Platinium *Taq* DNA polymerase Kit (Invitrogen). Gene-specific primers (Table S1)
5   were used to detect viral mRNAs. The absence of DNA template was verified by replacing the RT/*Taq* mix by the *Taq* DNA polymerase only.

Northern blotting. MDBK cells were mock-infected or infected with BoHV-4 V. test strain at a MOI of 4 PFU/cell. After an incubation period of 90 min, cells were washed with PBS and
10   then overlaid with MEM containing 5% FCS. Cells were scrapped at 4, 8 and 24h p.i.. RNA was then purified from the cells and analysed by northern blotting according to standard protocol (Sambrook & Russell, 2001). The entire ORF73 was used as probe to detect the RNAs encoding ORF73.

15   **Production of BoHV-4 ORF73 recombinant strains.** BoHV-4 recombinants were produced using BAC cloning and prokaryotic recombination technologies as described before (Gillet *et al.*, 2005). The V. test BAC G plasmid was used as parental plasmid (Gillet *et al.*, 2005). The V. test BAC G ΔORF73 and the V. test BAC G ORF73 revertant plasmids were produced using a two step galactokinase (*gal*K) positive/negative selection in bacteria (Warming *et al.*, 2005, Fig. 1).
20   The first recombination process (*gal*K positive selection) consisted to replace ORF73 by the *gal*K gene resulting in the V. test BAC G ΔORF73 plasmid. Recombination was achieved using the H1-*gal*K-H2 cassette (Fig. 1). It consisted of the *gal*K gene flanked by 60-bp sequences corresponding to ORF73 flanking regions. This cassette was produced by PCR using p*gal*K vector (Warming *et al.*, 2005) as template and *gal*K-H2endfw and *gal*K-H1startrev as forward
25   and reverse primers, respectively (Table S1). The second recombination process (*gal*K negative selection) consisted to restore ORF73 to generate a revertant plasmid. Recombination was achieved using the H1-ORF73-H2 cassette (Fig. 1), consisting of ORF73 flanked by 60-bp sequences corresponding to ORF73 flanking regions. This cassette was produced by PCR using BoHV-4 V. test genome as template and H2endfw and H1startrev as forward and reverse
30   primers, respectively (Table S1). Reconstitution of infectious virus from BAC plasmids was obtained by transfection in EBL cells. To excise the BAC cassette, reconstituted viruses were

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propagated in EBL-NLS-Cre cells expressing Cre recombinase to generate the corresponding excised strain (Fig. 1).

Virus purification. Virus grown on MDBK cells was semi-purified as described previously
5   (Vanderplasschen *et al.*, 1993). Briefly, after removal of the cell debris, the virus present in the cell supernatant was pelleted by ultracentrifugation through a 30% (w/v) sucrose cushion.

Southern blotting. Southern blot analysis was performed as described previously using ORF73 and *gal*K cassette as probes (Costes *et al.*, 2008).
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Plaque size assay. MDBK cells were infected at a MOI of 0.5 PFU/cell. After an incubation period of 90 min, cells were washed with PBS and then overlaid with MEM containing 5% FCS and 0.6% (w/v) carboxymethylcellulose (CMC, Sigma) in order to obtain isolated plaques as described elsewhere (Vanderplasschen *et al.*, 1993). At successive intervals after infection,
15   plaques were stained by indirect immunofluorescent staining (see below) and observed by epifluorescent microscopy.

Multi-step growth curves. Triplicate cultures of MDBK cells were infected at a MOI of 0.5 PFU/cell. After an incubation period of 90 min, cells were washed with PBS and then overlaid
20   with MEM containing 5% FCS. Supernatant of infected cultures was harvested at successive intervals after infection and the amount of infectious virus was determined by plaque assay on MDBK cells as described previously (Vanderplasschen *et al.*, 1993).

Episome persistence assay. BOMAC cells were infected at a MOI of 0.5 PFU/cell. After an
25   incubation period of 90 min, cells were washed with PBS and then overlaid with MEM containing 5% FCS. The cells were then passaged every other day (1:2 split ratio) for 20 days. At each passage, a fraction of the cells was fixed in PBS containing 4% (w/v) paraformaldehyde (Merck) at 4°C for 30 min. Fixed cells were then washed with PBS, resuspended in the same buffer and analysed by flow cytometry for EGFP protein expression (EGFP is encoded by the
30   BAC cassette).

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Animals. Specific-pathogen-free New-Zealand white rabbits were housed individually throughout this study. Four groups were used, each comprising three rabbits. Animals from group 1 were mock-infected intravenously with PBS. Animals from groups 2, 3 and 4 were inoculated intravenously with 10^8 PFU of V. test BAC G excised, V. test BAC G ΔORF73 excised and V.

5   test BAC G ORF73 revertant excised strains, respectively. At the end of the experiment, rabbits were euthanized and a necropsy examination was performed during which the spleen was collected.

Isolation of peripheral blood mononuclear cells and preparation of spleen cell

10   **suspension.** Blood samples were collected and peripheral blood mononuclear cells (PBMC) were separated by Ficoll (Ficoll-Paque Plus, GE Healthcare) density gradient as described previously (Dewals *et al.*, 2008). Immediately after euthanasia, spleen was removed and half-part of it was homogenized using a tissue grinder (VWR), passed through a stainless steel sieve and washed in FCS-free MEM before further analyses.

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Viral genome detection by Real time-PCR. DNA was purified from the spleen and PBMC using the QIAamp DNA Mini kit (Qiagen). Real-time PCR was performed as described elsewhere (Boudry *et al.*, 2007). A 103 bp fragment corresponding to BoHV-4 ORF8 was amplified with the forward primer 8startfw and the reverse primer 8middlerev (Table S1) in the

20   presence of the fluorescent probe 5'-FAM-AACACGTCAACA AGCAAGCCATCCACTG-TAMRA-3'. Purified BoHV-4 V. test genome was used to establish standard curves. PCR amplifications and fluorescence reactions were carried out in a iCycler system (Bio-Rad) under the following conditions: initial activation of the *Taq* polymerase (Bio-Rad) at 94°C for 5 min followed by 50 cycles at 94°C for 1 min, 50 cycles at 51°C for 30 sec and 50 cycles at 72°C for 1

25   min. For cellular quantitative amplification, a 178 bp fragment of the rabbit beta-globin gene was used (Genbank accession no. V00882) and amplified as described earlier (Boudry *et al.*, 2007).

Infectious centre assay. Viral detection in spleen cell suspension was assayed by infectious centre assay (ICA) as follows. 5.10^5 MDBK cells grown in 6 well cluster dishes (Becton

30   Dickinson) were co-cultured for 4 days at 37°C with 5.10^6, 5.10^5 or 5.10^4 spleen cells in MEM containing 5% FCS, 2% PS, 0.6% CMC and 5.10^{-5}M of β-mercaptoethanol (Merck). Cells were

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then fixed and stained for indirect immunofluorescent detection of viral antigen as described below.

Quantification of anti-BoHV-4 antibodies by ELISA. Nunc Maxisorp ELISA plates (Nalge Nunc) were coated for 18 h at 37°C with 0.1% Tween 20 (v/v) - disrupted BoHV-4 virions (2.10^6 PFU/well), blocked in PBS containing 0.1% Tween-20 (v/v) and 3% BSA (w/v), and incubated with rabbit sera (diluted 1/300 in PBS containing 0.1% Tween-20 (v/v) and 3% BSA (w/v)). Bound antibodies were detected with Alkaline Phosphatase conjugated goat anti-rabbit Ig polyclonal antibody (Sigma). Washing were performed with PBS containing 0.1% Tween-20 (v/v) and 3% BSA (w/v). Nitrophenylphosphatate (Sigma) was used as substrate and absorbance was read at 405nm using a Benchmark ELISA plate reader (Thermo).

Indirect immunofluorescent staining. Indirect immunofluorescent staining was performed as described previously (Gillet *et al.* 2005). Mouse monoclonal antibody (Mab) 35 (diluted 1:100) raised against the BoHV-4 glycoprotein complex gp6/gp10/gp17 and Alexa Fluor 568 goat anti-mouse IgG (H+L) (Invitrogen, $2\mu g\ ml^{-1}$) were used as primary and secondary antibodies, respectively.

Microscopy analysis. Samples were examined using an epifluorescence microscope TE2000-S Nikon and a Leica DC300F CCD camera system.

Flow cytometry. Flow cytometry acquisitions and analyses were performed using a three lasers Becton Dickinson fluorescence-activated cell sorter (FACSAria).

Statistical analysis. The linear model $y = \mu + group_i + time_j + group_i*time_j + experiment_k + error_{ijkl}$ was used to analyse viral multi-step growth curves and rabbit anti-BoHV-4 humoral responses. For multi-step growth curves comparison, the experiment factor was not significant and was therefore not included in the model. For analysis of anti-BoHV-4 humoral responses, correlation between successive measurements made on the same animal was taken into account using compound symmetry structure. Anova-2 test was used to determine the significance of the data obtained with the episome persistence assay ($P<0.0005$).

