Modified influenza virus

The present invention covers an influenza B virus M gene comprising a modification of at least one nucleotide proximate to the N-terminus of the M gene, more specifically at any one of nucleotide positions 265 to 294 of the M gene as well as an influenza B virus comprising said modified M gene.

Background of the invention

Epidemics and pandemics caused by viral diseases are still claiming human lives and are impacting global economy. Influenza is responsible for millions of lost work days and visits to the doctor, hundreds of thousands of hospitalizations worldwide (Couch 1993, Ann. NY. Acad. Sci 685;803,), tens of thousands of excess deaths (Collins & Lehmann 1953 Public Health Monographs 213:1; Glezen 1982 Am.J.Public Health 77:712) and billions of Euros in terms of health-care costs (Williams *et al.* 1988, Ann. Intern. Med. 108:616). Both influenza A and B viruses have in the past been responsible for these epidemics in humans, thus besides influenza A also influenza B virus surface antigens are an essential component of any vaccine effective in reducing influenza morbidity. Influenza viruses belong to the Orthomyxoviridae family and are characterized by segmental negative-strand RNA genomes that add up to total sizes of 13.6 to 14.6kb, respectively. Genomic viral RNA must be packaged into viral particles in order for the virus to be transmitted. The process by which progeny viral particles are assembled and the protein/protein interactions occur during assembly are similar within RNA viruses. The formation of virus particles ensures the efficient transmission of the RNA genome from one host cell to another within a single host or among different host organisms. The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M1). The segmented genome of influenza A virus consists of eight molecules of linear, single-stranded RNAs of negative polarity, which encodes eleven (some influenza A strains ten) polypeptides, including: the RNA-dependent RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix membrane proteins (M1, M2 or BM2); two surface

glycoproteins which project from the lipid containing envelope: hemagglutinin (HA) and neuraminidase (NA); the nonstructural protein (NS1) and nuclear export protein (NEP). Most influenza A strains also encode an eleventh protein (PB1-F2) believed to have proapoptotic properties, whereas only influenza B viruses express the NB protein that might contribute to viral virulence (Hatta and Kawaoka, 2003, J. Virol., 77, 6050-6054). There are further minor differences between influenza A and B viruses in their expression strategies of gene products encoded by the viral NA and M gene segments (Lamb and Horvath, 1991, Trends Genet. 7:261-266). Significant biological and epidemiological differences are indicated by the almost exclusive confinement of influenza B viruses to humans, although there have already been studies isolating influenza B virus from seals indicating that there might also be a bigger reservoir of different organisms. Influenza A viruses have a very broad reservoir in many avian and mammalian species.

Transcription and replication of the genome takes place in the nucleus and assembly occurs via budding from the plasma membrane. The viruses can reassort genes during mixed infections. Influenza virus adsorbs via HA to sialyloligosaccharides in cell membrane glycoproteins and glycolipids. Following endocytosis of the virion, a conformational change in the HA molecule occurs within the cellular endosome which facilitates membrane fusion, thus triggering uncoating. The nucleocapsid migrates to the nucleus where viral mRNA is transcribed. Viral mRNA is transcribed by a unique mechanism in which viral endonuclease cleaves the capped 5'- terminus from cellular heterologous mRNAs which then serve as primers for transcription of viral RNA templates by the viral transcriptase. Transcripts terminate at sites 15 to 22 bases from the ends of their templates, where oligo(U) sequences act as signals for the addition of poly(A) tracts. Of the eight viral RNA molecules so produced during influenza A replication, six are monocistronic messages that are translated directly into the proteins representing HA, NA, NP and the viral polymerase proteins, PB2, PB1 and PA. The other two transcripts undergo splicing, each yielding two mRNAs which are translated in different reading frames to produce M1, M2, NS1 and NEP. In other words, the eight viral RNA segments code for eleven proteins: nine structural and 2 nonstructural (NS1 and the recently identified PB1-F2) proteins. Influenza B uses a different coding strategy

for 2 proteins, namely NB and BM2. The former is translated from an overlapping reading frame of the NA gene and the latter is expressed via an overlapping stop-start codon from the M gene.

Vaccination is presently seen as the best way to protect humans against influenza. When healthy adults get immunized, currently available vaccines prevent clinical disease in 70–90% of cases. This level is reduced to 30–70% in those over the age of 65 and drops still further in those over 65 living in nursing homes (Strategic Perspective 2001: The Antiviral Market. Datamonitor. p. 59). The virus's frequent antigenic changes further contribute to a large death toll because not even annual vaccination can guarantee protection.
Vaccination is accomplished with commercially available, chemically inactivated (killed) or live attenuated influenza virus vaccines. Unfortunately, inactivated vaccines can hardly induce cross-protective immunity and therefore the vaccine strain must exactly fit to the antigenic properties of the future unknown pandemic strain.

Replication deficient influenza A viruses are supposed to overcome the safety issues in view of viral shedding. These can be influenza A mutants having deletions of the NS1 protein. The absence of the NS1 protein renders this virus replication-deficient in the respiratory tract of vaccinated mammalians. Upon intranasal administration, the vaccine virus is able to initiate abortive infection in mucosal tissues, without the effect of viral shedding. At the same time the virus stimulates local cytokine response and evokes a B- and T- cell mediated protective immune response.
Influenza B viruses mostly require laborious and time consuming adaptation to reach sufficient growth on Vero cells. Influenza B NS1 mutants which are able to replicate to high titres on Vero cells in addition to an interferon sensitive phenotype due to the abrogated function of NS1 are not described in the literature. Currently, the only published influenza B virus completely lacking the NS1 ORF does not replicate efficiently in Vero cells (titres of $1,7\text{-}2,5*10^2$ FFU/ml using an moi of 0,1 and no detectable titres at moi of 0,001, respectively. Dauber et. al ; Journal of Virology, Feb. 2004, p. 1865–1872). An influenza B NS1 deletion mutant consisting of the amino-terminal 16 aa is also highly attenuated in replication with maximum titres of approx. 10^4

FFU/ml.(Hai et. Al; Journal of Virology ; Nov2008, p.10580-90

Reflecting the need to develop vaccine formulations of high safety containing influenza B antigenic compounds, there is a great demand in developing influenza B strains which are attenuated due to the abrogated function of NS1 but still show high growth properties in cell culture.

Summary

Clinical isolates of influenza B viruses normally require laborious and time consuming adaptation to reach high growth on Vero cells. For influenza A viruses not only wild type viruses but also mutants expressing a truncated NS1 protein of e.g. 38 amino acids or mutants completely lacking the NS1 ORF are able to replicate to high titres on interferon deficient Vero cells. This finding was used to produce a replication deficient attenuated influenza A live vaccine.
Up to now, this concept could not be applied for influenza B viruses due to the incompatibility of mutants with non functional NS1 protein to grow to high titres in tissue culture.
The inventors have now surprisingly shown that growth capabilities can be highly increased by modifying selected nucleotides proximal to the N-terminal region of the M gene of the influenza B virus genome. The M gene of influenza B viruses is of approx. 1.076bp length, comprising the M1 and BM2 ORFs.

Further the disclosure also covers recombinant influenza B viruses comprising said modified M gene. Preferably the B viruses are replication deficient or at least attenuated, for example due to deletions within the NS1 protein.

Specifically a recombinant influenza B strain comprising a modification at any one of amino acid positions 82 to 90, preferably at any one of amino acid positions 85 to 87, preferably at amino acid position 86 in the M1 protein., a modified NS1 segment coding for a NS1 protein lacking a functional RNA binding domain and carboxy terminal domain and optionally a silent mutation at nucleotide position 950 of the M gene is covered by

the present disclosure.

Further, vaccine formulations containing the inventive virus and methods for the prophylactic treatment of influenza as well as methods of making the inventive virus are covered.
The present disclosure also covers an isolated nucleic acid encoding the influenza virus M gene of the disclosure and its production.

In a first aspect, there is provided an influenza B virus M gene comprising at least one nucleotide modification which results in an amino acid substitution at amino acid position 86 in the M1 protein.

