AVIAN POLYNUCLEOTIDE VACCINE FORMULA

The present invention relates to a vaccine formula allowing the vaccination of avian species, in particular chickens. It also relates to a corresponding method of vaccination.

Associations of vaccines against a number of viruses responsible for pathologies in chicken have already been proposed in the past.

The associations developed so far were prepared from inactivated vaccines or live vaccines. Their use poses problems of compatibility between valencies and of stability. It is indeed necessary to ensure both the compatibility between the different vaccine valencies, whether from the point of view of the different antigens used from the point of view of the formulations themselves. The problem of the conservation of such combined vaccines and also of their safety especially in the presence of an adjuvant also exists. These vaccines are in general quite expensive.

Patent applications WO-A-90 11092, WO-A-92 19183, WO-A-94 21797 and WO-A-95 20660 have made use of the recently developed technique of polynucleotide vaccines. It is known that these vaccines use a plasmid capable of expressing, in the host cells, the antigen inserted into the plasmid. All the routes of administration have been proposed (intraperitoneal, intravenous, intramuscular, transcutaneous, intradermal, mucosal and the like). Various vaccination means can also be used, such as DNA deposited at the surface of gold particles and projected so as to penetrate into the animal's skin (Tang et al., Nature, 356, 152-154, 1992) and liquid jet injectors which make it possible to transfect at the same time the skin, the muscle, the fatty tissues and the mammary tissues (Furth et al., Analytical Biochemistry, 205, 365-368, 1992).

The polynucleotide vaccines may also use both naked DNAs and DNAs formulated, for example, inside lipids or cationic liposomes.

The invention therefore proposes to provide a multivalent vaccine formula which makes it possible to ensure vaccination against a number of pathogenic avian viruses.

Another objective of the invention is to provide such a vaccine formula combining different valencies while exhibiting all the criteria required for mutual compatibility and stability of the valencies.

Another objective of the invention is to provide such a vaccine formula which makes it possible to combine different valencies in the same vehicle.

Another objective of the invention is to provide such a vaccine which is easy and inexpensive to use.

Yet another objective of the invention is to provide a method for vaccinating Gallinaceans which makes it possible to obtain protection, including multivalent protection, with a high level of efficiency and of long duration, as well as good safety and an absence of residues.

The subject of the present invention is therefore an avian vaccine formula comprising at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one avian pathogen valency, these valencies being selected from the group consisting of Marek's disease virus (MDV), Newcastle's disease virus (NDV), infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), infectious anaemia virus (CAV), infectious laryngotracheitis virus (ILTV), encephalomyelitis virus (AEV or avian leukosis virus ALV), pneumovirosis virus, and avian plague virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the Marek's disease virus, HN and F

for the Newcastle disease virus, VP2 for the infectious bursal disease virus, S, M and N for the infectious bronchitis virus, C + NS1 for the infectious anaemia virus, gB and gD for the infectious laryngotracheitis virus, env and gag/pro for the encephalomyelitis virus, F and G for the pneumovirosis virus and HA, N and NP for the avian plague virus.

Valency in the present invention is understood to mean at least one antigen providing protection against the virus for the pathogen considered, it being possible for the valency to contain, as subvalency, one or more natural or modified genes from one or more strains of the pathogen considered.

Pathogenic agent gene is understood to mean not only the complete gene but also the various nucleotide sequences, including fragments which retain the capacity to induce a protective response. The notion of a gene covers the nucleotide sequences equivalent to those described precisely in the examples, that is to say the sequences which are different but which encode the same protein. It also covers the nucleotide sequences of other strains of the pathogen considered, which provide cross-protection or a protection specific for a strain or for a strain group. It also covers the nucleotide sequences which have been modified in order to facilitate the in vivo expression by the host animal but encoding the same protein.

Preferably, the vaccine formula according to the invention comprises three valencies chosen from Marek, infectious bursal, infectious anaemia and Newcastle. The infectious bronchitis valency can also preferably be added thereto.

On this basis of 3, 4 or 5 valencies, it will be possible to add one or more of the avian plague, laryngotracheitis, pneumovirosis and encephalomyelitis valencies.

As regards the Marek valency, two genes may be used encoding gB and gD, in different plasmids or in

one and the same plasmid. The use of the gB gene alone is however preferred.

For the Newcastle valency, the two HN and F chains, integrated into two different plasmids or into one and the same plasmid, are preferably used.

For the infectious bronchitis valency, the use of the S gene is preferred. Optionally, but less preferably, S and M can be associated in a single plasmid or in different plasmids.

For the infectious anaemia valency, the two C and NS1 genes are preferably associated in the same plasmid.