RESULTS

BoHV-4 ORF73 is expressed as an IE gene.

To determine if BoHV-4 ORF73 is a bona fide gene, it was determined whether it is transcribed during BoHV-4 infection. RT-PCR was performed using first-strand cDNA made from BoHV-4-
5   infected and mock-infected MDBK cells (Fig. 2a). In contrast to mock-infected cells, cDNA from infected cells generated a 762 bp PCR product (Fig. 2a) corresponding to ORF73 as determined by sequencing. When RT was omitted from the reactions, no PCR product was detected, indicating that it did not result from amplification of contaminant viral DNA. All together, these data demonstrated that ORF73 is transcribed during BoHV-4 replication *in vitro*.
10 A similar conclusion was reached for ORF71 based on the data presented in Fig. 2a. Next, CHX and PAA were used to identify ORF73 and ORF71 kinetic class of transcription. This experiment revealed that neither CHX nor PAA inhibited ORF73 and ORF71 expression suggesting that both ORFs are expressed as IE genes. Bo5, ORF21 and ORF22 were used as controls in this experiment; the results presented in Fig. 2a confirmed that they are IE, E and L genes,
15 respectively. The absence of contaminant viral DNA in the mRNA preparations was confirmed by the absence of a PCR product when the reverse transcriptase was omitted from the reactions (Fig. 2a). The expression of BoHV-4 ORF73 as an IE gene is consistent with earlier reports describing the expression of ORF73 orthologues encoded by rhadinoviruses early after infection (Ebrahimi *et al.*, 2003; Martinez-Guzman *et al.*, 2003; Sarid *et al.*, 1999).
20

BoHV-4 ORF73 is expressed as polycistronic mRNAs encompassing ORF71. To characterise ORF73 transcript(s), Northern blot analysis was performed on total RNA extracted from BoHV-4-infected and mock-infected MDBK cells using full-length ORF73 as probe (Fig. 2b). A weak band of approximately 2.5 kb was detected as early as 4h p.i. (Fig. 2b). At 8h p.i., four bands
25 were detected with approximate size of 2.0, 2.5, 3.0 and 4.5 kb (Fig. 2b). One additional band of approximately 7.5 kb was detected 24h p.i. (Fig. 2b). These results suggest that ORF73 expression involves transcripts of different sizes. No signal was detected in RNA extracted from mock-infected cells (Fig. 2b).

ORF71 is expressed as a polycistronic RNA encompassing at least ORF73. The
30 polyadenylation signal (AATAAA) of the transcript is located 104 bp downstream of ORF71 stop codon. RT-PCR reactions were performed on cytoplasmic RNA extracted from BoHV-4-infected MDBK cells in order to further characterise polycistronic RNA molecules encoding

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ORF71 and ORF73 (Fig. 2, panels c and d). PCR products corresponding to ORF71- 73 (1454 bp), ORF73- end of ORF75 (1759 bp) and ORF71- end of ORF75 (2453 bp) were amplified (Fig. 2d). None of the PCR reactions involving primers hybridizing upstream of primer 75 endrev generated a product (Fig. 2d), while all reactions generated the expected products

5   when performed on control viral DNA. Absence of contaminant viral DNA was confirmed by the absence of PCR product when RT was omitted from the reactions. All together, the data presented above suggest that ORF71 and ORF73 are expressed as a bicistronic mRNA. At least two mRNAs were identified by combined Northern blot/RT-PCR approach; one encompassing both ORF71 and ORF73, the second encompassing ORF71, ORF73 and part of ORF75 (Fig. 2b

10   and d). Longer RNAs were identified by Northern blot approach but not by RT-PCR. These molecules could represent pre-mRNAs.

Production and characterisation of BoHV-4 ORF73 recombinants. In order to investigate the importance of ORF73 in virus replication *in vitro* and the biology of the infection *in vivo*, a

15   BoHV-4 strain deleted for ORF73 and a derived revertant strain were produced (Fig. 1). The V. test BAC G plasmid was used as parental plasmid to generate the V. test BAC G ΔORF73 plasmid in which the entire ORF73 had been replaced by a *gal*K cassette as described in the Methods. Then, a revertant plasmid was generated. Infectious viruses were efficiently reconstituted by transfection of BAC plasmids into permissive cells. Finally, reconstituted

20   infectious viruses were propagated in EBL-NLS-Cre cells to generate BAC-excised strains (Fig. 1). The molecular structures of the recombinant strains produced were confirmed by a combined *Hin*dIII restriction endonuclease and Southern blot approach (Fig. 3) and by sequencing of the regions used to target recombination. Moreover, the absence of polar effect of ORF73 deletion on the expression of ORF71 was demonstrated by RT-PCR. All recombinants tested expressed

25   ORF71 comparably. Finally, presence or absence of the BAC cassette was monitored by analysis of EGFP expression by viral plaques (Fig. 4a). All approaches confirmed that the recombinants produced have the correct molecular structure.

ORF73 deletion does not affect viral growth *in vitro*. In order to address the role of ORF73 in

30   BoHV-4 growth *in vitro*, V. test BAC G, V. test BAC G ΔORF73 and V. test BAC G ORF73 revertant excised strains were compared using multi-step growth and plaque size assays (Fig. 4). Corresponding non excised strains were also compared. All viruses tested exhibited similar

growth curves (p≤0.05) and comparable plaque sizes. Taken together, these results indicate that ORF73 deletion does not affect BoHV-4 replication *in vitro*.

ORF73 deletion affects viral persistence *in vitro* in a "latency-like" model. BOMAC cells

5   have been shown to support a BoHV-4 non replicative persistent infection mimicking latency (Donofrio & van Santen, 2001). Using this "latency-like" model, it was investigated whether ORF73 deletion affects the persistence of BoHV-4 infection in BOMAC cells. BOMAC cells were infected with the V. test BAC G, V. test BAC G ΔORF73 and V. test BAC G ORF73 revertant strains and viral persistence was monitored over time by flow cytometry quantification

10 of EGFP expressing cells (Fig. 5a). Independently of the recombinants, about 90% of the cells were positive for EGFP expression on day 2 p.i.. For all three recombinants tested, the percentage of EGFP positive cells decreased gradually over time. However, while comparable data were obtained for V. test BAC G and V. test BAC G ORF73 revertant strains, a faster decay of positive cells was observed for cells infected with V. test BAC G ΔORF73 strain (Fig. 5a). For

15 example, on day 10 p.i. while V. test BAC G and V. test BAC G ORF73 revertant infected cultures contained approximately 50% of EGFP positive cells, the percentage of EGFP positive cells in BOMAC cells infected with V. test BAC G ΔORF73 strain was close to 0.

At least two hypotheses that are not mutually exclusive could explain the faster decay of EGFP positive cells in V. test BAC G ΔORF73 infected culture in comparison to V. test BAC G

20 and V. test BAC G ORF73. Firstly, it is likely that the deletion of ORF73 impaired the persistence of the viral episome in the culture through cell division. Secondly, it is possible that the absence of ORF73 could induce lytic replication (by release of the inhibition on ORF50) of BoHV-4 in BOMAC cells and consequently eradication of infected cells from the culture. To test the latter hypothesis, BOMAC cells were infected with V. test BAC G, V. test BAC G ΔORF73

25 and V. test BAC G ORF73 revertant strains, then analysed 48 h p.i. by flow cytometry for expression of the structural protein gB. The data presented in Fig. 5b demonstrate that independently of the viral strain used for the infection, no expression of BoHV-4 gB could be detected in infected BOMAC cells (Fig. 5b, right column). In contrast, infection of MDBK cells used as positive control led to a large proportion of gB expressing cells (Fig. 5b, left column). All

30 recombinants tested led to comparable percentages of gB expressing cells. All together, the results presented above support the fact that BoHV-4 ORF73 plays a role in latency and justified the completion of *in vivo* experiments.

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BoHV-4 ORF73 is essential for viral persistence *in vivo*. To investigate the importance of
BoHV-4 ORF73 during latency *in vivo*, the rabbit model was used. Rabbits were inoculated with
the V. test BAC G, V. test BAC G ΔORF73 and V. test BAC G ORF73 revertant excised strains
5   (Fig. 6a and b). Independently of the groups, all rabbits remained healthy for the course of the
experiment. None of the rabbits showed clinical symptoms or lesions throughout the experiment.
No lesion was observed at necropsy 64 days post-inoculation. To address viral persistence,
real-time PCR were performed on DNA extracted from PBMC over time and from the spleen 64
days post-inoculation (Fig. 6a). After an eclipse phase of 12 days, viral copies ranging from 10^1
10   to 10^2 viral copies per 10^5 copies of beta-globin were detected in PBMC from all rabbits infected
with the V. test BAC G and V. test BAC G ORF73 revertant excised strains (Fig. 6a).
Interestingly, none of the rabbits infected with the V. test BAC G ΔORF73 excised strain had
detectable viral genome in PBMC throughout the experiment (Fig. 6a). Real-time PCR
performed on the spleen confirmed the essential role of ORF73 for viral persistence *in vivo*.
15   Indeed, while rabbits infected with the V. test BAC G and V. test BAC G ORF73 revertant
excised strains contained at least 10^3 viral copies per 10^5 cellular beta-globin copies, none of the
rabbits infected with the V. test BAC G ΔORF73 excised strain exhibited detectable amount of
viral genome (Fig. 6a). To confirm these real-time PCR results, viral isolations were attempted
by co-cultivation of spleen cells with permissive cells as described in Methods. Viral plaques
20   were observed for the 3 rabbits of the group infected with the V. test BAC G excised strain and
for 2 of the 3 rabbits infected with the V. test BAC G ORF73 revertant excised strain. In contrast,
no virus was isolated from spleen cells of rabbits infected with V. test BAC G ΔORF73 excised
strain. Viral isolation assays performed with the same sources of spleen cells, but frozen-thawed
before the assay, did not led to viral isolation. Taken together, the results presented above
25   demonstrate that ORF73 is essential for viral latency.

