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(54) Title: NEW DEV VECTORS

(57) Abstract: The present invention relates to the field of animal health. Particularly, the present invention relates to an attenuated Duck Enteritis Virus (DEV). More particularly, the present invention relates to an attenuated DEV that shows no or a reduced pathogenicity in duck and chicken. Furthermore, the present invention relates to a composition, comprising the attenuated DEV of the invention as vector-vaccine for poultry, and the use thereof.

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NEW DEV VECTORS

Cross-Reference to Related Application

This application claims the priority of PCT/CN2023/114895 filed on August 25, 2023 entitled by "ATTENUATED DEVs", the entirety of which is incorporated by reference herein.

Technical Field

The present invention relates to the field of animal health. Particularly, the present invention relates to an attenuated Duck Enteritis Virus (DEV). More particularly, the present invention relates to an attenuated DEV that shows no or a reduced pathogenicity in poultry. Furthermore, the present invention relates to a composition, comprising the attenuated DEV of the invention as vector-vaccine for poultry, and the use thereof.

Technical background

Duck plague, also known as duck virus enteritis, is an acute and septic infection in *Anseriformes*, such as ducks, geese, etc. It is caused by duck enteritis virus (DEV), which naturally infects ducks and geese. However, DEV does not only cause duck plague in ducks and geese, it also infects and kills chicken. DEV is also known as Anatid herpesvirus 1, duck herpesvirus 1, duck viral enteritis virus (DVEV) or duck plague virus (DPV). The complete nucleotide sequence of DEV has been determined and is available online (see for instance Genbank Accession No. JQ673560). The viral genome contains about 162Kb, encoding nearly 80 distinct proteins. Several strains of DEV have been isolated, such as the Jansen strain, the CSC strain, the CHv strain, the VAC strain, and the 2085 strain. The complete sequences of several DEV strains are available in Genbank, such as the VAC strain: ID EU082088.2; the Anatid isolate C-KCE: ID KF263690.1; the Anatid strain CHv: ID JQ647509.1; the Anatid strain 2085: ID JF999965; the Anatid strain CV: ID KJ549663.1 or the Anatid strain CSC: ID JQ673560.1.

As a member of the *Herpesvirus* family, DEV genome has a stable double-stranded DNA structure and contains a plurality of non-essential regions for virus replication, which can accommodate the insertion of a plurality of heterologous genes. DEV strains that are attenuated through traditional passaging in chicken embryos or duck embryos have become a promising vaccine live vector system for developing vaccines against avian diseases. However, attenuation through traditional passaging in chicken embryos or duck embryos results in unspecific and mainly unknown genetic modifications with the risk, that such modifications can get lost and the attenuated virus returns to virulence. At present, expressing heterologous genes with DEV as a vector is mainly studied for developing vaccines against diseases in ducks. It has been reported that DEV vectored-vaccine which expresses a heterologous gene would be safe in duck, but it showed virulence for chicken (see Wang, J., (2015). Construction of a recombinant duck enteritis virus (DEV) expressing hemagglutinin of H5N1 avian influenza virus based on an infectious clone of DEV vaccine strain and evaluation of its efficacy in ducks and chickens. Virology Journal, 12(1).). Developing a safe and effective live DEV vector that is

genetically modified for developing vaccines against diseases in poultry remains a challenge in the art.

Brief Description of the Invention

The present invention is based on the surprising finding that the DEV comprising an inactive gene in its genome, such as i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; or v) UL23 gene, alone or in combination with other DEV genes such as for example the combinations of: vi) UL24 gene and UL2 gene; vii) UL40 gene and UL2 gene; or viii) UL23 gene and UL41 gene, results in a reduced or no mortality in duck and chicken as compared to the non-modified DEV. While wild-type DEV is lethal in young ducks and chickens, the DEVs of the invention are safe and can effectively deliver and express a gene of interest *in vivo*. Particularly, such DEVs are (i) attenuated *in vivo*, particularly in duck and chicken, and (ii) are stable and capable of expressing foreign genes in a manner suitable for inducing protective immunity, including at very early stage (i.e., at day 0, day 1, day 2, or day 3 post-hatch). Furthermore, these attenuated DEVs retain a fast growth rate, allowing high titer production. Such DEVs thus represent very potent vectors for vaccinating non-human animals, particularly poultry, and for conferring early protective immunity.

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV), wherein one or more genes of the DEV genome selected from the group consisting of US3, UL24, UL40, UL39, and UL23 is inactivated.

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV), comprising an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; and v) UL23 gene, alone or in combination with any other non-essential gene of DEV.

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV), comprising inactivated gene(s) selected from any one of i)-viii), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; v) UL23 gene; vi) UL24 gene and UL2 gene; vii) UL40 gene and UL2 gene; or viii) UL23 gene and UL41 gene.

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV), comprising inactivated gene(s) as defined above, wherein the attenuated DEV further comprises a heterologous polynucleotide coding for a heterologous antigen of a pathogen.

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV) as a live vector vaccine in poultry, wherein the attenuated DEV comprises an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; and v) UL23 gene.

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV) as a live vector vaccine in poultry, wherein the attenuated DEV comprises an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; and v) UL23 gene, alone or in combination with any other non-essential gene of DEV.

In one aspect, the present invention provides a composition, comprising the attenuated DEV of the present invention.

In one aspect, the present invention provides the attenuated DEV of the present invention, the composition of the present invention, or the vector vaccine of the present invention, for the use in
5 method for inducing a protective immune response in poultry against a poultry pathogen, wherein such method comprises or consists of one or more administration of the attenuated DEV of the present invention, the composition of the present invention, or the vector vaccine of the present invention to poultry.

In one aspect, the present invention provides a method of vaccinating a poultry by inducing a
10 protective immune response in a poultry against a pathogen, comprising at least one administration of the composition of the present invention.

In one aspect, the present invention provides use of the attenuated DEV of the present invention in the manufacture of a composition for vaccinating a poultry by inducing a protective immune response in a poultry against a pathogen.

15 **Brief Description of the Drawings**

Figure 1: shows gene structure map of plasmid pB12.

Figure 2: illustrates schematic diagrams of (A) bacterial artificial chromosome rDEV4 BAC, (B) recombinant construct with deleted gene rDEV4 ΔUL39 and (C) recombinant construct with inserted H9HA gene rDEV4 ΔUL39 UL26-H9HA-UL27.

20 Figure 3: shows the result of transfection of DEFs (duck embryo fibroblasts) to rescue rDEV4 ΔUL39.

Figure 4: shows the result of RFLP analysis of rDEV4 ΔUL39 UL26-H9HA-UL27 by Xho I digestion.

Figure 5: shows the result of mini-F deletion to rescue rDEV4 ΔUL39 UL26-H9HA-UL27 by co-transfection.

25 Figure 6: shows the result of the genetic stability test of rDEV4 ΔUL39 UL26-H9HA-UL27 by PCR.

Figure 7: shows the result of the expression test of H9HA from rDEV4 ΔUL39 UL26-H9HA-UL27 by IFA.

Figure 8: shows the result of HI Ab level of different groups induced by different rDEV4 H9HA vaccine candidate strains.

30 **Detailed Description**

Before the aspects of the present invention are described, it must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless

the context clearly dictates otherwise. Thus, for example, a reference to "a gene" includes a plurality of genes, a reference to the "virus" is a reference to one or more viruses and equivalents thereof known to those skilled in the art, and so forth. The term “and/or” is intended to encompass any combinations of the items connected by this term, equivalent to listing all the combinations individually. For example, “A, B and/or C” encompasses “A”, “B”, “C”, “A and B”, “A and C”, “B and C”, and “A and B and C”. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the virus strains, the cell lines, vectors, and methodologies as reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The invention relates to a Duck Enteritis Virus (DEV), wherein said virus has specific inactive gene(s). The invention indeed shows that by inactivating such gene(s), viable, stable and replicative DEVs can be obtained, and that such viruses may be used to create recombinant DEVs by insertion of foreign genetic material. The results further show that such foreign genetic material is highly expressed from such viruses upon cell infection, and that such expression remains stable over time. Moreover, and strikingly, while native DEV as well as many other deleted DEV constructs produced by the inventors were found pathogenic or lethal in young duck and chicken (at day 0, day 1, day 2, or day 3 post-hatch), inactivation of specific gene(s) in the DEV genome generates attenuated viruses with respect to duck and chicken which can be used safely to express antigens in young animals, especially in duck and chicken. As shown in the Examples, as compared with 100% morbidity and 100% mortality caused by non-modified DEV strain in duck and chicken, the morbidity and mortality caused by the attenuated DEVs of the present invention was surprisingly reduced to less than 30%, mostly even surprisingly reduced to 0%. Such attenuated DEVs could be used as a safe live virus vector in the future, especially for both of duck and chicken.

Attenuated DEV

In one aspect, the present invention provides an attenuated Duck Enteritis Virus (DEV), wherein one or more genes of the DEV genome selected from the group consisting of US3, UL24, UL40, UL39, and UL23 is inactivated.

The term "virus" designates in particular a viral particle comprising a nucleic acid molecule (e.g., a genome) encapsulated in a capsid or capsule. The term "virus" also designates a viral vector or an isolated viral genome.

A "gene" designates a nucleic acid molecule or sequence which comprises an open reading frame encoding a product, such as a polypeptide (e.g., a peptide, protein, etc.) or an RNA.

The term “DEV” as used herein refers to all viruses belonging to species of Duck enteritis virus (DEV) in the genus *Mardivirus* within subfamily *Alphaherpesvirinae* of the family *Herpesviridae*.

The DEVs of the invention may be prepared from any DEV species or strain. In a preferred embodiment, the DEV of the invention is derived or prepared from a parental strain selected from
5 the Jansen strain, the VAC strain (ID EU082088.2), the C-KCE strain (ID KF263690.1), the CHv strain (ID JQ647509.1), the 2085 strain (ID JF999965), the CV strain (ID KJ549663.1) or the CSC strain (ID JQ673560.1), or any DEV strain having at least 90% sequence identity to the Jansen strain, the VAC strain (ID EU082088.2), the C-KCE strain (ID KF263690.1), the CHv strain (ID JQ647509.1), the 2085 strain (ID JF999965), the CV strain (ID KJ549663.1) or the CSC strain (ID JQ673560.1), more preferably
10 at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%. In a preferred embodiment, the DEV of the invention is derived or prepared from the DEV4 strain, which is deposited at China Center for Type Culture Collection (CCTCC) on August 4, 2023 under CCTCC NO: V202378, or any DEV strain having at least 90% sequence identity to the DEV4 strain, more preferably at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

15 The inactivated gene(s) in the DEV genome is non-essential gene(s).

As used herein, a "non-essential gene/region" is a gene/region in the attenuated DEV genome in which inactivation (including mutation, interruption, replacement or deletion) on that gene/region or insertion of a heterologous polynucleotide into that gene/region does not prevent the attenuated DEV from replicating in a host cell.

20 The term "attenuated" as used herein refers to a virus that is essentially not virulent in an animal, such as a poultry (e.g. duck or chicken), i.e. does not cause or causes reduced illness, especially does not cause death, in an animal, such as a poultry (e.g. duck or chicken) as compared to the non-modified wildtype parent virus. More particularly, an attenuated virus can typically replicate in an animal, such as a poultry (e.g. duck or chicken) without causing death thereof. More particularly,
25 the attenuated DEV of the present invention has no or lower virulence in an animal, such as a poultry, such as a duck or a chicken, than the corresponding non-modified wildtype parent DEV which does not comprise the inactivated gene(s) in its genome. More particularly, an attenuated virus designates a virus that is not virulent in a duck. More particularly, an attenuated virus designates a virus that is not virulent in a chicken. More particularly, an attenuated virus designates a virus that is not virulent
30 in both of a duck and a chicken. More particularly, an attenuated virus designates a virus that is not virulent in a duck when injected at a dose of $10^{4.0}$-$10^{7.0}$TCID$_{50}$/duck, such as $10^{6.0}$TCID$_{50}$/duck. More particularly, an attenuated virus designates a virus that is not virulent in a chicken when injected at a dose of $10^{4.0}$-$10^{7.0}$TCID$_{50}$/chicken, such as $10^{6.0}$TCID$_{50}$/chicken. More particularly, an attenuated virus designates a virus that is not virulent in a duck at a dose of $10^{4.0}$-$10^{7.0}$TCID$_{50}$/duck, such as
35 $10^{6.0}$TCID$_{50}$/duck in at least 10% injected ducks, in at least 20% injected ducks, in at least 30% injected ducks, in at least 40% injected ducks, in at least 50% injected ducks, in at least 60% injected ducks, in at least 70% injected ducks, more preferably in at least 80% injected ducks, even more preferably in at least 90%, 95%, 97%, 98%, 99% or more. More particularly, an attenuated virus designates a virus

that is not virulent in a chicken at a dose of $10^{4.0}$-$10^{7.0}$TCID$_{50}$/chicken, such as $10^{6.0}$TCID$_{50}$/chicken in at least 10% injected chickens, in at least 20% injected chickens, in at least 30% injected chickens, in at least 40% injected chickens, in at least 50% injected chickens, in at least 60% injected chickens, in at least 70% injected chickens, more preferably in at least 80% injected chickens, even more preferably in at least 90%, 95%, 97%, 98%, 99% or more. In some embodiments, an attenuated virus more particularly designates a virus that is not virulent in an embryo when injected at a dose of $10^{4.0}$-$10^{7.0}$TCID$_{50}$/egg, such as $10^{6.0}$TCID$_{50}$/egg. Most preferred an attenuated virus designates a virus that is not virulent in an embryo at a dose of $10^{4.0}$-$10^{7.0}$TCID$_{50}$/egg, such as $10^{6.0}$TCID$_{50}$/egg in at least 10% injected eggs, in at least 20% injected eggs, in at least 30% injected eggs, in at least 40% injected eggs, in at least 50% injected eggs, in at least 60% injected eggs, in at least 70% injected eggs, more preferably in at least 80% injected eggs, even more preferably in at least 90%, 95%, 97%, 98%, 99% or more. The attenuated viruses of the invention are also not virulent for injection post-hatch, including at Day 0, Day 1, Day 2, Day 3 post-hatch (i.e., between 0.1 and 72 hours post-hatch).

The attenuated DEV has a reduced or no mortality and/or morbidity in an animal, such as a poultry, e.g. a duck and/or a chicken. More particularly, the attenuated DEV has a reduced or no mortality and/or morbidity in both of a duck and a chicken. More particularly, the attenuated DEV of the present invention has a reduced or no mortality and/or morbidity as compared to the non-modified DEV in an animal, especially in poultry, such as duck and/or chicken. More particularly, the attenuated DEV of the present invention has a reduced or no mortality and/or morbidity as compared to the non-modified DEV, in both of duck and chicken. The phrase "DEV has a reduced or no mortality and/or morbidity in an animal, especially in poultry, such as duck and/or chicken (as compared to the non-modified DEV)" means that the DEV of the present invention is attenuated with respect to an animal, especially poultry, such as duck and/or chicken. Thus, the DEV of the present invention is attenuated with respect to an animal, especially poultry, such as duck and/or chicken. More particularly, the mortality and/or morbidity in an animal caused by the attenuated DEV of the present invention is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70%. More particularly, the mortality and/or morbidity in an animal caused by the attenuated DEV of the present invention is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70% of that caused by the non-modified DEV. More particularly, the mortality and/or morbidity in a poultry caused by the attenuated DEV of the present invention is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70%. More particularly, the mortality and/or morbidity in a poultry caused by the attenuated DEV of the present invention is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70% of that caused by the non-modified DEV. More particularly, the mortality and/or morbidity in a duck and/or a chicken caused by the attenuated DEV of the present invention is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70%. More particularly, the mortality and/or morbidity in a duck and/or a chicken caused by the attenuated DEV of the present invention is 0%, less than 5%, less than 10%, less than 20%, less

than 30%, less than 40%, less than 50%, less than 60%, or less than 70% of that caused by the non-modified DEV.

The attenuation of the DEV (with respect to poultry, such as duck and/or chicken) is caused by the inactivation of one or more genes of DEV genome as indicated below. That is, the inactivation of 5 the selected genes indicated in the present invention causes the attenuation of DEV. Within the context of the invention, a DEV with an "inactive" gene designates a DEV that cannot express a functional protein or RNA encoded by said gene. An inactive gene thus designates a mutated, an interrupted, a replaced or a deleted gene that cannot encode a wild-type protein encoded by said gene.

10 In some embodiments, the inactivated gene in the DEV genome is selected from the group consisting of US3, UL24, UL40, UL39, and UL23. In some embodiments, the inactivated gene is selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; or v) UL23 gene, alone or in combination with the inactivation of one or more further non-essential genes of DEV. In some embodiments, the one or more further non-essential gene of DEV is different from the 15 first inactivated gene and is selected from i) UL2 gene, ii) UL41 gene; iii) US3 gene; iv) UL24 gene; v) UL40 gene; vi) UL39 gene; or vii) UL23 gene. In some embodiments, the inactivated gene(s) is/are selected from any one of i)-viii), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; v) UL23 gene; vi) UL24 gene and UL2 gene; vii) UL40 gene and UL2 gene; viii) UL23 gene and UL41 gene.