In a second aspect, there is provided a recombinant influenza B virus comprising a modification at amino acid position 86 of the M1 protein.

In a third aspect, there is provided a vaccine composition comprising an immunogenically-inducing effective amount of virus as defined above in admixture with a pharmaceutically acceptable carrier.

In a fourth aspect, there is provided a method of making a recombinant influenza B virus as defined above wherein the method comprises introducing a recombinant vector that expresses the influenza virus M86V protein in a reverse genetics system.

In a fifth aspect, there is provided a method of increasing the growth rate of replication deficient influenza B viruses wherein said influenza B viruses are comprising the M86V protein.

In a sixth aspect, there is provided an isolated nucleic acid encoding the influenza virus M86V protein.

In a seventh aspect, there is provided the influenza B virus as defined above for use in the manufacture of a medicament.

In an eighth aspect, there is provided the use of the influenza B virus as defined above in the manufacture of a medicament in the treatment of an influenza virus infection.

Figures:

Fig 1: Schematic translation profile of influenza B NS genes of wild-type, NS14 , NS38, NS57 and NS80 (a) and wild-type and ΔNS1(b)

Fig 2: RT-PCR products of the NS gene of indicated viruses expressing an NS1 protein of 14, 38, 57, 80 amino acids or wild-type NS1 respectively.

Fig 3: Growth of influenza B/Malaysia viruses expressing an NS1 protein of 14, 38, 57 or 80 amino acids or wild-type NS1 respectively on Vero (a) and A549 cells(b)

Fig 4: Viral titres of influenza B/Malaysia mutants with NS1 proteins of 38 or 80 amino acids or wt NS1 containing wt M gene or M1-M86V gene 6 days post transfection (a) and B/Florida mutants containing ΔNS1 gene or with NS1 proteins of 38 or 80 amino acids or wt NS1 containing wt M gene or M1-M86V gene 5 days post transfection (b).

Fig 5: Amino acid comparison of the original B/Malaysia/2506/04-like swab M gene with the M1-M86V gene and other sequences published in the genebank.

Fig 6. Growth of influenza B/Florida viruses containing ΔNS1 gene or wild-type NS1 on Vero (a) and A549 cells (b).

Fig 7: Amino acid sequence of the B/Vienna/33/06 M1 protein containing the M86V mutation (marked with bold letters, SEQ ID No. 1) (a) and B/Thüringen/02/06 M1 protein containing the M86V mutation (marked with bold letters, SEQ ID No. 2) (b).

Fig. 8: Overview of constructed influenza B NS mutants. Genetic composition of all constructed influenza B Mutants (a); nucleotide sequence of B/Vienna/33/06 M gene M1-M86V, SEQ ID No. 3 (b); nucleotide sequence of B/Vienna/33/06 M gene M1-M86V and C950T, SEQ ID No. 4 (c); nucleotide sequence of B/Thüringen/02/06 M gene M1-wt, SEQ ID No. 5 (d); nucleotide sequence of B/Thüringen/02/06 M gene M1-M86V, SEQ ID No. 6 (e); nucleotide sequence of B/Vienna/33/06 M gene M1-C950T, SEQ ID

No. 7 (f); nucleotide sequence of B/Vienna/33/06 NS gene of NS14, SEQ ID No. 8 (g); nucleotide sequence of B/Vienna/33/06 NS gene of NS38, SEQ ID No. 9 (h); nucleotide sequence of B/Vienna/33/06 NS gene of NS57, SEQ ID No. 10, (i); nucleotide sequence of B/Vienna/33/06 NS gene of NS64, SEQ ID No. 11 (j); nucleotide sequence of B/Vienna/33/06 NS gene of NS80, SEQ ID No. 12 (k);
nucleotide sequence of B/Vienna/33/06 NS gene of ΔNS1-B, SEQ ID No. 13 (l);
nucleotide sequence of B/Thüringen/02/06 NS gene of ΔNS1-B, SEQ ID No. 14 (m);
Fig. 9: Schematic translation profile of IL2 expressing influenza B Vectors with NS1 proteins of 38, 80, 104 and 145 aa length, respectively (a); genetic composition of all constructed IL2 expressing influenza B Vectors (b); nucleotide sequence of B/Vienna/33/06 NS gene of NS1-38IL2, SEQ ID No. 15 (c); nucleotide sequence of B/Vienna/33/06 NS gene of NS1-80IL2, SEQ ID No. 16 (d); nucleotide sequence of B/Vienna/33/06 NS gene of NS1-104IL2, SEQ ID No. 17 (e); nucleotide sequence of B/Vienna/33/06 NS gene of NS1-145IL2, SEQ ID No. 18 (f);
Fig 10: RT-PCR products of influenza B/NS1-wt, B/NS1-38 and B/NS1-38IL2 viruses after 5 passages on Vero cells.

Detailed description of the invention

The present invention provides a nucleotide sequence change of the M gene of influenza B virus which can increase the growth capabilities of the influenza B strain. Such changes can include deletions and substitutions. It has been successfully shown by the inventors that at least one a single amino acid change in the M1 protein of influenza B can lead to high growth properties in cell culture, especially in Vero cells. This specifically can enable the rescue of viruses expressing NS1 proteins of reduced length compared to wt NS1 proteins or mutants bearing a complete deletion in the NS1 ORF.

Specifically, influenza B virus M gene according to the present invention comprises a modification of at least one nucleotide of any one of nucleotide positions 265 to 294 of the M gene, preferably of at least one nucleotide at any one of nucleotide positions 277 to 285, more preferred of at least one nucleotide at any one of nucleotide positions280 to 282.

Specifically, the modified M gene contains nucleotides GTG at positions 280 to 282 instead of ATG of the corresponding sequence of the isolated virus. Alternatively the modified M gene can also contain nucleotides GTA, GTC, GTT at positions 280 to 282. Of course, the embodiment also covers the respective RNA codons, GUG, GUA, GUC, GUU.

According to an alternative embodiment of the present invention the influenza B virus M gene comprises at least one nucleotide modification which results in at least one amino acid substitution at any one of amino acid positions 82 to 90, preferably at any one of amino acid positions 85 to 87, preferably at amino acid position 86 in the M1 protein. The substituted amino acid can be any amino acid; a non-polar, hydrophobic amino acid being preferred. Specifically, the amino acid change leads to the change of Methionine to Valine at amino acid position 86.

An M1 protein comprising at least one amino acid substitution at any one of amino acid positions 82 to 90, preferably at any one of amino acid positions 85 to 87, preferably at amino acid position 86 in the M1 protein is of course covered by the invention. The substituted amino acid can be any amino acid; a non-polar, hydrophobic amino acid being preferred. Specifically, the amino acid change leads to the change of Methionine to Valine at amino acid position 86. The M1 protein can be generated by any method as known in the art.

The term "amino acid substitution" refers to the presence of a modified or different amino acid at a particular location in the parenteral amino acid sequence of that molecule. The amino acid substitute occurs relative to any other amino acid that could have occupied that position. The polypeptide that results from the amino acid sequence change may include changes in post-translational modifications such as glycosylations, acetylations, phosphorylations or any other amino acid modification as well as the amino acid substitution.

A recombinant influenza B virus comprising the influenza virus M gene of the invention is also covered by the present invention.

Within other aspects a replication-deficient influenza viruses containing a modified M gene according to the invention can further contain a modification within the NS gene, specifically lack of part of or lack of the entire NS1 protein (ΔNS1). Due to the truncation or lack of expression of the NS1 protein, such viruses can replicate only in interferon-deficient cells but have lost their ability to grow in common hosts and organisms.