Finally, the humoral immune response induced by the ORF73 deleted strain was
compared to those observed with the wild type parental and the revertant strains (Fig. 6b).
Independently of the viral strain used for the inoculation, anti-BoHV-4 antibodies were detected
as early as 6 days post-inoculation. All infected rabbits exhibited comparable humoral immune
30   responses. The statistical test used to analyse the data suggested that for some time points, rabbits
infected with V. test BAC G ΔORF73 excised strain exhibited a higher response ($p \leq 0.05$) (Fig.
6b) when compared to the V. test BAC G and V. test BAC G ORF73 revertant excised strains.

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This statistical difference was not observed when the Student's *t*-test was used. However, these data demonstrate that the immune response raised against the ORF73 deleted recombinant is at least comparable to the responses induced by the wild type and the revertant strains. Absence of response in the mock-infected group confirmed absence of cross-contamination between the groups (Fig. 6b).

5   Several features make BoHV-4 a good candidate as a gene delivery vector and previous studies showed evidence that BoHV-4 can successfully be used to transduce expression of foreign proteins (Gillet *et al.*, 2005; Donofrio *et al.*, 2006). BoHV-4 (*i*) possesses a less complex genome than other herpesviruses (Zimmermann *et al.*, 2001), (*ii*) can accommodate large

10 amounts of foreign genetic material (Gillet *et al.*, 2005), (*iii*) in contrast to most gammaherpesviruses, is able to replicate in a broad range of host species both *in vitro* and *in vivo* (Thiry *et al.*, 1992), and (*iv*) BoHV-4 exhibits limited or no pathogenicity in natural and experimental hosts (Thiry *et al.*, 1992). However, the property of BoHV-4 to establish latency and to reactivate makes wild type strains inappropriate to develop safe recombinant vectors.

15 Indeed, throughout their live, infected animals could reactivate and contaminate naive animals. The results of the present study demonstrate that ORF73 deletion abolishes the property of BoHV-4 to establish persistent infection at least in a laboratory animal model (Fig. 6). Consequently, BoHV-4 ORF73 deleted strains represent excellent basis vectors for the development of BoHV-4 vectors in vaccinology.

20   In conclusion, the results of the present study demonstrate that despite its very small size, BoHV-4 ORF73 is a functional homologue of larger rhadinovirus ORF73 orthologues that is essential for latency *in vivo*. BoHV-4 ORF73 deleted strains represent excellent basis vectors for the development of BoHV-4 vectors in vaccinology.

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Table S1. Oligonucleotides used for PCR amplification

Primers	Sequence	Coordinates according to Genbank accession no. NC_002665
Bo5endfw	5'-TCATGTCCTGAGTGGGTCTATG-3'	19414-19435
Bo5startrev	5'-GCTACAGAAAATGGCCAGTAAAG-3'	20541-20563
21middlefw	5'-CATGGAGAGGCCATCAGGAG-3'	29044-29063
21endrev	5'-CTTAACCACAGAACTCCTTTGC-3'	29521-29542
22middlefw	5'-CCGGGTGAAACAAGTTCCTG-3'	31114-31133
22endrev	5'-<u>GG</u>TCAGAGAACATATGATAACATC-3'	31654-31675
71endfw	5'-<u>CCAAGCTT</u>CTAGAGAGTGATTTGTAGTGT-3'	98883-98903
71startrev	5'-<u>AAGGATCC</u>GATGGTTACCAGGGATGTGTTG-3'	99411-99431
73endfw	5'-TCACTGATCAACGGGTATTCC-3'	99576-99596
73startrev	5'-ATGCCGGGCCGCCACCTC-3'	100320-100337
75endrev	5'-GGGTACACCACAAAGTCCC-3'	101317-101335
75middlerev	5'-GAGCAGACCATCATCTATAGG-3'	101851-101871
75startrev	5'-ATGGATCAAAGACATCTTGTGG-3'	104724-104745
Bo14startrev	5'-GGAGAGTCCTGTCTAGAACC-3'	105949-105968
Bo15startrev	5'-GGTGAGTAATGGGACCATGG-3'	106581-106600
*gal*K-H2endfw	5'-<u>TCTTTAAAAAACAACCATAGAACATATCATGAA GAC ATTCAAAAATATTCAAATCAGGTG</u>TCAGCACTGTCCTG CTCCTT -3'	99517-99575
*gal*K-H1startrev	5'-<u>GACATCTAGTGGTGGCTCGTATAGTTACACCAGTCTT GTGAATTTTCTTTCTGCCAAAGG</u>CCTGTTGACAATTAAT CATCGGCA -3'	100338-100397
H2endfw	5'-TCTTTAAAAAACAACCATAGAACATATCATGAAGAC ATTCAAAAATATTCAAATCAGGTG -3'	99517-99575
H1startrev	5'-GACATCTAGTGGTGGCTCGTATAGTTACACCAGTCT TGTGAATTTTCTTTCTGCCAAAGG-3'	100338-100397
*gal*Kstartfw	5'-GCCAACGCATTTGGCTACC-3'	
*gal*Kendrev	5'-CGGCAGGCACCAGCTCTTC-3'	
8startfw	5'- CAAATAGTTCATTAGCTGCCTCTCC -3'	11571-11595
8middlerev	5'- TCATCAGTAACAGTTGGAATAGTGG -3'	11649-11673

Underlined: extrabases added. In bold: mutation specific to the BoHV-4 strain used in the present study (V. test, Genbank accession no. Z79633).

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Supplementary Table S2. GenBank accession numbers of BoHV-4 sequenced regions

Strain	ORF 16	Bo10	ORF 71	Intergenic region 71–73	ORF 73	Bo17
V. test	AY847335	Z84818	Z46385*	Z46385*	Z46385*	AF321105*†
LVR 140	AY847332	AY847310	AY847339	AY847325	AY847346	AF465330*‡
MOVAR	AY847334	AY847305	AY847341	AY847327	AY847348	AF465331*‡
DN599	AY847331	AY847309	U23760*	U23760*	AY847345	AF465332*‡
66-p-347	AF318573*§	AF318573*§	AF318573*§	AF318573*§	AF318573*§	AF318573*§
Buf.	AY847330	AY847308	AY847338	AY847324	AY847344	AY143157*‡
130	AY857329	AY847307	AY847337	AY847323	AY847343	AY143156*‡
198	AY847328	AY847306	AY847336	AY847322	AY847342	AY143155*‡
M40	AY847333	AY847311	AY847340	AY847326	AY847347	AY143158*†

Supplementary Table S1. Primers used to amplify BoHV-4 genome sequences

The position of each primer is described according to the sequence of strain 66-p-347
(Zimmermann et al., 2001).

Region	Nucleotide position	Type	Sequence (5'→3')
Intergenic region 71–73	98886–98903	Forward	GAGAGTGATTTGTAGTGT
and ORF 73	98972–98992	Forward	CAAAGAGATCTAGGTTGTCTGG
	100128–100107	Reverse	AGTTAGGACACGTTCACCAACG
	100337–100318	Reverse	ATGCCGGGCCGCCACCTCAG

5

LEGEND TO FIGURES

Fig. 1. Flowchart of stages performed to produce BoHV-4 ORF73 recombinants using BAC cloning and prokaryotic recombination technologies.

Fig. 2. (a) Determination of ORF73 kinetic class of transcription. MDBK cells were mock-infected or infected with BoHV-4 V. test strain, in absence or presence of CHX or PAA. Twenty-four hours p.i., expression of IE Bo5, E ORF21, L ORF22, ORF71 and ORF73 was studied using an RT-PCR approach as described in Methods. mRNA and cDNA represent PCR products generated when the RT was omitted from the reactions and RT-PCR products, respectively. (b) Northern blot analysis was performed on total RNA harvested from mock-infected and infected MDBK cells 4, 8 and 24 h p.i. with the BoHV-4 V. test strain. ORF73 was used as probe. To optimize the visualization of the bands in the 24h p.i. sample, the corresponding line was exposed for a shorter period (right column). Weak and intense bands identified on the blot are marked with open arrows and arrows, respectively. (c) and (d) Characterisation of ORF73 transcripts by RT-PCR. Cytoplasmic RNA extracted 24 h p.i. was analysed by RT-PCR using the pairs of primers described in panel (c). For each pair of primers the size of the expected amplicon is indicated. The results of the PCR are presented in panel (d). PCR suggesting the polycistronic nature of ORF73 transcript are marked with asterisk.