For the infectious laryngotracheitis valency, the use of the gB gene alone is preferred. Optionally, but less preferably, the two gB and gD genes can be associated in different plasmids or in one and the same plasmid.

For the pneumovirosis valency, the use of the two F and G genes, in a single plasmid or in different plasmids, is preferred

For the avian plague valency, the use of the HA gene is preferred. Optionally, but less preferably, it is possible to use the associations HA and NP or HA and N in different plasmids or in one and the same plasmid. Preferably, the HA sequences from more than one influenza virus strain, in particular from the different strains found in the field, are preferably associated in the same vaccine. On the other hand, NP provides cross-protection and the sequence from a single virus strain will therefore be satisfactory.

For the encephalomyelitis valency, the use of env is preferred.

The vaccine formula according to the invention can be presented in a dose volume of between 0.1 and 1 ml and in particular between 0.3 and 0.5 ml.

The dose will be generally between 10 ng and 1 mg, preferably between 100 ng and 500 μg and preferably between 0.1 μg and 50 μg per plasmid type.

Use will be preferably made of naked plasmids, simply placed in the vaccination vehicle which will be in general physiological saline and the like. It is of course possible to use all the polynucleotide vaccine forms described in the prior art and in particular formulated in liposomes.

Each plasmid comprises a promoter capable of ensuring the expression of the gene inserted, under its control, into the host cells. This will be in general a strong eukaryotic promoter and in particular a cytomegalovirus early CMV-IE promoter of human or murine origin, or optionally of another origin such as rats, pigs and guinea pigs.

More generally, the promoter may be either of viral origin or of cellular origin. As viral promoter other than CMV-IE, there may be mentioned the SV40 virus early or late promoter or the Rous sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the gene's own promoter.

As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as, for example, the desmin promoter (Bolmont et al., Journal of Submicroscopic Cytology and Pathology, 1990, 22, 117-122; and Zhenlin et al., Gene, 1989, 78, 243-254), or alternatively the actin promoter.

When several genes are present in the same plasmid, these may be presented in the same transcription unit or in two different units.

The combination of the different vaccine valencies according to the invention may be preferably achieved by mixing the polynucleotide plasmids expressing the antigen(s) of each valency, but it is also possible to envisage causing antigens of several valencies to be expressed by the same plasmid.

The subject of the invention is also monovalent vaccine formulae comprising one or more plasmids encoding one or more genes from one of the viruses above, the genes being those described above. Besides

their monovalent character, these formulae may possess the characteristics stated above as regards the choice of the genes, their combinations, the composition of the plasmids, the dose volumes, the doses and the like.

The monovalent vaccine formulae may also be used (i) for the preparation of a polyvalent vaccine formula as described above, (ii) individually against the actual pathology, (iii) associated with a vaccine of another type (live or inactivated whole, recombinant, subunit) against another pathology, or (iv) as booster for a vaccine as described below.

The subject of the present invention is in fact also the use of one or more plasmids according to the invention for the manufacture of an avian vaccine intended to vaccinate animals first vaccinated by means of a first conventional vaccine (monovalent or multivalent) of the type in the prior art, in particular selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having (that is to say containing or capable of expressing) the antigen(s) encoded by the plasmids or antigen(s) providing cross-protection.

Remarkably, the polynucleotide vaccine has a potent booster effect which results in an amplification of the immune response and the acquisition of a long-lasting immunity.

In general, the first-vaccination vaccines can be selected from commercial vaccines available from various veterinary vaccine producers.

The subject of the invention is also a vaccination kit grouping together a vaccine formula according to the invention and a first-vaccination vaccine as described above. It also relates to a vaccine formula according to the invention accompanied by a leaflet indicating the use of this formula as a booster for a first vaccination as described above.

The subject of the present invention is also a method of avian vaccination, comprising the

administration of an effective vaccine formula as described above. This vaccination method comprises the administration of one or more doses of the vaccine formula, it being possible for these doses to be administered in succession over a short period of time and/or in succession at widely spaced intervals.

The vaccine formulae according to the invention can be administered in the context of this method of vaccination, by the different routes of administration proposed in the prior art for polynucleotide vaccination and by means of known techniques of administration.

The intramuscular route, the in ovo route, the intraocular route, nebulization and drinking water will be targeted in particular.

The efficiency of presentation of the antigens to the immune system varies according to the tissues. In particular, the mucous membranes of the respiratory tree serve as barrier to the entry of pathogens and are associated with lymphoid tissues which support local immunity. In addition, the administration of a vaccine by contact with the mucous membranes, in particular the buccal mucous membrane, the pharyngeal mucous membrane and the mucous membrane of the bronchial region, is certainly of interest for mass vaccination.