The NS1 protein of influenza A virus is a multifunctional protein that consists of approximately 230 amino acids and is early and abundantly synthesized in infection. It counters cellular antiviral activities and is a virulence factor. By the activity of its carboxy terminal region, the NS1 protein is able to inhibit the host mRNA´s processing mechanisms. Second, it facilitates the preferential translation of viral mRNA by direct interaction with the cellular translation initiation factor. Third, by binding to dsRNA and interaction with putative cellular kinase(s), the NS1 protein is able to prevent the activation of interferon (IFN-) inducible dsRNA-activated kinase (PKR), 2´5´ oligoadenylate synthetase system and cytokine transcription factors. Fourth, the N terminal part of NS1 binds to RIG-I and inhibits downstream activation of IRF-3, preventing the transcriptional induction of IFN-β. Therefore the NS1 protein inhibits the expression of INF-α or INF-ß genes, delays the development of apoptosis in the infected cells, and prevents the formation of the antiviral state in neighbouring cells.
Influenza B viruses express from an unspliced transcript of the viral NS segment a 281-amino acid nonstructural protein termed NS1-B that shares with its NS1-A counterpart the ability to bind the same RNA targets and to inhibit PKR activation in vitro. In contrast to influenza A, influenza B NS1 does not inhibit pre-mRNA splicing but binds to the interferon-stimulated gene 15 (ISG15) product and inhibit its conjugation to cellular targets
Influenza A viruses containing modifications within the NS1 protein are known in the art. For example, WO 99/64571 describes the complete knock out of the NS1 gene segment, WO 99/64068 discloses various NS1 gene segments that have been partially deleted. These publications are incorporated herein in full by reference. Only few influenza B viruses containing modifications in the NS1 protein are currently known and none of those viruses bearing an interferon sensitive phonotype due to the abrogated

function in NS1 grows to high titres in Vero cells. Here, the construction of such viruses with modifications in the NS1 protein is described.

According to the present invention the modification within the NS1 protein can be a deletion, an insertion or substitution of at least one amino acid resulting in a replication deficient influenza virus.

Preferably the modified influenza B NS1 protein comprises a deletion of at least 50% of the NS1 amino acids, preferably of at least 70%, more preferably of at least 90%. Alternatively, the functionality of the NS1 protein can be completely diminished.
The NS1 protein of the influenza virus according to the invention can lack the functional RNA binding domain. The primary function of this domain located at the amino end of the NS1 protein (amino acids 1- 93) is binding dsRNA and inhibiting the activation of PKR (Dauber et al, J Virol. 2006 Dec;80(23):11667-77).
According to the invention, the term functional carboxy terminal domain can comprise the region within the NS1 protein that enables the inhibition of the host mRNA´s processing mechanisms, i.e. its activity suppresses the IFN response of the host cells.

Influenza B-NS1 seems to lack a carboxy terminal effector domain with similar function than influenza A-NS1. The C-terminal domain of the NS1 protein (amino acid positions 84 to281) of influenza B virus is mainly responsible for inhibition of the IFN-α/β response (Dauber et al, J Virol. 2006 Dec;80(23):11667-77).
According to the invention, the carboxy terminal domain of the influenza B NS1 protein might be rendered non functional. This domain can be completely or partially deleted as well as amino acids can be substituted or inserted and the remaining domain can be tested for functionality as described in the art (Dauber et al, J Virol. 2006 Dec;80(23):11667-77).

An inventive mutation of the M gene enables the generation of influenza B mutants which express a truncated NS1 protein (e.g. shorter than 80 amino acids) therefore bearing an interferon sensitive phenotype. Such mutants could be used especially as live attenuated vaccine strains of high safety and efficacy.

The inventive modification within the M gene can specifically increase the growth capabilities of said replication deficient influenza B. For example, an influenza B virus expressing a NS1 protein of 80 amino acids (NS1-80) containing the M1-M86V mutation achieved titres in a range of $5*10^5$ to $5*10^7$ $TCID_{50}$ whereas a similar virus with wt M1 protein grows to only $3*10^3$-$3*10^5$ $TCID_{50}$. Viruses containing a NS1 protein shorter than 80 amino acids i.e. 14, 38, 57 or 64 amino acids were only rescued using the inventive modification in the M gene and grew to titres ranging from $1*10^4$-$3*10^8$ $TCID^{50}$. The M1-M86V mutation can also be introduced into the backbone of another influenza B virus of different genetic subtype (for example Jiangsu/10/03-like) to prove, that the improved growth capacity especially of NS1 truncation mutants is rather universal than strain specific. In this backbone, the inventive M1-M86V mutation enabled the generation of a ΔNS1-B virus, in which the NS1 ORF is completely deleted, growing to high titres in the range of 10^7 to 10^8 $TCID_{50}$/ml in Vero cells.

According to a specific embodiment of the invention the recombinant influenza virus can comprise

a) a modification at amino acid position 86 of the M1 protein
b) a modified NS1 segment coding for an NS1 protein lacking a functional RNA binding domain and functional carboxy terminal domain and
c) optionally a silent mutation at nucleotide position 950 of the M gene.

As an alternative embodiment of the invention, the recombinant replication deficient influenza B virus can also be used as a vehicle for expressing heterologous sequences, e.g. for the expression of chemokines or cytokines or fragments thereof.

More specifically it can be a recombinant influenza virus comprising

a. a modification at amino acid position 86 of the M1 protein
b. a modified NS1 segment coding for a NS1 protein lacking a functional RNA binding domain and functional carboxy terminal domain and
c. a heterologous sequence inserted between the splice donor site and the

splice acceptor site of the NS1 gene segment.
optionally a silent mutation at nucleotide position 950 of the M gene.

According to a preferred embodiment of the invention the heterologous sequence expresses cytokines or chemokines or fragments or derivatives thereof.
Cytokines are small secreted proteins which mediate and regulate immunity, inflammation and hematopoiesis. The largest group of cytokines are those which promote proliferation and differentiation of immune cells. Included within this group are interleukins, which are cytokines produced by leukocytes, and interferons, which may be produced by a variety of cell types.

Interferons (IFN) are a family of naturally occurring glycoproteins produced by cells of the immune system of vertebrates, including mammals, birds, reptiles and fish, in response to challenge by agents such as bacteria, viruses, parasites and tumour cells. In humans there are three major classes of interferons. The type I interferons include 14 IFN-alpha subtypes and single IFN-beta, omega, kappa and epsilon isoforms. Type II interferons consist of IFN-gamma and a recently discovered third class consists of IFN-lambda with three different isoforms.
Th1 cells secrete mainly IL-2, IFN-γ, and TNF-β, whereas Th2 cells which are relevant in humoral immune responses secrete cytokines such as IL-4, IL-5, and IL-10. Th2-type cytokines mediate delayed type hypersensitivity responses against intracellular pathogens and inhibit the Th1 responses.
Chemokines, originally derived from chemoattractant cytokines, actually comprise more than 50 members and represent a family of small, inducible, and secreted proteins of low molecular weight (6-12 kDa in their monomeric form) that play a decisive role during immunosurveillance and inflammatory processes. Depending on their function in immunity and inflammation, they can be distinguished into two classes. Inflammatory chemokines are produced by many different tissue cells as well as by immigrating leukocytes in response to bacterial toxins and inflammatory cytokines like IL-1, TNF and interferons. Their main function is to recruit leukocytes for host defence and in the process of inflammation. Homing chemokines, on the other hand, are expressed constitutively in defined areas of the lymphoid tissues. They direct the traffic and homing

of lymphocytes and dendritic cells within the immune system. These chemokines, as illustrated by BCA-I, SDF-1 or SLC, control the relocation and recirculation of lymphocytes in the context of maturation, differentiation, activation and ensure their correct homing within secondary lymphoid organs.

According to the present invention it has been shown that biologically active cytokines or chemokines or derivatives or fragments thereof can be stably and efficiently expressed using an open reading frame different from the ORF expressing the NS1 protein. Alternatively additional leader sequences other than the natural signal peptides can be fused to the cytokines or chemokines which may further support efficient secretion of the protein and show a highly efficient induction of immune response in vivo.
Surprisingly, chemokines and cytokines can also be efficiently expressed when the amino acid sequence corresponding to the mature cytokine/chemokine is fused to a part of the NS1 protein via an amino acid sequence acting as a signal peptide , For example, this can be a part of the mouse IgKappa signal peptide.