Fig. 3. Structural analysis of BoHV-4 recombinant strains. The DNA of the various intermediates was analysed by *Hin*dIII restriction (left panel) and further tested by Southern blot (right panel) using probes corresponding to ORF73 and *gal*K cassette. Arrows and open arrows indicate restriction fragments containing the ORF73 and the *gal*K cassette, respectively. Markers sizes (MS) in kb are indicated on the left.

Fig. 4. Characterisation of BoHV-4 V. test strain and derived recombinant strains.
(a) Epifluorescent analysis of viral plaques. MDBK cells were infected (MOI of 0.5 PFU/cell) with BoHV-4 V. test (i-iii), V. test BAC G (iv-vi), V. test BAC G excised (vii-ix), V. test BAC G ΔORF73 (x-xii), V. test BAC G ΔORF73 excised (xiii-xv), V. test BAC G ORF73 revertant (xvi-xviii) and V. test BAC G ORF73 revertant excised strains (xix-xxi). The cells were then overlaid with MEM containing 5% FCS and 0.6% carboxymethylcellulose to obtain isolated plaques.

Three days p.i., plaques were revealed by indirect immunofluorescent staining using Mab 35 and Alexa Fluor 568-conjugated goat anti-mouse as primary and secondary antibodies, respectively. Each set of three horizontal panels represents analysis of the same plaques. EGFP and Alexa Fluor 568 fluorescences are shown in the first and the second column, respectively; while the

5   merged EGFP and Alexa Fluor 568 signals are shown in the third column. The side of each panel corresponds to 75 µm of the specimen. (b) Replication kinetics of recombinant strains. Replication kinetics of V. test BAC G ΔORF73 excised strain (triangle) and V. test BAC G ORF73 revertant excised strain (cross) were compared with those of the parental V. test BAC G excised strain (square) as described in Methods. The data presented are the means +/- SD of

10   triplicate measurements.

Fig. 5. BoHV-4 episome persistence and replication in BOMAC cells. (a) BOMAC cells were mock-infected (open circle) or infected with V. test BAC G (square), V. test BAC G ΔORF73 (triangle) or V. test BAC G ORF73 (cross). Episome persistence was then measured over time as

15   described in Methods. Each value represents the mean +/- SD of triplicate measurements of the percentage of EGFP-positive cells based on the analysis of 10,000 cells. (b) 48 h p.i., BOMAC and MDBK cells (used as positive control for viral replication) were stained with Mab 35 raised against BoHV-4 gB and analysed by flow cytometry to investigate BoHV-4 replication. The results presented are based on the analysis of 10,000 cells.

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Fig. 6. Rabbit infection with BoHV-4 ORF73 recombinants. Groups consisting of 3 rabbits were mock-infected or infected with V. test BAC G, V. test BAC G ΔORF73 and V. test BAC G ORF73 revertant excised strains. (a) Real-Time PCR relative quantification of BoHV-4 genome. DNA was extracted from the PBMC at the different times post-inoculation and from the spleen

25   64 days post-inoculation. Data are expressed as the number of BoHV-4 ORF8 gene copies per 10^5 rabbit beta-globin gene copies for each rabbit. (b) Rabbit anti-BoHV-4 humoral response. Sera were collected at different times post-inoculation and the titre of anti-BoHV-4 antibodies was estimated by ELISA. Assays were made in triplicate for each rabbit. Each value represents the mean +/- SD of the data obtained for the three rabbits of each group.

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LITERATURE REFERENCES

Ballestas, M. E., Chatis, P. A. & Kaye, K. M. (1999). Efficient persistence of extrachromosomal
KSHV DNA mediated by latency-associated nuclear antigen. *Science* **284**, 641-4.

Barbera, A. J., Chodaparambil, J. V., Kelley-Clarke, B., Joukov, V., Walter, J. C., Luger, K. &
Kaye, K. M. (2006). The nucleosomal surface as a docking station for Kaposi's sarcoma
herpesvirus LANA. *Science* **311**, 856-61.

Boudry, C., Markine-Goriaynoff, N., Delforge, C., Springael, J. Y., de Leval, L., Drion, P.,
Russell, G., Haig, D. M., Vanderplasschen, A. F. & Dewals, B. (2007). The A5 gene of
alcelaphine herpesvirus 1 encodes a constitutively active G-protein-coupled receptor that is
non-essential for the induction of malignant catarrhal fever in rabbits. *J Gen Virol* **88**,
3224-33.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B. & Cullen, B. R. (2005). Kaposi's
sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected
cells. *Proc Natl Acad Sci U S A* **102**, 5570-5.

Calderwood, M., White, R. E., Griffiths, R. A. & Whitehouse, A. (2005). Open reading frame 73
is required for herpesvirus saimiri A11-S4 episomal persistence. *J Gen Virol* **86**, 2703-8.

Costes, B., Fournier, G., Michel, B., Delforge, C., Raj, V. S., Dewals, B., Gillet, L., Drion, P.,
Body, A., Schynts, F., Lieffrig, F. & Vanderplasschen, A. (2008). Cloning of the Koi
Herpesvirus Genome as an Infectious Bacterial Artificial Chromosome Demonstrates That
Disruption of the Thymidine Kinase Locus Induces Partial Attenuation in Cyprinus carpio
koi. 10.1128/JVI.00211-08. *J. Virol.* **82**, 4955-4964.

Dewals, B., Boudry, C., Farnir, F., Drion, P.-V. & Vanderplasschen, A. (2008). Malignant
Catarrhal Fever Induced by *Alcelaphine herpesvirus 1* is Associated with Proliferation of
CD8+ T Cells Supporting a Latent Infection. *PLoS ONE* **3**, e1627.

Dewals, B., Thirion, M., Markine-Goriaynoff, N., Gillet, L., de Fays, K., Minner, F., Daix, V.,
Sharp, P. M. & Vanderplasschen, A. (2006). Evolution of Bovine herpesvirus 4:
recombination and transmission between African buffalo and cattle. *J Gen Virol* **87**, 1509-
19.

Donofrio, G., Cavirani, S., Vanderplasschen, A., Gillet, L. & Flammini, C. F. (2006).
Recombinant bovine herpesvirus 4 (BoHV-4) expressing glycoprotein D of BoHV-1 is

27

immunogenic and elicits serum-neutralizing antibodies against BoHV-1 in a rabbit model. *Clin Vaccine Immunol* **13**, 1246-54.

Donofrio, G. & van Santen, V. L. (2001). A bovine macrophage cell line supports bovine herpesvirus-4 persistent infection. *J Gen Virol* **82**, 1181-1185.

Ebrahimi, B., Dutia, B. M., Roberts, K. L., Garcia-Ramirez, J. J., Dickinson, P., Stewart, J. P., Ghazal, P., Roy, D. J. & Nash, A. A. (2003). Transcriptome profile of murine gammaherpesvirus-68 lytic infection. *J Gen Virol* **84**, 99-109.

Fowler, P., Marques, S., Simas, J. P. & Efstathiou, S. (2003). ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency. *J Gen Virol* **84**, 3405-16.

Friborg, J., Jr., Kong, W., Hottiger, M. O. & Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* **402**, 889-94.

Gillet, L., Daix, V., Donofrio, G., Wagner, M., Koszinowski, U. H., China, B., Ackermann, M., Markine-Goriaynoff, N. & Vanderplasschen, A. (2005). Development of bovine herpesvirus 4 as an expression vector using bacterial artificial chromosome cloning. *J Gen Virol* **86**, 907-17.

Krithivas, A., Young, D. B., Liao, G., Greene, D. & Hayward, S. D. (2000). Human herpesvirus 8 LANA interacts with proteins of the mSin3 corepressor complex and negatively regulates Epstein-Barr virus gene expression in dually infected PEL cells. *J Virol* **74**, 9637-45.

Lan, K., Kuppers, D. A., Verma, S. C. & Robertson, E. S. (2004). Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen inhibits lytic replication by targeting Rta: a potential mechanism for virus-mediated control of latency. *J Virol* **78**, 6585-94.

Lan, K., Kuppers, D. A., Verma, S. C., Sharma, N., Murakami, M. & Robertson, E. S. (2005). Induction of Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen by the lytic transactivator RTA: a novel mechanism for establishment of latency. *J Virol* **79**, 7453-65.

Li, H., Gailbreath, K., Flach, E. J., Taus, N. S., Cooley, J., Keller, J., Russell, G. C., Knowles, D. P., Haig, D. M., Oaks, J. L., Traul, D. L. & Crawford, T. B. (2005). A novel subgroup of rhadinoviruses in ruminants. *J Gen Virol* **86**, 3021-3026.

Li, M., Lee, H., Yoon, D. W., Albrecht, J. C., Fleckenstein, B., Neipel, F. & Jung, J. U. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional cyclin. *J Virol* **71**, 1984-91.