Consequently, the mucosal routes of administration form part of a preferred mode of administration for the invention, using in particular neubilization or spray or drinking water. It will be possible to apply the vaccine formulae and the vaccination methods according to the invention in this context.

The subject of the invention is also the method of vaccination consisting in making a first vaccination as described above and a booster with a vaccine formula according to the invention.

In a preferred embodiment of the process according to the invention, there is administered in a first instance, to the animal, an effective dose of the vaccine of the conventional, especially inactivated,

live, attenuated or recombinant, type, or alternatively a subunit vaccine so as to provide a first vaccination, and, after a period preferably of 2 to 6 weeks, the polyvalent or monovalent vaccine according to the invention is administered.

The invention also relates to the method of preparing the vaccine formulae, namely the preparation of the valencies and mixtures thereof, as evident from this description.

The invention will now be described in greater detail with the aid of the embodiments of the invention taken with reference to the accompanying drawings.

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Sequence listing SEQ ID No.

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SEQ ID No. 27: Oligonucleotide AB140
SEQ ID No. 28: Oligonucleotide AB141
SEQ ID No. 29: Oligonucleotide AB164
SEQ ID No. 30: Oligonucleotide AB165
SEQ ID No. 31: Oligonucleotide AB160
SEQ ID No. 32: Oligonucleotide AB161
SEQ ID No. 33: Oligonucleotide AB150
SEQ ID No. 34: Oligonucleotide AB151
SEQ ID No. 35: Oligonucleotide AB152
SEQ ID No. 36: Oligonucleotide AB153
SEQ ID No. 37: Oligonucleotide AB142
SEQ ID No. 38: Oligonucleotide AB143
SEQ ID No. 39: Oligonucleotide AB144
SEQ ID No. 40: Oligonucleotide AB145
SEQ ID No. 41: Oligonucleotide AB156
SEQ ID No. 42: Oligonucleotide AB158
SEQ ID No. 43: Oligonucleotide AB146
SEQ ID No. 44: Oligonucleotide AB147

EXAMPLES

Example 1: Culture of the viruses

The viruses are cultured on the appropriate cellular system until a cytopathic effect is obtained. The cellular systems to be used for each virus are well known to persons skilled in the art. Briefly, the cells sensitive to the virus used, which are cultured in Eagle's minimum essential medium (MEM medium) or another appropriate medium, are inoculated with the viral strain studied using a multiplicity of infection of 1. The infected cells are then incubated at 37°C for the time necessary for the appearance of a complete cytopathic effect (on average 36 hours).

Example 2: Extraction of the viral genomic DNAs

After culturing, the supernatant and the lysed cells are harvested and the entire viral suspension is centrifuged at 1000 g for 10 minutes at +4°C so as to remove the cellular debris. The viral particles are then harvested by ultracentrifugation at 400,000 g for 1 hour at +4°C. The pellet is taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA). This concentrated viral suspension is treated with proteinase K (100 µg/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA is then extracted with a phenol/chloroform mixture and then precipitated with 2 volumes of absolute ethanol. After leaving overnight at -20°C, the DNA is centrifuged at 10,000 g for 15 minutes at +4°C. The DNA pellet is dried and then taken up in a minimum volume of sterile ultrapure water. It can then be digested with restriction enzymes.

Example 3: Isolation of the viral genomic RNAs

The RNA viruses were purified according to techniques well known to persons skilled in the art. The genomic viral RNA of each virus was then isolated using the "guanidium thiocyanate/phenol-chloroform" extraction technique described by P. Chromczynski and N. Sacchi (Anal. Biochem., 1987. **162**, 156-159).

Example 4: Molecular biology techniques

All the constructions of plasmids were carried out using the standard molecular biology techniques described by J. Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). All the restriction fragments used for the present invention were isolated using the "Geneclean" kit (BIO 101 Inc. La Jolla, CA).

Example 5: RT-PCR technique

Specific oligonucleotides (comprising restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized such that they completely cover the coding regions of the genes which are to be amplified (see specific examples). The reverse transcription (RT) reaction and the polymerase chain reaction (PCR) were carried out according to standard techniques (Sambrook J. *et al.*, 1989). Each RT-PCR reaction was performed with a pair of specific amplimers and taking, as template, the viral genomic RNA extracted. The complementary DNA amplified was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before being digested with restriction enzymes.

Example 6: Plasmid pVR1012

The plasmid pVR1012 (Figure No. 1) was obtained from Vical Inc., San Diego, CA, USA. Its construction has been described in J. Hartikka *et al.* (Human Gene Therapy, 1996, **7**, 1205-1217).

