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(54) **METHOD FOR PRODUCING THE FLU VIRUS**

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(57) **ABSTRACT**

The invention relates to a method for producing flu virus according to which:

a) immunizing a hen by administering a flu vaccine to the hen,
b) triggering embryogenesis in one or more eggs of the immunized hen,
c) infecting the one or more embryonated eggs by inoculating a flu virus into the allantoic cavity of the eggs,
d) incubating the one or more infected embryonated eggs under temperature and humidity conditions that allow replication of the virus, and
e) harvesting the allantoic fluid of the one or more incubated eggs containing the virus.

FIGURE 1

```
ctaaattgta agcgttaata ttttgttaaa attcgcgtta aatttttgtt aaatcagctc     60
atttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga    120
gatagggttg agtgttgttc cagtttggaa caagagtcca ctattaaaga acgtggactc    180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc    240
ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaagggag    300
cccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aagggaagaa    360
agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac    420
cacacccgcc gcgcttaatg cgccgctaca gggcgcgtcc cattcgccat tcaggctgcg    480
caactgttgg gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg    540
gggatgtgct gcaaggcgat taagttgggt aacgccaggg ttttcccagt cacgacgttg    600
taaaacgacg gccagtgaat tgtaatacga ctcactatag ggcgaattgg gtgacccttt    660
acaagaataa aagaagaaac aactgtgaaa tagtttataa atgtaattcg tatgcagaaa    720
acgataatat attttggtat gagaaatcta aaggagacat agtttgtata gacatgcgct    780
cttccgatga gatattcgat gcttttctaa tgtatcatat agctacaaga tatgcctatc    840
atgatgatga tatatatcta caaatagtgt tatattattc taataatcaa aatgttatat    900
cttatattac gaaaaataaa tacgttaagt atataagaaa taaaactaga gacgatattc    960
ataaagtaaa aatattagct ctagaagact ttacaacgga agaaatatat tgttggatta   1020
gtaatatata acagcgtagc tgcacggttt tgatcatttt ccaacaatat aaaccaatga   1080
aggaggacga ctcatcaaac ataaataaca ttcacggaaa atattcagta tcagatttat   1140
cacaagatga ttatgttatt gaatgtatag acggatcttt tgattcgatc aagtatagag   1200
atataaaggt tataataatg aagaataacg gttacgttaa ttgtagtaaa ttatgtaaaa   1260
tgcggaataa atacttttct agatggttgc gtctttctac ttctaaagca ttattagaca   1320
tttacaataa taagtcagta gataatgcta ttgttaaagt ctatggtaaa ggtaagaaac   1380
```

FIGURE 1 (cont'd)

```
ttattataac aggattttat ctcaaacaaa atatgatacg ttatgttatt gagtggatag   1440
gggatgattt tacaaacgat atatacaaaa tgattaattt ctataatgcg ttattcggta   1500
acgatgaatt aaaaatagta tcctgtgaaa acactctatg cccgtttata gaacttggta   1560
gatgctatta tggtaaaaaa tgtaagtata tacacggaga tcaatgtgat atctgtggtc   1620
tatatatact acaccctacc gatattaacc aacgagtttc tcacaagaaa acttgtttag   1680
tagatagaga ttctttgatt gtgtttaaaa gaagtaccag taaaaagtgt ggcatatgca   1740
tagaagaaat aaacaaaaaa catatttccg aacagtattt tggaattctc ccaagttgta   1800
aacatatttt ttgcctatca tgtataagac gttgggcaga tactaccaga aatacagata   1860
ctgaaaatac gtgtcctgaa tgtagaatag tttttccttt cataataccc agtaggtatt   1920
ggatagataa taaatatgat aaaaaaatat tatataatag atataagaaa atgattttta   1980
caaaaatacc tataagaaca ataaaaatat aattacattt acggaaaata gctggtttta   2040
gtttaccaac ttagagtaat tatcatattg aatctatatt gctaattagc taataaaaac   2100
ccgggttaat taattagtca tcaggcaggg cgagaacgag actatctgct cgttaattaa   2160
ttagagcttc tttattctat acttaaaaag tgaaaataaa tacaaaggtt cttgagggtt   2220
gtgttaaatt gaaagcgaga aataatcata aattatttca ttatcgcgat atccgttaag   2280
tttgtatcgt aatggagaaa atcgtgctgc tgctggccat cgtgagcctg gtgaaaagcg   2340
atcagatctg catcggctac cacgccaaca acagcacaga gcaagtggac acaatcatgg   2400
aaaagaacgt gaccgtgaca cacgcccagg acatcctgga aaagacacac aacgggaagc   2460
tgtgcgatct ggatggagtg aagcctctga tcctgagaga ttgcagcgtg gccggatggc   2520
tgctggggaa cccaatgtgc gacgaattca tcaacgtgcc cgaatggagc tacatcgtgg   2580
agaaggccaa cccagccaac gacctgtgct acccagggaa cctgaacgac tacgaagaac   2640
tgaaacacct gctgagcaga atcaaccact ttgagaaaat ccagatcatc cccaaaagca   2700
gctggtccga tcacgaagcc agcagcggag tgagcagcgc ctgcccatac cagggaaagt   2760
ccagcttttt tagaaacgtg gtgtggctga tcaaaaagaa cagcgcctac ccaacaatca   2820
```

FIGURE 1 (cont'd)

agagaagcta caacaacacc aaccaggaag atctgctggt gctgtggggg atccaccacc 2880
ctaacgatgc cgccgagcag acaaggctgt accagaaccc aaccacctac atctccgtgg 2940
ggacaagcac actgaaccag agactggtgc caaaaatcgc catcagatcc aaagtgaacg 3000
ggcagagcgg aagaatggag ttcttctgga caatcctgaa acccaacgat gccatcaact 3060
tcgagagcaa cggaaacttc atcgccccag aatacgccta caaaatcgtg aagaaagggg 3120
acagcgccat catgaaaagc gaactggaat acggcaactg caacaccaag tgccagaccc 3180
caatgggggc catcaacagc agcatgccat tccacaacat ccaccctctg accatcgggg 3240
aatgccccaa atacgtgaaa agcaacagac tggtgctggc caccgggctg agaaacagcc 3300
ctcagagaga gaccagagga ctgtttggag ccatcgccgg ctttatcgag ggaggatggc 3360
agggaatggt ggatggctgg tacggatacc accacagcaa cgagcagggg agcggatacg 3420
ccgccgacaa agaatccacc cagaaggcca tcgacggcgt gaccaacaaa gtgaacagca 3480
tcatcgacaa aatgaacacc cagtttgagg ccgtgggaag ggagtttaac aacctggaaa 3540
ggagaatcga gaacctgaac aagaagatgg aggacggatt cctggatgtg tggacctaca 3600
acgccgaact gctggtgctg atggaaaacg agagaaccct ggactttcac gacagcaacg 3660
tgaagaacct gtacgacaaa gtgaggctgc agctgaggga taacgccaag gagctgggca 3720
acggctgctt cgagttctac cacaaatgcg ataacgaatg catggaaagc atcagaaacg 3780
gaacctacaa ctacccccag tacagcgaag aagccagact gaaaagagaa gaaatctccg 3840
gagtgaaact ggaatccatc ggaacctacc agatcctgag catctacagc acagtggcct 3900
cctccctggc cctggccatc atgatggccg gactgagcct gtggatgtgc tccaacggaa 3960
gcctgcagtg cagaatctgc atctgactcg agtttttatt gactagttaa tcataagata 4020
aataatatac agcattgtaa ccatcgtcat ccgttatacg ggaataata ttaccataca 4080
gtattattaa attttcttac gaagaatata gatcggtatt tatcgttagt ttattttaca 4140
tttattaatt aaacatgtct actattacct gttatggaaa tgacaaattt agttatataa 4200
tttatgataa aattaagata ataataatga aatcaaataa ttatgtaaat gctactagat 4260