28

Lim, C., Gwack, Y., Hwang, S., Kim, S. & Choe, J. (2001). The transcriptional activity of cAMP response element-binding protein-binding protein is modulated by the latency associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J Biol Chem* **276**, 31016-22.

Lu, F., Day, L., Gao, S. J. & Lieberman, P. M. (2006). Acetylation of the latency-associated nuclear antigen regulates repression of Kaposi's sarcoma-associated herpesvirus lytic transcription. *J Virol* **80**, 5273-82.

Markine-Goriaynoff, N., Georgin, J. P., Goltz, M., Zimmermann, W., Broll, H., Wamwayi, H. M., Pastoret, P. P., Sharp, P. M. & Vanderplasschen, A. (2003). The core 2 beta-1,6-N-acetylglucosaminyltransferase-mucin encoded by bovine herpesvirus 4 was acquired from an ancestor of the African buffalo. *J Virol* **77**, 1784-92.

Martinez-Guzman, D., Rickabaugh, T., Wu, T. T., Brown, H., Cole, S., Song, M. J., Tong, L. & Sun, R. (2003). Transcription program of murine gammaherpesvirus 68. *J Virol* **77**, 10488-503.

Naeem, K., Caywood, D. D., Werdin, R. E. & Goyal, S. M. (1990). Evaluation of pregnant rabbits as a laboratory model for bovid herpesvirus-4 infection. *Am J Vet Res* **51**, 640-4.

Osorio, F. A., Reed, D. E. & Rock, D. L. (1982). Experimental infection of rabbits with bovine herpesvirus-4: acute and persistent infection. *Vet Microbiol* **7**, 503-13.

Radkov, S. A., Kellam, P. & Boshoff, C. (2000). The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat Med* **6**, 1121-7.

Renne, R., Barry, C., Dittmer, D., Compitello, N., Brown, P. O. & Ganem, D. (2001). Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J Virol* **75**, 458-68.

Roizman, B. & Pellett, P. E. (2007). The family *Herpesviridae* :a brief introduction. In *Fields Virology (fifth edition)*, pp. 2479-2499. Edited by H. P. M. Knipe D.M. Philadelphia: Lippincott Williams & Wilkins, a Wolters Kluwer Business.

Sambrook, J. & Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual. In *Chapter 7 : Extraction, Purification and Analysis of mRNA from Eukaryotic Cells*, Third Edition edn, pp. 7.1-7.45. Edited by N. Irwin. New-York: Cold Spring Harbor Lab.

Sarid, R., Flore, O., Bohenzky, R. A., Chang, Y. & Moore, P. S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). *J Virol* **72**, 1005-12.

29

Sarid, R., Wiezorek, J. S., Moore, P. S. & Chang, Y. (1999). Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. *J Virol* **73**, 1438-46.

Stabel, J. R. & Stabel, T. J. (1995). Immortalization and characterization of bovine peritoneal macrophages transfected with SV40 plasmid DNA. *Vet Immunol Immunopathol* **45**, 211-20.

Thiry, E., Bublot, M., Dubuisson, J., Van Bressem, M. F., Lequarre, A. S., Lomonte, P., Vanderplasschen, A. & Pastoret, P. P. (1992). Molecular biology of bovine herpesvirus type 4. *Vet Microbiol* **33**, 79-92.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., Peter, M. E. & Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**, 517-21.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. & Higgins, D. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. 10.1093/nar/25.24.4876. *Nucl. Acids Res.* **25**, 4876-4882.

Vanderplasschen, A., Bublot, M., Pastoret, P. P. & Thiry, E. (1993). Restriction maps of the DNA of cervid herpesvirus 1 and cervid herpesvirus 2, two viruses related to bovine herpesvirus 1. *Arch Virol* **128**, 379-88.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. & Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* **33**, e36.

Weck, K. E., Kim, S. S., Virgin, H. I. & Speck, S. H. (1999). Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. *J Virol* **73**, 3273-83.

Ye, F. C., Zhou, F. C., Yoo, S. M., Xie, J. P., Browning, P. J. & Gao, S. J. (2004). Disruption of Kaposi's sarcoma-associated herpesvirus latent nuclear antigen leads to abortive episome persistence. *J Virol* **78**, 11121-9.

Zimmermann, W., Broll, H., Ehlers, B., Buhk, H. J., Rosenthal, A. & Goltz, M. (2001). Genome sequence of bovine herpesvirus 4, a bovine Rhadinovirus, and identification of an origin of DNA replication. *J Virol* **75**, 1186-94.

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Claims

1) Mutant BoHV-4 genome characterised in that it does not encode a functional ORF73 protein.

2) Mutant BoHV-4 virus characterised in that it is not capable of making a functional ORF73 protein.

3) Mutant BoHV-4 virus according to claim 2 characterised in that it has a mutation in the ORF73 gene.

4) Mutant BoHV-4 virus according to claim 3 characterised in that said mutation is a deletion or an insertion.

5) Mutant BoHV-4 virus according to any of claims 2-4, characterised in that said virus comprises a second deletion.

6) Mutant BoHV-4 virus according to claim 5, characterised in that said second deletion is a deletion in the in the ORF71-gene encoding a v-FLIP, the ORF16-gene encoding a v-Bcl-2 homologue, the Bo10 gene encoding glycoprotein gp80 or the ORF21-gene encoding thymidine kinase.

7) Mutant BoHV-4 virus according to any of claims 2-6, characterised in that said virus comprises a heterologous DNA sequence.

8) Mutant BoHV-4 virus according to claim 7, characterised in that said heterologous DNA sequence encodes an immunogenic protein of another virus or microorganism pathogenic to animals that are sensitive to BoHV-4 infection, preferably to ruminants, and/or a cytokine.

9) Mutant BoHV-4 virus according to claim 8, characterized in that said another virus or micro-organism is selected from the group of Bovine Herpesvirus, Bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria* sp., *Babesia* sp., *Trypanosoma species*, *Anaplasma* sp., *Neospora caninum*, *Staphylococcus aureus*, *Streptococcus agalactiae*, Mycoplasma, *E. coli*, *Enterobacter*, *Klebsiella, Citrobacter* and *Streptococcus dysgalactiae*.

10) Mutant BoHV-4 virus according to any of claims 7-9, characterised in that said heterologous DNA sequence is inserted in the ORF73 gene.

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11) Mutant BoHV-4 genome according to claim 1 or mutant BoHV-4 virus according to any of claims 2-10 for use in a vaccine.

12) Vaccine for combating BoHV-4 infection, characterised in that said vaccine comprises a mutant BoHV-4 genome according to claim 1 or a mutant BoHV-4 virus according to any of claims 2-10, and a pharmaceutically acceptable carrier.

13) Mutant BoHV-4 carrier vaccine, characterised in that said vaccine comprises a mutant BoHV-4 virus according to any of claims 7-10, and a pharmaceutically acceptable carrier.

14) Vaccine according to claim 12 or 13, characterized in that said vaccine is in a freeze-dried form.

15) Use of a mutant BoHV-4 genome according to claim 1 or a mutant BoHV-4 virus according to any of claims 2-10 for the manufacture of a vaccine for the protection of susceptible animals against BoHV-4 infection.

16) Use of a mutant BoHV-4 virus according to any of claims 7-10 for the manufacture of a BoHV-4 carrier vaccine.

17) A cell comprising a mutant BoHV-4 genome according to claim 1

18) A cell comprising a mutant BoHV-4 virus according to any of claims 2-10.

19) Method for the production of a mutant BoHV-4 genome according to claim 1 or a mutant BoHV-4 virus according to any of claims 2-10, said method comprising the step of mutating the ORF73 gene so that it no longer encodes a functional ORF73 protein.

20) Diagnostic test kit for differentiating BoHV-4 field-virus infected animals from animals vaccinated with a mutant BoHV-4 vaccine comprising a mutant BoHV-4 virus according to any of claims 8-10, characterized in that said kit comprises antibodies against said immunogenic protein.