Thus, in another aspect, the present invention relates to an attenuated Duck Enteritis Virus 20 (DEV), wherein any one of i)-viii), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; v) UL23 gene; vi) UL24 gene and UL2 gene; vii) UL40 gene and UL2 gene; viii) UL23 gene and UL41 gene is inactivated in the DEV genome.

UL41, US3, UL24, UL40, UL39, UL23, and UL2 are highly conserved between DEV strains. It is understood that the skilled artisan may easily identify the exact location of the UL41, US3, UL24, 25 UL40, UL39, UL23, and UL2 gene in any DEV strain using the information contained in the present application and general common knowledge, or by sequence alignment. For example, the exact location of the UL41, US3, UL24, UL40, UL39, UL23, and UL2 gene may be identified by reference to a DEV strain with Genbank accession No. EU082088.2.

The DEV comprising one or more inactivated genes in its genome and/or a heterologous 30 polynucleotide coding for a heterologous antigen of a pathogen as described below is also called a recombinant or modified DEV herein. The recombinant/modified DEV of the present invention can be used a vector. The terms “recombinant/modified DEV”, “rDEV” and “recombinant/modified DEV vector” are used interchangeably herein.

The term “recombinant” or “modified” refers to a DEV that has been altered, rearranged, or 35 modified by genetic engineering. However, the term does not refer to alterations in polynucleotide, amino acid sequence, or nucleotide sequence that result from naturally occurring events, such as spontaneous mutations.

In some embodiments, the gene is inactivated by mutation, interruption, replacement or deletion of a portion of or the whole sequence of the gene. In some embodiments, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the sequence of the gene is replaced or deleted.

5 In a particular embodiment, the gene is inactive as a result of one or more mutations in the coding sequence, particularly point mutations in the coding sequence that prevent the expression of a full length protein. Such mutations may cause substitution of essential amino acid residue(s) in the encoded protein, resulting in an inactive protein.

In a particular embodiment, the gene is inactive as a result of one or more interruptions in the 10 coding sequence that prevent the expression of a full length protein. Such interruptions may introduce a stop or non-sense codon in the sequence, resulting in an inactive protein.

In another embodiment, the gene is inactive as a result of a deletion of a portion of the (coding) sequence of said gene or the whole (coding) sequence of said gene, more particularly of at least 20% of the (coding) sequence of the gene, more preferably at least 30%, at least 40%, at least 50%, at 15 least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%, up to 100%. Such deletion removes the coding sequence and thus prevents the expression of a wild-type protein.

In another embodiment, the gene is inactive as a result of a replacement of a portion of the (coding) sequence of said gene or the whole (coding) sequence of said gene with a heterologous polynucleotide, more particularly of at least 20% of the (coding) sequence of the gene, more 20 preferably at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%, up to 100%. Such replacement removes the coding sequence and thus prevents the expression of a wild-type protein.

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the US3 gene. In a specific embodiment, the attenuated DEV 25 of the invention has a deletion of the whole sequence of the US3 gene. A specific example of such a construct is e.g. rDEV4 ΔUS3 (see Example 2).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL24 gene. In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of the whole sequence of the UL24 gene. A specific 30 example of such a construct is e.g., rDEV4 ΔUL24 (see Example 2), rDEV4 H9HA ΔUL24 (see Example 3) or rDEV4 ΔUL24 UL26-H9HA-UL27 (see Example 3).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL40 gene. In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of the whole sequence of the UL40 gene. A specific 35 example of such a construct is e.g., rDEV4 ΔUL40 (see Example 2) or rDEV4 H9HA ΔUL40 (see Example 3).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL39 gene. In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of the whole sequence of the UL39 gene. A specific example of such a construct is e.g., rDEV4 ΔUL39 (see Example 2), rDEV4 H9HA ΔUL39 (see Example 3) or rDEV4 ΔUL39 UL26-H9HA-UL27 (see Example 3).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL23 gene. In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of the whole sequence of the UL23 gene. A specific example of such a construct is e.g., rDEV4 ΔUL23 (see Example 2), or rDEV4 H9HA ΔUL23 (see Example 3).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL24 gene, and a portion of or the whole sequence of the UL2 gene. In a more preferred embodiment, the attenuated DEV of the invention comprises a deletion of the whole sequence of the UL24 gene, and a replacement of the whole sequence of the UL2 gene. A specific example of such a construct is e.g., rDEV4 ΔUL24 H9HA ΔUL2 (see Example 3).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL40 gene, and a portion of or the whole sequence of the UL2 gene. In a more preferred embodiment, the attenuated DEV of the invention comprises a deletion of the whole sequence of the UL40 gene, and a replacement of the whole sequence of the UL2 gene. A specific example of such a construct is e.g., rDEV4 ΔUL40 H9HA ΔUL2 (see Example 3).

In a specific embodiment, the attenuated DEV of the invention has a deletion or replacement of a portion of or the whole sequence of the UL23 gene, and a portion of or the whole sequence of the UL41 gene. In a more preferred embodiment, the attenuated DEV of the invention comprises a replacement of the whole sequence of the UL23 gene, and a deletion of the whole sequence of the UL41 gene. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL23 ΔUL41 (see Example 3).

In a particular embodiment, the attenuated DEV is a live virus vector. A “live virus vector” is virus (in the present case a DEV) that is competent to replicate in a host when such host is infected with the live virus or the genomic nucleic acid of such virus and wherein such virus encodes, delivers and express a heterologous polynucleotide sequence in such host.

In a particular embodiment, the attenuated DEV further comprises a heterologous polynucleotide coding for a heterologous antigen of a pathogen.

The term "heterologous polynucleotide" in relation to a virus designates a polynucleotide which is not found naturally in the genome of the virus, or which is found naturally in said genome but in a different form or at a different position.

An "antigen" as used herein refers to, but is not limited to, components which elicit an immune response in a host.

In a particular embodiment, the heterologous polynucleotide is inserted into a non-essential gene or region of the attenuated DEV. In a particular embodiment, the heterologous polynucleotide is inserted into or in replacement of a portion of or the whole sequence of the non-essential gene or region of the attenuated DEV. In a particular embodiment, the non-essential gene or region of the DEV genome is selected from the group consisting of UL2 gene, UL24 gene, UL39 gene, UL26-UL27 intergenic region, UL40 gene, and UL23 gene.

In a particular embodiment, the heterologous polynucleotide is located in a gene or a region selected from the group consisting of UL2 gene, UL24 gene, UL39 gene, UL26-UL27 intergenic region, UL40 gene, and UL23 gene of DEV genome. In a particular embodiment, the heterologous polynucleotide is inserted into or in replacement of a portion of or the whole sequence of one or more genes of DEV genome selected from the group consisting of UL2 gene, UL24 gene, UL39 gene, UL40 gene, UL23 gene, or inserted into UL26-UL27 intergenic region of DEV genome.

In some embodiments, the heterologous polynucleotide is located in UL23 gene. In a particular embodiment, the heterologous polynucleotide is inserted into the UL23 gene sequence of the DEV viral genome, in addition to the existing UL23 gene sequence (thus rendering the gene inactive by interrupting the gene sequence), or inserted into the UL23 gene region of the DEV viral genome after the deletion of a portion of or the whole sequence of the UL23 gene (thus rendering the gene inactive by deleting the gene sequence), or in replacement of a portion of or the whole sequence of the UL23 gene (thus rendering the gene inactive by replacing the gene sequence), or located in a mutated UL23 gene sequence.

In some embodiments, the heterologous polynucleotide is located in UL2 gene. In a particular embodiment, the heterologous polynucleotide is inserted into the UL2 gene sequence of the DEV viral genome, in addition to the existing UL2 gene sequence (thus rendering the gene inactive by interrupting the gene sequence), or inserted into the UL2 gene region of the DEV viral genome after the deletion of a portion of or the whole sequence of the UL2 gene (thus rendering the gene inactive by deleting the gene sequence), or in replacement of a portion of or the whole sequence of the UL2 gene (thus rendering the gene inactive by replacing the gene sequence), or located in a mutated UL2 gene sequence.

In some embodiments, the heterologous polynucleotide is located in UL24 gene. In a particular embodiment, the heterologous polynucleotide is inserted into the UL24 gene sequence of the DEV viral genome, in addition to the existing UL24 gene sequence (thus rendering the gene inactive by interrupting the gene sequence), or inserted into the UL24 gene region of the DEV viral genome after the deletion of a portion of or the whole sequence of the UL24 gene (thus rendering the gene inactive by deleting the gene sequence), or in replacement of a portion of or the whole sequence of the UL24 gene (thus rendering the gene inactive by replacing the gene sequence), or located in a mutated UL24 gene sequence.

In some embodiments, the heterologous polynucleotide is located in UL39 gene. In a particular embodiment, the heterologous polynucleotide is inserted into the UL39 gene sequence of the DEV viral genome, in addition to the existing UL39 gene sequence (thus rendering the gene inactive by interrupting the gene sequence), or inserted into the UL39 gene region of the DEV viral genome after the deletion of a portion of or the whole sequence of the UL39 gene (thus rendering the gene inactive by deleting the gene sequence), or in replacement of a portion of or the whole sequence of the UL39 gene (thus rendering the gene inactive by replacing the gene sequence), or located in a mutated UL39 gene sequence.

In some embodiments, the heterologous polynucleotide is located in UL26-UL27 intergenic region. In a particular embodiment, the heterologous polynucleotide is inserted into the UL26-UL27 intergenic region of the DEV viral genome.

In some embodiments, the heterologous polynucleotide is located in UL40 gene. In a particular embodiment, the heterologous polynucleotide is inserted into the UL40 gene sequence of the DEV viral genome, in addition to the existing UL40 gene sequence (thus rendering the gene inactive by interrupting the gene sequence), or inserted into the UL40 gene region of the DEV viral genome after the deletion of a portion of or the whole sequence of the UL40 gene (thus rendering the gene inactive by deleting the gene sequence), or in replacement of a portion of or the whole sequence of the UL40 gene (thus rendering the gene inactive by replacing the gene sequence), or located in a mutated UL40 gene sequence.

In an alternative embodiment, the attenuated DEV of the invention has an inactive gene, preferably a deleted gene, and contains a heterologous polynucleotide coding for an antigen of a pathogen located in a different gene or region. In this case, the heterologous polynucleotide may be cloned in replacement of a portion of or the whole sequence of said different gene, or it may be inserted within said different gene, or inserted into said different gene after the deletion of a portion of or the whole sequence of said different gene (thus rendering the different gene also inactive). For example, the DEV of the invention has an inactive UL24 gene, and contains a heterologous polynucleotide coding for an antigen of a pathogen located in UL2 gene, in replacement of a portion of or the whole sequence of the UL2 gene or inserted within UL2 gene (thus rendering UL2 gene also inactive).

Furthermore, the attenuated DEVs of the invention may comprise several heterologous polynucleotides coding for one or more antigens of one or more pathogens. In this regard, the several heterologous polynucleotides may be inserted in the same position in the virus, under the control of a single or several distinct promoters. Alternatively, the heterologous polynucleotides may be inserted into different cloning sites of the virus.

In some embodiments, the heterologous polynucleotide is expressed after the attenuated DEV has been transfected into a suitable host cell.

In one aspect, the present invention provides the attenuated DEV of the present invention for use as a vector vaccine in poultry (such as duck or chicken). The term "vector vaccine" is a vaccine that uses virus (in the present case a DEV) as a vector to deliver and express a polynucleotide sequence coding for an antigen, wherein such antigen provides protection against a pathogen. The virus that is used as a vector shows no or only limited pathogenicity to the target species in which the virus is used as a vector.

Thus, in one aspect, the present invention also provides an attenuated Duck Enteritis Virus (DEV) as a live vector vaccine in poultry (such as duck or a chicken), wherein the attenuated DEV comprises an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; and v) UL23 gene.

In one aspect, the present invention also provides an attenuated Duck Enteritis Virus (DEV) as a live vector vaccine in poultry (such as duck or a chicken), wherein the attenuated DEV comprises an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; v) UL23 gene, alone or in combination with any other non-essential gene of DEV.

In one aspect, the present invention also provides an attenuated Duck Enteritis Virus (DEV) as a live vector vaccine in poultry (such as duck or a chicken), wherein the attenuated DEV comprises inactivated gene(s) selected from any one of i)-viii), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; v) UL23 gene; vi) UL24 gene and UL2 gene; vii) UL40 gene and UL2 gene; or viii) UL23 gene and UL41 gene.

Virus construction and cloning may be accomplished by techniques known per se in the art. Gene cloning and plasmid construction are well known to one person of ordinary skill in the art and may be essentially performed by standard molecular biology techniques (Molecular Cloning: A Laboratory Manual. 4th Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, 2012). Typically, the recombinant viruses may be prepared by homologous recombination between the viral genome and a construct (e.g., a homology plasmid) comprising the nucleic acid to be inserted, flanked by nucleotides from the insertion site to allow recombination. Cloning can be made with or without the deletion of endogenous sequences.

In one aspect, the present invention provides a method of making the attenuated DEV of the invention, comprising the inactivation of one or more genes of the DEV genome as indicated above. The inactivation of the one or more selected genes leads to a reduced or no mortality of the attenuated DEV in poultry as compared to the non-modified DEV.

Heterologous polynucleotide

The attenuated DEV of the invention may contain any heterologous polynucleotide coding for an antigen of a pathogen. The pathogen may be or the antigen may be derived from viruses, bacteria, fungi, protozoa, etc.

In some embodiments, the pathogen may be a poultry (such as duck or chicken) pathogen. In some embodiments, the antigen may be an antigen of a poultry (such as duck or chicken) pathogen. In some embodiments, the pathogen is or the antigen is derived from avian influenza virus. In some embodiments, the pathogen is or the antigen is derived from avian influenza virus selected from the group consisting of Influenza A virus, Influenza B virus, Influenza C virus, and Influenza D virus. Preferentially, the pathogen is or the antigen is derived from Influenza A virus. More preferentially, the pathogen is or the antigen is derived from avian influenza A virus of subtype H9. Most preferentially, the pathogen is or the antigen is derived from avian influenza A virus of subtype H9N2.

In some embodiments, the antigen of a pathogen is the HA protein of the subtype H9 avian influenza virus (H9 HA protein). In some embodiments, the antigen of a pathogen is the HA protein of the subtype H9N2 avian influenza virus.

In some embodiments, the H9 HA protein has an amino acid sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9% or 100% sequence identity with SEQ ID NO: 1.

In some embodiments, the complete H9 HA coding sequence has a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9% or 100% sequence identity with SEQ ID NO: 2.

"Sequence identity" between two polypeptide/nucleotide sequences indicates the percentage of amino acids/nucleotides that are identical between the sequences. Methods for evaluating the level of sequence identity between amino acid or nucleotide sequences are known in the art. For example, sequence analysis software is often used to determine the identity of amino acid/nucleotide sequences. For example, identity can be determined by using the BLAST program in the NCBI database. For determination of sequence identity, see, e.g., Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987 and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991.

As used herein, it is in particular understood that the term “sequence identity with the sequence of SEQ ID NO:X” is equivalent to the term “sequence identity with the sequence of SEQ ID NO:X over the length of SEQ ID NO: X” or to the term “sequence identity with the sequence of SEQ ID NO:X over the whole length of SEQ ID NO: X”, respectively. In this context, “X” is any integer, such as 1 or 2, so that “SEQ ID NO: X” represents any of the SEQ ID NOs mentioned herein.

In some embodiments, the heterologous polynucleotide is generally operably linked to a promoter. The promoter may be any natural or synthetic promoter, derived from cellular or viral genes. Examples of suitable promoters include, for instance, an immediate early cytomegalovirus (CMV) promoter, mouse CMV promoter, guinea pig CMV promoter, an SV40 promoter, Human Herpesvirus Type III glycoprotein B (HHV3gB) promoter, Pseudorabies Virus promoters such as that of glycoprotein X promoter, Herpes Simplex Virus- 1 alpha 4 promoter, a Marek's Disease Virus glycoprotein A (or gC) promoter, a Marek's Disease Virus glycoprotein B promoter, a Marek's Disease Virus glycoprotein E promoter, a Marek's Disease Virus glycoprotein I promoter, an Infectious Laryngotracheitis Virus glycoprotein B, an Infectious Laryngotracheitis Virus glycoprotein E promoter, an Infectious Laryngotracheitis Virus glycoprotein D promoter, an Infectious Laryngotracheitis Virus glycoprotein I promoter, vaccinia H6, and a combination thereof. In some embodiments, the heterologous polynucleotide coding for an antigen of a pathogen is generally operably linked to mCMV promoter. In some embodiments, the heterologous polynucleotide coding for H9 HA is generally operably linked to mCMV promoter.

In some embodiments, the heterologous polynucleotide is operably linked to a transcription terminator. The transcription terminator may be derived from human Herpes Simplex Virus (HSV), thymidine kinase (TK) gene, from the glycoprotein B (gB) gene of Feline Herpesvirus (FHV), from the immediate early (IE) gene of human cytomegalovirus (hCMV), strain AD 169 or from simian virus 40 (SV40), or may be a synthetic terminator. In some embodiments, the heterologous polynucleotide is operably linked to an SV40 polyA signal. In some embodiments, the heterologous polynucleotide coding for H9 HA is generally operably linked to an SV40 polyA signal.