According to the present invention the heterologous sequence preferably codes for interleukin 2 (IL-2) or a fragment or derivative thereof. IL-2 comprises secretory signal sequences and is an immunomodulatory, T-cell derived molecule required for the clonal expansion of antigen-activated T-cells. The secretion of IL-2 by CD4+ T lymphocytes has multiple biological effects, such as the induction of proliferation of T-helper and T-killer cells and the stimulation of T-cells to produce other cytokines. Furthermore, IL-2 can also activate B-cells, NK cells and macrophages. When IL-2 is expressed from recombinant viruses infecting non-lymphoid cells, its secretion could significantly decrease the pathogenesis of viral infection and modify the immune response. It is also known that IL-2 acts as immune adjuvant.
According to the present invention any fragment or derivative of the cytokines and chemokines is included that is still biologically active, i.e. shows immunomodulatory activities.
Alternatively, the cytokines/chemokines can also be selected from the group consisting of IL-15, GM-CSF, CCL3 or CCL20 or derivatives or fragments thereof.

Alternatively, it can be also any epitope or immunomodulatory region derived from Mycobacterium tuberculosis, for example ESAT-6.

Alternatively the heterologous sequences can also comprise chimeric proteins being cytokines or chemokines or fragments or derivatives thereof fused to antigenic proteins or antigenic peptides. Fusion can be either directly or via peptide linker sequences having a length of at least 4 amino acids, preferably at least 5 amino acids. For example, the linker sequences according to the invention are GGGS or GGGGS.
Examples for IL-2 chimeric proteins are known in the art. Exemplarily, this could be IL-2-PE40 (wherein PE is Pseudomonas exotoxin A), DAB389-IL-2 (where DAB is diphtheria toxin) or IL-2 Bax (wherein Bax is a proapoptotic protein of human origin) (Aqeilan R. et al., Biochem.J., 2003, 129-140).

According to the present invention the nucleotide sequences of the heterologous sequences which are introduced into the replication deficient influenza vector show at least 80% identity with their native sequences, preferably at least 85% identity, more preferred at least 90% identity. Any optimization of the nucleotide sequence in view of codon usage is included thereby.

Alternatively, the heterologous sequence can comprise B-cell or T-cell-epitopes, for example a B cell epitope from influenza hemagglutinin (HATB) , for example the A loop epitope from the influenza virus hemagglutinin (HA) or parts thereof, or peptides representing one of the immunodominant epitopes of HA corresponding to amino acid sequence 150 to 159 (Caton et al., 1982, Cell, 417-427).
The epitope can also be derived from melanoma-associated endogenous retrovirus (MERV) as described in WO06/119527.

According to a specific embodiment, the NS1 gene segment can contain a functional natural splice donor and acceptor splice site, i.e. the splice donor and acceptor sites are kept as natural sites, i.e. the nucleotides are not modified by artificial techniques.

Any nucleotide modifications at the splice sites occurring naturally due to modifications

of influenza viruses based on environmental adaptations or natural strain developments are natural modifications and do not fall under the term synthetic or artificial modifications.

Alternatively, the sequences surrounding the splice donor and/or upstream of the acceptor site can be altered, Preferably, alteration or modification can be performed within 3 nucleotides 5' to the and/or 8 nucleotides 3' to the 5' border of the NS intron, as well as 100 nucleotides 5' to the and/or 2 nucleotides 3' to the 3' border of the NS intron.. This is preferably by introducing synthetic sequences in order to modify splicing activity.
If e.g. insertion of a heterologous sequence increases NS intron size it may be preferable to modify the sequences surrounding the splice donor and/or acceptor site in order to increase splicing efficacy and thus genetic stability of the recombinant NS segment.

For example, it can be modified in that either the sequence surrounding the splice donor site is altered to increase the homology to the 5'end of the human U1 snRNA and/or the sequence upstream of the splice acceptor site containing the branch point (Plotch et al. 1986, Proc Natl Acad Sci U S A. 83:5444-8; Nemeroff et al. 1992, Mol Cell Biol. 12:962-70) and the pyrimidine stretch is replaced by a sequence that enhances splicing of the NS segment.

In order to optimize splicing, the a preferred sequence introduced 5' of the splice acceptor site comprises a lariat consensus sequence and a pyrimidine stretch.
In view of stability of the virus vector and the expression rate of the heterologous sequence it can be important to introduce the synthetic/modified sequence containing a lariat consensus sequence and a pyrimidine stretch at a specific position within the NS gene, e.g. directly upstream of the slice acceptor site.

Furthermore, it may be necessary to vary the distance between the lariat consensus sequence and the pyrimidine stretch to modify the splicing rate of the NS segment (Plotch S. and Krug R., 1986, Proc.Natl.Acad.Sci., 83, 5444-5448; Nemeroff M. et al.,

1992, Mol.Cell.Biol., 962-970).

For example, the recombinant influenza B strain can comprise an amino acid sequence as shown in SEQ ID. No. 1 or 2 or a nucleotide sequence as shown in any one of SEQ ID Nos. 3 to 18 or a derivative thereof having at least 98% sequence identity, preferably at least 99% sequence identity, preferably at least 99,5%.

According to the invention, the term "recombinant" does cover all influenza strains that have been produced using recombinant techniques like, for example, reverse genetics technology. Therefore the influenza strains according to the invention contain a modification within the M gene but need not contain any further modifications within the nucleotide or amino acid sequence compared to the parental strain.
Also a vaccine composition comprising an immunogenically-inducing effective amount of virus in admixture with a pharmaceutically acceptable carrier is covered. Adjuvants can also be contained in the composition.
According to the invention the term "immunogenic" means that the virus is capable of eliciting humoral or cellular immune response, and preferably both. An immunogenic entity is antigenic, too. An immunogenically inducing effective amount of virus elicits humoral or cellular immune response, or both, when administered to an animal, preferably to a human.

The vaccine composition may be used for prophylactic treatment of influenza disease comprising administering to a human patient in need of treatment an immunologically inducing effective amount of the composition.

Vaccine compositions can contain complete influenza B virus according to the invention but also reassortant strains wherein part of the viral segments are derived from different influenza B strains and segments, especially M1 protein is derived from the recombinant influenza B virus according to the invention. The inventive M1-M86V mutation could be further introduced into any other influenza B strain.

The compositions may be used in methods or as medicaments in preventing, managing,

neutralizing, treating and/or ameliorating influenza virus infection. The use of an influenza virus M molecule according to the invention in the manufacture of a medicament for treatment of an influenza virus infection is of course included.
The immunogenic compositions may comprise either a live or inactivated influenza B virus of the invention. The virus can be inactivated by methods well known to those of skill in the art. Common methods use formalin and heat for inactivation.
A live immunogenic formulation may be preferred due to increased immunogenicity. Production of such live recombinant immunogenic formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in embryonated eggs (e.g., embryonated chicken eggs) followed by purification.

The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S.
The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition (e.g., immunogenic or vaccine formulation) is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. The formulation should be selected according to the mode of administration. The particular formulation may also depend on whether the virus is live or inactivated.

The term adjuvant refers to a compound or mixture that enhances the immune response to an antigen.

The prophylactic and/or therapeutic effect of the immunogenic formulations of the invention is based, in part, upon achieving or inducing an immune response (e.g., a humoral immune response). In one aspect, the immunogenic formulations induce a detectable serum titer of an antibody against antigens of the influenza B virus in either the subject or an animal model thereof (e.g. mouse, ferret, rat or canine model). The

serum titer of an antibody can be determined using techniques known to one of skill in the art, e.g., immunoassays such as ELISAs or hemagglutinin inhibition tests.