FIGURE 1 (cont'd)

```
tatgtgaatt acgaggaaga aagtttacga actggaaaaa attaagtgaa tctaaaatat   4320
tagtcgataa tgtaaaaaaa ataaatgata aaactaacca gttaaaaacg gatatgatta   4380
tatacgttaa ggatattgat cataaaggaa gagatacttg cggttactat gtacaccaag   4440
atctggtatc ttctatatca aattggatat ctccgttatt cgccgttaag gtaaataaaa   4500
ttattaacta ttatatatgt aatgaatatg atatacgact tagcgaaatg gaatctgata   4560
tgacagaagt aatagatgta gttgataaat tagtaggagg atacaatgat gaaatagcag   4620
aaataatata tttgtttaat aaatttatag aaaaatatat tgctaacata tcgttatcaa   4680
ctgaattatc tagtatatta aataatttta taaattttaa taaaaaatac aataacgaca   4740
taaaagatat taaatcttta attcttgatc tgaaaaacac atctataaaa ctagataaaa   4800
agttattcga taaagataat aatgaatcga acgatgaaaa attggaaaca gaagttgata   4860
agctaatttt tttcatctaa atagtattat tttattgaag tacgaagttt tacgttagat   4920
aaataataaa ggtcgatttt tattttgtta aatatcaaat atgtcattat ctgataaaga   4980
tacaaaaaca cacggtgatt atcaaccatc taacgaacag atattacaaa aaatacgtcg   5040
gactatggaa aacgaagctg atagcctcaa tagaagaagc attaaagaaa ttgttgtaga   5100
tgttatgaag aattgggatc atcctctcaa cgaagaaata gataaagttc taaactggaa   5160
aaatgataca ttaaacgatt tagatcatct aaatacagat gataatatta aggaaatcat   5220
acaatgtctg attagagaat ttgcgtttaa aaagatcaat tctattatgt atagttatgc   5280
tatggtaaaa ctcaattcag ataacgaaac attgaaagat aaaattaagg attattttat   5340
agaaactatt cttaaagaca aacgtggtta taaacaaaag ccattaccct agagcggccg   5400
ccaccgcggt ggagctccag cttttgttcc ctttagtgag ggttaatttc gagcttggcg   5460
taatcatggt catagctgtt tcctgtgtga aattgttatc cgctcacaat tccacacaac   5520
atacgagccg gaagcataaa gtgtaaagcc tggggtgcct aatgagtgag ctaactcaca   5580
ttaattgcgt tgcgctcact gcccgctttc cagtcgggaa acctgtcgtg ccagctgcat   5640
taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta ttgggcgctc ttccgcttcc   5700
```

FIGURE 1 (cont'd)

tcgctcactg actcgctgcg ctcggtcgtt cggctgcggc gagcggtatc agctcactca 5760

aaggcggtaa tacggttatc cacagaatca ggggataacg caggaaagaa catgtgagca 5820

aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt tgctggcgtt tttccatagg 5880

ctccgccccc ctgacgagca tcacaaaaat cgacgctcaa gtcagaggtg gcgaaacccg 5940

acaggactat aaagatacca ggcgtttccc cctggaagct ccctcgtgcg ctctcctgtt 6000

ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc cttcgggaag cgtggcgctt 6060

tctcatagct cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc caagctgggc 6120

tgtgtgcacg aaccccccgt tcagcccgac cgctgcgcct tatccggtaa ctatcgtctt 6180

gagtccaacc cggtaagaca cgacttatcg ccactggcag cagccactgg taacaggatt 6240

agcagagcga ggtatgtagg cggtgctaca gagttcttga agtggtggcc taactacggc 6300

tacactagaa ggacagtatt tggtatctgc gctctgctga agccagttac cttcggaaaa 6360

agagttggta gctcttgatc cggcaaacaa accaccgctg gtagcggtgg tttttttgtt 6420

tgcaagcagc agattacgcg cagaaaaaaa ggatctcaag aagatccttt gatcttttct 6480

acggggtctg acgctcagtg gaacgaaaac tcacgttaag ggattttggt catgagatta 6540

tcaaaaagga tcttcaccta gatcctttta aattaaaaat gaagttttaa atcaatctaa 6600

agtatatatg agtaaacttg gtctgacagt taccaatgct taatcagtga ggcacctatc 6660

tcagcgatct gtctatttcg ttcatccata gttgcctgac tccccgtcgt gtagataact 6720

acgatacggg agggcttacc atctggcccc agtgctgcaa tgataccgcg agacccacgc 6780

tcaccggctc cagatttatc agcaataaac cagccagccg gaagggccga gcgcagaagt 6840

ggtcctgcaa ctttatccgc ctccatccag tctattaatt gttgccggga agctagagta 6900

agtagttcgc cagttaatag tttgcgcaac gttgttgcca ttgctacagg catcgtggtg 6960

tcacgctcgt cgtttggtat ggcttcattc agctccggtt cccaacgatc aaggcgagtt 7020

acatgatccc ccatgttgtg caaaaaagcg gttagctcct tcggtcctcc gatcgttgtc 7080

agaagtaagt tggccgcagt gttatcactc atggttatgg cagcactgca taattctctt 7140

FIGURE 1 (cont'd)

actgtcatgc catccgtaag atgcttttct gtgactggtg agtactcaac caagtcattc 7200

tgagaatagt gtatgcggcg accgagttgc tcttgcccgg cgtcaatacg ggataatacc 7260

gcgccacata gcagaacttt aaaagtgctc atcattggaa aacgttcttc ggggcgaaaa 7320

ctctcaagga tcttaccgct gttgagatcc agttcgatgt aacccactcg tgcacccaac 7380

tgatcttcag catcttttac tttcaccagc gtttctgggt gagcaaaaac aggaaggcaa 7440

aatgccgcaa aaaagggaat aagggcgaca cggaaatgtt gaatactcat actcttcctt 7500

tttcaatatt attgaagcat ttatcagggt tattgtctca tgagcggata catatttgaa 7560

tgtatttaga aaaataaaca aataggggtt ccgcgcacat ttccccgaaa agtgccac 7618

(SEQ ID NO: 2)