One embodiment of the invention provides an attenuated DEV comprising and (capable of expressing) a heterologous polynucleotide coding for the H9 HA protein. In some embodiments, the heterologous polynucleotide encoding the H9 HA protein is operably linked to the mouse CMV promoter and therefore the expression of the H9 HA protein is regulated by the mouse CMV promoter. In some embodiments, the heterologous polynucleotide encoding the H9 HA protein is operably linked to the SV40 polyA signal and therefore the expression of H9 HA protein is regulated by the SV40 polyA signal. In some embodiments, the heterologous polynucleotide encoding the H9 HA protein is operably linked to the mouse CMV promoter and the SV40 polyA signal, and therefore the expression of H9 HA protein is regulated by the mouse CMV promoter and the SV40 polyA signal.

In some embodiments, the attenuated DEV comprising and (capable of expressing) a heterologous polynucleotide coding for the H9 HA protein comprises an expression cassette containing in 5' to 3' direction in the following order, a) a promoter, b) a heterologous polynucleotide, c) a transcription terminator. In some embodiments, the attenuated DEV comprising and (capable of expressing) a heterologous polynucleotide coding for the H9 HA protein comprises an expression cassette containing in 5' to 3' direction in the following order, a) the mouse CMV promoter, b) the heterologous polynucleotide encoding the H9 HA protein, c) a transcription terminator. In some embodiments, the attenuated DEV comprising and (capable of expressing) a heterologous

polynucleotide coding for the H9 HA protein comprises an expression cassette containing in 5' to 3' direction in the following order, a) the mouse CMV promoter, b) the heterologous polynucleotide encoding the H9 HA protein, c) the SV40 polyA signal.

In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against the corresponding pathogen. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against avian influenza virus, particularly avian influenza A virus of subtype H9, more particularly avian influenza A virus of subtype H9N2. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against the corresponding pathogen in poultry. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against avian influenza virus, particularly avian influenza A virus of subtype H9, more particularly avian influenza A virus of subtype H9N2 in poultry. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against the corresponding pathogen in duck. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against avian influenza virus, particularly avian influenza A virus of subtype H9, more particularly avian influenza A virus of subtype H9N2 in duck. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the protection against the corresponding pathogen in chicken. In some embodiments, the attenuated DEV of the present invention containing a heterologous polynucleotide coding for an antigen of a pathogen is capable of providing the efficacy of at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% in the

protection against avian influenza virus, particularly avian influenza A virus of subtype H9, more particularly avian influenza A virus of subtype H9N2 in chicken.

Preferred DEVs

A preferred attenuated DEV of the invention, wherein the US3 gene of the DEV genome is inactive as a result of a deletion or replacement of a portion of or the whole sequence of the US3 gene. A specific example of such a construct is e.g., rDEV4 ΔUS3 (see Example 2).

A preferred attenuated DEV of the invention, wherein the UL24 gene of the DEV genome is inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL24 gene. A specific example of such a construct is e.g., rDEV4 ΔUL24 (see Example 2), rDEV4 H9HA ΔUL24 (see Example 3) or rDEV4 ΔUL24 UL26-H9HA-UL27 (see Example 3).

A preferred attenuated DEV of the invention, wherein the UL40 gene of the DEV genome is inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL40 gene. A specific example of such a construct is e.g., rDEV4 ΔUL40 (see Example 2) or rDEV4 H9HA ΔUL40 (see Example 3).

A preferred attenuated DEV of the invention, wherein the UL39 gene of the DEV genome is inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL39 gene. A specific example of such a construct is e.g., rDEV4 ΔUL39 (see Example 2), rDEV4 H9HA ΔUL39 (see Example 3) or rDEV4 ΔUL39 UL26-H9HA-UL27 (see Example 3).

A preferred attenuated DEV of the invention, wherein the UL23 gene of the DEV genome is inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL23 gene. A specific example of such a construct is e.g., rDEV4 ΔUL23 (see Example 2), or rDEV4 H9HA ΔUL23 (see Example 3).

A preferred attenuated DEV of the invention, wherein the UL24 gene and UL2 gene of the DEV genome are inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL24 gene, and a portion of or the whole sequence of the UL2 gene. A specific example of such a construct is e.g., rDEV4 ΔUL24 H9HA ΔUL2 (see Example 3).

A preferred attenuated DEV of the invention, wherein the UL40 gene and UL2 gene of the DEV genome are inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL40 gene, and a portion of or the whole sequence of the UL2 gene. A specific example of such a construct is e.g., rDEV4 ΔUL40 H9HA ΔUL2 (see Example 3).

A preferred attenuated DEV of the invention, wherein the UL23 gene and UL41 gene of the DEV genome are inactive as a result of a deletion or replacement of a portion of or the whole sequence of the UL23 gene, and a portion of or the whole sequence of the UL41 gene. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL23 ΔUL41 (see Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL23 gene, in replacement of a portion of or the whole sequence of the UL23 gene, rendering the UL23 gene inactive. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL23 (see Example 3).

5 A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL2 gene, in replacement of a portion of or the whole sequence of the UL2 gene, rendering the UL2 gene inactive, and further comprises an inactive UL24 gene, optionally a deleted UL24 gene. A specific example of such a construct is e.g., rDEV4 ΔUL24 H9HA ΔUL2 (see Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located 10 in the UL2 gene, in replacement of a portion of or the whole sequence of the UL2 gene, rendering the UL2 gene inactive, and further comprises an inactive UL40 gene, optionally a deleted UL40 gene. A specific example of such a construct is e.g., rDEV4 ΔUL40 H9HA ΔUL2 (see Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL24 gene, in replacement of a portion of or the whole sequence of the UL24 gene, rendering 15 the UL24 gene inactive. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL24 (see Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL39 gene, in replacement of a portion of or the whole sequence of the UL39 gene, rendering the UL39 gene inactive. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL39 (see 20 Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL26-UL27 intergenic region, and further comprises an inactive UL24 gene, optionally a deleted UL24 gene. A specific example of such a construct is e.g., rDEV4 ΔUL24 UL26-H9HA-UL27 (see Example 3).

25 A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL40 gene, in replacement of a portion of or the whole sequence of the UL40 gene, rendering the UL40 gene inactive. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL40 (see Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located 30 in the UL26-UL27 intergenic region, and further comprises an inactive UL39 gene, optionally a deleted UL39 gene. A specific example of such a construct is e.g., rDEV4 ΔUL39 UL26-H9HA-UL27 (see Example 3).

A preferred attenuated DEV of the invention comprises a heterologous polynucleotide located in the UL23 gene, in replacement of a portion of or the whole sequence of the UL23 gene, rendering 35 the UL23 gene inactive, and further comprises an inactive UL41 gene, optionally a deleted UL41 gene. A specific example of such a construct is e.g., rDEV4 H9HA ΔUL23 ΔUL41 (see Example 3).

In a preferred attenuated DEV of the invention, the heterologous polynucleotide encodes H9 HA protein.

Host cell

The invention also relates to a host cell, expressing the attenuated DEV as defined above. The invention also relates to a host cell, expressing the attenuated DEV and the heterologous polynucleotide as defined above. In some embodiments, the host cell is CEF cell (Liang Z.,et.al, Animal(Basel), 2022, 12(24):3523), EB66 cell (Alexander Nikolay, Applied Microbiology and Biotechnology (2018) 102:8725-8737), DEF cell (Chenghuai Yang, Arch virol 2015, 160:267-274), embryonated egg, or chicken kidney cell (Andres Rodrı´guez-Avila et.al, Avian diseases 2007, 51:905-911).

The attenuated DEV of the present invention may be propagated in any competent cell cultures. After the required growth of the viruses is achieved, the cells may be detached from the wells using a scraper or with trypsin and the infected cells may be separated from the supernatant by centrifugation.

Examples of competent cell include CEF, EB66, DEF, embryonated egg, chicken kidney cells, and the like. The cells or viruses may be cultured in a culture medium such as MEM containing 5% FBS at about 37° C for 1h to 6 days.

Composition

The invention also relates to a composition, which comprises the attenuated DEV of the present invention.

The term “composition” as used herein refers to a composition that comprises at least one antigen, which elicits an immune response in the host to which the composition is administered. Such immune response may be a cellular and/or antibody-mediated immune response to the composition of the invention. The host is also described as a “subject”. Preferably, any of the hosts or subjects described or mentioned herein is an animal. In some embodiments, the animal is avian species, particularly young avian species. In some embodiments, the animal is poultry, particularly young poultry. In some embodiments, the animal is duck, particularly young duck. In some embodiments, the animal is chicken, particularly chick.

An "immune response" to a composition is the development in the host of a cellular and/or antibody-mediated immune response to a composition of interest. Usually, an " immune response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, and/or cytotoxic T cells, directed specifically to an antigen or antigens included in the composition of interest. Preferably, the host will display either a therapeutic or protective immune response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced.

A "protective immune response" or "protective response" will be demonstrated by either a reduction or lack of clinical signs normally displayed by an infected host, a quicker recovery time and/or a lowered duration of infectivity or lowered pathogen titer in the tissues or body fluids or excretions of the infected host.

5 In case where the host displays a protective immune response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced, the composition of the invention is described as a "vaccine". In one aspect, the composition of the present invention is a vaccine.

In some embodiments, the composition of the present invention is a vector vaccine. In some 10 embodiments, the composition of the present invention is a vector vaccine in poultry (such as duck and/or chicken). In some embodiments, the composition of the present invention is a vector vaccine in duck. In some embodiments, the composition of the present invention is a vector vaccine in chicken. In some embodiments, the attenuated DEV is used as a vector in the vector vaccine.

Compositions of the invention may further comprise a pharmaceutically or veterinarily 15 acceptable carrier, excipient, vehicle, or adjuvant.

The pharmaceutically or veterinarily acceptable carriers or adjuvant or vehicles or excipients are well known to the one skilled in the art. For example, a pharmaceutically or veterinarily acceptable carrier or adjuvant or vehicle or excipient includes, but is not limited to, 0.9% NaCl (e.g., saline) solution or a phosphate buffer, poly-(L-glutamate), the Lactated Ringer's Injection diluent (sodium 20 chloride, sodium lactate, potassium chloride, and calcium chloride), or polyvinylpyrrolidone. The pharmaceutically or veterinarily acceptable carrier or vehicle or adjuvant or excipients may be any compound or combination of compounds facilitating the administration of the vector (or protein expressed from an inventive vector *in vitro*), or facilitating transfection or infection and/or improving the preservation of the vector (or protein).

25 In some embodiments, the composition of the invention comprises a lyoprotectant. In a particular embodiment, the composition of the invention comprises a preservative.

The composition of the invention may be liquid (solutions, suspensions, emulsions) or solid (powder, gel, paste, oil). The composition of the invention may be formulated for any administration route. Preferably, the composition may be formulated for oro-nasal, eye drop, spray, drinking water, 30 in ovo, intramuscular, subcutaneous, intradermal, or transdermal administration.

The composition of the invention may contain a suitable dose sufficient to elicit a protective response in a poultry (e.g., a duck or a chicken). Doses and dose volumes are herein discussed in the general description and can also be determined by the skilled artisan from this disclosure in conjunction with the knowledge in the art, without any undue experimentation. The viral vector may 35 be titrated based on any virus titration methods including, but not limited to, FFA (Focus Forming Assay) or FFU (Focus Forming Unit), $TCID_{50}$ (50% Tissue Culture Infective Dose), PFU (Plaque Forming

Units), and $FAID_{50}$ (50% Fluorescent Antibody Infectious Dose), and the VLPs produced *in vitro* can be titrated by hemagglutination assay, ELISA, and electron microscopy. In some embodiments, the attenuated DEV in the composition is present in a dose from 1×10^2 $TCID_{50}$/ml or $TCID_{50}$/g to $1x10^7$ $TCID_{50}$/ml or $TCID_{50}$/g. In some embodiments, the attenuated DEV in the composition is present in a dose from $1x10^4$ $TCID_{50}$/ml or $TCID_{50}$/g to $1x10^6$ $TCID_{50}$/ml or $TCID_{50}$/g. In some embodiments, the attenuated DEV in the composition is present in a dose of $1x10^6$ $TCID_{50}$/ml or $TCID_{50}$/g. In some embodiments, the dose volumes can be between about 0.01 and about 10 ml, between about 0.01 and about 5 ml.

The composition of the invention can be administered in a single dose or in repeated doses, depending on the vaccination protocol. The vector vaccine of the invention can be formulated as single doses or in repeated doses, depending on the vaccination protocol.

Use and Method

In one aspect, the present invention provides the attenuated DEV of the present invention, the composition of the present invention, or the vector vaccine of the present invention, for the use in a method for inducing a protective immune response in an animal against a pathogen, wherein such method comprises or consists of one or more administration of the attenuated DEV of the present invention, the composition of the present invention, or the vector vaccine of the present invention to the animal.

In one aspect, the present invention provides the attenuated DEV of the present invention, the composition of the invention, or the vector vaccine of the present invention for use in vaccinating an animal by inducing a protective immune response in an animal against a pathogen.

In one aspect, the present invention provides a method of vaccinating an animal by inducing a protective immune response in an animal against a pathogen, comprising or consisting of at least one administration of the attenuated DEV of the invention, the composition of the invention, or the vector vaccine of the invention.

In one aspect, the present invention provides use of the attenuated DEV of the present invention in the manufacture of a composition for vaccinating an animal by inducing a protective immune response in an animal against a pathogen.

The term "vaccinating" relates to an active immunization by the administration of an immunogenic composition to an animal (such as a poultry) to be immunized, thereby causing a protective immune response against the antigen included in such immunogenic composition.

In some embodiments, the animal is avian species, particularly young avian species. In some embodiments, the animal is poultry, particularly young poultry. In some embodiments, the animal is duck, particularly young duck. In some embodiments, the animal is chicken, particularly chick.

In some embodiments, the animal, particularly the avian species, preferably the poultry, more preferably the duck or the chicken is 0 day-old, 1 day-old, 2 day-old, 3 day-old, 4 day-old, 5 day-old, 6 day-old, or 7 day-old at the day of vaccination.

In some embodiments, the attenuated DEV, the composition or the vector vaccine is administrated at Day 0 post-hatch, Day 1 post-hatch, Day 2 post-hatch, Day 3 post-hatch, Day 4 post-hatch, Day 5 post-hatch, Day 6 post-hatch, or Day 7 post-hatch.

As indicated in the experimental section, the viruses of the invention are particularly advantageous for vaccinating young poultry (at Day 0, Day 1, Day 2, or Day 3 post-hatch). Indeed, the invention surprisingly shows that the attenuated DEVs of the invention are safe upon such early administration to poultry, while native or wild-type DEV is lethal to poultry. Such early administration, combined with the early onset of immunity caused by attenuated DEV, is particularly advantageous to induce early protective immunity, before poultry can be substantially exposed to pathogens.

In some embodiments, the pathogen is avian species pathogen. In some embodiments, the pathogen is a poultry pathogen. In some embodiments, the pathogen is a duck pathogen. In some embodiments, the pathogen is a chicken pathogen. In some embodiments, the pathogen is avian influenza virus. In some embodiments, the pathogen is avian influenza A virus. In some embodiments, the pathogen is avian influenza A virus of subtype H9. In some embodiments, the pathogen is avian influenza A virus of subtype H9N2.

The administration or the attenuated DEV, the composition or the vector vaccine of the invention results in lessening of the incidence of the particular pathogen infection in a poultry or in the reduction in the severity of clinical signs caused by or associated with the specific pathogen infection. Preferably, the administration, or the attenuated DEV, the composition or the vector vaccine of the invention results in lessening of the incidence of the particular avian influenza virus infection in a poultry or in the reduction in the severity of clinical signs caused by or associated with the specific avian influenza virus infection. It is to be understood that the administration, or the attenuated DEV, the composition or the vector vaccine of the invention may not be effective in all poultry administrated, but there is a significant portion (for example, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90%) of poultry effectively immunized.

In some embodiments, the attenuated DEV, the composition or the vector vaccine is administered by oro-nasal, eye drop, spray, drinking water, in ovo, intramuscular, subcutaneous, intradermal, or transdermal. In some embodiments, the attenuated DEV, the composition or the vector vaccine may be formulated for oro-nasal, eye drop, spray, drinking water, in ovo, intramuscular, subcutaneous, intradermal, or transdermal administration. However, depending on the nature and mode of action of a compound, the immunogenic composition may be administered by other routes as well.

In one aspect of the invention, the attenuated DEV, the composition or the vector vaccine is administered once and is efficacious by such single administration.

However, while a single dose administration is preferred, the attenuated DEV, the composition or the vector vaccine can also be administered twice or several times, with a first dose being administered prior to the administration of a second (booster) dose. Preferably, the second dose is administered at least 15 days after the first dose. More preferably, the second dose is administered between 15 and 40 days after the first dose. Even more preferably, the second dose is administered at least 17 days after the first dose. Still more preferably, the second dose is administered between 17 and 30 days after the first dose. Even more preferably, the second dose is administered at least 19 days after the first dose. Still more preferably, the second dose is administered between 19 and 25 days after the first dose. Most preferably the second dose is administered at least 21 days after the first dose. In a preferred aspect of the two-time administration regimen, both the first and second doses of the immunogenic composition are administered in the same amount. In addition to the first and second dose regimen, an alternate embodiment comprises further subsequent doses. For example, a third, fourth, or fifth dose could be administered in these aspects. Preferably, subsequent third, fourth, and fifth dose regimens are administered in the same amount as the first dose, with the time frame between the doses being consistent with the timing between the first and second doses mentioned above.