According to the invention also a method of generating a virus is also covered wherein the method comprises using a recombinant expression system that expresses the inventive influenza virus M molecule. According to the present invention the expression system can be any plasmid useful, e.g. as described by Hoffmann et al. ((Hoffmann et al. 2000, Proc Natl Acad Sci U S A. 97:6108-13) or a linear expression construct according to EP07450177.
For developing reassortants and/or expression of modified influenza virus strains a reverse genetics system on Vero cells can be used. The technology is already well known in the art (Pleschka S. et al., 1996, J. Virol., 70(6), 4188-4192, Neumann and Kawaoka, 1999, Adv. Virus Res., 53, 265-300, Hoffmann et al. 2000, Proc Natl Acad Sci U S A. 97:6108-13)

The cells used for the cultivation of viruses using a cultivation medium can be any cells that can grow in vitro in synthetic media and can be used for the propagation of viruses. Within the scope of the invention, the term "cells" means the cultivation of individual cells, tissues, organs, insect cells, avian cells, mammalian cells, hybridoma cells, primary cells, continuous cell lines, and/or genetically engineered cells, such as recombinant cells expressing a virus. These can be for example BSC-1 cells, LLC-MK cells, CV-1 cells, CHO cells, COS cells, murine cells, human cells, HeLa cells, 293 cells, VERO cells, MDBK cells, MDCK cells, MDOK cells, CRFK cells, RAF cells, TCMK cells, LLC-PK cells, PK15 cells, WI-38 cells, MRC-5 cells, T-FLY cells, BHK cells, SP2/0 cells, NS0, PerC6 (human retina cells), chicken embryo cells or derivatives, embryonated egg cells, embryonated chicken eggs or derivatives thereof. Preferably the cell line is a VERO cell line.

The cultivation medium used for the production of viruses can be any medium known from prior art that is applicable for virus cultivation. Preferably the medium is a synthetic medium. This can be for example basal media as Modified Eagle's media MEM, minimum essential media MEM, Dulbecco's modified Eagle's media D-MEM, D-MEM-

F12 media, William's E media, RPMI media and analogues and derivative thereof. These can also be specialty cell cultivation and virus growth media as VP-SFM, OptiPro™ SFM, AIM V® media, HyQ SFM4MegaVir™, EX-CELL ™ Vero SFM, EPISERF, ProVero, any 293 or CHO media and analogues and derivatives thereof. These media can be supplemented by any additive known from prior art that is applicable for cell and virus cultivation as for example animal sera and fractions or analogues thereof, amino acids, growth factors, hormones, buffers, trace elements, trypsin, sodium pyruvate, vitamins, L-glutamine and biological buffers. Preferable medium is OptiPRO™ SFM supplemented with L-glutamine and trypsin.

Using the inventive modification of the M gene the growth rate of influenza B NS1 truncation mutants can be increased at least by a factor of approx 100-1000 as demonstrated for the NS1-38 virus, when the viral titers ($TCID_{50}$) between the unmodified and improved M genes were compared. For influenza B viruses expressing a NS1 protein of 14, 57 or 80 amino acids, respectively, the mutation is in the M gene is absolutely required to generate such viruses. These viruses can grow up to a titre of approx 10^6-10^8 $TCID_{50}$

A further embodiment of the invention is an isolated nucleic acid encoding the inventive influenza virus M gene and/or a recombinant influenza virus B containing the modified M gene.
Further a method for preparing said nucleic acid according which method comprises introducing a nucleotide sequence into a vector encoding the inventive M molecule.
If a DNA vector is used, said vector is a transcription system for minus sense influenza RNA. For example it can be a vector as used by Hoffmann et al., 2000, Proc Natl Acad Sci U S A. 97:6108-13.

The foregoing description will be more fully understood with reference to the following examples. Such examples are, however, merely representative of methods of practicing one or more embodiments of the present invention and should not be read as limiting the scope of invention.

Examples:

Example 1:

Influenza B Viruses expressing truncated NS1 proteins for usage as a replication deficient live attenuated influenza vaccine.

A reverse genetic system for the construction of a Vero adapted influenza B strain (B/Vienna/33/06 subtyped as B/Malaysia/2506/04) as a representative of the B/Victoria/2/87 linage was generated (Hoffmann et. al 2000, PNAS 97(11):6108-13.). HA and NA were cloned from influenza B/Thüringen/02/06 (B/Jiangsu/10/03-like). By introducing a coding mutation in the M1 protein (change from methionine to valine at amino acid position 86), influenza mutants expressing a truncated version of the NS1 protein of 14, 38, 57 and 80 amino acids could be obtained and named B/Malaysia NS1-14, NS1-38, NS1-57 or NS1-80 respectively. The translation of NS1 was terminated by two consecutive, in frame stop codons and the non translated part downstream of the stop codons up to the splicing signal of NEP was deleted (Fig.1a). Due to the different lengths of the NS gene, the viruses containing NS1 proteins of different lengths could be distinguished according to their size by RT-PCR (Fig. 2). All generated mutants were replicating to high titres on Interferon deficient Vero cells (Fig.3a) but were attenuated on interferon competent A549 cells (Fig. 3b

The inventive modification within the M gene can specifically increase the growth capabilities of said replication deficient influenza B viruses. For example, an influenza B virus expressing a NS1 protein of 80 amino acids (NS1-80) containing the M1-M86V mutation grows to titres of approx. $5{,}62*10^4$ $TCID_{50}$ in contrast to titres of approx. $1{,}33*10^4$ $TCID_{50}$ of a similar virus which does not contain any modification in M1 when analyzed 6 days post transfection (Fig. 4a). An influenza B virus expressing a NS1 protein of 38 amino acids (NS1-38) could not be rescued at all without the adapting M1 mutation but grows to average titres of approx. $3{,}16*10^2$ $TCID_{50}$ when the M1-M86V mutation was introduced (Fig. 4a). This effect was even more pronounced in the 2^{nd} passage post transfection, reflected by titres (Table 1). The same rescue efficiency was observed with influenza B viruses expressing an NS1 protein of 14, or 57 amino acids, respectively, which were only rescued in the presence of the M1-M86V mutation (data not shown). Further adaptive passages on Vero cells resulted in high titres ranging from 6,5-8,5 logs $TCID_{50}$ which are required for efficient vaccine production, of all NS1

mutants (Fig. 3a). The inventive mutation might have no or only minor effect on the growth of wt NS1 viruses. The wt NS1 influenza B virus containing the non-modified M gene even grew to slightly higher titres than the analogous virus containing the M1-M86V mutation 6 days post transfection (Fig 4a) and in the 2nd passage after transfection (Table 1). This can be interpreted by the variation of growth between the different passages as demonstrated by the growth of the 1st Passage after transfection ($1,78*10^7$ $TCID_{50}$ with wt M gene in comparison to $2,82*10^7$ $TCID_{50}$ with M1-M86V, both containing wt NS gene)

Table 1

$TCID_{50}$ 2nd Passge after Transfection		
	wt M1	M1-M86V
wt NS	1,00E+07	1,00E+06
NS1-80	3,16E+05	1,78E+07
NS1-38	0,00E+00	2,31E+04

Viral titres (TCID50) of influenza B viruses with wt, NS80 or NS38 NS1 protein in combination with either wt or M1-M86V M gene 4 days post infection analyzed in the 2nd passage after transfection.

Therefore, only this novel mutation enables the generation of influenza B mutants which express a short NS1 protein (i.e. comprising less than the first N-terminal 80 amino acids) with non-functional NS1, therefore bearing an interferon sensitive phenotype. Such mutants could be consequently used as vaccine strains. This mutation was never described before nor was it found it the NIBSC sequencing database. Figure 5 shows a sequence comparison of the M gene original B/Malaysia/2506/04-like swab with the M1-M86V gene and other sequences published in the genebank.