FIGURE 2

M13F (SEQ ID NOS: 1 and 3)

```
  1 GTAAAACGAC GGCCAGTGAA TTGTAATACG ACTCACTATA GGGCGAATTG
    CATTTTGCTG CCGGTCACTT AACATTATGC TGAGTGATAT CCCGCTTAAC
 51 GGTGACCCTT TACAAGAATA AAAGAAGAAA CAACTGTGAA ATAGTTTATA
    CCACTGGGAA ATGTTCTTAT TTTCTTCTTT GTTGACACTT TATCAAATAT
101 AATGTAATTC GTATGCAGAA AACGATAATA TATTTTGGTA TGAGAAATCT
    TTACATTAAG CATACGTCTT TTGCTATTAT ATAAAACCAT ACTCTTTAGA
151 AAAGGAGACA TAGTTTGTAT AGACATGCGC TCTTCCGATG AGATATTCGA
    TTTCCTCTGT ATCAAACATA TCTGTACGCG AGAAGGCTAC TCTATAAGCT
201 TGCTTTTCTA ATGTATCATA TAGCTACAAG ATATGCCTAT CATGATGATG
    ACGAAAAGAT TACATAGTAT ATCGATGTTC TATACGGATA GTACTACTAC
251 ATATATATCT ACAAATAGTG TTATATTATT CTAATAATCA AAATGTTATA
    TATATATAGA TGTTTATCAC AATATAATAA GATTATTAGT TTTACAATAT
301 TCTTATATTA CGAAAAATAA ATACGTTAAG TATATAAGAA ATAAAACTAG
    AGAATATAAT GCTTTTTATT TATGCAATTC ATATATTCTT TATTTTGATC
351 AGACGATATT CATAAAGTAA AAATATTAGC TCTAGAAGAC TTTACAACGG
    TCTGCTATAA GTATTTCATT TTTATAATCG AGATCTTCTG AAATGTTGCC
401 AAGAAATATA TTGTTGGATT AGTAATATAT AACAGCGTAG CTGCACGGTT
    TTCTTTATAT AACAACCTAA TCATTATATA TTGTCGCATC GACGTGCCAA
451 TTGATCATTT TCCAACAATA TAAACCAATG AAGGAGGACG ACTCATCAAA
    AACTAGTAAA AGGTTGTTAT ATTTGGTTAC TTCCTCCTGC TGAGTAGTTT
```

FIGURE 2 (cont'd)

501 CATAAATAAC ATTCACGGAA AATATTCAGT ATCAGATTTA TCACAAGATG

GTATTTATTG TAAGTGCCTT TTATAAGTCA TAGTCTAAAT AGTGTTCTAC

551 ATTATGTTAT TGAATGTATA GACGGATCTT TTGATTCGAT CAAGTATAGA

TAATACAATA ACTTACATAT CTGCCTAGAA AACTAAGCTA GTTCATATCT

601 GATATAAAGG TTATAATAAT GAAGAATAAC GGTTACGTTA ATTGTAGTAA

CTATATTTCC AATATTATTA CTTCTTATTG CCAATGCAAT TAACATCATT

651 ATTATGTAAA ATGCGGAATA AATACTTTTC TAGATGGTTG CGTCTTTCTA

TAATACATTT TACGCCTTAT TTATGAAAAG ATCTACCAAC GCAGAAAGAT

701 CTTCTAAAGC ATTATTAGAC ATTTACAATA ATAAGTCAGT AGATAATGCT

GAAGATTTCG TAATAATCTG TAAATGTTAT TATTCAGTCA TCTATTACGA

751 ATTGTTAAAG TCTATGGTAA AGGTAAGAAA CTTATTATAA CAGGATTTTA

TAACAATTTC AGATACCATT TCCATTCTTT GAATAATATT GTCCTAAAAT

801 TCTCAAACAA AATATGATAC GTTATGTTAT TGAGTGGATA GGGGATGATT

AGAGTTTGTT TTATACTATG CAATACAATA ACTCACCTAT CCCCTACTAA

851 TTACAAACGA TATATACAAA ATGATTAATT TCTATAATGC GTTATTCGGT

AATGTTTGCT ATATATGTTT TACTAATTAA AGATATTACG CAATAAGCCA

901 AACGATGAAT TAAAAATAGT ATCCTGTGAA AACACTCTAT GCCCGTTTAT

TTGCTACTTA ATTTTTATCA TAGGACACTT TTGTGAGATA CGGGCAAATA

951 AGAACTTGGT AGATGCTATT ATGGTAAAAA ATGTAAGTAT ATACACGGAG

TCTTGAACCA TCTACGATAA TACCATTTTT TACATTCATA TATGTGCCTC

1001 ATCAATGTGA TATCTGTGGT CTATATATAC TACACCCTAC CGATATTAAC

TAGTTACACT ATAGACACCA GATATATATG ATGTGGGATG GCTATAATTG

FIGURE 2 (cont'd)

```
1051 CAACGAGTTT CTCACAAGAA AACTTGTTTA GTAGATAGAG ATTCTTTGAT
     GTTGCTCAAA GAGTGTTCTT TTGAACAAAT CATCTATCTC TAAGAAACTA
1101 TGTGTTTAAA AGAAGTACCA GTAAAAAGTG TGGCATATGC ATAGAAGAAA
     ACACAAATTT TCTTCATGGT CATTTTTCAC ACCGTATACG TATCTTCTTT
1151 TAAACAAAAA ACATATTTCC GAACAGTATT TTGGAATTCT CCCAAGTTGT
     ATTTGTTTTT TGTATAAAGG CTTGTCATAA AACCTTAAGA GGGTTCAACA
1201 AAACATATTT TTTGCCTATC ATGTATAAGA CGTTGGGCAG ATACTACCAG
     TTTGTATAAA AAACGGATAG TACATATTCT GCAACCCGTC TATGATGGTC
1251 AAATACAGAT ACTGAAAATA CGTGTCCTGA ATGTAGAATA GTTTTTCCTT
     TTTATGTCTA TGACTTTTAT GCACAGGACT TACATCTTAT CAAAAAGGAA
1301 TCATAATACC CAGTAGGTAT TGGATAGATA ATAAATATGA TAAAAAAATA
     AGTATTATGG GTCATCCATA ACCTATCTAT TATTTATACT ATTTTTTTAT
1351 TTATATAATA GATATAAGAA AATGATTTTT ACAAAAATAC CTATAAGAAC
     AATATATTAT CTATATTCTT TTACTAAAAA TGTTTTTATG GATATTCTTG
1401 AATAAAAATA TAATTACATT TACGGAAAAT AGCTGGTTTT AGTTTACCAA
     TTATTTTTAT ATTAATGTAA ATGCCTTTTA TCGACCAAAA TCAAATGGTT
1451 CTTAGAGTAA TTATCATATT GAATCTATAT TGCTAATTAG CTAATAAAAA
     GAATCTCATT AATAGTATAA CTTAGATATA ACGATTAATC GATTATTTTT
1501 CCCGGGTTAA TTAATTAGTC ATCAGGCAGG GCGAGAACGA GACTATCTGC
     GGGCCCAATT AATTAATCAG TAGTCCGTCC CGCTCTTGCT CTGATAGACG
1551 TCGTTAATTA ATTAGAGCTT CTTTATTCTA TACTTAAAAA GTGAAAATAA
     AGCAATTAAT TAATCTCGAA GAAATAAGAT ATGAATTTTT CACTTTTATT
```