The attenuated DEV, the composition or the vector vaccine of the invention may be administrated in a suitable dose sufficient to elicit a protective response in poultry. Doses and dose volumes are herein discussed in the general description and can also be determined by the skilled artisan from this disclosure in conjunction with the knowledge in the art, without any undue experimentation. In some embodiments, the attenuated DEV in the composition or the vector vaccine is present in a dose from 1×10^2 $TCID_{50}$/ml or $TCID_{50}$/g to $1x10^7$ $TCID_{50}$/ml or $TCID_{50}$/g. In some embodiments, the attenuated DEV in the composition or the vector vaccine is present in a dose from $1x10^4$ $TCID_{50}$/ml or $TCID_{50}$/g to $1x10^6$ $TCID_{50}$/ml or $TCID_{50}$/g. In some embodiments, the attenuated DEV in the composition or the vector vaccine is present in a dose of $1x10^6$ $TCID_{50}$/ml or $TCID_{50}$/g. In some embodiments, the dose volumes can be between about 0.01 and about 10 ml, between about 0.01 and about 5 ml.

The present invention further relates to vaccination kits for vaccinating a poultry by inducing a protective immune response in a poultry against a pathogen, which comprises an effective amount of the attenuated DEV, the composition or the vector vaccine as described above and a means for administering said attenuated DEV, the composition or the vector vaccine to said poultry. For example, such kit comprises an injection device filled with the attenuated DEV, the composition or the vector vaccine according to the invention and instructions for intradermic, subcutaneous, intramuscular, or in ovo injection. Alternatively, the kit comprises a spray/aerosol or eye drop device filled with the attenuated DEV, the composition or the vector vaccine according to the invention and instructions for oro-nasal administration, oral or mucosal administration.

The following clauses are also described herein and part of disclosure of the invention:

Clause 1. An attenuated Duck Enteritis Virus (DEV) (as a live vector vaccine in poultry, such as duck and/or chicken), wherein one or more genes of the DEV genome selected from the group consisting of US3, UL24, UL40, UL39, and UL23 is inactivated.

5 Clause 2. An attenuated Duck Enteritis Virus (DEV) (as a live vector vaccine in poultry, such as duck and/or chicken), comprising an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; and v) UL23 gene, in combination with the inactivation of one or more further non-essential genes of DEV.

Clause 3. The attenuated Duck Enteritis Virus (DEV) of clause 2, wherein the one or more further 10 non-essential gene of DEV is different from the first inactivated gene and is selected from i) UL2 gene, ii) UL41 gene; iii) US3 gene; iv) UL24 gene; v) UL40 gene; vi) UL39 gene; or vii) UL23 gene.

Clause 4. An attenuated Duck Enteritis Virus (DEV) (as a live vector vaccine in poultry, such as duck and/or chicken), comprising inactivated gene(s) selected from any one of i)-viii), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; v) UL23 gene; vi) UL24 gene and UL2 gene; vii) UL40 gene 15 and UL2 gene; or viii) UL23 gene and UL41 gene.

Clause 5. The attenuated DEV of any one of the preceding clauses, wherein the gene is inactivated by mutation, interruption, replacement or deletion of a portion of or the whole sequence of the gene.

Clause 6. The attenuated DEV of any one of the preceding clauses, wherein at least 40%, at least 20 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the sequence of the gene is replaced or deleted.

Clause 7. The attenuated DEV of any one of the preceding clauses, wherein the attenuated DEV further comprises (and is capable of expressing) a heterologous polynucleotide coding for a heterologous antigen of a pathogen.

25 Clause 8. The attenuated DEV of clause 7, wherein the heterologous polynucleotide is inserted into a non-essential gene or region of the attenuated DEV.

Clause 9. The attenuated DEV of clause 7, wherein the heterologous polynucleotide is inserted into or in replacement of a portion of or the whole sequence of the non-essential gene or region of the attenuated DEV.

30 Clause 10. The attenuated DEV of clause 7, wherein the non-essential gene or region of the attenuated DEV is selected from the group consisting of UL2 gene, UL24 gene, UL39 gene, UL26-UL27 intergenic region, UL40 gene, and UL23 gene of the DEV genome.

Clause 11. The attenuated DEV of clause 7, wherein the heterologous polynucleotide is located in a gene or a region selected from the group consisting of UL23 gene, UL2 gene, UL24 gene, UL39 gene, UL40 gene, and a UL26-UL27 intergenic region of DEV genome.

Clause 12. The attenuated DEV of clause 7, wherein the heterologous polynucleotide is inserted
5 into or in replacement of a portion of or the whole sequence of one or more genes selected from the group consisting of UL23 gene, UL2 gene, UL24 gene, UL39 gene, and UL40 gene of DEV genome, or inserted into UL26-UL27 intergenic region of DEV genome.

Clause 13. The attenuated DEV of any one of the preceding clauses, wherein said attenuated DEV comprises any one of the followings:

10 i) the heterologous polynucleotide located in the UL23 gene, in replacement of a portion of or the whole sequence of the UL23 gene;

ii) the heterologous polynucleotide located in the UL2 gene, in replacement of a portion of or the whole sequence of the UL2 gene, and further comprises an inactive UL24 gene, optionally a deleted UL24 gene;

15 iii) the heterologous polynucleotide located in the UL2 gene, in replacement of a portion of or the whole sequence of the UL2 gene, and further comprises an inactive UL40 gene, optionally a deleted UL40 gene;

iv) the heterologous polynucleotide located in the UL24 gene, in replacement of a portion of or the whole sequence of the UL24 gene;

20 v) the heterologous polynucleotide located in the UL39 gene, in replacement of a portion of or the whole sequence of the UL39 gene;

vi) the heterologous polynucleotide located in the UL26-UL27 intergenic region, and further comprises an inactive UL24 gene, optionally a deleted UL24 gene;

vii) the heterologous polynucleotide located in the UL40 gene, in replacement of a portion of or
25 the whole sequence of the UL40 gene;

viii) the heterologous polynucleotide located in the UL26-UL27 intergenic region, and further comprises an inactive UL39 gene, optionally a deleted UL39 gene; or

ix) the heterologous polynucleotide located in the UL23 gene, in replacement of a portion of or the whole sequence of the UL23 gene, and further comprises an inactive UL41 gene, optionally a
30 deleted UL41 gene.

Clause 14. The attenuated DEV of any one of clauses 7-13, wherein the pathogen is a poultry pathogen or the antigen is an antigen of a poultry pathogen.

Clause 15. The attenuated DEV of any one of clauses 7-13, wherein the pathogen is or the antigen is derived from avian influenza virus.

Clause 16. The attenuated DEV of any one of clauses 7-13, wherein the pathogen is or the antigen is derived from an avian influenza virus selected from the group consisting of an Influenza A virus, an Influenza B virus, an Influenza C virus, and an Influenza D virus.

Clause 17. The attenuated DEV of any one of clauses 7-13, wherein the pathogen is or the antigen is derived from avian influenza virus type H9.

Clause 18. The attenuated DEV of any one of clauses 7-13, wherein the pathogen is or the antigen is derived from an avian influenza virus H9N2.

Clause 19. The attenuated DEV of any one of clauses 7-13, wherein the heterologous antigen is the Hemagglutinin (HA) protein of the subtype H9N2 avian influenza virus.

Clause 20. The attenuated DEV of any one of clauses 7-19, wherein the amino acid sequence of the heterologous antigen has at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9% or 100% sequence identity with SEQ ID NO: 1.

Clause 21. The attenuated DEV of any one of clauses 7-19, wherein the sequence of the heterologous polynucleotide has at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9% or 100% sequence identity with SEQ ID NO: 2.

Clause 22. The attenuated DEV of any one of clauses 7-19, wherein the heterologous polynucleotide is operably linked to a promoter.

Clause 23. The attenuated DEV of clause 22, wherein the promoter is selected from the group consisting of an immediate early cytomegalovirus (CMV) promoter, mouse CMV promoter, guinea pig CMV promoter, an SV40 promoter, Human Herpesvirus Type III glycoprotein B (HHV3gB) promoter, Pseudorabies Virus promoters such as that of glycoprotein X promoter, Herpes Simplex Virus- 1 alpha 4 promoter, a Marek's Disease Virus glycoprotein A (or gC) promoter, a Marek's Disease Virus glycoprotein B promoter, a Marek's Disease Virus glycoprotein E promoter, a Marek's Disease Virus glycoprotein I promoter, an Infectious Laryngotracheitis Virus glycoprotein B, an Infectious Laryngotracheitis Virus glycoprotein E promoter, an Infectious Laryngotracheitis Virus glycoprotein D promoter, an Infectious Laryngotracheitis Virus glycoprotein I promoter, vaccinia H6, and a combination thereof.

Clause 24. The attenuated DEV of any one of clauses 7-23, wherein the heterologous polynucleotide is operably linked to an SV40 polyA signal.

Clause 25. The attenuated DEV of any one of clauses 7-23, wherein the heterologous polynucleotide is expressed after the attenuated DEV has been transfected into a suitable host cell.

Clause 26. The attenuated DEV of any one of clauses 1-25, wherein the attenuated DEV has a reduced or no mortality in poultry (such as duck or chicken) as compared to the non-modified wildtype DEV.

Clause 27. The attenuated DEV of any one of clauses 1-25, wherein the mortality in poultry caused by the attenuated DEV is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70% of that caused by the non-modified DEV.

Clause 28. The attenuated DEV of any one of clauses 1-25, wherein the mortality (in duck and/or chicken) caused by the attenuated DEV is 0%, less than 5%, less than 10%, less than 20%, less than 30%, less than 40%, less than 50%, less than 60%, or less than 70% of that caused by the non-modified DEV.

Clause 29. The attenuated DEV of any one of the preceding claims, wherein the inactivation of the selected genes causes the attenuation of DEV.

Clause 30. The attenuated DEV of any of the preceding clauses for use as vector vaccine in poultry.

Clause 31. The attenuated DEV of any of the preceding clauses for use as vector vaccine in duck.

Clause 32. The attenuated DEV of any of the preceding clauses for use as vector vaccine in chicken.

Clause 33. A composition, comprising the attenuated DEV of any one of clauses 7-29.

Clause 34. The composition of clause 33, further comprising a pharmaceutically or veterinarily acceptable carrier, excipient, vehicle or adjuvant.

Clause 35. The composition of clause 33 or 34, wherein the composition is a vaccine.

Clause 36. The composition of clause 33 or 34, wherein the composition is a vector vaccine in poultry.

Clause 37. The composition of clause 33 or 34, wherein the composition is a vector vaccine in duck.

Clause 38. The composition of clause 33 or 34, wherein the composition is a vector vaccine in chicken.

Clause 39. The composition of any one of clauses 33-38, wherein the attenuated DEV is used as a vector.

Clause 40. The attenuated DEV of any one of clauses 1-29, the composition of any one of clauses 33-39, or the vector vaccine of any one of clauses 30-32, for the use in a method for inducing a protective immune response in poultry (e.g. duck or chicken) against a poultry pathogen, wherein such method comprises or consists of one or more administration of the attenuated DEV of any one of clauses 1-29, the composition of any one of clauses 33-39, or the vector vaccine of any one of clauses 30-32 to poultry (e.g. duck or chicken).

Clause 41. The attenuated DEV of any one of clauses 1-29, the composition of any one of clauses 33-39, or the vector vaccine of any one of clauses 30-32, for use in vaccinating a poultry (e.g. duck or chicken) by inducing a protective immune response in a poultry (e.g. duck or chicken) against a pathogen.

Clause 42. A method of vaccinating a poultry (e.g. duck or chicken) by inducing a protective immune response in a poultry (e.g. duck or chicken) against a pathogen, comprising or consisting of at least one administration of the attenuated DEV of any one of clauses 1-29, the composition of any one of clauses 33-39, or the vector vaccine of any one of clauses 30-32 to poultry (e.g. duck or chicken).

Clause 43. Use of the attenuated DEV of any one of clauses 1-29 in the manufacture of a composition for vaccinating a poultry (e.g. duck or chicken) by inducing a protective immune response in a poultry (e.g. duck or chicken) against a pathogen.

Clause 44. The method and the use of any one of clauses 40-43, wherein the pathogen is avian influenza virus.

Clause 45. The method and the use of clause 44, wherein the pathogen is avian influenza virus, optionally avian influenza A virus, optionally avian influenza A virus of subtype H9, optionally avian influenza A virus of subtype H9N2.

Clause 46. The method and the use of any one of clauses of 40-45, wherein the poultry is a chicken or a duck.

Clause 47. The method and the use of any one of clauses of 40-46, wherein the poultry is 0 day-old, 1 day-old, 2 day-old, 3 day-old, 4 day-old, 5 day-old, 6 day-old, or 7 day-old at the day of vaccination.

Clause 48. The method and the use of any one of clauses 40-47, wherein the attenuated DEV, the composition or the vector vaccine is administrated at Day 0 post-hatch, Day 1 post-hatch, Day 2 post-hatch, Day 3 post-hatch, Day 4 post-hatch, Day 5 post-hatch, Day 6 post-hatch, or Day 7 post-hatch.

Clause 49. The method and the use of any one of clauses 40-48, wherein the attenuated DEV, the composition or the vector vaccine is administrated by oro-nasal, eye drop, spray, drinking water, in ovo, intramuscular, subcutaneous, intradermal, or transdermal.

Clause 50. A vaccination kit for vaccinating a poultry (e.g. duck or chicken) by inducing a protective immune response in a poultry (e.g. duck or chicken) against a pathogen, which comprises an effective amount of the attenuated DEV of any one of clauses 1-29, the composition of any one of clauses 33-39 or the vector vaccine of any one of clauses 30-32 and a means for administering the
5 attenuated DEV, said composition or said vector vaccine to said poultry.

Clause 51. A host cell, expressing the attenuated DEV of any one of clauses 1-29.

Clause 52. A host cell, expressing the attenuated DEV and the heterologous polynucleotide of any one of clauses 1-29.

Clause 53. The host cell of clause 51 or 52, wherein the host cell is CEF cell, EB66 cell or DEF cell.

10 Clause 54. A method of making the attenuated DEV of any one of clauses 1-29, comprising the inactivation of one or more genes of the DEV genome as defined in any one of clauses 1-29, wherein said inactivation of the one or more selected genes leads to a reduced or no mortality of the modified DEV in poultry (such as duck or chicken) as compared to the non-modified DEV.

15 **Examples**

The subsequent examples further illustrate the invention in an exemplified manner. It is understood that the invention is not limited to any of those examples as described below. A person skilled in the art understands that the performance, results and findings of these examples can be adapted and applied in a broader sense in view of the general description of the present invention.

20 **Example 1: Construction of bacterial artificial chromosome (BAC) of DEV**

The duck plague virus DEV4 strain used in this study is deposited at China Center for Type Culture Collection (CCTCC), Wuhan University, Wuhan 430072 P.R. China, on August 4, 2023 under CCTCC NO: V202378.

In order to attenuate the DEV4 strain and make it a safe vector, a bacterial artificial chromosome
25 system was constructed with the genome of the DEV4 strain for subsequent gene deletion and insertion. The specific steps include:

1. The sequences of the homologous arms on the left and right side of the insertion site (between UL44 and UL44.5 of the DEV4 genome) for mini-F element were amplified by PCR using the primers as shown in Table 1. The digestion sites were introduced simultaneously. The plasmids pB12
30 (constructed according to B. Karsten Tischer et al, 2007, Journal of Virology, p.13200-13208 and gene structure map of pB12 is shown in Figure 1) containing the mini-F gene fragments were digested with BamH I, to obtain the mini-F DNA fragments having BamH I on both sides. Then, the obtained DNA fragments of the left and right homologous arms and the mini-F DNA fragment were ligated to obtain the Mini-F transfer vector, and then transformed into E. coli TOP10 competent cells (purchased from

Tiangen). The plasmid with the mini F transfer vector was extracted and identified by enzyme digestion.

Table 1. PCR Primers

Primer	Sequence(5'-3')
Left arm-F	cgcgaattcaagtgctcgtatcccacgct(EcoR I)(SEQ ID NO:3)
Left arm-R	ggcggatcccttcacagttctacagtcat(BamH I) (SEQ ID NO:4)
Right arm-F	ggcggatcccgctcgaactacactaccat(BamH I) (SEQ ID NO:5)
Right arm-R	tgttcgtcgacaaaacaactggc(Sal I) (SEQ ID NO:6)

5 2. The linearized mini-F transfer vector obtained in above step 1 and the extracted DEV4 genomic DNA were co-transfected into DEFs according to the instructions of the commercial transfection kit Lipofectamine™ 3000 (purchased from Invitrogen), to construct the recombinant virus rDEV4 (i.e. recombinant DEV4)-BAC by homologous recombination. Upon screening and purification, the recombinant virus rDEV4-BAC was obtained (see Figure 2A).

10 3. The extracted rDEV4-BAC genomic DNA was electroporated into MegaX competent cell (purchased from Invitrogen, catalog No. C6400-03) according to the instruction of the competent cell. Then, the bacmid rDEV4-BAC was extracted and identified.