Example 2

<u>The inventive mutation in the M1 protein (M86V) enables the generation of influenza B viruses with truncated NS1 proteins on Vero cells in different influenza B lineages.</u>

To test the influence of the M1-M86V mutation in other influenza B strains, a reverse genetic system for the generation of influenza B/Thüringen/02/06 (B/Jiangsu/10/03-like)

as a representative of the B/Yamagata/16/88 linage was generated and the described mutation in the M1 protein (M86V) was introduced. To follow the WHO recommendation for the influenza vaccine strains for the season 2008/2009 in the northern hemisphere, HA and NA of a B/Florida/04/06-like virus were used. Influenza B mutants expressing a truncated version of the NS1 protein of 38 and 80 amino acids or a mutant with a complete deletion in the NS1 ORF (ΔNS1-B) (Fig.1b), in which NS2/NEP is expressed as a monicistronic RNA were obtained and named B/Florida NS1-38, NS1-80 or ΔNS1-B, respectively. As was the case with the mutants of B/Malaysia, a representative of the B/Victoria/2/87 lineage, the M1-M86V mutation had only a small impact on the rescue efficacy of NS1-80 and NS1-wt viruses of the B/Florida, a representative of the Yamagata lineage (Fig 4b). This was demonstrated by slightly increased titers 5 days post transfection as compared to mutants containing the wt M gene (Fig. 4b). Viral titers for the NS1-38 mutant containing the M1-M86V mutation were approximately 4 logs higher than wt M1 analogs. Due to the M1-M86V mutation, we were able to rescue a ΔNS1-B virus reaching titers of almost 4 logs 5 days post transfection (Fig 4b).
In order to demonstrate the replication-deficient phenotype of ΔNS1-B virus, we examined its ability to grow on IFN-deficient Vero (Fig. 6a) and IFN-competent A549 (Fig. 6b) cells in comparison to the corresponding wt virus. Both viruses showed comparable growth kinetics on Vero cells, reaching titers in the range of 10^7 to 10^8 $TCID_{50}$/ml. Replication of ΔNS1-B virus was completely restricted in IFN-competent A549 cells, which displayed no growth above the detection limit of 2×10^2 $TCID_{50}$/ml, while NS1-wt virus replicated to high titers of $3{,}15 \times 10^8$ $TCID_{50}$/ml. Mutant viruses NS1-80 and NS1-38 showed intermediate replication capacity (data not shown).
These data show that the adaptive M1 mutation does not only have a growth-optimizing effect in one viral strain but also seems to be effective in other influenza B lineages. Moreover, the data demonstrate that this mutation is essential for rescuing a ΔNS1-B virus, in which the NS1 ORF is completely deleted, growing to high titers in Vero cells. Therefore, this is a universal concept for the generation of replication deficient influenza B viruses in which the attenuation mechanism is based on the removal of NS1, the major interferon antagonist.

Example 3:

An influenza B virus vector expressing human Interleukin 2 from the NS gene using a bicistronic expression strategy as potential live attenuated influenza vaccine with improved immunogenicity especially in elderly.

An overlapping Stop-Start codon cassette (TAATG) followed by the coding sequence of human IL2 was inserted after amino acid position 38, 80, 104 or 145 of the NS1 protein of an influenza B virus, respectively. In addition, a synthetic sequence of 29 nucleotides comprising a lariat consensus sequence followed by an optimized splice site, a 20-base pyrimidine stretch segment (opt. splice) replaces the part between the stop codon of IL2 and the splice acceptor site of NS2 (EP7450176.8). (Fig 9a).
By introducing the inventive mutation in the M1 protein (M86V), we succeeded in rescuing an influenza B virus B/NS1-38IL2 in the backbone of influenza B/Thüringen/02/06 (B/Jiangsu/10/03-like). Without the inventive modification in the M1 protein, this virus was not rescued. Although growth of B/NS1-38IL2 was slightly lower compared to the "empty vector" B/NS1-38, a titer of more than 6 log10 TCID50/ml was achieved (Table 2). This stock was further passaged five times on Vero cells to check genetic stability. No appearance of deletion mutants were found as demonstrated by the presence of RT-PCR bands of the expected size of the NS gene without appearance of smaller PCR bands potentially reflecting deletion mutants (Fig 10).
Vero cells infected with B/NS1-38IL2 secreted high levels of more than 2,5 µg/ml IL2 in contrast to non detectable IL2 levels in non infected cells (mock), cells infected with B/NS1-38 or B/NS1-wt (Table 2). Such vectors could be used as live influenza vaccines with an increased immunogenicity especially in elderly as already demonstrated for influenza A (Ferko, Kittel et al 2006)

Table 2. Replication in Vero cells and expression levels of human IL2 of indicated viruses

Virus	Titer [TCID50/ml]	Human IL2 ELISA [pg/ml]

B/NS1-wt	2.88E+07	<19
B/NS1-38	1.78E+08	<19
B/NS1-38IL2	2,31E+06	2687
Mock	-	<19

We investigated the immunogenicity of the generated viruses of all 3 examples. From these data we conclude, that the inventive M1-M86V mutation does not negatively influence the immunogenicity of the constructed viruses as demonstrated by comparable immunogenicity data of respective influenza A viruses.

Claims:

1. An influenza B virus M gene comprising at least one nucleotide modification which results in an amino acid substitution at amino acid position 86 in the M1 protein.

2. The influenza B virus M gene according to claim 1 comprising nucleotides GTG, GTA, GTC, GTT, GUG, GUA, GUC, GUU at positions 280 to 282.

3. A recombinant influenza B virus comprising a modification at amino acid position 86 of the M1 protein.

4. The influenza B virus according to claim 3 wherein the amino acid at position 86 of the M1 protein is Valine.

5. The influenza B virus according to claims 3 or 4 wherein said virus is a reassortant virus.

6. The influenza B virus according to any one of claims 3 to 5 wherein the virus is attenuated or replication deficient, preferably it is completely replication deficient.

7. The influenza B virus according to any one of claims 3 to 6 wherein the virus comprises a modification within the NS gene.

8. The influenza B virus according to any one of claims 3 to 7 comprising a modified NS1 segment which codes for a NS1 protein lacking a functional RNA binding domain and a functional carboxy terminal domain.

9. The influenza B virus according to any one of claims 3 to 8 comprising
    a. a modification at amino acid position 86 of the M1 protein
    b. a modified NS1 segment coding for a NS1 protein lacking a functional RNA binding domain and functional carboxy terminal domain and
    c. optionally a silent mutation at nucleotide position 950 of the M gene.

10. The influenza B virus according to any one of claims 3 to 8 comprising
    a. a modification at amino acid position 86 of the M1 protein
    b. a modified NS1 segment coding for a NS1 protein lacking a functional RNA binding domain and functional carboxy terminal domain and
    c. a heterologous sequence inserted between the splice donor site and the splice acceptor site of the NS1 gene segment and
    d. optionally a silent mutation at nucleotide position 950 of the M gene.

11. The influenza B virus according to any one of claims 3 to 10 comprising a nucleotide sequence as shown in any one of SEQ ID Nos. 3, 4, 7 to 12, or 15.

12. The influenza B virus according to any one of claims 3 to 10 comprising a nucleotide sequence as shown in any one of SEQ ID Nos. 6, 16 to 18.

13. The influenza B virus according to any one of claims 3 to 10 comprising an amino acid sequence having at least 98% sequence identity with SEQ ID. No 1.

14. The influenza B virus according to any one of claims 3 to 10 comprising an amino acid sequence as shown in SEQ ID No. 2 or a derivative thereof having at least 98% sequence identity.

15. A vaccine composition comprising an immunogenically-inducing effective amount of virus according to any one of claims 3 to 14 in admixture with a pharmaceutically acceptable carrier.

16. A method of making a recombinant influenza B virus according to any one of claims 3 to 14 wherein the method comprises introducing a recombinant vector that expresses the influenza virus M86V protein in a reverse genetics system.

17. A method of increasing the growth rate of replication deficient influenza B viruses wherein said influenza B viruses are comprising the M86V protein.

18. An isolated nucleic acid encoding the influenza virus M86V protein.

19. The influenza B virus according to any of claims 3 to 14 for use in the manufacture of a medicament.

20. Use of the influenza B virus according to any one of claims 3 to 14 in the manufacture of a medicament in the treatment of an influenza virus infection.