FIGURE 2 (cont'd)

```
1601 ATACAAAGGT TCTTGAGGGT TGTGTTAAAT TGAAAGCGAG AAATAATCAT
     TATGTTTCCA AGAACTCCCA ACACAATTTA ACTTTCGCTC TTTATTAGTA

                                        (SEQ ID NO: 1)  M  E  K ·
1651 AAATTATTTC ATTATCGCGA TATCCGTTAA GTTTGTATCG TAATGGAGAA
     TTTAATAAAG TAATAGCGCT ATAGGCAATT CAAACATAGC ATTACCTCTT

     . I  V  L   L  L  A  I   V  S  L   V  K  S   D  Q  I  C ·
1701 AATCGTGCTG CTGCTGGCCA TCGTGAGCCT GGTGAAAAGC GATCAGATCT
     TTAGCACGAC GACGACCGGT AGCACTCGGA CCACTTTTCG CTAGTCTAGA

     .. I  G  Y   H  A  N   N  S  T  E   Q  V  D   T  I  M
1751 GCATCGGCTA CCACGCCAAC AACAGCACAG AGCAAGTGGA CACAATCATG
     CGTAGCCGAT GGTGCGGTTG TTGTCGTGTC TCGTTCACCT GTGTTAGTAC

      E  K  N  V   T  V  T   H  A  Q   D  I  L  E   K  T  H ·
1801 GAAAAGAACG TGACCGTGAC ACACGCCCAG GACATCCTGG AAAAGACACA
     CTTTTCTTGC ACTGGCACTG TGTGCGGGTC CTGTAGGACC TTTTCTGTGT

     . N  G  K   L  C  D  L   D  G  V   K  P  L   I  L  R  D ·
1851 CAACGGGAAG CTGTGCGATC TGGATGGAGT GAAGCCTCTG ATCCTGAGAG
     GTTGCCCTTC GACACGCTAG ACCTACCTCA CTTCGGAGAC TAGGACTCTC
```

FIGURE 2 (cont'd)

```
     .. C  S  V   A  G  W   L  L  G  N   P  M  C   D  E  F
1901 ATTGCAGCGT GGCCGGATGG CTGCTGGGGA ACCCAATGTG CGACGAATTC
     TAACGTCGCA CCGGCCTACC GACGACCCCT TGGGTTACAC GCTGCTTAAG

      I  N  V  P   E  W  S   Y  I  V   E  K  A  N   P  A  N·
1951 ATCAACGTGC CCGAATGGAG CTACATCGTG GAGAAGGCCA ACCCAGCCAA
     TAGTTGCACG GGCTTACCTC GATGTAGCAC CTCTTCCGGT TGGGTCGGTT

     . D  L  C   Y  P  G  N   L  N  D   Y  E  E   L  K  H  L·
2001 CGACCTGTGC TACCCAGGGA ACCTGAACGA CTACGAAGAA CTGAAACACC
     GCTGGACACG ATGGGTCCCT TGGACTTGCT GATGCTTCTT GACTTTGTGG

     .. L  S  R   I  N  H   F  E  K  I   Q  I  I   P  K  S
2051 TGCTGAGCAG AATCAACCAC TTTGAGAAAA TCCAGATCAT CCCCAAAAGC
     ACGACTCGTC TTAGTTGGTG AAACTCTTTT AGGTCTAGTA GGGGTTTTCG

      S  W  S  D   H  E  A   S  S  G   V  S  S  A   C  P  Y·
2101 AGCTGGTCCG ATCACGAAGC CAGCAGCGGA GTGAGCAGCG CCTGCCCATA
     TCGACCAGGC TAGTGCTTCG GTCGTCGCCT CACTCGTCGC GGACGGGTAT
```

FIGURE 2 (cont'd)

```
      . Q  G  K   S  S  F  F   R  N  V   V  W  L   I  K  K  N ·
2151 CCAGGGAAAG TCCAGCTTTT TTAGAAACGT GGTGTGGCTG ATCAAAAAGA
     GGTCCCTTTC AGGTCGAAAA AATCTTTGCA CCACACCGAC TAGTTTTTCT
      .. S  A  Y   P  T  I   K  R  S  Y   N  N  T   N  Q  E
2201 ACAGCGCCTA CCCAACAATC AAGAGAAGCT ACAACAACAC CAACCAGGAA
     TGTCGCGGAT GGGTTGTTAG TTCTCTTCGA TGTTGTTGTG GTTGGTCCTT
       D  L  L  V   L  W  G   I  H  H   P  N  D  A   A  E  Q ·
2251 GATCTGCTGG TGCTGTGGGG GATCCACCAC CCTAACGATG CCGCCGAGCA
     CTAGACGACC ACGACACCCC CTAGGTGGTG GGATTGCTAC GGCGGCTCGT
      . T  R  L   Y  Q  N  P   T  T  Y   I  S  V   G  T  S  T ·
2301 GACAAGGCTG TACCAGAACC CAACCACCTA CATCTCCGTG GGGACAAGCA
     CTGTTCCGAC ATGGTCTTGG GTTGGTGGAT GTAGAGGCAC CCCTGTTCGT
      .. L  N  Q   R  L  V   P  K  I  A   I  R  S   K  V  N
2351 CACTGAACCA GAGACTGGTG CCAAAAATCG CCATCAGATC CAAAGTGAAC
     GTGACTTGGT CTCTGACCAC GGTTTTTAGC GGTAGTCTAG GTTTCACTTG
       G  Q  S  G   R  M  E   F  F  W   T  I  L  K   P  N  D ·
2401 GGGCAGAGCG GAAGAATGGA GTTCTTCTGG ACAATCCTGA AACCCAACGA
     CCCGTCTCGC CTTCTTACCT CAAGAAGACC TGTTAGGACT TTGGGTTGCT
      . A  I  N   F  E  S  N   G  N  F   I  A  P   E  Y  A  Y ·
2451 TGCCATCAAC TTCGAGAGCA ACGGAAACTT CATCGCCCCA GAATACGCCT
     ACGGTAGTTG AAGCTCTCGT TGCCTTTGAA GTAGCGGGGT CTTATGCGGA
```

FIGURE 2 (cont'd)

```
     .. K  I  V   K  K  G   D  S  A  I   M  K  S   E  L  E
2501 ACAAAATCGT GAAGAAAGGG GACAGCGCCA TCATGAAAAG CGAACTGGAA
     TGTTTTAGCA CTTCTTTCCC CTGTCGCGGT AGTACTTTTC GCTTGACCTT
      Y  G  N  C   N  T  K   C  Q  T   P  M  G  A   I  N  S ·
2551 TACGGCAACT GCAACACCAA GTGCCAGACC CCAATGGGGG CCATCAACAG
     ATGCCGTTGA CGTTGTGGTT CACGGTCTGG GGTTACCCCC GGTAGTTGTC
     . S  M  P   F  H  N  I   H  P  L   T  I  G   E  C  P  K ·
2601 CAGCATGCCA TTCCACAACA TCCACCCTCT GACCATCGGG GAATGCCCCA
     GTCGTACGGT AAGGTGTTGT AGGTGGGAGA CTGGTAGCCC CTTACGGGGT
     .. Y  V  K   S  N  R   L  V  L  A   T  G  L   R  N  S
2651 AATACGTGAA AAGCAACAGA CTGGTGCTGG CCACCGGGCT GAGAAACAGC
     TTATGCACTT TTCGTTGTCT GACCACGACC GGTGGCCCGA CTCTTTGTCG
      P  Q  R  E   T  R  G   L  F  G   A  I  A  G   F  I  E ·
2701 CCTCAGAGAG AGACCAGAGG ACTGTTTGGA GCCATCGCCG GCTTTATCGA
     GGAGTCTCTC TCTGGTCTCC TGACAAACCT CGGTAGCGGC CGAAATAGCT
     . G  G  W   Q  G  M  V   D  G  W   Y  G  Y   H  H  S  N ·
2751 GGGAGGATGG CAGGGAATGG TGGATGGCTG GTACGGATAC CACCACAGCA
     CCCTCCTACC GTCCCTTACC ACCTACCGAC CATGCCTATG GTGGTGTCGT
     .. E  Q  G   S  G  Y   A  A  D  K   E  S  T   Q  K  A
2801 ACGAGCAGGG GAGCGGATAC GCCGCCGACA AAGAATCCAC CCAGAAGGCC
     TGCTCGTCCC CTCGCCTATG CGGCGGCTGT TTCTTAGGTG GGTCTTCCGG
```