4. The bacmid rDEV4-BAC was electroporated into gs1783 competent cells containing redE/T recombinase (Wang et al, 2015, Virology Journal, 12:126) to obtain GS1783-DEV4-BAC strain.

15 **Example 2: Construction of DEV strains having deleted virulence genes**

Based on the constructed rDEV4-BAC, different virulence-associated genes of DEV4 were deleted, including US3, UL24, UL40, UL39, and UL23 (hereinafter referred to as X in general), so as to construct DEV strains having deleted virulence genes, including rDEV4 ΔUS3, rDEV4 ΔUL24, rDEV4 ΔUL40, rDEV4 ΔUL39, and rDEV4 ΔUL23.

20 "Δ" refers to the inactivation (such as deletion or replacement) of the gene herein, for example, ΔUL24 refers to deletion or replacement of UL24.

The construction method comprises the following steps.

1. The DNA fragment I_SceI-Kana-X (X represents the virulence gene to be deleted) containing I_SceI site, Kana resistance gene and 50bp homologous arms of upstream and downstream of the 25 virulence gene to be deleted was obtained by Not I digestion from the plasmid pKan which was synthesized by GenScript company.

2. GS1783-DEV4-BAC (obtained in Example 1) competent cells for electroporation were prepared by conventional method. Then, the I_SceI-Kana-X fragment was electroporated into the GS178-DEV4-BAC competent cells. Recombinant clones were then selected on chloramphenicol and

kanamycin double-resistant LB agar plates. The recombinant bacmid DNA was extracted and analyzed by both PCR and RFLP methods. Thus, the GS1783-DEV4-BAC-ΔX-Kana strain and the recombinant bacmid rDEV4-BAC-ΔX-Kana were obtained. In the 2nd step of Red recombination, 2% arabinose was used to induce expression of the homing endonuclease I-SceI, resulting in the cleavage of the I-SceI
5 restriction site upstream of the kanamycin gene and, ultimately, the excision of the kanamycin cassette. The recombinant bacmid DNA was extracted and analyzed by both PCR and RFLP methods. Then, the GS1783-DEV4-BAC-ΔX strain and the bacmid rDEV4-BAC-ΔX were obtained.

3. To rescue the recombinant rDEV4 ΔX (see Figure 3) and to delete the mini-F sequence, the recombinant bacmid DNA was extracted and co-transfected with mini-F homologous arm DNA into
10 DEFs (prepared from 11-day-old or 12 day-old clean duck embryos (purchased from Harbin Veterinary Research Institute) according to conventional methods) using lipofectamine 3000 (Invitrogen). After co-transfection, cells were observed to check the formation of both GFP-positive and -negative plaques. Limiting dilution or plaque purification was performed to separate the GFP-negative recombinant virus, in which mini-F containing EGFP gene was removed via intra-molecular
15 homologous recombination mechanism. Thus, the rDEV4 ΔX was obtained (see Figure 2B), including rDEV4 ΔUS3, rDEV4 ΔUL24, rDEV4 ΔUL40, rDEV4 ΔUL39, and rDEV4 ΔUL23.

Example 3: Construction of recombinant DEV containing HA gene of subtype H9N2 of avian influenza virus

Based on DEV strains having deleted virulence genes, the HA gene of subtype H9N2 of avian
20 influenza virus was inserted into different sites. The following recombinant viruses were finally constructed: rDEV4 H9HA ΔUL23, rDEV4 ΔUL24 H9HA ΔUL2, rDEV4 ΔUL40 H9HA ΔUL2, rDEV4 H9HA ΔUL24, rDEV4 H9HA ΔUL39, rDEV4 ΔUL24 UL26-H9HA-UL27, rDEV4 H9HA ΔUL40, rDEV4 ΔUL39 UL26-H9HA-UL27, and rDEV4 H9HA ΔUL23 ΔUL41.

In this example, the construction method will be described using the construction of rDEV4
25 ΔUL39 UL26-H9HA-UL27 as an example. Particularly, the construction method comprises the following steps.

1. The heterologous gene fragment comprising HA gene and Kana resistant gene UL26-mCMV-H9HA-kana-UL27 with 50bp homologous arms on both sides was obtained by PCR amplification using the plasmid puc57-mCMV-H9HA-kana-SV40 (constructed by Nanjing GenScript) as
30 a template and L26L27HA-F and L26L27HA-R as primers (see Table 2). The primers used for the construction of other rDEV4 H9HA viruses are also listed in Table 2.

Table 2. PCR primers

Primer	Sequence(5'-3')
L26L27HA-F	TGTACGTGTTTTAAATACGAGCGTATAGTTTAATCGGGAGGCAGCTTCATAACTCCGCCCGTTTTATGACT (SEQ ID NO:7)
L26L27HA	AATGCATTCGGCCTGGCAAATATGACAACTTTAGCAATTACTCGTGGCACGAAAAAACCTCCCACACCT

-R	CCC(SEQ ID NO:8)
L2HA-F	ACGACCTTCCAGGTATTCATTGGCCTTTTTAAAATGATCACATAAGATGAATGGCAACTCCGCCCGTTTTATGACT (SEQ ID NO:9)
L2HA-R	CTCCCGTCTCCGTCCCAGGCGCCGAAACGACGCAGGCCTTGTGGTGCGCCAGCGGGAAAAAACCTCCCACACCTCCC (SEQ ID NO:10)
L39HA-F	GTTGAAGCTCCATCTTATGTTCTATTGCTGCGGTAGGAACCGTTGCAAATGAAAAAACCTCCCACACCTCCC (SEQ ID NO:11)
L39HA-R	TCTTTATAATCGACGCGGCTGGGAAGTTGGTACACGAACGCGGCGACATTAACTCCGCCCGTTTTATGACT (SEQ ID NO:12)
L40HA-F	GAATAACATATTCGAATATATAAGAGTAGAAAATTAAGGCGTATAGAGGGGAAAAAACCTCCCACACCTCCC (SEQ ID NO:13)
L40HA-R	CTGCGCTCTCTAAATTTGCAACGGTTCCTACCGCAGCAATAGAACATAAGAACTCCGCCCGTTTTATGACT (SEQ ID NO:14)
L24HA-F	TCAACACTGCCAGACAATAGGATGGTAATATGCGTTTCTGTAATAATGCAGAAAAAACCTCCCACACCTCCC (SEQ ID NO:15)
L24HA-R	GGTTTTGCCAGTTCCATACGGCCCGTCTAGGTATACGCGGACGAGGCATAAACTCCGCCCGTTTTATGACT (SEQ ID NO:16)
L23HA-F	TGCCGAGCCAATGGCGTATTGGAGAAATCATTTTGAAGATGTAATAAAGGAACTCCGCCCGTTTTATGACT (SEQ ID NO:17)
L23HA-R	CATAGTTTCAGAACCGCCCATCAGAGCTTTATTTAAAACAAATATATTTAGAAAAAACCTCCCACACCTCCC (SEQ ID NO:18)

2. GS1783-DEV4-BAC-ΔUL39 (obtained in Example 2) competent cells for electroporation were prepared by conventional method. Then, the heterologous gene fragment UL26-mCMV-H9HA-kana-UL27 was electroporated into the GS1783-DEV4-BAC-ΔUL39 competent cells. Recombinant clones were then selected on chloramphenicol and kanamycin double-resistant LB agar plates. The recombinant bacmid DNA was extracted and analyzed by both PCR and RFLP (see Figure 4) methods. Thus, the recombinant blacmid rDEV4-BAC-ΔUL39-UL26-H9HA-kana-UL27 was obtained. In the 2nd step of Red recombination, 2% arabinose was used to induce expression of the homing endonuclease I-SceI, resulting in the cleavage of the I-SceI restriction site upstream of the kanamycin gene and, ultimately, the excision of the kanamycin cassette. The recombinant bacmid DNA was extracted and analyzed by both PCR and RFLP methods. Then, the bacmid rDEV4-BAC-ΔUL39-UL26-H9HA-UL27 was obtained.

3. To rescue the recombinant rDEV4-ΔUL39-UL26-H9HA-UL27 and to delete the mini-F sequence, the recombinant bacmid DNA was extracted and co-transfected with mini-F homologous arm DNA into DEFs (prepared from 11-day-old or 12 day-old clean duck embryos (purchased from Harbin Veterinary Research Institute) according to conventional methods) using lipofectamine 3000 (Invitrogen). After transfection, cells were observed to check the formation of both GFP-positive and -negative plaques (see Figure 5). Limiting dilution or plaque purification was performed to separate the GFP-negative recombinant virus, in which mini-F containing EGFP gene was removed via intra-molecular homologous recombination mechanism. Thus, the recombinant DEV containing HA

gene of subtype H9N2 of avian influenza virus rDEV4-ΔUL39-UL26-H9HA-UL27 was obtained (see Figure 2C).

4. According to above steps 1-3, additional recombinant viruses expressing the H9N2 HA gene were constructed, including rDEV4 H9HA ΔUL23, rDEV4 ΔUL24 H9HA ΔUL2, rDEV4 ΔUL40 H9HA ΔUL2, rDEV4 H9HA ΔUL24, rDEV4 H9HA ΔUL39, rDEV4 ΔUL24 UL26-H9HA-UL27, rDEV4 H9HA ΔUL40, and rDEV4 H9HA ΔUL23 ΔUL41.

Example 4: Preparation and *in vitro* characterization of the seed batch of the recombinant virus

4.1. Preparation of seed batch of recombinant DEVs having deleted virulence genes rDEV4 ΔX (X represents the deleted gene(s))

1.4E7 DEFs were inoculated on a 10cm cell culture dish. The medium was 10ml of MEM containing 5% FBS. After 24 hours of incubation at 37°C in a 5% CO2 incubator, the monolayer of DEFs covered more than 90% of the dish. The cells were inoculated with the DEV strains having deleted virulence genes, respectively. The cells were continued to be incubated at 37°C in a 5% CO2 incubator for about 4 days until all the cells were infected. The cell supernatant was harvested and centrifuged at 3000 rpm for 10 min. The supernatant was separated into 1 ml or 4 ml freezing tubes, and stored at -80°C for later use.

4.2. Preparation of seed batch of recombinant DEVs containing the HA gene of subtype H9N2 of avian influenza virus

1.4E7 DEFs were inoculated on a 10cm cell culture dish. The medium was 10ml of MEM+5% FBS. After 24 hours of incubation at 37°C in a 5% CO2 incubator, the monolayer of DEFs covered more than 90% of the dish. Then the cells were inoculated with the recombinant DEVs expressing HA gene of subtype H9N2 of avian influenza virus, respectively. The cells were continued to incubate at 37°C in a 5% CO2 incubator for about 4 days until all cells were infected. The cell supernatant was harvested and centrifuged at 3000 rpm for 10 min. The supernatant was separated into 1 ml or 4 ml freezing tubes, and stored at -80°C for later use.

Next, 1.4E7 CEF cells were inoculated on a 10cm cell culture dish. The medium was 10ml of MEM+5% FBS. After 24 hours of incubation at 37°C in a 5% CO2 incubator, the monolayer of CEF cells covered more than 90% of the dish. The cells were inoculated with the recombinant DEVs that have been passaged to P7 in DEF, respectively. The cells were continued to be incubated at 37°C in a 5% CO2 incubator for about 4 days until all cells were infected. The cell supernatant was harvested and centrifuged at 3000 rpm for 10 min. The supernatant was separated into 1 ml or 4 ml freezing tubes, and stored at -80°C.

4.3. *In vitro* identification of the rDEVs vector

1. The rDEVs with deleted virulence genes were continuously passaged to 15th generation in DEF. The DNA of the recombinant viruses in the 5th, 10th, and 15th generations was extracted by QIAamp

DNA Mini Kit (QIAGEN). The extracted DNA was amplified by PCR using primers to identify the deletion of virulence gene(s). PCR products were identified by sequencing, indicating that the virulence genes were successfully deleted.

Table 3. PCR primers

Identification Primer	**Sequence(5'-3')**
IDENΔUS3-F	TGTTGTCAAAGGGCGAGTTCGT (SEQ ID NO:19)
IDENΔUS3-R	TTGCTGCGGCTATCTCCCATT (SEQ ID NO:20)
IDENΔUL24-F	GTTGATTATACAAGCCGATAGT (SEQ ID NO:21)
IDENΔUL24-R	GTCAGTTGTTGCTATGTCACCT (SEQ ID NO:22)
IDENΔUL40-F	CTTTCTATATAGTGTCTTAGCT (SEQ ID NO:23)
IDENΔUL40-R	GTCGACCACAGTCAATCAACCT (SEQ ID NO:24)
IDENΔUL39-F	ATGTTTCGGGCGGTCCATAATT (SEQ ID NO:25)
IDENΔUL39-R	CTACTTCTTGTCGTATCTGGCT (SEQ ID NO:26)
IDENΔUL23-F	GTCGCTGCGCCAATACATTT (SEQ ID NO:27)
IDENΔUL23-R	AGTTGGCCGGTTAGCTTTACCT (SEQ ID NO:28)

5 2. The harvested rDEVs having deleted virulence genes were subjected to the detection of sterility and mycoplasma. Sterility detection was performed by a conventional method, and mycoplasma detection was performed by qPCR. Results showed that these recombinant viruses had no exogenous microbial contamination.

3. Determination of titers of rDEVs having deleted virulence genes: the titer of the rDEVs having
10 deleted virulence genes was determined on DEFs (TCID50).

Table 4. TCID50 of rDEVs

rDEVs with deleted virulence genes	**TCID50/ml**
rDEV4 ΔUS3	1.37E4
rDEV4 ΔUL24	1.12E8
rDEV4 ΔUL40	1.29E7
rDEV4 ΔUL39	1.44E7
rDEV4 ΔUL23	5.99E6

4.4. *In vitro* identification of the rDEVs containing the HA gene of subtype H9N2 of avian influenza virus

15 1. Stability of the HA gene. The rDEVs containing the HA gene of subtype H9N2 of avian influenza virus were continuously passaged to 15th generation in CEF. The DNA of the recombinant viruses in the 5th, 10th, and 15th generations was extracted by QIAamp DNA Mini Kit (QIAGEN). The extracted DNA was amplified by PCR using primers to identify the insertion of HA gene (see Figure 6). PCR products were identified by sequencing, indicating that the HA gene was stably present in the
20 genome.

Table 5. PCR primers

Recombinant virus	PCR primer	Sequence of PCR primer
rDEV4 H9HA ΔUL23	IdenUL23HA-F	GTCGCTGCGCCAATACATTT (SEQ ID NO:29)
	IdenUL23HA-R	AGTTGGCCGGTTAGCTTTACCT (SEQ ID NO:30)
rDEV4 ΔUL24 H9HA ΔUL2	IdenL2HA-F	ATGGTACTGGGGTGTCCATAGT (SEQ ID NO:31)
	IdenL2HA-R	GAAACGCGAATATGCATCGCTT (SEQ ID NO:32)
rDEV4 ΔUL40 H9HA ΔUL2	IdenL2HA-F	ATGGTACTGGGGTGTCCATAGT(SEQ ID NO:33)
	IdenL2HA-R	GAAACGCGAATATGCATCGCTT (SEQ ID NO:34)
rDEV4 H9HA ΔUL24	IdenL24HA-F	GTTGATTATACAAGCCGATAGT (SEQ ID NO:35)
	IdenL24HA-R	GTCAGTTGTTGCTATGTCACCT (SEQ ID NO:36)
rDEV4 H9HA ΔUL39	IdenL39HA-F	ATGTTTCGGGCGGTCCATAATT (SEQ ID NO:37)
	IdenL39HA-R	CTACTTCTTGTCGTATCTGGCT (SEQ ID NO:38)
rDEV4 ΔUL24 UL26-H9HA-UL27	IdenL26L27HA-F	TGAAGATGGCGATTCGGCAGAT (SEQ ID NO:39)
	IdenL26L27HA-R	CGATTAAATGGTTGCGTGCCTT (SEQ ID NO:40)
rDEV4 H9HA ΔUL40	IdenL40HA-F	CTTTCTATATAGTGTCTTAGCT (SEQ ID NO:41)
	IdenL40HA-R	GTCGACCACAGTCAATCAACCT (SEQ ID NO:42)
rDEV4 ΔUL39 UL26-H9HA-UL27	IdenL26L27HA-F	TGAAGATGGCGATTCGGCAGAT (SEQ ID NO:43)
	IdenL26L27HA-R	CGATTAAATGGTTGCGTGCCTT (SEQ ID NO:44)
rDEV4 H9HA ΔUL23 ΔUL41	IdenUL23HA-F	GTCGCTGCGCCAATACATTT (SEQ ID NO:45)
	IdenUL23HA-R	AGTTGGCCGGTTAGCTTTACCT (SEQ ID NO:46)
	IdenUL41-F	ATCACGGACCGGTAGTCTTAGT (SEQ ID NO:47)
	IdenUL41-R	ATCATCGACACCGAAGCTATAT (SEQ ID NO:48)

2. Detection of sterility and mycoplasma. The harvested rDEVs containing the HA gene of subtype H9N2 of avian influenza virus were subjected to detection of sterility and mycoplasma. Sterility detection was performed by a conventional method, and mycoplasma detection was performed by qPCR. Results showed that these recombinant viruses had no exogenous microbial contamination.