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ggtgggaaag aatttgacct agactctgcc ttggaatgga taaaaaacaa aagatgctta 180
actgatatac aaaaagcact aattggtgcc tctatatgct ttttaaaacc caaagaccag 240
gaaagaaaaa gaagattcat cacagagccc ttatcaggaa tgggaacaac agcaacaaaa 300
aagaaaggcc tgattctggc tgagagaaaa atgagaagat gtgtgagctt tcatgaagca 360
tttgaaatag cagaaggcca tgaaagctca gcgctactat actgtctcat ggtcatgtac 420
ctgaatcctg gaaattattc aatgcaagta aaactaggaa cgctctgtgc tttatgcgag 480
aaacaagcat cacattcaca cagggctcat agcagagcag cgagatcttc agtgcctgga 540
gtgagacgag aaatgcagat ggtctcagct atgaacacag caaaaacaat gaatggaatg 600
ggaaaaggag aagacgtcca aaagttggca gaagagctgc aaagcaacat tggagtgctg 660
agatctcttg gggcaagtca aaagaatggg gaagggattg caaaggatgt aatggaagtg 720
ctaaagcaga gctccatggg aaattcagct cttgtgaaga aatatctata atgctcgaac 780
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caatagggca tttgaatcaa ataaaaagag gaataaacat gaaaatacga ataaaaggtc 900
caaacaaaga gacaataaac agagaggtat caattttgag acacagttat caaaaagaaa 960
tccaggccaa agaaacaatg aaggaagtac tctctgacaa catggaggta ttgagtgacc 1020
acataataat tgaggggctt tctgccgaag agataataaa aatgggtgaa acagttttgg 1080
agatagaaga attgcattaa attcaatttt actgtatttc ttactatgca tttaagcaaa 1140
ttgtaatcaa tgtcagcaaa taaactggaa aaagtgcgtt gtttctact 1189

<210> 8
<211> 558
<212> DNA
<213> Influenza B virus

<400> 8
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tgcatagatt gaatgcatat gaccagagtg gaaggcttgt tgctaaactt gttgccactg 180
atgatcttac agtggaggat gaagaagatg gccatcggat cctcaactca ctcttcgagc 240
gtcttaatga aggacattca aagccaattc gagcagctga aactgcggtg ggagtcttat 300
cccaatttgg tcaagagcac cgattatcac cagaagaggg agacaattag actggtcacg 360
gaagaacttt atcttttaag taaaagaatt gatgataaca tattattcca caaaacagta 420
atagctaaca gctccataat agctgacatg gttgtatcat tatcattatt agaaacattg 480
tatgaaatga aggatgtggt tgaagtgtac agcaggcagt gcttgtgaat ttaaaataaa 540

aatcctcttg ttactact 558

<210> 9
<211> 630
<212> DNA
<213> Influenza B virus

<400> 9
agcagaagca gaggatttgt ttagtcactg gcaaacagga aaaaatggcg aacaacatga 60

ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagatg atgacccaat ggatacaagt 180

ccttatcaac tctgcataga ttgaatgcat atgaccagag tggaaggctt gttgctaaac 240

ttgttgccac tgatgatctt acagtggagg atgaagaaga tggccatcgg atcctcaact 300

cactcttcga gcgtcttaat gaaggacatt caaagccaat tcgagcagct gaaactgcgg 360

tgggagtctt atcccaattt ggtcaagagc accgattatc accagaagag ggagacaatt 420

agactggtca cggaagaact ttatctttta agtaaaagaa ttgatgataa catattattc 480

cacaaaacag taatagctaa cagctccata atagctgaca tggttgtatc attatcatta 540

ttagaaacat tgtatgaaat gaaggatgtg gttgaagtgt acagcaggca gtgcttgtga 600

atttaaaata aaaatcctct tgttactact 630

<210> 10
<211> 687
<212> DNA
<213> Influenza B virus

<400> 10
agcagaagca gaggatttgt ttagtcactg gcaaacagga aaaaatggcg aacaacatga 60

ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagagc ccttgactac cctggtcaag 180

accgcctaaa cagactaaag agaaaattag agtcatgatg acccaatgga tacaagtcct 240

tatcaactct gcatagattg aatgcatatg accagagtgg aaggcttgtt gctaaacttg 300

ttgccactga tgatcttaca gtggaggatg aagaagatgg ccatcggatc ctcaactcac 360

tcttcgagcg tcttaatgaa ggacattcaa agccaattcg agcagctgaa actgcggtgg 420

gagtcttatc ccaatttggt caagagcacc gattatcacc agaagaggga gacaattaga 480

ctggtcacgg aagaacttta tcttttaagt aaaagaattg atgataacat attattccac 540

aaaacagtaa tagctaacag ctccataata gctgacatgg ttgtatcatt atcattatta 600

gaaacattgt atgaaatgaa ggatgtggtt gaagtgtaca gcaggcagtg cttgtgaatt 660

taaaataaaa atcctcttgt tactact 687

<210> 11
<211> 708
<212> DNA
<213> Influenza B virus

<400> 11

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ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagagc ccttgactac cctggtcaag 180

accgcctaaa cagactaaag agaaaattag agtcaagaat aaagactcac aacaaatgat 240

gacccaatgg atacaagtcc ttatcaactc tgcatagatt gaatgcatat gaccagagtg 300

gaaggcttgt tgctaaactt gttgccactg atgatcttac agtggaggat gaagaagatg 360

gccatcggat cctcaactca ctcttcgagc gtcttaatga aggacattca aagccaattc 420

gagcagctga aactgcggtg ggagtcttat cccaatttgg tcaagagcac cgattatcac 480

cagaagaggg agacaattag actggtcacg gaagaacttt atcttttaag taaaagaatt 540

gatgataaca tattattcca caaaacagta atagctaaca gctccataat agctgacatg 600

gttgtatcat tatcattatt agaaacattg tatgaaatga aggatgtggt tgaagtgtac 660

agcaggcagt gcttgtgaat ttaaaataaa aatcctcttg ttactact 708

<210> 12
<211> 756
<212> DNA
<213> Influenza B virus

<400> 12
agcagaagca gaggatttgt ttagtcactg gcaaacagga aaaaatggcg aacaacatga 60

ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagagc ccttgactac cctggtcaag 180

accgcctaaa cagactaaag agaaaattag agtcaagaat aaagactcac aacaaaagtg 240

agcctgaaag taaaaggatg tcccttgaag agagaaaagc aatttgatga cccaatggat 300

acaagtcctt atcaactctg catagattga atgcatatga ccagagtgga aggcttgttg 360

ctaaacttgt tgccactgat gatcttacag tggaggatga agaagatggc catcggatcc 420

tcaactcact cttcgagcgt cttaatgaag gacattcaaa gccaattcga gcagctgaaa 480

ctgcggtggg agtcttatcc caatttggtc aagagcaccg attatcacca gaagagggag 540

acaattagac tggtcacgga agaactttat cttttaagta aaagaattga tgataacata 600

ttattccaca aaacagtaat agctaacagc tccataatag ctgacatggt tgtatcatta 660

tcattattag aaacattgta tgaaatgaag gatgtggttg aagtgtacag caggcagtgc 720

ttgtgaattt aaaataaaaa tcctcttgtt actact 756

<210> 13
<211> 443
<212> DNA
<213> Influenza B virus

<400> 13
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ccacaacaca aattgagtgg aggatgaaga agatggccat cggatcctca actcactctt 120

cgagcgtctt aatgaaggac attcaaagcc aattcgagca gctgaaactg cggtgggagt 180

cttatcccaa tttggtcaag agcaccgatt atcaccagaa gagggagaca attagactgg 240

tcacggaaga actttatctt ttaagtaaaa gaattgatga taacatatta ttccacaaaa 300

cagtaatagc taacagctcc ataatagctg acatggttgt atcattatca ttattagaaa 360

cattgtatga aatgaaggat gtggttgaag tgtacagcag gcagtgcttg tgaatttaaa 420

ataaaaatcc tcttgttact act 443

<210> 14
<211> 442
<212> DNA
<213> Influenza B virus

<400> 14
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cacaacacaa attgagtgga ggatgaagaa gatggccatc ggatcctcaa ttcactcttc 120