FIGURE 2 (cont'd)

```
      I  D  G  V   T  N  K   V  N  S   I  I  D  K   M  N  T·
2851 ATCGACGGCG TGACCAACAA AGTGAACAGC ATCATCGACA AAATGAACAC
     TAGCTGCCGC ACTGGTTGTT TCACTTGTCG TAGTAGCTGT TTTACTTGTG
     . Q  F  E   A  V  G  R   E  F  N   N  L  E   R  R  I  E·
2901 CCAGTTTGAG GCCGTGGGAA GGGAGTTTAA CAACCTGGAA AGGAGAATCG
     GGTCAAACTC CGGCACCCTT CCCTCAAATT GTTGGACCTT TCCTCTTAGC
     .. N  L  N   K  K  M   E  D  G  F   L  D  V   W  T  Y
2951 AGAACCTGAA CAAGAAGATG GAGGACGGAT TCCTGGATGT GTGGACCTAC
     TCTTGGACTT GTTCTTCTAC CTCCTGCCTA AGGACCTACA CACCTGGATG
      N  A  E  L   L  V  L   M  E  N   E  R  T  L   D  F  H·
3001 AACGCCGAAC TGCTGGTGCT GATGGAAAAC GAGAGAACCC TGGACTTTCA
     TTGCGGCTTG ACGACCACGA CTACCTTTTG CTCTCTTGGG ACCTGAAAGT
     . D  S  N   V  K  N  L   Y  D  K   V  R  L   Q  L  R  D·
3051 CGACAGCAAC GTGAAGAACC TGTACGACAA AGTGAGGCTG CAGCTGAGGG
     GCTGTCGTTG CACTTCTTGG ACATGCTGTT TCACTCCGAC GTCGACTCCC
     .. N  A  K   E  L  G   N  G  C  F   E  F  Y   H  K  C
3101 ATAACGCCAA GGAGCTGGGC AACGGCTGCT TCGAGTTCTA CCACAAATGC
     TATTGCGGTT CCTCGACCCG TTGCCGACGA AGCTCAAGAT GGTGTTTACG
      D  N  E  C   M  E  S   I  R  N   G  T  Y  N   Y  P  Q·
3151 GATAACGAAT GCATGGAAAG CATCAGAAAC GGAACCTACA ACTACCCCCA
     CTATTGCTTA CGTACCTTTC GTAGTCTTTG CCTTGGATGT TGATGGGGGT
```

FIGURE 2 (cont'd)

```
      . Y  S  E   E  A  R  L   K  R  E   E  I  S   G  V  K  L ·
3201 GTACAGCGAA GAAGCCAGAC TGAAAAGAGA AGAAATCTCC GGAGTGAAAC
     CATGTCGCTT CTTCGGTCTG ACTTTTCTCT TCTTTAGAGG CCTCACTTTG

      .. E  S  I   G  T  Y   Q  I  L  S   I  Y  S   T  V  A
3251 TGGAATCCAT CGGAACCTAC CAGATCCTGA GCATCTACAG CACAGTGGCC
     ACCTTAGGTA GCCTTGGATG GTCTAGGACT CGTAGATGTC GTGTCACCGG

       S  S  L  A   L  A  I   M  M  A   G  L  S  L   W  M  C ·
3301 TCCTCCCTGG CCCTGGCCAT CATGATGGCC GGACTGAGCC TGTGGATGTG
     AGGAGGGACC GGGACCGGTA GTACTACCGG CCTGACTCGG ACACCTACAC

      . S  N  G   S  L  Q  C   R  I  C   I  *
3351 CTCCAACGGA AGCCTGCAGT GCAGAATCTG CATCTGACTC GAGTTTTTAT
     GAGGTTGCCT TCGGACGTCA CGTCTTAGAC GTAGACTGAG CTCAAAAATA

3401 TGACTAGTTA ATCATAAGAT AAATAATATA CAGCATTGTA ACCATCGTCA
     ACTGATCAAT TAGTATTCTA TTTATTATAT GTCGTAACAT TGGTAGCAGT

3451 TCCGTTATAC GGGGAATAAT ATTACCATAC AGTATTATTA AATTTTCTTA
     AGGCAATATG CCCCTTATTA TAATGGTATG TCATAATAAT TTAAAAGAAT
```

FIGURE 2 (cont'd)

3501 CGAAGAATAT AGATCGGTAT TTATCGTTAG TTTATTTTAC ATTTATTAAT

GCTTCTTATA TCTAGCCATA AATAGCAATC AAATAAAATG TAAATAATTA

3551 TAAACATGTC TACTATTACC TGTTATGGAA ATGACAAATT TAGTTATATA

ATTTGTACAG ATGATAATGG ACAATACCTT TACTGTTTAA ATCAATATAT

3601 ATTTATGATA AAATTAAGAT AATAATAATG AAATCAAATA ATTATGTAAA

TAAATACTAT TTTAATTCTA TTATTATTAC TTTAGTTTAT TAATACATTT

3651 TGCTACTAGA TTATGTGAAT TACGAGGAAG AAAGTTTACG AACTGGAAAA

ACGATGATCT AATACACTTA ATGCTCCTTC TTTCAAATGC TTGACCTTTT

3701 AATTAAGTGA ATCTAAAATA TTAGTCGATA ATGTAAAAAA AATAAATGAT

TTAATTCACT TAGATTTTAT AATCAGCTAT TACATTTTTT TTATTTACTA

3751 AAAACTAACC AGTTAAAAAC GGATATGATT ATATACGTTA AGGATATTGA

TTTTGATTGG TCAATTTTTG CCTATACTAA TATATGCAAT TCCTATAACT

3801 TCATAAAGGA AGAGATACTT GCGGTTACTA TGTACACCAA GATCTGGTAT

AGTATTTCCT TCTCTATGAA CGCCAATGAT ACATGTGGTT CTAGACCATA

3851 CTTCTATATC AAATTGGATA TCTCCGTTAT TCGCCGTTAA GGTAAATAAA

GAAGATATAG TTTAACCTAT AGAGGCAATA AGCGGCAATT CCATTTATTT

3901 ATTATTAACT ATTATATATG TAATGAATAT GATATACGAC TTAGCGAAAT

TAATAATTGA TAATATATAC ATTACTTATA CTATATGCTG AATCGCTTTA

3951 GGAATCTGAT ATGACAGAAG TAATAGATGT AGTTGATAAA TTAGTAGGAG

CCTTAGACTA TACTGTCTTC ATTATCTACA TCAACTATTT AATCATCCTC

4001 GATACAATGA TGAAATAGCA GAAATAATAT ATTTGTTTAA TAAATTTATA

CTATGTTACT ACTTTATCGT CTTTATTATA TAAACAAATT ATTTAAATAT

FIGURE 2 (cont'd)