3. Determination of titers of rDEVs containing the HA gene of subtype H9N2 of avian influenza virus: the titers of rDEVs containing the HA gene of subtype H9N2 of avian influenza virus was determined on CEFs (TCID50).

Table 6. TCID50 of rDEVs

rDEV containing the HA gene of subtype H9N2 of avian influenza virus	CEF based TCID50/ml
rDEV4 H9HA ΔUL23	2.37E6
rDEV4 ΔUL24 H9HA ΔUL2	1.74E7
rDEV4 ΔUL40 H9HA ΔUL2	4.16E6
rDEV4 H9HA ΔUL24	1.03E7
rDEV4 H9HA ΔUL39	4.39E7

rDEV4 ΔUL24 UL26-H9HA-UL27	3.15E6
rDEV4 H9HA ΔUL40	6.58E6
rDEV4 ΔUL39 UL26-H9HA-UL27	2.88E5
rDEV4 H9HA ΔUL23 ΔUL41	5.08E5

4. Expression of the rDEVs containing the HA gene of subtype H9N2 of avian influenza virus

The rDEV containing the HA gene of subtype H9N2 of avian influenza virus was inoculated into CEFs in 48-well plate at a dilution of 10^{-2}-10^{-4}. 4 hours after inoculation, the medium was replaced with MEM+5% FBS containing 0.75% methylcellulose, and incubation was continued at 37°C in a 5% CO2 incubator for 4 days. The expression of DEV virus and HA gene was detected using an indirect immunofluorescence assay (IFA).

Indirect immunofluorescence assay comprises the following steps. The cell culture medium was removed. The surface was washed slightly with PBS once, each well was added with 96% cold ethanol and fixed at room temperature for 10 minutes. The ethanol was discarded. The wells were dried in air. The wells were then added with appropriate dilutions of chicken anti-DEV serum and polyclonal rabbit antibody against H9N2 HA (Sino Biological Inc, Catalog# 11229-RP02), respectively, and incubated at 37 °C for 1 hour. Then the antibody was discarded. The wells were washed three times with PBS and added with appropriate amounts of anti-chicken IgG and anti-rabbit IgG (Alexa Fluor 594 goat anti-chicken IgG (H+L) and Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (Invitrogen)), and incubated at 37 °C for 1 hour. Then the antibody was discarded. The wells were washed three times with PBS and observed under a fluorescence-inverted microscope. All of the rDEVs containing the HA gene of subtype H9N2 of avian influenza virus showed specific fluorescence for DEV and H9HA. The results indicate the successful expression of HA protein from the recombinant viruses in CEF (see Figure 7).

Example 5: Safety of the recombinant DEV vectors in duck

5.1. Experimental design

To verify the safety of the rDEVs in duck, 80 1-day-old clean ducks were randomly divided into 8 groups, with 10 ducks per group. Groups 1 to 6 were the test groups, and inoculated with the corresponding material to be tested subcutaneously through the neck, respectively; Group 7 was the positive control group, and inoculated with DEV4 virus subcutaneously through the neck; Group 8 was the negative control group, and subcutaneously injected with the same volume of dilution through the neck. The specific experimental design and grouping are shown in Table 7.

Table 7. Experimental design and grouping

Group	Number of animals	Inoculation materials	Inoculation dose	Inoculation route
1	10	rDEV4 ΔUL23	0.5ml/duck,	Subcutaneously

2	10	rDEV4 ΔUS3	$10^{6.0}TCID_{50}$ /duck	through neck
3	10	rDEV4 ΔUL24		
4	10	rDEV4 ΔUL39		
5	10	rDEV4 ΔUL40		
6	10	rDEV4 H9HA ΔUL23		
7	10	DEV4		
8	10	MEM+5%FBS	0.5ml/duck	

On the day of beginning the test, as shown in Table 7, ducks in each test group were subcutaneously inoculated into the neck with 0.5 ml of the materials or dilutions to be tested. After inoculation, all the ducks including those in the negative control group, were observed once a day for 14 consecutive days. Abnormal symptoms, including but not limited to: mental depression, retracted heads and necks, shuffled feathers, drooping wings, numbness and weakness of both feet, tears, palpebral edemas, outflowing of nasal secretions, swelling of the heads and the necks to varying degrees, following a fluctuation feeling with touch and the like were recorded. On days 7 and 14 after inoculation, blood samples of all the animals were collected in pro-coagulation tubes. Serum was separated by centrifugation. DEV sero-conversion in the experimental ducks was detected with indirect immunofluorescence assay IFA.

5.2. Morbidity and mortality of the experimental ducks after inoculation

After inoculation of the experimental ducks, all the ducks were clinically observed once a day for 14 consecutive days. The experimental ducks in Groups 1-5, and 8 (negative control group) showed no abnormal clinical symptom or death during the whole experiment; one duck of Group 6 (rDEV4 H9HA ΔUL23) died on Day 6 after inoculation; the ducks of Group 7 (positive control group) showed clinical symptom on Day 3 after inoculation, and all the ducks died on Day 5 day after inoculation. Pathological changes of DEV infection, including typical symptoms such as annular hemorrhage at junctions of glandular stomachs and oesophaguses, and ring-like hemorrhage rings in intestines and the like were observed in all the dead experimental ducks from autopsy. The morbidity and mortality of the experimental ducks in each group are shown in Table 8.

Table 8. Morbidity and mortality of the experimental ducks in each group

Group	Number of animals	Inoculation materials	Morbidity	Mortality
1	10	rDEV4 ΔUL23	0%	0%
2	10	rDEV4 ΔUS3	0%	0%
3	10	rDEV4 ΔUL24	0%	0%
4	10	rDEV4 ΔUL39	0%	0%
5	10	rDEV4 ΔUL40	0%	0%
6	10	rDEV4 H9HA ΔUL23	10%	10%
7	10	DEV4	100%	100%
8	10	MEM+5%FBS	0%	0%

5.3. DEV sero-conversion in the experimental ducks after inoculation

The collected serum samples were tested by IFA to detect the DEV sero-conversion of the inoculated ducks. The results showed that the experimental ducks in Group 8 were negative for anti-DEV antibody during the whole experiment; the serum of all healthy living ducks in other groups became positive for anti-DEV antibody on Day 14 after inoculation. DEV sero-conversion of the experimental ducks in each group is shown in Table 9.

Table 9. DEV sero-conversion of the experimental ducks in each group

Group	Number of animals	Inoculation materials	Numbers of ducks positive for anti-DEV antibody / Total number of ducks in the group*
1	10	rDEV4 ΔUL23	10/10
2	10	rDEV4 ΔUS3	10/10
3	10	rDEV4 ΔUL24	10/10
4	10	rDEV4 ΔUL39	10/10
5	10	rDEV4 ΔUL40	10/10
6	10	rDEV4 H9HA ΔUL23	9/9
7	10	DEV4	Not applicable
8	10	MEM+5%FBS	0/10

Note: Not applicable means that all of the ducks dead.

5.4. Summary

We investigated the safety of the rDEVs in ducks. Three aspects were detected and analyzed, including morbidity, mortality and DEV sero-conversion in 1-day-old clean ducks. Inoculation into 1-day-old ducks with rDEVs including rDEV4 ΔUL23, rDEV4 ΔUS3, rDEV4 ΔUL24, rDEV4 ΔUL39 and rDEV4 ΔUL40 did not cause any morbidity and mortality, and resulted in the production of anti-DEV antibody. rDEV4 H9HA ΔUL23 also showed significantly attenuated virulence, resulting in 10% mortality after inoculation into 1-day-old ducks. The original DEV4 virus without genetic modification caused 100% mortality in the experimental ducks. In summary, genes UL23, US3, UL24, UL39, and UL40 are DEV virulence associated genes. These rDEVs are completely or partially attenuated as compared with the wild-type DEV strain by deleting different virulence-associated genes, offering the possibility of being used as safe live virus vectors for use in duck.

Example 6: Horizontal spreading ability of the recombinant DEVs among ducks

6.1. Experimental design

In order to verify the horizontal spreading ability of the recombinant DEVs in the ducks, 75 1-day-old clean ducks were randomly divided into 5 groups. On the day of the experiment (i.e., the hatching-out day of ducks), 50 1-day-old experimental ducks were randomly divided into 5 groups, with 10 ducks in each group. Groups 1-3 were inoculation groups, and corresponding materials to be tested were inoculated to the ducks subcutaneously through the neck. Group 4 was a positive control group, and a DEV4 virus was inoculated to the ducks subcutaneously through the neck. Group 5 was

a negative control group, and the same volume of MEM solution containing 5% FBS (a diluent of the material to be tested, hereinafter referred to as MEM + 5% FBS) was injected into the ducks subcutaneously through the neck. In addition, on the day of inoculation, 25 uninoculated 1-day-old experimental ducks of the same batch were placed in separate isolators, and randomly divided into 5 groups, with 5 ducks in each group, as contact ducks. After 24 h of inoculating the experimental ducks, the contact ducks in each group were transferred into the corresponding groups. Specific experimental design and grouping are shown in Table 10.

Table 10. Experimental design and grouping

Group	Number of animals	Inoculation material	Inoculation dose	Inoculation route
1	10+5*	rDEV4 ΔUL24	$10^{6.0}TCID_{50}$/duck	Subcutaneously through neck
2	10+5*	rDEV4 ΔUL40		
3	10+5*	rDEV4 H9HA ΔUL23		
4	10+5*	DEV4		
5	10+5*	MEM+5%FBS	0.5ml/duck	

Note: * 10 for immunized ducks, and 5 for contact ducks; and the contact ducks were fed in the same environment after 24 h of inoculation.

On the day of the experiment, as shown in Table 10, the experimental ducks in Groups 1-3 were subcutaneously inoculated with 0.5 ml of the corresponding recombinant DEVs into the necks at an inoculation dose of $10^{6.0}TCID_{50}$/duck. The ducks in Group 4 were subcutaneously inoculated with 0.5 ml of DEV4 through the neck at an inoculation dose of $10^{6.0}TCID_{50}$/duck. The ducks in Group 5 were subcutaneously injected with 0.5 ml of MEM + 5% FBS into the necks.

After inoculation, all the experimental ducks including those in the negative control group were observed once a day for 21 consecutive days; and abnormal symptoms of the experimental ducks were recorded, including: mental depression, retracted heads and necks, shuffled feathers, drooping wings, numbness and weakness of both feet, tears, palpebral edemas, outflowing of nasal secretions, swelling of the heads and the necks to varying degrees, following a fluctuation feeling with touch, etc. On Day 14 and Day 21 after inoculation, blood samples of all the experimental ducks were collected in pro-coagulation tubes. Serum was separated to detect DEV sero-conversion.

6.2. Morbidity and mortality of the experimental ducks after inoculation

After inoculation, all the experimental ducks were clinically observed once a day for 21 consecutive days. The experimental ducks in Group 5 (the negative control group) were in good health during the whole experiment, and the experiment was valid. The inoculated ducks and the contact ducks in Group 4 all died during the experiment. The experimental ducks in Groups 1-3 were in good health during the whole experiment, which was consistent with the clinical results of the

previous experiment (Example 5),. The contact ducks were in good health during the whole experiment after contact with the inoculated ducks.

6.3. DEV sero-conversion in the experimental ducks after inoculation

The experimental ducks in Group 5 (the negative control group) were negative for the antibody against DEV during the whole experiment; and on Day 14 after inoculating the experimental ducks in other groups, the serum of all the inoculated ducks turned positive for the antibody against DEV. On Day 13 and Day 20 after contact (i.e., on Day 14 and Day 21 after inoculation), the serum of all the contact ducks was negative for the antibody against DEV. The results of DEV sero-conversion in the experimental ducks are shown in Table 11.

Table 11. DEV sero-conversion of the experimental ducks in each group

Group	**Number of animals***	**Inoculation material**	**Number of days after inoculation**	
			14	**21**
1	10	rDEV4 ΔUL24	10/10	10/10
	5	Not applicable	0/5	0/5
2	10	rDEV4 ΔUL40	10/10	10/10
	5	Not applicable	0/5	0/5
3	10	rDEV4 H9HA ΔUL23	10/10	10/10
	5	Not applicable	0/5	0/5
4	10	DEV4	All dead	All dead
	5	Not applicable	All dead	All dead
5	10	MEM+5%FBS	0/10	0/10
	5	Not applicable	0/5	0/5

Note: * 10 for immunized ducks, and 5 for contact ducks; and the contact ducks were fed in the same environment after 24 h of inoculation.

6.4. Conclusion

Antibodies can be produced after a live DEV vector of rDEV4 ΔUL24, rDEV4 ΔUL40, or rDEV4 H9HA ΔUL23 is inoculated to the 1-day-old experimental ducks. Such vectors did not cause clinical abnormality and pathological change in tissues and organs of the inoculated ducks. There was no horizontal spreading. Therefore, such vectors can be used as safe vectors in the future.

Example 7: Safety of the recombinant DEVs in chicken

7.1. Introduction

In this example, the safety of the recombinant DEVs, including rDEV4 ΔUL23, rDEV4 ΔUS3, rDEV4 ΔUL24, rDEV4 ΔUL39, rDEV4 ΔUL40, rDEV4 H9HA ΔUL23, rDEV4 ΔUL40 H9HA ΔUL2, rDEV4 ΔUL24 H9HA ΔUL2, rDEV4 H9HA ΔUL39, rDEV4 H9HA ΔUL40, rDEV4 H9HA ΔUL24, rDEV4 H9HA

ΔUL23 ΔUL41, rDEV4 ΔUL24 UL26-H9HA-UL27 and rDEV4 ΔUL39 UL26-H9HA-UL27, in chicken was verified.

7.2. Experimental design

On the day of the experiment, 160 1-day-old SPF experimental chickens were randomly divided into 16 groups, with 10 chickens in each group. Groups 1-14 were inoculation groups, and corresponding materials to be tested were inoculated to the chickens subcutaneously through the neck. Group 15 was a positive control group, and a DEV4 virus was inoculated to the chickens in Group 15 subcutaneously through the neck. Group 16 was a negative control group, and the same volume of MEM + 5% FBS (a diluent of the material to be tested) was injected into the chickens subcutaneously through the neck. Specific experimental design and grouping are shown in Table 12.

Table 12. Experimental design and grouping

Group	Number of animals	Inoculation material	Inoculation dose	Inoculation route
1	10	rDEV4 ΔUL23	0.5ml, $10^{6.0}$ TCID50 / chicken	Subcutaneously through neck
2	10	rDEV4 ΔUS3		
3	10	rDEV4 ΔUL24		
4	10	rDEV4 ΔUL39		
5	10	rDEV4 ΔUL40		
6	10	rDEV4 H9HA ΔUL23		
7	10	rDEV4 ΔUL40 H9HA ΔUL2		
8	10	rDEV4 ΔUL24 H9HA ΔUL2		
9	10	rDEV4 H9HA ΔUL39		
10	10	rDEV4 H9HA ΔUL40		
11	10	rDEV4 H9HA ΔUL24		
12	10	rDEV4 H9HA ΔUL23 ΔUL41		
13	10	rDEV4 ΔUL24 UL26-H9HA-UL27		
14	10	rDEV4 ΔUL39 UL26-H9HA-UL27		
15	10	DEV4		
16	10	MEM+5%FBS	0.5ml/chicken	

On the day of the experiment, as shown in Table 12, the experimental chickens in each group were subcutaneously inoculated with 0.5 ml of the materials to be tested or their diluents into the necks. After inoculation, all the experimental chickens including those in the negative control group were observed once a day for 21 consecutive days; and abnormal symptoms of the experimental chickens were recorded, including: mental depression, retracted heads and necks, shuffled feathers, drooping wings, numbness and weakness of both feet, tears, palpebral edemas, outflowing of nasal secretions, swelling of the heads and the necks to varying degrees, following a fluctuation feeling with touch, etc.

7.3. Morbidity and mortality of the experimental chickens after inoculation

After inoculating the experimental chickens, all the experimental chickens in Group 15 (the positive control group) died. The experimental chickens in Group 16 (the negative control group) showed no abnormal clinical symptoms or death during the whole experiment. The experimental chickens in Groups 3, 4, and 5 (rDEV4 ΔUL24, rDEV4 ΔUL39, rDEV4 ΔUL40,) and Groups 7-14
5 expressing H9N2 HA showed no abnormal clinical symptom or death during the whole experiment, with both of the morbidity and the mortality of 0%. The inoculated chickens in other experimental groups (rDEV4 ΔUL23) showed some level of morbidity and mortality. However, both morbidity and mortality was significantly reduced when compared to the DEV4 challenge group. Results of the morbidity and mortality of the experimental chickens in each group are shown in Table 13.