21) Diagnostic test kit for differentiating BoHV-4 field-virus infected animals from animals vaccinated with a mutant BoHV-4 vaccine according to claim 12 or 13 characterized in that said kit comprises PCR-primers against a fragment of ORF73 that is not present in mutant BoHV-4 virus according to any of claims 2-10.
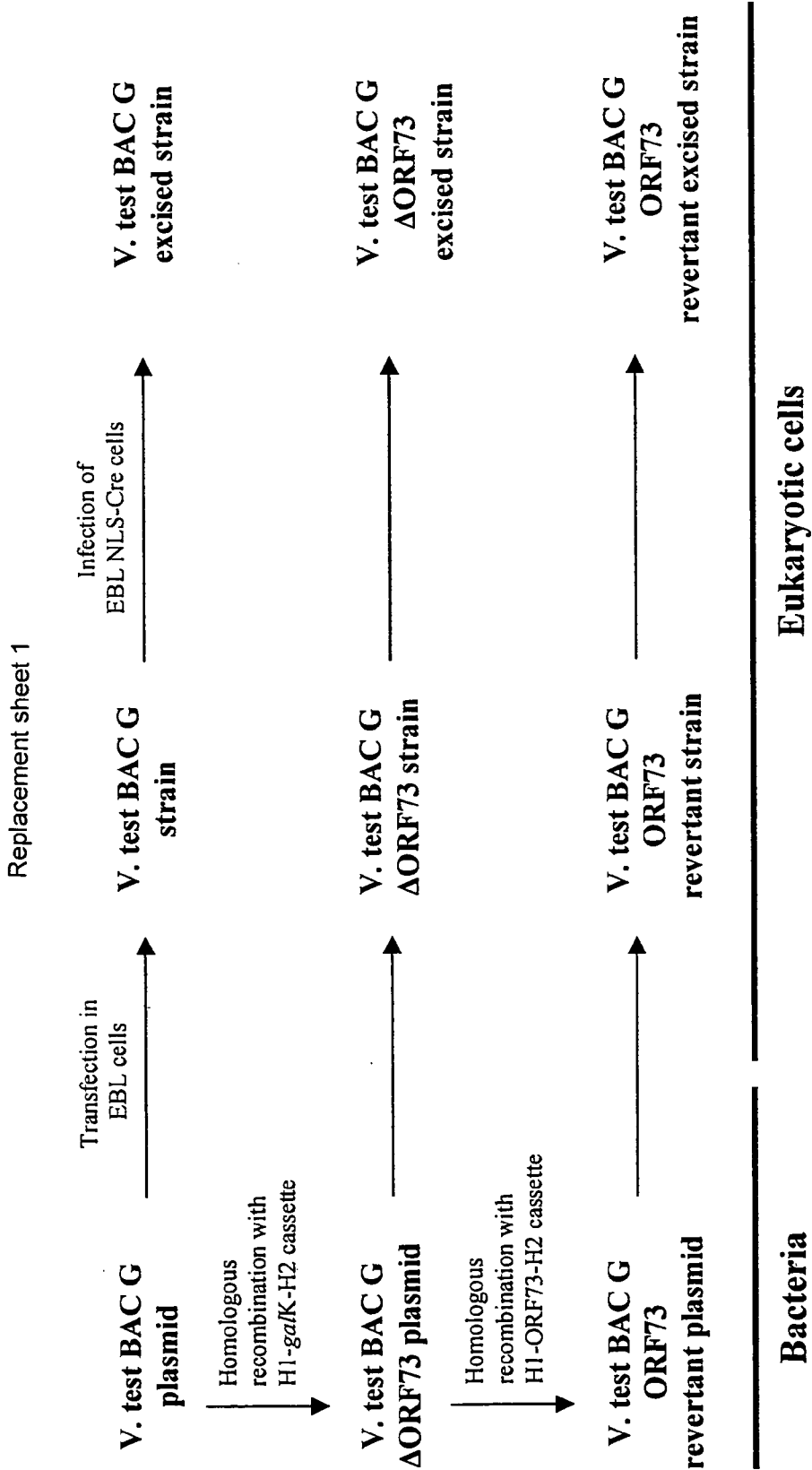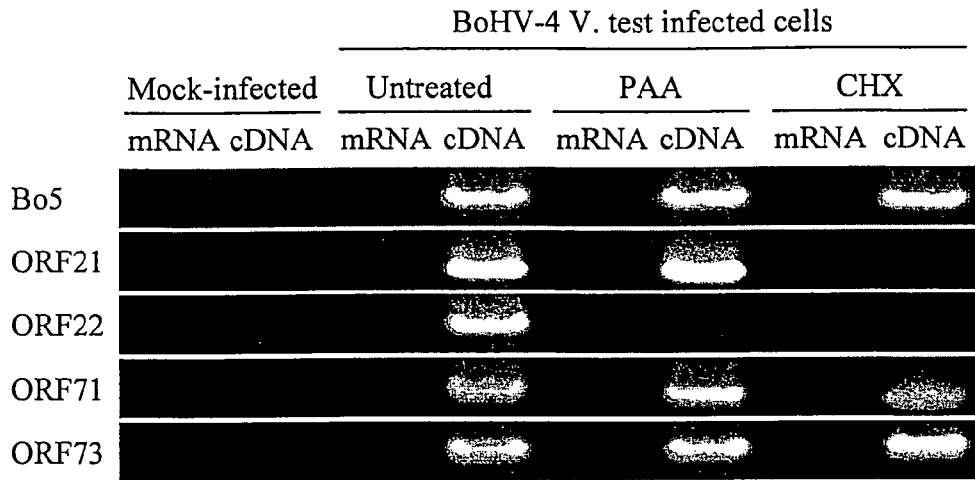
Replacement sheet 1
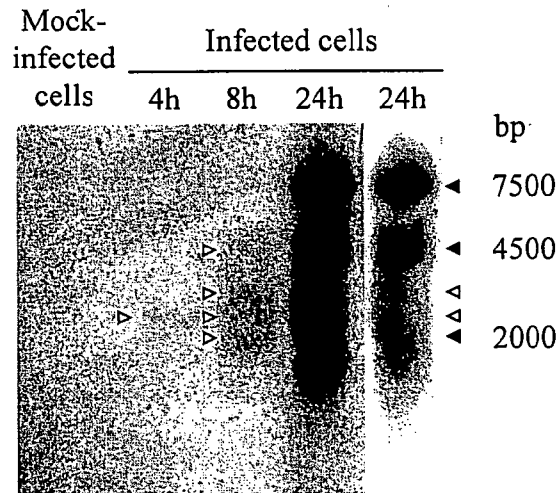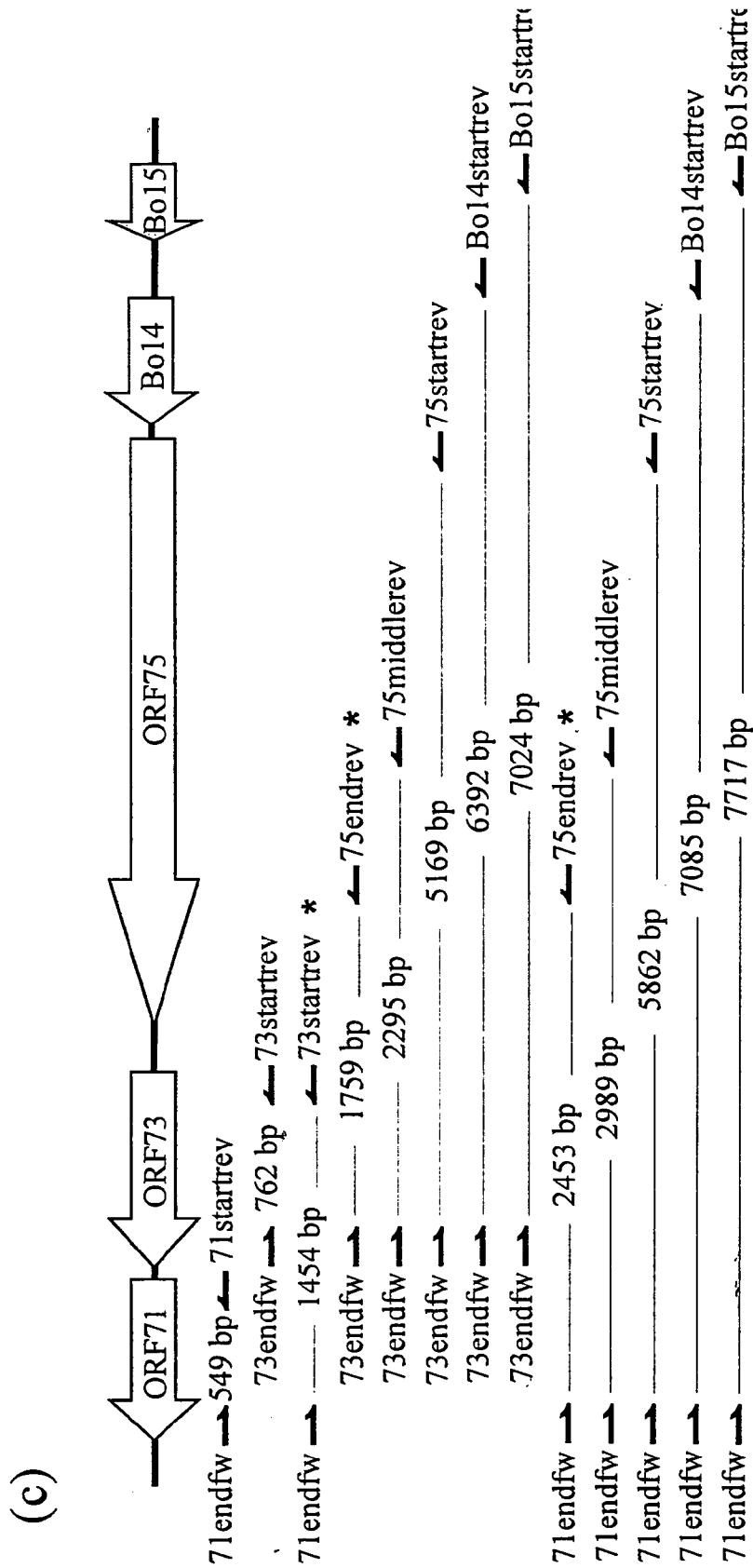