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4051 GAAAAATATA TTGCTAACAT ATCGTTATCA ACTGAATTAT CTAGTATATT
     CTTTTTATAT AACGATTGTA TAGCAATAGT TGACTTAATA GATCATATAA
4101 AAATAATTTT ATAAATTTTA ATAAAAAATA CAATAACGAC ATAAAAGATA
     TTTATTAAAA TATTTAAAAT TATTTTTTAT GTTATTGCTG TATTTTCTAT
4151 TTAAATCTTT AATTCTTGAT CTGAAAAACA CATCTATAAA ACTAGATAAA
     AATTTAGAAA TTAAGAACTA GACTTTTTGT GTAGATATTT TGATCTATTT
4201 AAGTTATTCG ATAAAGATAA TAATGAATCG AACGATGAAA AATTGGAAAC
     TTCAATAAGC TATTTCTATT ATTACTTAGC TTGCTACTTT TTAACCTTTG
4251 AGAAGTTGAT AAGCTAATTT TTTTCATCTA AATAGTATTA TTTTATTGAA
     TCTTCAACTA TTCGATTAAA AAAAGTAGAT TTATCATAAT AAAATAACTT
4301 GTACGAAGTT TTACGTTAGA TAAATAATAA AGGTCGATTT TTATTTTGTT
     CATGCTTCAA AATGCAATCT ATTTATTATT TCCAGCTAAA AATAAAACAA
4351 AAATATCAAA TATGTCATTA TCTGATAAAG ATACAAAAAC ACACGGTGAT
     TTTATAGTTT ATACAGTAAT AGACTATTTC TATGTTTTTG TGTGCCACTA
4401 TATCAACCAT CTAACGAACA GATATTACAA AAAATACGTC GGACTATGGA
     ATAGTTGGTA GATTGCTTGT CTATAATGTT TTTTATGCAG CCTGATACCT
4451 AAACGAAGCT GATAGCCTCA ATAGAAGAAG CATTAAAGAA ATTGTTGTAG
     TTTGCTTCGA CTATCGGAGT TATCTTCTTC GTAATTTCTT TAACAACATC
4501 ATGTTATGAA GAATTGGGAT CATCCTCTCA ACGAAGAAAT AGATAAAGTT
     TACAATACTT CTTAACCCTA GTAGGAGAGT TGCTTCTTTA TCTATTTCAA
4551 CTAAACTGGA AAAATGATAC ATTAAACGAT TTAGATCATC TAAATACAGA
     GATTTGACCT TTTTACTATG TAATTTGCTA AATCTAGTAG ATTTATGTCT
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FIGURE 2 (cont'd)

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4601 TGATAATATT AAGGAAATCA TACAATGTCT GATTAGAGAA TTTGCGTTTA
     ACTATTATAA TTCCTTTAGT ATGTTACAGA CTAATCTCTT AAACGCAAAT

4651 AAAAGATCAA TTCTATTATG TATAGTTATG CTATGGTAAA ACTCAATTCA
     TTTTCTAGTT AAGATAATAC ATATCAATAC GATACCATTT TGAGTTAAGT

4701 GATAACGAAA CATTGAAAGA TAAAATTAAG GATTATTTTA TAGAAACTAT
     CTATTGCTTT GTAACTTTCT ATTTTAATTC CTAATAAAAT ATCTTTGATA

4751 TCTTAAAGAC AAACGTGGTT ATAAACAAAA GCCATTACCC TAGAGCGGCC
     AGAATTTCTG TTTGCACCAA TATTTGTTTT CGGTAATGGG ATCTCGCCGG

4801 GCCACCGCGG TGGAGCTCCA GCTTTTGTTC CCTTTAGTGA GGGTTAATTT
     CGGTGGCGCC ACCTCGAGGT CGAAAACAAG GGAAATCACT CCCAATTAAA

4851 CGAGCTTGGC GTAATCATGG TCATAGCTGT TTCCT
     GCTCGAACCG CATTAGTACC AGTATCGACA AGGA

                         M13R
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METHOD FOR PRODUCING THE FLU VIRUS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. provisional application 61/063,659, filed Apr. 2, 2008.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The invention relates to a method for producing the flu virus using eggs originating from hens immunized against the flu and also to the use of such a method for the manufacture of a flu vaccine.

[0004] 2. Summary of the Related Art

[0005] Three types of flu virus (A, B and C) are currently known, the type A viruses being responsible for animal and human conditions while the type B and type C viruses are especially pathogenic for humans. The type A viruses are subdivided into subtypes according to the antigenic structure of hemagglutinin (HA) and of neuraminidase (NA) which are the principal glycoproteins of the viral envelope. Sixteen subtypes of HA (H1 to H16) and 9 subtypes of NA (N1 to N9) stand out. The subtype of a type A virus is therefore defined by the HA subtype and the NA subtype which are present in the viral envelope. Wild birds constitute the reservoir of all influenza A subtypes. Certain subtypes of influenza virus type A endemically or epidemically (annual epidemics) infect domestic birds (various subtypes including H5N1 and H9N2), horses (principally H3N8), pigs (principally H1N1, H3N2 and H1N2) and also humans (principally H1N1 and H3N2). Dogs, cats and other wild species can also occasionally be infected with certain subtypes (H3N8 and H5N1 in dogs; H5N1 in cats).

[0006] In the veterinary field, poultry farms, and more particularly chicks, chickens, hens and roosters, represent in terms of number the largest population liable to be affected by the flu virus. The avian flu strains of subtypes H5 and H7 may be of two pathotypes: a low path (or LP) pathotype and a high path (or HP) pathotype. The HP strains are responsible for avian flu and derive from the LP strains H5 and H7 after mutations/insertions in particular at the hemagglutinin cleavage site (presence of multiple basic amino acids). Up until now, strict hygiene measures and regular controls are strongly recommended in farms in order to prevent avian flu, and in particular infection with the H5 and H7 subtypes.

[0007] In humans, immunization is recommended against the seasonal circulating viral strains responsible for epidemics that are more or less substantial according to the years. Most of the current vaccines are produced using embryonated hen eggs, these eggs being infected with three different flu virus strains (two strains of type A flu virus having the H3N2 and H1N1 subtype and one strain of type B virus). Eggs fro hens that have not been immunized against the flu are used in order to prevent any phenomenon of interference which could be harmful to the replication of the virus. It is in fact known that the maternal antibodies are transferred to the chicks after having spent time in the egg and protect them against microbial infections during the first days of life, but, in return, they are responsible for deficient immunity if chicks are prematurely immunized against a microbial agent while protective maternal antibodies still exist against this agent. H. Stone et al. (1992, Avian Dis. 36: 1048-1051) have shown that newborn chicks can be passively immunized against Newcastle disease by inoculating yolk from eggs originating from hens immunized against the Newcastle disease virus (NDV). However, if, following the administration of the egg yolks, the chicks are immunized with the NDV virus, a decrease in the immune response to the vaccine is observed. It is also known, according to the studies by Hamal et al. (2006, Poultry Science 65: 1364-1372), that the rates of transfer of protective maternal antibodies to newborn chicks, in particular the transfer of antibodies directed against the NDV virus or the infectious bronchitis virus (IBV), is between 30% and 40% (percentage of the amount of antibodies in the hen's plasma circulating in the blood of the three-day-old chick), which indicates that a large amount of the maternal antibodies is sequestered in the egg. This is confirmed by the studies of J. R. Beck et al. (2003, Avian Dis. 47:1196-1199), which show that all the eggs contain anti-HA antibodies, approximately 3 weeks after having immunized hens with a strain of inactivated flu virus. Finally, it is known, according to the studies by Fontaine et al. (1963, Pathobiologie, 11/9: 611-613), that if embryonated eggs are inoculated with anti-flu serum, the eggs are protected against infection by the flu virus. All these reasons have led those skilled in the art to consider that if eggs from hens immunized against the flu were used, said eggs would, due to the transfer of the maternal antibodies directed against the flu into the eggs, become incapable of producing flu viruses.