Example 7: Construction of the plasmid pAB045 (MDV gB gene)

A PCR reaction was carried out with the Marek's disease virus (MDV) (RB1B strain) (L. Ross *et al.*, J. Gen. Virol., 1989, **70**, 1789-1804) genomic DNA, prepared according to the technique in Example 2, and with the following oligonucleotides:

AB062 (37 mer) (SEQ ID No. 1)

5' AAAACTGCAGACTATGCACTATTTTAGGCGGAATTGC 3'

AB063 (35 mer) (SEQ ID No. 2)

5' GGAAGATCTTTACACAGCATCATCTTTCTGAGTCTG 3'

so as to isolate the gene encoding the gB glycoprotein from the MDV virus in the form of a PstI-BglII fragment. After purification, the 2613 bp PCR product was digested with PstI and BglI in order to isolate a 2602 bp PstI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously

digested with PstI and BglII, to give the plasmid pAB045 (7455 bp) (Figure No. 2).

Example 8: Construction of the plasmid pAB080 (MDV gD gene)

A PCR reaction was carried out with the Marek's disease virus (MDV) (RB1B strain) (L. Ross *et al.*, J. Gen. Virol., 1989, **72**, 949-954) genomic DNA, prepared according to the technique in Example 2, and with the following oligonucleotides:

AB148 (29 mer) (SEQ ID No. 3)

5' AAACTGCAGATGAAAGTATTTTTTTTTAG 3'

AB149 (32 mer) (SEQ ID No. 4)

5' GGAAGATCTTTATAGGCGGGAATATGCCCGTC 3'

so as to isolate the gene encoding the gD glycoprotein from the MDV virus in the form of a PstI-BglII fragment. After purification, the 1215 bp PCR product was digested with PstI and BglII in order to isolate a 1199 bp PstI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BglII, to give the plasmid pAB080 (6051 bp) (Figure No. 3).

Example 9: Construction of the plasmid pAB046 (NDV HN gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the Newcastle disease virus (NDV) (Texas GB strain) genomic RNA, prepared according to the technique of Example 3, and with the following oligonucleotides:

AB072 (39 mer) (SEQ ID No. 5)

5' AGAATGCGGCCGCGATGGGCTCCAGATCTTCTACCAG 3'

AB094 (34 mer) (SEQ ID No. 6)

5' CGCGGATCCTTAAATCCCATCATCCTTGAGAATC 3'

so as to isolate the gene encoding the HN glycoprotein from the NDV virus, Texas GB strain (Figure No. 4 and SEQ ID No. 7) in the form of an NotI-BamHI fragment. After purification, the 1741 bp RT-PCR product was digested with NotI and BamHI in order to isolate a 1723

bp NotI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with NotI and BamHI, to give the plasmid pAB046 (6616 bp) (Figure No. 5).

Example 10: Construction of the plasmid pAB047 (NDV F gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the Newcastle disease virus (NDV) (Texas GB strain) genomic RNA, prepared according to the technique of Example 3, and with the following oligonucleotides:

AB091 (37 mer) (SEQ ID No.8)

5' AGAATGCGGCCGCGATGGGCTCCAGATCTTCTACCAG 3'

AB092 (34 mer) (SEQ ID No. 9)

5' TGCTCTAGATCATATTTTTGTAGTGGCTCTCATC 3'

so as to isolate the gene encoding the F glycoprotein from the NDV virus, Texas GB strain (Figure No. 6 and SEQ ID No. 10) in the form of an NotI-XbaI fragment. After purification, the 1684 bp RT-PCR product was digested with NotI and XbaI in order to isolate a 1669 bp NotI-XbaI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with NotI and XbaI, to give the plasmid pAB047 (6578 bp) (Figure No. 7).

Example 11: Construction of the plasmid pAB048 (IBDV VP2 gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the infectious bursal disease virus (IBDV) (Faragher strain) genomic RNA, prepared according to the technique of Example 3, and with the following oligonucleotides:

AB093 (33 mer) (SEQ ID No. 11)

5' TCAGATATCGATGACAAACCTGCAAGATCAAAC 3'

AB094 (38 mer) (SEQ ID No. 12)

5' AGAATGCGGCCGCTTACCTCCTTATAGCCCGGATTATG 3'

so as to isolate the sequence encoding the VP2 protein from the IBDV virus, Faragher strain (Figure No. 8 and

SEQ ID No. 13) in the form of an EcoRV-NotI fragment. After purification, the 1384 bp RT-PCR product was digested with EcoRV and NotI in order to isolate a 1367 bp EcoRV-NotI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with EcoRV and NotI, to give the plasmid pAB048 (6278 bp) (Figure No. 9).