gagcgtctta atgaaggaca ttcaaagcca attcgagcag ctgaaactgc ggtgggagtc 180

ttatcccaat ttggtcaaga gcaccgatta tcaccagaag agggagacaa ttagactggt 240

cacggaagaa ctttatcttt taagtaaaag aattgatgat aacatattgt tccacaaaac 300

agtaatagct aacagctcca taatagctga catggttgta tcattatcat tattagaaac 360

attgtatgaa atgaaggatg tggttgaagt gtacagcagg cagtgcttgt gaatttaaaa 420

taaaaatcct cttgttacta ct 442

<210> 15
<211> 1023
<212> DNA
<213> Influenza B virus

<400> 15
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ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagata atgtacagga tgcaactcct 180

gtcttgcatt gcactaagtc ttgcacttgt cacaaacagt gcacctactt cttcgtcgac 240

aaagaaaaca cagctacaac tggagcattt actgctggat ttacagatga ttttgaatgg 300

aattaataat tacaagaatc ccaaactcac caggatgctc acatttaagt tttacatgcc 360

caagaaggcc acagaactga aacatcttca gtgtctagaa gaagaactca aacctctgga 420

ggaagtgcta aatttagctc aaagcaaaaa ctttcactta agacccaggg acttaatcag 480

caatatcaac gtaatagttc tggaactaaa gggatctgaa acaacattca tgtgtgaata 540

tgctgatgag acagcaacca ttgtagaatt tctgaacaga tggattacct tttgtcaaag 600

catcatctca acactaactt gataatacta accttcttct ctttcttctc ctgacagtgg 660

aggatgaaga agatggccat cggatcctca actcactctt cgagcgtctt aatgaaggac 720

attcaaagcc aattcgagca gctgaaactg cggtgggagt cttatcccaa tttggtcaag 780

agcaccgatt atcaccagaa gagggagaca attagactgg tcacggaaga actttatctt 840

ttaagtaaaa gaattgatga taacatatta ttccacaaaa cagtaatagc taacagctcc 900

ataatagctg acatggttgt atcattatca ttattagaaa cattgtatga aatgaaggat 960

gtggttgaag tgtacagcag gcagtgcttg tgaatttaaa ataaaaatcc tcttgttact 1020

act 1023

<210> 16
<211> 1149
<212> DNA
<213> Influenza B virus

<400> 16
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ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagagc ccttgactac cctggtcaag 180

accgcctaaa cagactaaag agaaaattag agtcaagaat aaagactcac aacaaaagtg 240

agcctgaaag taaaaggatg tcccttgaag agagaaaagc aatttaatgt acaggatgca 300

actcctgtct tgcattgcac taagtcttgc acttgtcaca aacagtgcac ctacttcttc 360

gtcgacaaag aaaacacagc tacaactgga gcatttactg ctggatttac agatgatttt 420

gaatggaatt aataattaca agaatcccaa actcaccagg atgctcacat ttaagtttta 480

catgcccaag aaggccacag aactgaaaca tcttcagtgt ctagaagaag aactcaaacc 540

tctggaggaa gtgctaaatt tagctcaaag caaaaacttt cacttaagac ccagggactt 600

aatcagcaat atcaacgtaa tagttctgga actaaaggga tctgaaacaa cattcatgtg 660

tgaatatgct gatgagacag caaccattgt agaatttctg aacagatgga ttacctttg 720

tcaaagcatc atctcaacac taacttgata atactaacct tcttctcttt cttctcctga 780

cagtggagga tgaagaagat ggccatcgga tcctcaactc actcttcgag cgtcttaatg 840

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gtcaagagca ccgattatca ccagaagagg gagacaatta gactggtcac ggaagaactt 960

tatcttttaa gtaaaagaat tgatgataac atattattcc acaaaacagt aatagctaac 1020

agctccataa tagctgacat ggttgtatca ttatcattat tagaaacatt gtatgaaatg 1080

aaggatgtgg ttgaagtgta cagcaggcag tgcttgtgaa tttaaaataa aaatcctctt 1140

gttactact 1149

<210> 17
<211> 1221
<212> DNA
<213> Influenza B virus

<400> 17
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ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagagc ccttgactac cctggtcaag 180

accgcctaaa cagactaaag agaaaattag agtcaagaat aaagactcac aacaaaagtg 240

agcctgaaag taaaaggatg tcccttgaag agagaaaagc aattggagta aaaatgatga 300

aagtactcct atttatgaat ccgtctgctg gaattgaagg gtttgagcca tactgttaat 360

gtacaggatg caactcctgt cttgcattgc actaagtctt gcacttgtca caaacagtgc 420

acctacttct tcgtcgacaa agaaaacaca gctacaactg gagcatttac tgctggattt 480

acagatgatt ttgaatggaa ttaataatta caagaatccc aaactcacca ggatgctcac 540

atttaagttt tacatgccca agaaggccac agaactgaaa catcttcagt gtctagaaga 600

agaactcaaa cctctggagg aagtgctaaa tttagctcaa agcaaaaact ttcacttaag 660

acccagggac ttaatcagca atatcaacgt aatagttctg gaactaaagg gatctgaaac 720

aacattcatg tgtgaatatg ctgatgagac agcaaccatt gtagaatttc tgaacagatg 780

gattaccttt tgtcaaagca tcatctcaac actaacttga taatactaac cttcttctct 840

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acggaagaac tttatctttt aagtaaaaga attgatgata acatattatt ccacaaaaca 1080

gtaatagcta acagctccat aatagctgac atggttgtat cattatcatt attagaaaca 1140

ttgtatgaaa tgaaggatgt ggttgaagtg tacagcaggc agtgcttgtg aatttaaaat 1200

aaaaatcctc ttgttactac t 1221

<210> 18
<211> 1344
<212> DNA
<213> Influenza B virus

<400> 18
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ccacaacaca aattgaggtg ggtccgggag caaccaatgc caccataaac tttgaagcag 120

gaattctgga gtgctatgaa aggctttcat ggcaaagagc ccttgactac cctggtcaag 180

accgcctaaa cagactaaag agaaaattag agtcaagaat aaagactcac aacaaaagtg 240

agcctgaaag taaaaggatg tcccttgaag agagaaaagc aattggagta aaaatgatga 300

aagtactcct atttatgaat ccgtctgctg gaattgaagg gtttgagcca tactgtatga 360

aaagttcctc aaatagcaac tgtacgaaat acaattggac cgattaccct tcaacaccag 420

ggaggtgcct tgatgacata gaagaagaac cagaggatgt tgatggccca actgaaatat 480

aatgtacagg atgcaactcc tgtcttgcat tgcactaagt cttgcacttg tcacaaacag 540

tgcacctact tcttcgtcga caaagaaaac acagctacaa ctggagcatt tactgctgga 600

tttacagatg attttgaatg gaattaataa ttacaagaat cccaaactca ccaggatgct 660

cacatttaag ttttacatgc ccaagaaggc cacagaactg aaacatcttc agtgtctaga 720

agaagaactc aaacctctgg aggaagtgct aaatttagct caaagcaaaa actttcactt 780

aagacccagg gacttaatca gcaatatcaa cgtaatagtt ctggaactaa agggatctga 840

aacaacattc atgtgtgaat atgctgatga gacagcaacc attgtagaat ttctgaacag 900

atggattacc ttttgtcaaa gcatcatctc aacactaact tgataatact aaccttcttc 960

tctttcttct cctgacagtg gaggatgaag aagatggcca tcggatcctc aactcactct 1020

tcgagcgtct taatgaagga cattcaaagc caattcgagc agctgaaact gcggtgggag 1080

tcttatccca atttggtcaa gagcaccgat tatcaccaga agagggagac aattagactg 1140

gtcacggaag aactttatct tttaagtaaa agaattgatg ataacatatt attccacaaa 1200

acagtaatag ctaacagctc cataatagct gacatggttg tatcattatc attattagaa 1260

acattgtatg aaatgaagga tgtggttgaa gtgtacagca ggcagtgctt gtgaatttaa 1320

aataaaaatc ctcttgttac tact 1344