[0008] Since the beginning of the 2000s, the economic consequence of avian flu in domestic bird farms has not ceased to increase with the appearance of highly contagious and pathogenic avian virus strains which decimate entire poultry farms. The typing of the HAs of highly pathogenic virus strains shows that almost all of them have the H5 or H7 subtype. It is now feared that virus strains having the H5 or H7 subtype will adapt to humans and may be responsible for a real flu pandemic in humans; serious cases of human flu, admittedly isolated, involving these subtypes have already been reported.

[0009] Faced with the risk that the supply of eggs may no longer always be ensured for manufacturing the flu vaccine, new methods of producing vaccines against the flu are currently directed toward the use of cell culture systems.

SUMMARY OF THE INVENTION

[0010] Despite new methods of producing vaccines, there still exists a need to be able to produce, under any circumstances, in a short period of time and in very large amount, flu virus for manufacturing the flu vaccine. The present invention meets this need by describing a method for producing flu virus based, against all expectations, on the use of eggs originating from hens immunized beforehand against the flu.

[0011] A subject of the invention is in fact:

[0012] A method for producing flu virus comprising:

- **[0013]** a) immunizing a hen by administering a flu vaccine to the hen,
- **[0014]** b) triggering embryogenesis in one or more eggs of the immunized hen,
- **[0015]** c) infecting the one or more embryonated eggs by inoculating a flu virus into the allantoic cavity of the eggs,
- **[0016]** d) incubating the one or more infected embryonated eggs under temperature and humidity conditions that allow replication of the virus, and
- **[0017]** e) harvesting the allantoic fluid of the one or more incubated eggs containing the virus.

[0018] Preferably, the vaccine protects the hens against avian flu.

[0019] Typically, the flu vaccine comprises, in its composition, the hemagglutinin of a flu virus in the form of protein and/or of a gene encoding this protein.

[0020] According to one aspect, the composition of the flu vaccine contains an inactivated whole flu virus.

[0021] According to another aspect, the composition of the flu vaccine contains a product derived from a whole flu virus.

[0022] According to yet another aspect, the composition of the vaccine also contains an adjuvant.

[0023] In another aspect, the composition of the flu vaccine contains an attenuated flu virus.

[0024] According to another aspect, the flu vaccine comprises a vector comprising a nucleic acid fragment encoding the hemagglutinin of a flu virus.

[0025] According to another aspect, the composition of the vaccine also contains an adjuvant.

[0026] Preferably, the vector is a poxvirus.

[0027] In a specific aspect, the vector also comprises a nucleic acid fragment encoding the neuraminidase of a flu virus.

[0028] According to one embodiment of the method according to the invention, the flu virus hemagglutinin in the form of protein and/or of a gene encoding this protein contained in the composition of the vaccine which is used to immunize the hens against the flu and the hemagglutinin of the flu virus which is used to infect the allantoic cavity of the embryonated eggs from the immunized hens are of different subtypes.

[0029] According to another embodiment, the flu virus hemagglutinin in the form of protein and/or of a gene encoding this protein contained in the composition of the vaccine which is used to immunize the hens against the flu and the hemagglutinin of the flu virus which is used to infect the allantoic cavity of the embryonated eggs from the immunized hens are of the same subtype.

[0030] According to yet another embodiment, the flu virus hemagglutinin in the form of protein and/or of a gene encoding this protein contained in the composition of the vaccine which is used to immunize the hens against the flu and the hemagglutinin of the flu virus which is used to infect the allantoic cavity of the embryonated eggs from the immunized hens are identical.

[0031] According to yet another embodiment, the flu virus contained in the composition of the vaccine which is used to immunize the hens against the flu is identical to the flu virus which is used to infect the allantoic cavity of the embryonated eggs from the immunized hens.

[0032] In a particularly preferred embodiment, the flu virus hemagglutinin in the form of protein and/or of a gene encoding this protein contained in the composition of the vaccine which is used to immunize the hens against the flu and the hemagglutinin of the flu virus which is used to infect the allantoic cavity of the embryonated eggs from the immunized hens are independently selected from those of the H5, H6, H7 or H9 subtype.

[0033] In another particularly preferred embodiment, the flu virus hemagglutinin in the form of protein and/or of a gene encoding this protein contained in the composition of the vaccine which is used to immunize the hens against the flu and the hemagglutinin of the flu virus which is used to infect the allantoic cavity of the embryonated eggs from the immunized hens are independently of the H5 or H7 subtype.

[0034] In a specific aspect, the method according to the invention comprises an additional step of purification of the virus.

[0035] In another specific aspect, the method according to the invention comprises an additional step of inactivation of the virus.

[0036] The invention also comprises a flu vaccine obtained using a method according to the invention.

[0037] A subject of the invention is also the use of a method according to the invention for the manufacture of a vaccine for use in preventing the flu.

[0038] In a specific aspect, the use of the method according to the invention serves to manufacture a vaccine for use in preventing pandemic human flu.

[0039] In another specific aspect, the use of a method according to the invention serves to manufacture a vaccine for use in preventing epidemic human flu.

[0040] In yet another aspect, the use of a method according to the invention serves to manufacture a vaccine for use in preventing the flu in members of the equine family, members of the porcine family, members of the canine family, members of the feline family, mustelids and avian species.

[0041] A subject of the invention is also the use of the eggs from hens immunized against the flu for the production of a flu virus.

[0042] Finally, a subject of the invention is the use of the eggs from hens immunized against the flu for the manufacture of a flu vaccine.

[0043] In one aspect, the eggs from immunized hens contain antibodies against the hemagglutinin of flu virus.

[0044] In a further aspect, the eggs from immunized hens contain antibodies against H5, H6, H7 or H9 subtype.

[0045] All patents, patent applications, and publications referred to herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

[0046] FIG. **1**. represents the complete nucleotide sequence of the donor plasmid pJY1394.1.

[0047] FIG. **2**. represents the nucleotide sequence of the insert in the donor plasmid pJY1394.1 comprising the arms flanking the insertion locus F8, and also the H6 vaccinia promoter followed by the synthetic gene modified at the cleavage site encoding the HA of the A/chicken/Indonesia/7/03 strain.

DETAILED DESCRIPTION OF THE INVENTION

[0048] The phrase "flu virus" denotes both flu virus originating from a wild-type strain and flu virus originating from a reassortant strain which results from the reassortment of the genomic segments of one or more wild-type strains with a "master" strain selected for its strong growth potential in eggs. The reassortant strain acquires characteristics of the "master" strain but keeps at least the characteristics of the HA and of the NA of the wild-type strain, which means that the identity of the protein structure of the HA and of the NA of the reassortant strain with respect to the HA and to the NA of the wild-type strain, determined by means of an overall alignment program, is at least 95%, preferably at least 98%, more preferably at least 99%, and even more preferably 100%. The reassortant strain can be obtained by coinfection of a sensitive cell with the wild-type strain and the "master" strain, followed by the appropriate means for selecting the desired reassortant strain. It can also be obtained by reverse genetics from the nucleic acids of the wild-type strain and of the "master" strain and expression in multiplasmid expression systems as described in WO 01/83794 and WO 03/091401 and in Proc. Nat. Acad. Sci. USA 96:9345-9350 (1999). In the case of the high path H5 and H7 strains, a modification of the cleavage site comprising the multiple basic amino acids may be carried out so as to render the reassortant strain low path.