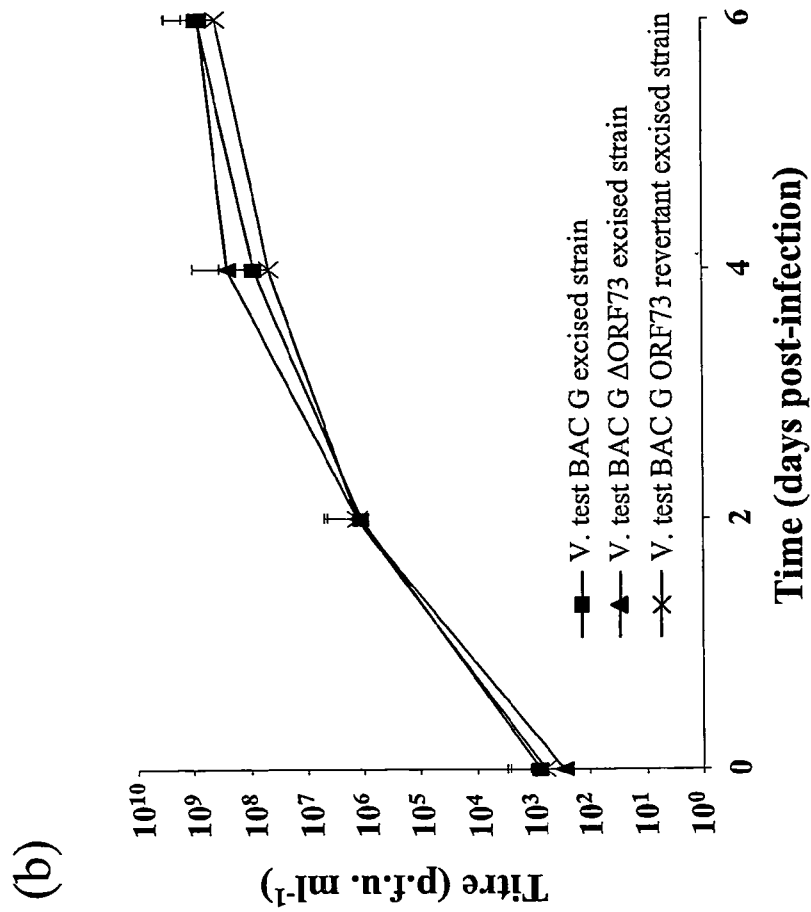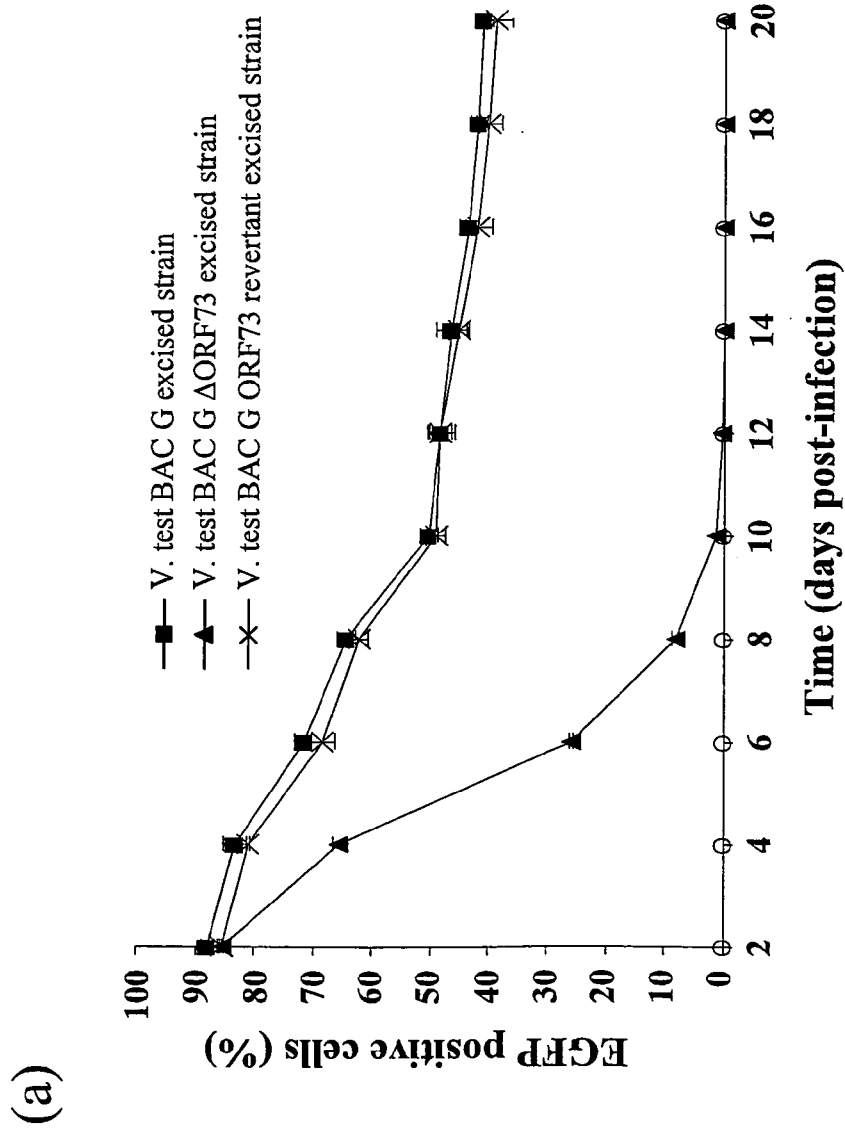Figure 1

(a)

(b)

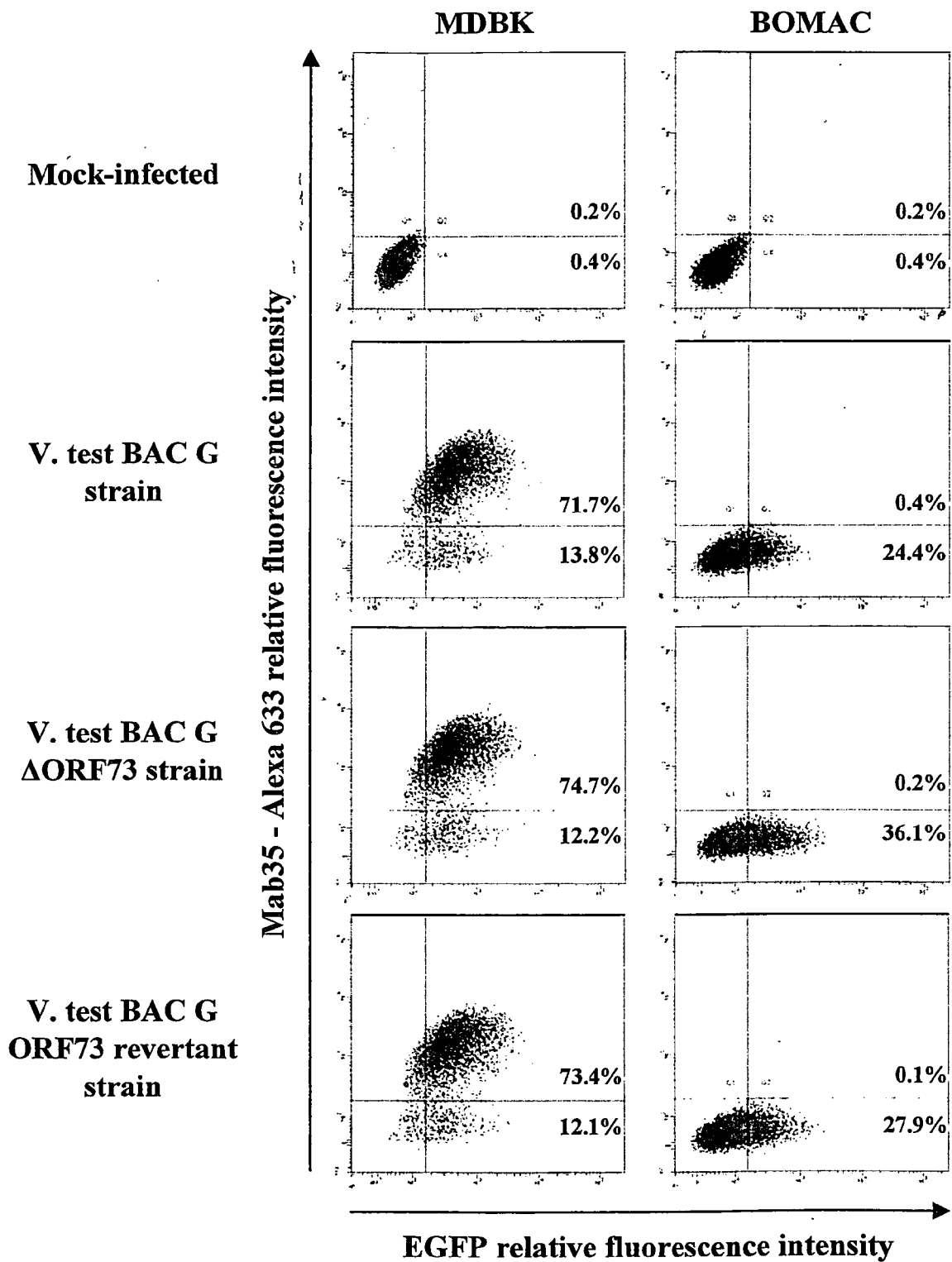Figure 2 a and b

Figure 2 c

(b)