Example 12: Construction of the plasmid pAB049 (IBV S1 gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the chicken infectious bronchitis virus (IBV) (Massachusetts 41 strain) genomic RNA, prepared according to the technique of Example 3, and with the following oligonucleotides:

AB095 (32 mer) (SEQ ID No. 14)

5' ACGCGTCGACATGTTGGTAACACCTCTTTTAC 3'

AB096 (35 mer) (SEQ ID No. 15)

5' GGAAGATCTTCATTAACGTCTAAAACGACGTGTTC 3'

so as to isolate the sequence encoding the S1 subunit of the S glycoprotein from the IBV virus, Massachusetts 41 strain (Figure No. 10 and SEQ ID No. 16) in the form of a SalI-BglII fragment. After purification, the 1635 bp RT-PCR product was digested with SalI and BglII in order to isolate a 1622 bp SalI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BglII, to give the plasmid pAB049 (6485 bp) (Figure No. 11).

Example 13: Construction of the plasmid pAB050 (IBV M gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the chicken infectious bronchitis virus (IBV) (Massachusetts 41 strain) genomic RNA, prepared according to the technique of Example 3, and with the following oligonucleotides:

AB097 (37 mer) (SEQ ID No. 17)

5' ATAAGAATGCGGCCGCATGTCCAACGAGACAAATTGTAC 3'

AB098 (38 mer) (SEQ ID No. 18)

5' ATAAGAATGCGGCCGCTTTAGGTGTAAAGACTACTCCC 3'

so as to isolate the gene encoding the M glycoprotein from the IBV virus, Massachusetts 41 strain (Figure No. 12 and SEQ ID No. 19) in the form of a NotI-NotI fragment. After purification, the 710 bp RT-PCR product was digested with NotI in order to isolate a 686 bp NotI-NotI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with NotI, to give the plasmid pAB050 (5602 bp) which contains the IBV M gene in the correct orientation relative to the promoter (Figure No. 13).

Figure 14: Construction of the plasmid pAB051 (IBV N gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the chicken infectious bronchitis virus (IBV) (Massachusetts 41 strain) genomic RNA, prepared according to the technique of Example 3, and with the following oligonucleotides:

AB099 (34 mer) (SEQ ID No. 20)

5' AAAACTGCAGTCATGGCAAGCGGTAAGGCAACTG 3'

AB100 (33 mer) (SEQ ID No. 21)

5' CGCGGATCCTCAAAGTTCATTCTCTCCTAGGGC 3'

so as to isolate the gene encoding the N protein from the IBV virus, Massachusetts 41 strain (Figure No. 14 and SEQ ID No. 22) in the form of a PstI-BamHI fragment. After purification, the 1250 bp RT-PCR product was digested with PstI and BamHI in order to isolate a 1233 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB051 (6092 bp) (Figure No. 15).

Example 15: Construction of the plasmid pAB054 (VAC VP1 gene)

A PCR reaction was carried out with the chicken anaemia virus (CAV) (Cuxhaven-1 strain) genomic DNA (B. Meehan *et al.*, Arch. Virol., 1992, **124**, 301-319), prepared according to the technique of Example 2, and with the following oligonucleotides:

CD064 (39 mer) (SEQ ID No. 23)

5' TTCTTGCGGCCGCCATGGCAAGACGAGCTCGCAGACCGA 3'

CD065 (38 mer) (SEQ ID No. 24)

5' TTCTTGCGGCCGCTCAGGGCTGCGTCCCCCAGTACATG 3'

so as to isolate the gene encoding the CAV VP1 capsid protein in the form of an NotI-NotI fragment. After purification, the 1377 bp PCR product was digested with NotI in order to isolate a 1359 bp NotI-NotI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with NotI, to give the plasmid pAB054 (6274 bp) which contains the CAV VP1 gene in the correct orientation relative to the promoter (Figure No. 16).

Example 17: Construction of the plasmid pAB055 (CAV VP2 gene)

A PCR reaction was carried out with the chicken anaemia virus (CAV) (Cuxhaven-1 strain) genomic DNA (B. Meehan *et al.*, Arch. Virol., 1992, **124**, 301-319), prepared according to the technique of Example 2, and with the following oligonucleotides:

CD066 (39 mer) (SEQ ID No. 25)

5' TTCTTGCGGCCGCCATGCACGGGAACGGCGGACAACCGG 3'

AB105 (32 mer) (SEQ ID No. 26)

5' CGCGGATCCTCACACTATACGTACCGGGGCGG 3'

so as to isolate the gene encoding the CAV virus VP2 protein in the form of an NotI-BamHI fragment. After purification, the 674 bp PCR product was digested with NotI and BamHI in order to isolate a 659 bp NotI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with NotI and

BamHI, to give the plasmid pAB055 (5551 bp) (Figure No. 17).