10 **Table 13. Morbidity and mortality of the experimental chickens in each group**

Group	Number of animals	Inoculation material	Morbidity	Mortality
1	10	rDEV4 ΔUL23	40%	30%
2	10	rDEV4 ΔUS3	10%	10%
3	10	rDEV4 ΔUL24	0%	0%
4	10	rDEV4 ΔUL39	0%	0%
5	10	rDEV4 ΔUL40	0%	0%
6	10	rDEV4 H9HA ΔUL23	10%	10%
7	10	rDEV4 ΔUL40 H9HA ΔUL2	0%	0%
8	10	rDEV4 ΔUL24 H9HA ΔUL2	0%	0%
9	10	rDEV4 H9HA ΔUL39	0%	0%
10	10	rDEV4 H9HA ΔUL40	0%	0%
11	10	rDEV4 H9HA ΔUL24	0%	0%
12	10	rDEV4 H9HA ΔUL23 ΔUL41	0%	0%
13	10	rDEV4 ΔUL24 UL26-H9HA-UL27	0%	0%
14	10	rDEV4 ΔUL39 UL26-H9HA-UL27	0%	0%
15	10	DEV4	100%	100%
16	10	MEM+5%FBS	0%	0%

7.4. DEV sero-conversion in the experimental chickens after inoculation

The experimental chickens in Group 16 was negative for the DEV antibody during the whole experiment. On Day 21 after inoculation, the serum of the experimental chickens in other groups
15 became positive for the DEV antibody. It was verified that the chickens in all the experimental groups were successfully inoculated without missing. The DEV sero-conversion in the experimental chickens also proved that these recombinant viruses had a certain degree of replication *in vivo*.

7.5. Conclusion

The wild-type DEV4 has strong pathogenicity to the chickens, and can result in a morbidity of 100% in the 1-day-old SPF chickens. By deleting different genes, the strain can be attenuated to significant degrees, making it a safe live virus vector for use in chicken.

We also verified the effect of successive passages on the virulence of the recombinant viruses. 5 The results showed that successive passages would not significantly affect the virulence of the recombinant viruses.

Example 8: Horizontal spreading ability of the recombinant DEVs among chickens

8.1. Experimental design

This example aims to evaluate the horizontal spreading ability of the constructed recombinant 10 DEVs among the 1-day-old SPF chickens. 150 SPF chickens were randomly divided into 10 groups with 15 chickens in each group, and transferred to corresponding isolators for feeding after being hatched (1 day old). On the day of the experiment (i.e., the hatching-out day of chickens), 100 1-day-old chickens were randomly divided into 10 groups, with 10 chickens in each group. Groups 1-8 were test groups for live rDEV vectors, and corresponding materials to be tested were inoculated into the 15 chickens subcutaneously through the neck. Group 9 was a positive control group, and the DEV4 virus was inoculated into the chickens subcutaneously through the neck. Group 10 was a negative control group, and the same volume of MEM solution containing 5% FBS (a diluent of the material to be tested, hereinafter referred to as MEM + 5% FBS) was injected into the chickens subcutaneously through the neck. In addition, on the day of inoculation, 50 homologous 1-day-old SPF chickens of 20 the same batch were selected and placed in separate isolators, and randomly divided into 10 groups, with 5 chickens in each group, as contact chickens. After 24 h of inoculating immunized chickens, the contact chickens in each group were transferred into the corresponding groups. Specific experimental design and grouping are shown in Table 14.

Table 14. Experimental design and grouping

Group	Number of animals	Inoculation material	Inoculation dose	Inoculation route
1	10+5*	rDEV4 ΔUL24	$10^{6.0}$TCID50/ chicken	Subcutaneously through neck
2	10+5*	rDEV4 ΔUL39		
3	10+5*	rDEV4 ΔUL40		
4	10+5*	rDEV4 H9HA ΔUL23		
5	10+5*	rDEV4 ΔUL40 H9HA ΔUL2		
6	10+5*	rDEV4 ΔUL24 H9HA ΔUL2		
7	10+5*	rDEV4 H9HA ΔUL39		
8	10+5*	rDEV4 H9HA ΔUL40		
9	10+5*	DEV4		
10	10+5*	MEM+5%FBS	0.5ml/chicken	

Note: * 10 for immunized chickens, and 5 for contact chickens; and the contact chickens were fed in the corresponding groups after 24 h of inoculating the immunized chickens.

On the day of the experiment, as shown in Table 14, the experimental chickens in Groups 1-8 were subcutaneously inoculated with 0.5 ml of the corresponding recombinant DEV into the necks at 5 an inoculation dose of $10^{6.0}TCID_{50}$/chicken. The chickens in Group 9, as a positive control group, were subcutaneously inoculated with 0.5 ml of DEV4 into the necks at an inoculation dose of $10^{6.0}TCID_{50}$/chicken. The chickens in Group 10, as a negative control group, were subcutaneously injected with 0.5 ml of MEM + 5% FBS into the necks.

After inoculation, all the experimental chickens (the immunized chickens and the contact 10 chickens) including those in the negative control group were observed once a day for 21 consecutive days; and abnormal symptoms of the experimental chickens were recorded, including: mental depression, retracted heads and necks, shuffled feathers, drooping wings, numbness and weakness of both feet, tears, palpebral edemas, outflowing of nasal secretions, swelling of the heads and the necks to varying degrees, following a fluctuation feeling with touch, etc.

15 On Day 14 after inoculation, blood samples of all the immunized chickens were collected. Serum was separated to detect DEV sero-conversion. On Day 14 and Day 21 after inoculation, blood samples of the contact chickens were collected. Serum was separated to detect DEV sero-conversion.

8.2. DEV sero-conversion in the experimental chickens after inoculation

The experimental chickens in Group 10 (the negative control) were negative for the antibody 20 against DEV during the whole experiment; and on Day 14 after inoculating the serum of the immunized chickens in other groups became positive for the antibody against DEV. On Day 13 and Day 20 after contact (i.e., on Day 14 and Day 21 after inoculating the immunized chickens), the serum of all the contact chickens in all the groups was negative for the antibody against DEV. The results of DEV sero-conversion in the experimental chickens in each group are specifically shown in Table 15.

25 **Table 15. DEV sero-conversion of experimental chickens in each group**

Group	Number of animals*	Inoculation material	Number of days after inoculation (Chickens positive for DEV antibody/experimental chickens of each group)	
			14	21
1	10	rDEV4 ΔUL24	10/10	Not applicable
	5	Not applicable	0/5	0/5
2	10	rDEV4 ΔUL39	9/9	Not applicable
	5	Not applicable	0/5	0/5
3	10	rDEV4 ΔUL40	10/10	Not applicable
	5	Not applicable	0/5	0/5
4	10	rDEV4 H9HA ΔUL23	9/9	Not applicable

	5	Not applicable	0/5	0/5
5	10	rDEV4 ΔUL40 H9HA ΔUL2	10/10	Not applicable
	5	Not applicable	0/5	0/5
6	10	rDEV4 ΔUL24 H9HA ΔUL2	10/10	Not applicable
	5	Not applicable	0/5	0/5
7	10	rDEV4 H9HA ΔUL39	10/10	Not applicable
	5	Not applicable	0/5	0/5
8	10	rDEV4 H9HA ΔUL40	10/10	Not applicable
	5	Not applicable	0/5	0/5
9	10	DEV4	Not applicable	Not applicable
	5	Not applicable	0/5	0/5
10	10	MEM+5%FBS	0/10	Not applicable
	5	Not applicable	0/5	0/5

Note: * 10 for immunized chickens, and 5 for contact chickens; and the contact chickens were fed in the corresponding groups after 24 h of inoculating the immunized chickens.

8.3. Conclusion

According to laboratory detection, no sero-conversion was detected in all the contact chickens on Day 13 and Day 20 after contact with the inoculated chickens, which proved that the recombinant DEVs with deletion of these genes or insertion of the H9N2 HA gene involved in this example did not horizontally spread among the chickens. In particular, these recombinant viruses, which neither cause diseases and death in the chickens nor cause horizontal spreading among chicken flocks, can be used as safe vectors in the future.

Example 9: Efficacy of vaccine candidate strains containing live rDEV vectors expressing H9N2-HA

9.1. Experimental design

In this example, 8 vaccine candidate strains containing live rDEV vectors expressing the HA gene of avian influenza virus (subtype H9) were inoculated subcutaneously to 1-day-old SPF chickens through the neck. On Day 28 after inoculation, the SPF chickens were challenged with an avian influenza virus (subtype H9) challenge strain (A/chicken/Jiangsu/TX10/2010 strain). It aims to evaluate the immunogenicity of vaccine candidate strains upon the challenge.

The recombinant viruses tested in this example included rDEV4ΔUL40 H9HAΔUL2, rDEV4ΔUL24 H9HAΔUL2, rDEV4 H9HAΔUL39, rDEV4 H9HAΔUL40, rDEV4 H9HA ΔUL24, rDEV4 H9HA ΔUL23 ΔUL41, rDEV4 ΔUL24 UL26-H9HA-UL27, and rDEV4 ΔUL39 UL26-H9HA-UL27.

On the day of the experiment (i.e., the hatching-out day of the SPF chickens), 90 1-day-old SPF chickens were randomly divided into 9 groups, with 10 chickens in each group. Groups 1-8 were test groups for the vaccine candidate strains containing the live rDEV vectors, and Group 9 was a control group. As shown in Table 16, all the experimental chickens were subcutaneously inoculated with

corresponding materials to be tested through the neck. After inoculation, all the experimental chickens were clinically observed for 28 consecutive days. On Day 28, all experimental chickens were inoculated with 0.2 ml of H9 subtype avian influenza virus by nasal drip, with a challenge dose of $10^{6.0}EID_{50}$/chicken. Experimental design and grouping are shown in Table 16.

5 **Table 16. Experimental design and grouping**

Group	Number of animals	Inoculation material	Immunizion dose ($TCID_{50}$)	Challenge information
1	10	rDEV4ΔUL40 H9HAΔUL2	$1.58\times10^{6.0}$	H9 subtype avian influenza virus $10^{6.0}EID_{50}$/0.2ml/chicken, nasal drip
2	10	rDEV4ΔUL24 H9HAΔUL2	$1.01\times10^{6.0}$	
3	10	rDEV4 H9HAΔUL39	$1.58\times10^{6.0}$	
4	10	rDEV4 H9HAΔUL40	$0.89\times10^{6.0}$	
5	10	rDEV4 ΔUL24 H9HA	$0.36\times10^{5.0}$	
6	10	rDEV4 H9HA ΔUL23 ΔUL41	$0.75\times10^{5.0}$	
7	10	rDEV4 ΔUL24 UL26-H9HA-UL27	$0.89\times10^{5.0}$	
8	10	rDEV4 ΔUL39 UL26-H9HA-UL27	$1.58\times10^{5.0}$	
9	10	MEM+5%FBS	Not applicable	

After inoculation, all the experimental chickens were observed once a day for 28 consecutive days. Abnormal symptoms of the experimental chickens were observed, including, but not limited to: mental depression, retracted heads and necks, shuffled feathers, drooping wings, numbness and weakness of both feet, tears, palpebral edemas, outflowing of nasal secretions, swelling of the heads
10 and the necks to varying degrees, following a fluctuation feeling with touch, etc.

On Day 28 after inoculation (before challenge), blood samples of all the chickens were collected; and serum was collected by centrifugation for detection with an HI antibody of the H9 subtype avian influenza.

On Day 5 after the challenge, cotton throat and cloacal swabs of all the experimental chickens
15 were collected for H9 virus isolation. If it was negative for the virus isolation, it was determined that the vaccine candidate strains provided protection to the experimental chickens. Specific operation steps were as follows: on Day 5 after the challenge, the cotton throat and cloacal swabs of all the experimental chickens were collected. The collected cotton swabs were placed in a centrifuge tube containing 1.6 ml of a six-antibiotics swab buffer. All cotton swab samples were inoculated into the
20 chicken embryos for virus isolation. The operation steps were as follows: each cotton swab sample was inoculated to five 9-11-day-old SPF chicken embryos through allantoic cavities with 0.2 ml per embryo, then the embryos were incubated for 96 h. Then the HA titers of all allantoic fluids were determined. As long as the HA titer of allantoic fluid of one chicken embryo among the chicken embryos inoculated with each cotton swab sample was no less than 1:16, it could be determined as
25 positive for H9 virus isolation. The samples that were negative for virus isolation were determined again after blind passage for 1 generation. If it was still negative for virus isolation after the blind

passage, then it was determined as negative for H9 virus isolation; if it was positive for virus isolation after the blind passage, then it was determined as positive for H9 virus isolation.

9.2. Level of anti-H9 antibody in the experimental chickens

On Day 28 after inoculation, blood of the experimental chickens in each group was collected;
5 and serum was separated to detect the level of anti-H9 antibody. Results showed that the serums of all the experimental chickens in Group 9 (the control group) were negative for the haemagglutination inhibition (HI) antibody of H9; the average HI antibody titer of the experimental chickens in Group 3 was lower than 4 log2; and the average HI antibody titers of the experimental chickens in other groups were higher than or equal to 4 log2. Average HI antibody titers in the serum of the
10 experimental chickens in each group are shown in Table 17 and Figure 8.

Table 17. Average HI antibody titers in the serum of the experimental chickens in each group

Group	Inoculation material	Average HI antibody titer (log2)
1	rDEV4ΔUL40 H9HAΔUL2	5.8
2	rDEV4ΔUL24 H9HAΔUL2	4.7
3	rDEV4 H9HAΔUL39	3.5
4	rDEV4 H9HAΔUL40	6.1
5	rDEV4 H9HA ΔUL24	4
6	rDEV4 H9HA ΔUL23 ΔUL41	6
7	rDEV4 ΔUL24 UL26-H9HA-UL27	4.2
8	rDEV4 ΔUL39 UL26-H9HA-UL27	5.8
9	MEM+5%FBS	2

9.3. Protection rate of the recombinant viruses to H9N2

On Day 5 after the challenge, cotton throat and cloacal swabs of all the experimental chickens
15 were collected for H9N2 virus isolation. Results showed that 10 experimental chickens in Group 9 (a challenge control group) were positive for virus isolation, that is, the positive rate of virus isolation from the experimental chickens in the challenge control group was 100 %; and the challenge control was valid. The results of H9N2 virus isolation from the cotton swab samples and the protection rates for H9N2 in each group are shown in Table 18.

20 **Table 18. H9N2 virus isolation and protection rates for H9N2**

Group	Inoculation material	Positive rate of virus isolation	Protection rate
1	rDEV4ΔUL40 H9HAΔUL2	50%(5/10)	50%
2	rDEV4ΔUL24 H9HAΔUL2	70%(7/10)	30%
3	rDEV4 H9HAΔUL39	50%(5/10)	50%
4	rDEV4 H9HAΔUL40	60%(6/10)	40%

5	rDEV4 H9HAΔ UL24	50%(5/10)	50%
6	rDEV4 H9HA ΔUL23Δ UL41	30%(3/10)	70%
7	rDEV4 Δ UL24 UL26-H9HA-UL27	40%(4/10)	60%
8	rDEV4 Δ UL39 UL26-H9HA-UL27	10%(1/10)	90%
9	MEM+5%FBS	100%(10/10)	0%

9.4. Conclusion

The vaccine candidate strains containing live rDEV vectors expressing the HA gene of avian influenza virus (subtype H9) can provide protection to H9N2 to varying degrees up to 90%, proving
5 that these different insertion sites are effective.

What we claim is:

1. An attenuated Duck Enteritis Virus (DEV), wherein one or more genes of the DEV genome selected from the group consisting of US3, UL24, UL40, UL39, and UL23 is inactivated.

2. An attenuated Duck Enteritis Virus (DEV), comprising an inactivated gene selected from any one of i)-v), i) US3 gene; ii) UL24 gene; iii) UL40 gene; iv) UL39 gene; and v) UL23 gene, in combination with the inactivation of one or more further non-essential genes of DEV.

3. The attenuated DEV of claim 2, wherein the one or more further non-essential gene of DEV is different from the first inactivated gene and is selected from i) UL2 gene, ii) UL41 gene; iii) US3 gene; iv) UL24 gene; v) UL40 gene; vi) UL39 gene; or vii) UL23 gene.

4. An attenuated Duck Enteritis Virus (DEV), comprising inactivated gene(s) selected from any one of i)-viii),

i) US3 gene;

ii) UL24 gene;

iii) UL40 gene;

iv) UL39 gene;

v) UL23 gene;

vi) UL24 gene and UL2 gene;

vii) UL40 gene and UL2 gene; or

viii) UL23 gene and UL41 gene.

5. The attenuated DEV of any one of the preceding claims, wherein the inactivation of the selected genes causes the attenuation of DEV.

6. The attenuated DEV of any one of the preceding claims, wherein the gene is inactivated by mutation, interruption, replacement or deletion of a portion of or the whole sequence of the gene.

7. The attenuated DEV of any one of the preceding claims, wherein at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the sequence of the gene is replaced or deleted.

8. The attenuated DEV of any one of the preceding claims, wherein the attenuated DEV further comprises a heterologous polynucleotide coding for a heterologous antigen.

9. The attenuated DEV of claim 8, where the heterologous antigen is an antigen of a poultry pathogen.

10. The attenuated DEV of claim 8 or 9, wherein the heterologous polynucleotide is inserted into a non-essential gene of the attenuated DEV.

11. The attenuated DEV of any one of claims 8-10, wherein the heterologous polynucleotide is expressed after the attenuated DEV has been transfected into a suitable host cell.

5 12. The attenuated DEV of any of the preceding claims for use as vector vaccine in poultry.