Figure 4 b

Figure 5 a

(b)

Figure 5 b

(a)

PBMC over time
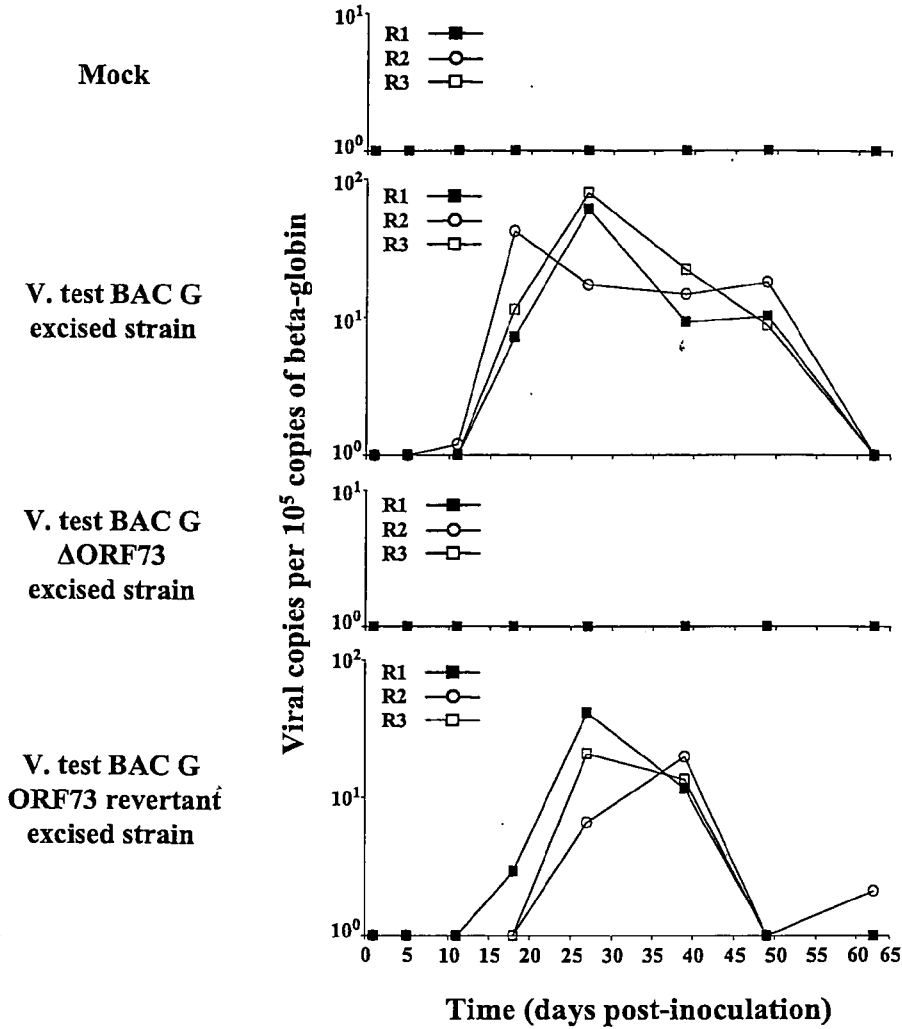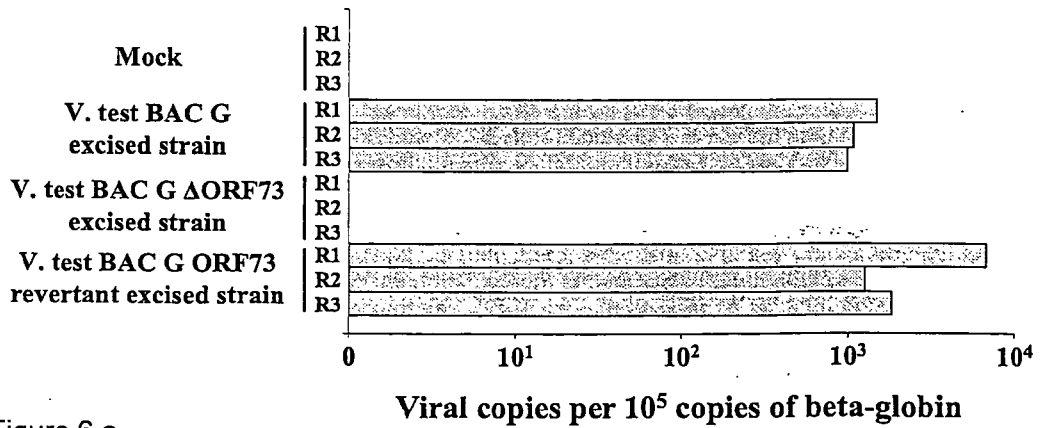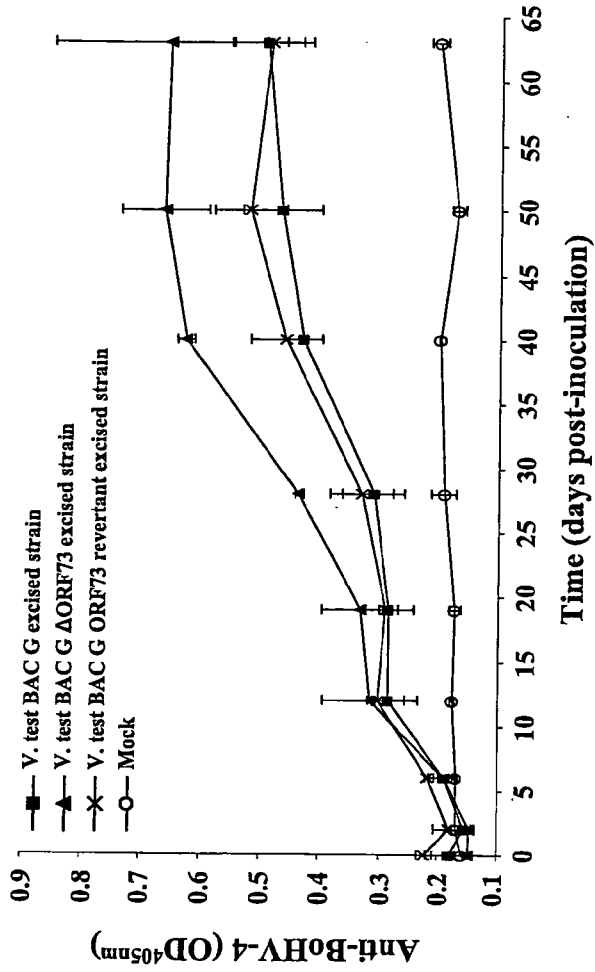
Time (days post-inoculation)

Spleen 64 days post-inoculation

Viral copies per 10⁵ copies of beta-globin

Figure 6 a

Figure 6 b

(b)

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N7/04   C12N15/36   A61K39/245
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GILLET L ET AL: "Development of bovine herpesvirus 4 as an expression vector using bacterial artificial chromosome cloning" JOURNAL OF GENERAL VIROLOGY, vol. 86, April 2005 (2005-04), pages 907-917, XP002593831 ISSN: 0022-1317 page 915, left-hand column, lines 8-20 -/--	1-21

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
8 October 2010	22/10/2010

Name and mailing address of the ISA/	Authorized officer
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LOMONTE P ET AL: "ANALYSIS OF BOVINE HERPESVIRUS 4 GENOMIC REGIONS LOCATED OUTSIDE THE CONSERVED GAMMAHERPESVIRUS GENE BLOCKS" JOURNAL OF GENERAL VIROLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, SPENCERS WOOD, GB, vol. 76, 1 January 1995 (1995-01-01), pages 1835-1841, XP001053327 ISSN: 0022-1317 page 1838, right-hand column, lines 19-30 table 1	1-21
Y	DEWALS BENJAMIN ET AL: "Evolution of Bovine herpesvirus 4: recombination and transmission between African buffalo and cattle" JOURNAL OF GENERAL VIROLOGY, vol. 87, no. Part 6, June 2006 (2006-06), pages 1509-1519, XP002593832 ISSN: 0022-1317 page 1512, right-hand column, paragraph 3 - page 1514, left-hand column, paragraph 1	1-21
X,P	THIRION M ET AL: "Bovine herpesvirus 4 ORF73 is dispensable for virus growth in vitro, but is essential for virus persistence in vivo." THE JOURNAL OF GENERAL VIROLOGY OCT 2010 LNKD- PUBMED:20592111, vol. 91, no. Pt 10, 30 June 2010 (2010-06-30), pages 2574-2584, XP002603988 ISSN: 1465-2099 page 2574 - page 2584	1-21