Example 18: Construction of the plasmid pAB076 (ILTV gB gene)

A PCR reaction was carried out with the chicken infectious laryngotracheitis virus (ILTV) (SA-2 strain) genomic DNA (K. Kongsuwan *et al.*, Virology, 1991, **184**, 404-410), prepared according to the technique of Example 2, and with the following oligonucleotides:

AB140 (38 mer) (SEQ ID No. 27)

5' TTCTTGCGGCCGCATGTCTTGAAAATGCTGATC 3'

AB141 (36 mer) (SEQ ID No. 28)

5' TTCTTGCGGCCGCTTATTCGTCTTCGCTTTCTTCTG 3'

so as to isolate the gene encoding the ILTV virus gB glycoprotein in the form of an NotI-NotI fragment. After purification, the 2649 bp PCR product was digested with NotI in order to isolate a 2631 bp NotI-NotI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with NotI, to give the plasmid pAB076 (7546 bp) which contains the ILTV gB gene in the correct orientation relative to the promoter (Figure No. 18).

Example 20: Construction of the plasmid pAB089 (ILTV gD gene)

A PCR reaction was carried out with the chicken infectious laryngotracheitis virus (ILTV) (SA-2 strain) genomic DNA (M. Johnson *et al.*, 1994, Genbank sequence accession No. = L31965), prepared according to the technique of Example 2, and with the following oligonucleotides:

AB164 (33 mer) (SEQ ID No. 29)

5' CCGGTCGACATGGACCGCCATTTATTTTGAGG 3'

AB165 (33 mer) (SEQ ID No. 30)

5' GGAAGATCTTTACGATGCTCCAAACCAGTAGCC 3'

so as to isolate the gene encoding the ILTV virus gD glycoprotein in the form of an SalI-BglII fragment. After purification, the 1134 bp PCR product was

digested with SalI and BglII in order to isolate a 1122 bp SalI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI-BglII, to give the plasmid pAB089 (5984 bp) (Figure No. 19).

Example 21: Construction of the plasmid pAB086 (AEV env gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the avian encephalomyelitis virus (AEV) (Type C) genomic RNA (E. Bieth *et al.*, Nucleic Acids Res., 1992, **20**, 367), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB160 (54 mer) (SEQ ID No. 31)

5'TTTGATATCATGGAAGCCGTCATTAAGGCATTTCTGACTGGATACCCTGGGAA G3'

AB161 (31 mer) (SEQ ID No. 32)

5'TTTGGATCCTTATACTATTCTGCTTTCAGGC 3'

so as to isolate the sequence encoding the AEV virus Env glycoprotein in the form of an EcoRV-BamHI fragment. After purification, the 1836 bp RT-PCR product was digested with EcoRV and BamHI in order to isolate a 1825 bp EcoRV-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with EcoRV and BamHI, to give the plasmid pAB086 (6712 bp) (Figure No. 20).

Example 22: Construction of the plasmid pAB081 (AEV gag/pro gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the avian encephalomyelitis virus (AEV) (Type C) genomic RNA (E. Bieth *et al.*, Nucleic Acids Res., 1992, **20**, 367), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB150 (31 mer) (SEQ ID No. 33)

5'ACGCGTCGACATGGAAGCCGTCATTAAGGTG 3'

AB151 (32 mer) (SEQ ID No. 34)

5'TGCTCTAGACTATAAATTTGTCAAGCGGAGCC 3'

so as to isolate the sequence encoding the AEV virus Gag and Pro proteins in the form of an SalI-XbaI fragment. After purification, the 2125 bp RT-PCR product was digested with SalI-XbaI in order to isolate a 2111 bp SalI-XbaI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and XbaI, to give the plasmid pAB081 (6996 bp) (Figure No. 21).

Example 23: Construction of the plasmid pAB082 (Pneumovirus G gene)

An RT-PCR reaction according to the technique of Example 5 was carried out with the turkey rhinotracheitis virus (TRV) (2119 strain) genomic RNA (K. Juhasz *et al.*, J. Gen. Virol., 1994, **75**. 2873-2880), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB152 (32 mer) (SEQ ID No. 35)

5'AAACTGCAGAGATGGGGTCAGAGCTCTACATC 3'

AB153 (31 mer) (SEQ ID No. 36)

5' CGAAGATCTTTATTGACTAGTACAGCACCAC 3'

so as to isolate the gene encoding the TRV virus G glycoprotein in the form of a PstI-BglII fragment. After purification, the 2165 bp RT-PCR product was digested with PstI and BglII in order to isolate a 1249 bp PstI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BglII, to give the plasmid pAB082 (6101 bp) (Figure No. 22).