13. The attenuated DEV of any of the proceeding claims for use as vector vaccine in duck.

14. The attenuated DEV of any of the proceeding claims for use as vector vaccine in chicken.

15. A composition, comprising the attenuated DEV of any one of claims 1-11.

16. The composition of claim 15, wherein the composition is a vaccine.

10 17. A method of vaccinating a poultry by inducing a protective immune response in a poultry against a pathogen, comprising at least one administration of the composition of claim 15 or 16, or the vector vaccines of any one of claims 12-14.

18. The composition of claim 15 or 16, or the vector vaccine of any one of claims 12-14, for the use in a method for inducing a protective immune response in poultry against a poultry pathogen, wherein
15 such method comprises or consists of one or more administration of the composition of claim 15 or 16, or the vector vaccine of any one of claims 12-14 to poultry.

19. The method of claim 17 or the use of claim 18, wherein the poultry is a duck or a chicken.

20. The method of claim 17 or 19, or the use of claim 18 or 19, wherein the poultry is 1 day-old, 2 day-old, 3 day-old, 4 day-old, 5 day-old, 6 day-old, or 7 day-old at the day of vaccination.

20 21. The method of any one of claims 17, 19 and 20, or the use of any one of claims 18-20, wherein the administration is by oro-nasal, eye drop, spray, drinking water, in ovo, intramuscular, subcutaneous, intradermal, or transdermal.

22. A host cell, expressing the attenuated DEV of any one of claims 1-11.

23. A host cell expressing the attenuated DEV and the heterologous polynucleotide of any one of
25 claims 8-11.

24. The host cell of claim 22 or 23, wherein the host cell is a CEF cell, EB66 cell or DEF cell.

Figure 1

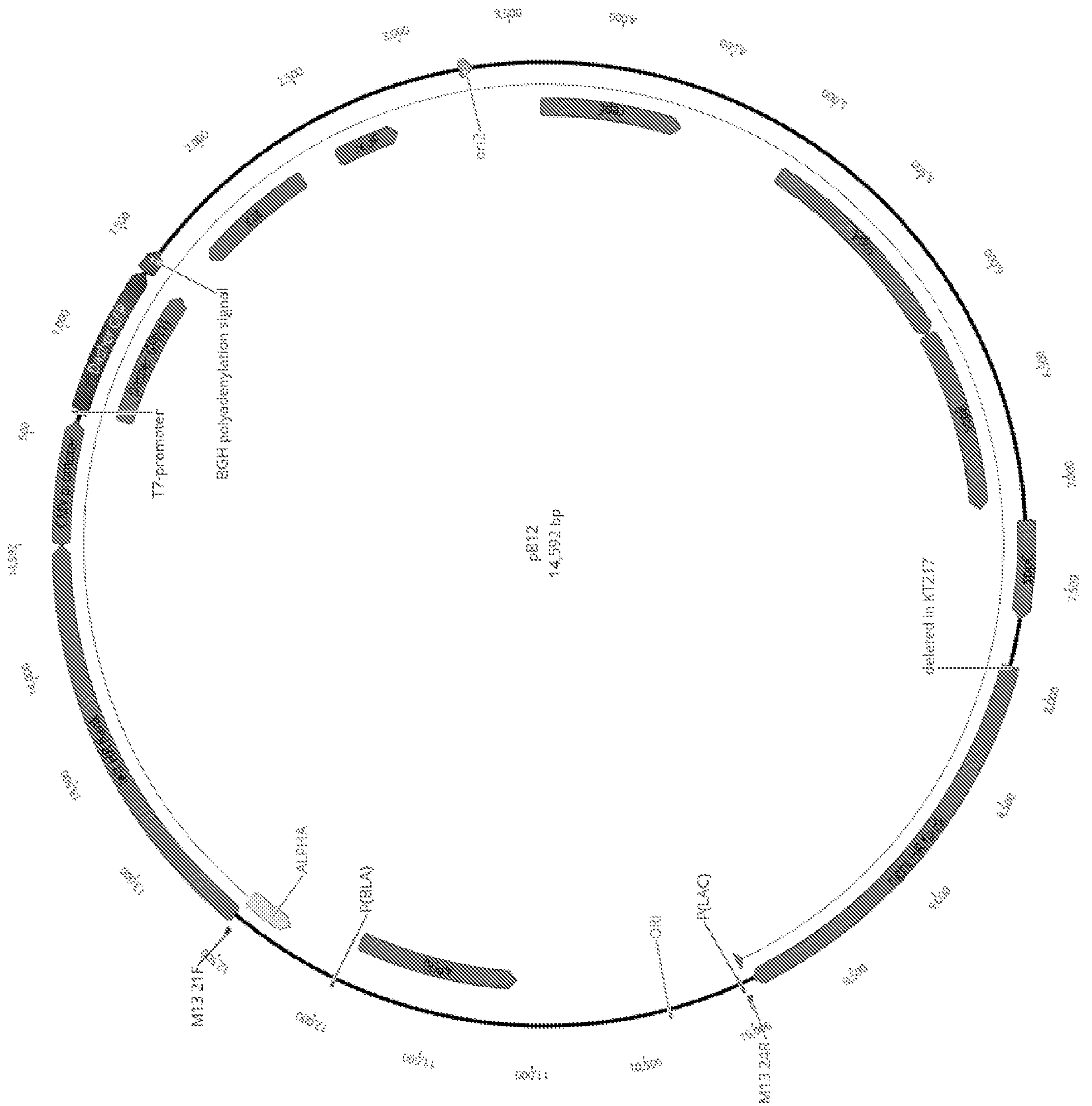

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Figure 2

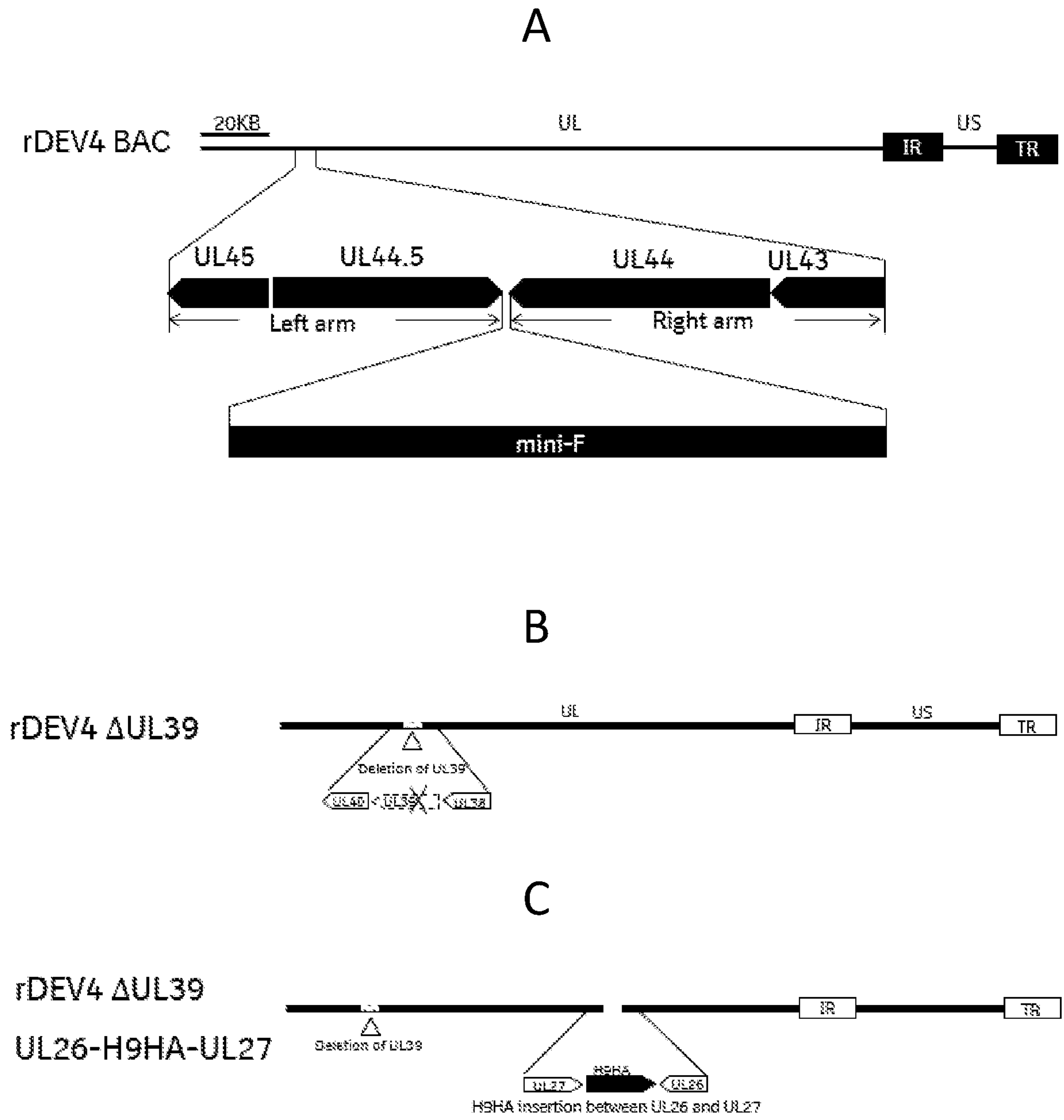


Figure 3

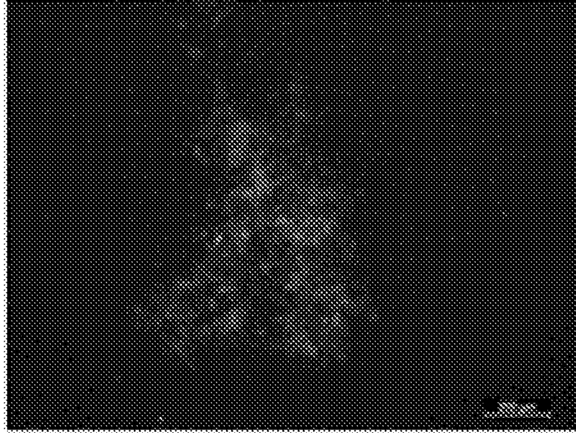


Figure 4

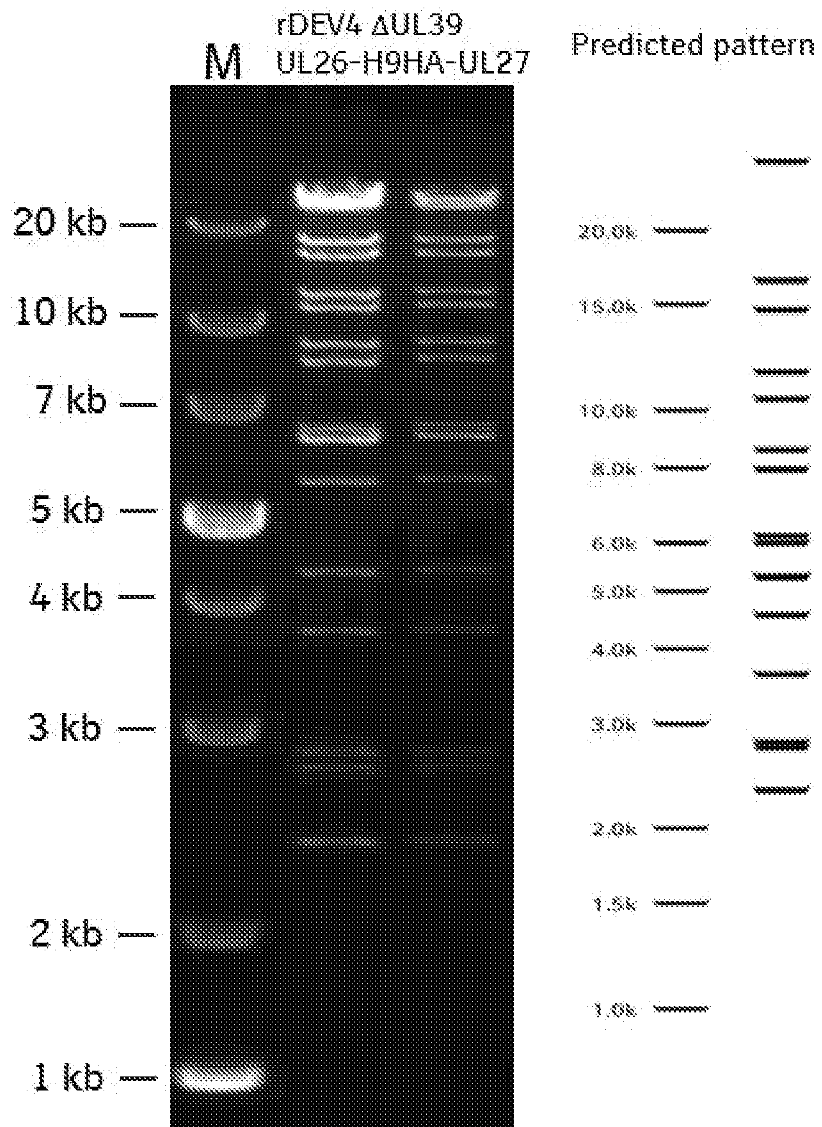
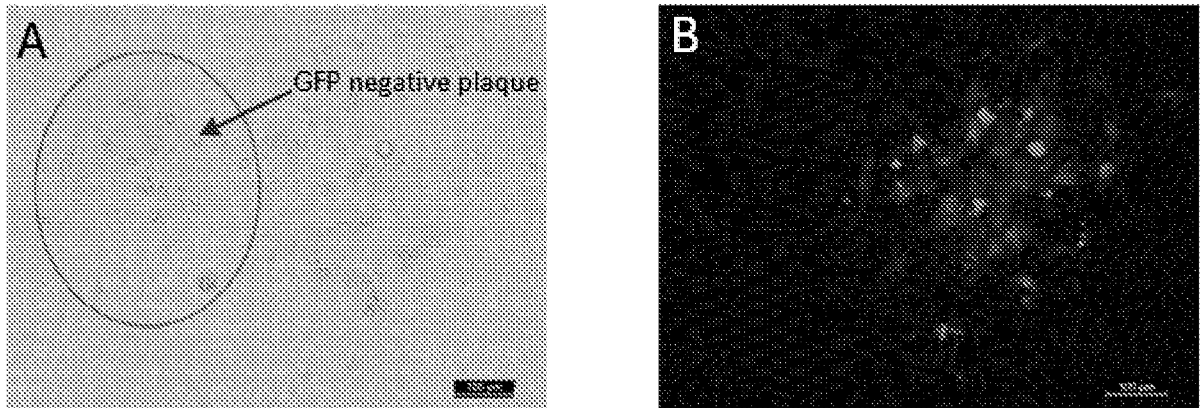


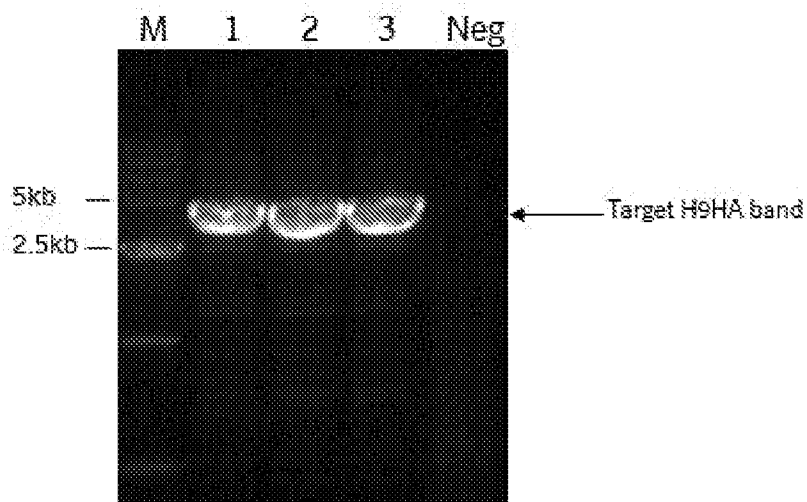
Figure 5



A: observation under common microscope

B: observation under fluorescence microscope for the same microscopic field of A

Figure 6



M: GeneRuler 1kb Plus DNA Ladder (Thermo Scientific, Catalog# SM1333)

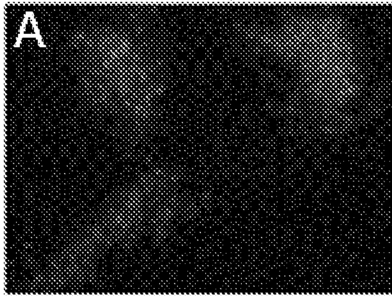
1: rDEV4 ΔUL39 UL26-H9HA-UL27 of passage 5th

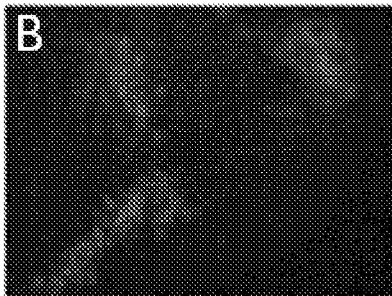
2: rDEV4 ΔUL39 UL26-H9HA-UL27 of passage 10th

3: rDEV4 ΔUL39 UL26-H9HA-UL27 of passage 10th

Neg: PCR negative control

Figure 7





A: IFA test by DEV chicken serum

B: IFA test by commercial H9N2 HA ployclonal antibody (Sino Biological Inc, Catalog# 11229-RP02)

Figure 8