Example 24: Construction of the plasmid pAB077 (avian plague HA gene, H2N2 strain)

An RT-PCR reaction according to the technique of Example 5 was carried out with the avian **plague** virus (AIV) (H2N2 Postdam strain) genomic RNA (J. Schäfer *et al.*, Virology, 1993, **194**, 781-788), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB142 (33 mer) (SEQ ID No. 37)

5' AAACTGCAGCAATGGCCATCATTTATCTAATTC 3'

AB143 (31 mer) (SEQ ID No. 38)

5'CGAAGATCTTCATATGCAGATTCTGCATTGC 3'

so as to isolate the gene encoding the HA glycoprotein from the avian plague virus (H2N2 strain) in the form of a PstI-BglII fragment. After purification, the 1709 bp RT-PCR product was digested with PstI and BglII in order to isolate a 1693 bp PstI-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BglII, to give the plasmid pAB077 (6545 bp) (Figure No. 23).

Example 25: Construction of the plasmid pAB078 (avian plague HA gene, H7N7 strain)

An RT-PCR reaction according to the technique of Example 5 was carried out with the avian plague virus (AIV) (H7N7 Leipzig strain) genomic RNA (C. Rohm *et al.*, Virology, 1995, **209**, 664-670), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB144 (31 mer) (SEQ ID No. 39)

5'AAACTGCAGATGAACACTCAAATCCTGATAC 3'

AB145 (31 mer) (SEQ ID No. 40)

5' TTTGGATCCTTATATACAAATAGTGCACCGC 3'

so as to isolate the gene encoding the HA glycoprotein from the avian plague virus (H7N7 strain) in the form of a PstI-BamHI fragment. After purification, the 1707 bp RT-PCR product was digested with PstI and BamHI in order to isolate a 1691 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB078 (6549 bp) (Figure No. 24).

Example 26: Construction of the plasmid pAB088 (avian plague NP gene, H1N1 strain)

An RT-PCR reaction according to the technique of Example 5 was carried out with the avian influenza virus (AIV) (H1N1 Bavaria strain) genomic RNA

(M. Gammelin *et al.*, Virology, 1989, **170**, 71-80), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB156 (32 mer) (SEQ ID No. 41)

5' CCGGTCGACATGGCGTCTCAAGGCACCAAACG 3'

AB158 (30 mer) (SEQ ID No. 42)

5' CGCGGATCCTTAATTGTCATACTCCTCTGC 3'

so as to isolate the gene encoding the avian influenza virus NP nucleoprotein in the form of a SalI-BamHI fragment. After purification, the 1515 bp RT-PCR product was digested with SalI and BamHI in order to isolate a 1503 bp SalI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB088 (6371 bp) (Figure No. 25).

Example 27: Construction of the plasmid pAB079 (avian plague N gene, H7N1 strain)

An RT-PCR reaction according to the technique of Example 5 was carried out with the avian **plague** virus (AIV) (H7N1 Rostock strain) genomic RNA (J. McCauley, 1990, Genbank sequence accession No. = X52226), prepared according to the technique of Example 3, and with the following oligonucleotides:

AB146 (35 mer) (SEQ ID No. 43)

5' CGCGTCGACATGAATCCAAATCAGAAAATAATAAC 3'

AB147 (31 mer) (SEQ ID No. 44)

5' GGAAGATCTCTACTTGTCAATGGTGAATGGC 3'

so as to isolate the gene encoding the N glycoprotein from the avian plague virus (H7N1 strain) in the form of an SalI-BglII fragment. After purification, the 1361 bp RT-PCR product was digested with SalI and BglII in order to isolate a 1350 bp Sal1-BglII fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with Sal1I and BglII, to give the plasmid pAB079 (6212 bp) (Figure No. 26).

Example 28: Preparation and purification of the plasmids

For the preparation of the plasmids intended for the vaccination of animals, any technique may be used which makes it possible to obtain a suspension of purified plasmids predominantly in the supercoiled form. These techniques are well known to persons skilled in the art. There may be mentioned in particular the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in J. Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Reference may also be made to patent applications PCT WO 95/21250 and PCT WO 96/02658 which describe methods for producing, on an industrial scale, plasmids which can be used for vaccination. For the purposes of the manufacture of vaccines (see Example 17), the purified plasmids are resuspended so as to obtain solutions at a high concentration (> 2 mg/ml) which are compatible with storage. To do this the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

Example 29: Manufacture of the associated vaccines

The various plasmids necessary for the manufacture of an associated vaccine are mixed starting with their concentrated solutions (Example 16). The mixtures are prepared such that the final concentration of each plasmid corresponds to the effective dose of each plasmid. The solutions which can be used to adjust the final concentration of the vaccine may be either a 0.9% NaCl solution, or PBS buffer.

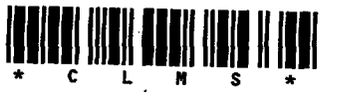
Specific formulations such as liposomes, cationic lipids, may also be used for the manufacture of the vaccines.

Example 30: Vaccination of chickens

The chickens are vaccinated with doses of 10, 50 or 100 µg per plasmid. The injections can be

performed with a needle by the intramuscular route. The sites of injection are the carina (for chickens more than 2 weeks old) and the thigh (for 1-day-old or older chickens). In this case, the vaccinal doses are administered in the volume of 0.1 to 0.3 ml.

In adult chickens (more than 20 weeks old) the injections are also performed by the intramuscular route using a liquid jet injection apparatus (with no needle) which has been specially designed for the vaccination of chickens (for example AVIJET apparatus). In this case, the injected volume is 0.3 ml. The injection may be performed in the carina or at the level of the thigh. Likewise, in adult chickens, the injections may be performed with a needle by the intramuscular route, in the carina or in the thigh, in a volume of 0.3 ml. The injection of the plasmid vaccines can also be done *in ovo*. In this case, special formulations as mentioned in Example 29 may be used. The volume injected into the 18-day embryonated egg is between 50 μl and 200 μl.



CLAIMS

1. Avian vaccine formula, comprising at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one avian pathogen valency, these valencies being selected from the group consisting of Marek's disease virus, Newcastle disease virus, infectious bursal disease virus, infectious anaemia virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the Marek's disease virus, HN and F for the Newcastle disease virus, VP2 for the infectious bursal disease virus, C + NS1 for the infectious anaemia virus.

2. Vaccine formula according to Claim 1, wherein, for the valency of the Marek's disease virus, it comprises the gB gene alone.

3. Formula according to Claim 1, which comprises the Newcastle disease virus HN and F genes in the same plasmid or in different plasmids.

4. Vaccine formula according to Claim 1, wherein the plasmid for the infectious anaemia virus comprises C + NS1 in the same plasmid.

5. Vaccine formula according to any one of Claims 1 to 4, which comprises, in addition, at least one valency selected from the group consisting of infectious bronchitis virus, infectious laryngotracheitis virus, encephalomyelitis virus, pneumovirosis virus, and avian **plague** virus, the plasmids comprising, for these valencies, one or more of the genes selected from the group consisting of S, M and N for the infectious bronchitis virus, gB and gD for the infectious laryngotracheitis virus, env and gag/pro for the encephalomyelitis virus, F and G for the pneumovirosis virus and HA, N and NP for the avian plague virus.

6. Vaccine formula according to Claim 5, wherein for the infectious bronchitis virus valency, it comprises the S gene alone.

7. Vaccine formula according to Claim 5, wherein for the laryngotracheitis valency, the formula comprises the gB gene alone.

8. Vaccine formula according to Claim 5, wherein for the pneumovirosis valency, the formula comprises the two F and G genes in different plasmids or in one and the same plasmid.

9. Vaccine formula according to Claim 5, wherein, for the avian plague valency, the formula comprises the HA gene alone.

10. Vaccine formula according to Claim 5, wherein for the encephalomyelitis valency, the vaccine formula comprises the env gene.

11. Vaccine formula according to any one of Claims 1 to 10, which comprises from 10 ng to 1 mg, preferably from 100 ng to 500 μg, still more preferably from 0.1 μg to 50 μg of each plasmid.

12. Use of one or more plasmids as described in any one of Claims 1 to 11, for the manufacture of an avian vaccine intended to vaccinate animals first vaccinated by means of a first vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having the antigen(s) encoded by the plasmid(s) or antigen(s) providing cross-protection.

13. Vaccination kit grouping together a vaccine formula according to any one of Claims 1 to 11, and an avian vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having the antigen encoded by the polynucleotide vaccine or an antigen providing cross-protection, for an administration of the latter in first vaccination or as booster with the vaccine formula.

14. Vaccine formula according to any one of Claims 1 to 11, accompanied by a leaflet indicating that this formula can be used as booster for a first avian vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having the antigen encoded by the polynucleotide vaccine or an antigen providing cross-protection.