[0049] For ease of language, the term "vaccinal strain" or "vaccinal virus" is used without distinction to denote the flu virus used for the manufacture of the flu vaccine; similarly, the term "infecting strain" or "infecting virus" is used to denote the flu virus used to infect biological material (eggs, animals).

[0050] Moreover, the term "eggs from immunized hens" is used to denote fertilized hen eggs originating from hens which have been immunized beforehand and brought into contact with roosters.

[0051] In general, the flu vaccine used for the immunization of the hens can be manufactured from any strain of flu virus. The vaccinal strain may be a type A virus, but also a type B or C virus. When it is a strain of type A virus, the virus may be any subtype of HA and/or any subtype of NA. It may, for example, be viral strains having the H1N1 or H3N2 subtype currently responsible for epidemic human type A flu.

[0052] There is a great advantage in immunizing hens with a flu virus provided that the immunization confers protection against avian flu.

[0053] Avian flu can pass unnoticed or can be characterized by a set of manifestations, often of respiratory and/or intestinal nature, of more or less great intensity, which more or less impair the general condition of the hens and which can result in the animal's death when the virus strain is highly pathogenic. Avian flu commonly results in a decrease or even a disappearance of the egg-laying activity. The low path virus strains belonging to the H6N2 or H9N2 or even H5 or H7 subtypes are commonly responsible for mild forms of avian flu, generally resulting in a decrease or a disappearance of egg production, but no great mortality. On the other hand, the high path virus strains belonging to the H5 and H7 subtypes (in particular H5N1, H5N2, H5N9, H7N1, H7N4 or H7N7) are highly virulent and cause a very high degree of mortality in hen farms.

[0054] HA is an antigen that is essential in the development of protective immunity against the flu. The vaccine used in the method according to the invention comprises in its composition at least the HA of a flu virus in the form of protein and/or of a gene encoding this protein.

[0055] The term "gene" is intended to mean a nucleic acid comprising nucleotide sequence corresponding to an open reading frame (ORF) and encoding a protein. The gene under the control of regulatory sequences for expression (promoter, enhancer, polyadenylation signal, transcription stop, etc.) can be inserted into the nucleic acid of a vector, in particular a plasmid or a virus. The regulatory sequences can be of exogen or endogen origin with respect to the ORF encoding the protein.

[0056] It may involve the HA of a human strain of flu virus but which is not pathogenic for hens. Preferably, it contains an HA of interest, i.e., an HA which has the same subtype as the HA of a viral strain which is responsible for an avian flu and against which it is sought to immunize and to protect the hens. Preferably, the degree of identity between the protein sequence of the HA present in the vaccine and that of the strain against which it is desired to protect the hens is at least 80%, preferably at least 90%, and even more preferably at least 95%, determined by means of an overall alignment program (such as the Blast program).

[0057] Conventionally, the vaccine comprising the HA is in the form of a composition containing inactivated whole flu virus, or a product derived from the inactivated whole flu virus.

[0058] The expression "product derived from an inactivated whole virus" is intended to mean an inactivated (i.e. noninfectious) vaccinal composition which is prepared from a strain of virus and which comprises at least the HA of said strain of virus. The product derived from a strain of whole virus may be fragmented (or split) virus and, in this case, reference is made to a "split" vaccine. Another product derived from a strain of whole virus is the HA of this strain as such or associated with the NA which has been obtained using extraction and purification methods and, in this case, reference is made to a "subunit" vaccine (EP 0 776 362). The HA may also be integrated secondarily into a virosome. The vaccinal composition containing inactivated whole flu virus, or a product derived from inactivated whole virus, may also contain one or more adjuvants or adjuvant formulations. As an example of nonlimiting adjuvant formulations, mention is made of water-in-oil or oil-in-water emulsions, such as the MF59 emulsion (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 page 183), liposome-based formulations, and formulations based on MPL (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 pages 1186-187), on avridine (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 page 148), on dimethyldioctadecylammonium bromide (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 page 157), on *Corynebacterium parvum*, on saponin, on lysolecithin, on pluronic derivatives (Hunter H. et al. 1991, vaccine, 9: 250-256) (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 page 200 and pages 297-311), on aluminum salts or on combinations thereof (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 pages 249-276). Preferably, the water-in-oil emulsions are composed of liquid paraffin, of a hydrophilic surfactant such as polysorbate 80 or polysorbate 85 and of a lipophilic surfactant such as sorbitan oleate or sorbitan trioleate.

[0059] Examples of emulsions used in the hen are described in Stone et al. (1983, Avian Dis., 27: 688-697; 1993; Avian Dis., 37: 399-405; 1991, Avian Dis., 35: 8-16); in M. Brugh et al. (1983, Am. J; Vet. Res., 44: 72-75); in Woodward L. et al. (1985, Vaccine, 3: 137-144); in (Vaccine Design—The Subunit and Adjuvant Approach Edited by M. Powell and M. Newman, Plenum Press, 1995 page 219). The vaccinal strain generally originates from a wild-type strain which has been isolated in hens, turkeys, ducks, geese or in other avian species, this strain most commonly being low path for hens. As an example of isolates (wild-type strains) used for the preparation of vaccines for protecting hens against avian flu subtype H5 or H7, mention is made of the A/turkey/Wisconsin/68 or A/chicken/Italy/22A/98 isolates which are viral strains having the H5N9 subtype, the A/turkey/England/N-28/73, A/chicken/Mexico/238/94/CPA, A/chicken/

Mexico/232/94/CPA or A/duck/Potsdam/1402/86 isolates which are viral strains having the H5N2 subtype, the A/goose/Guandong/1/1996 isolate which is an HP viral strain having the H5N1 subtype, the A/chicken/Italy/AG-473/1999 or A/chicken/Italy/1067/1999 isolates which are viral strains having the H7N1 subtype, the A/chicken/Pakistan/95 isolate which is an HP strain having the H7N3 subtype, and the A/duck/Potsdam/15/80 isolate which is a viral strain having the H7N7 subtype. As an example of H9N2 viral strains used for protecting domestic birds against avian flu subtype H9, mention is made of the A/chicken/Iran/AV12221/98 and A/chicken/UAE/415/99 isolates. As an example of an H6N2 viral strain used for protecting hens against avian flu subtype H6, mention is made of the A/turkey/Italy/90 isolate. The vaccinal strains used for the manufacture of vaccines may also be reassortants of wild-type strains obtained in particular by reverse genetics. Mention is in particular made of the Re-1 vaccinal strain which is a reassortant strain obtained by reverse recombination of the H5N1 wild-type strain A/goose/Guandong/1/96 with the "master" strain A/PR/8/34, which reproduces very well in eggs (Tian et al., 2005, Virology, 341: 153-162). Another example of a reassortant strain obtained by reverse genetics is the H5N3 strain obtained by genetic reassortment and containing the H5 hemagglutinin of the A/chicken/Vietnam/C58/04 H5N1 strain, the neuraminidase of the A/duck/Germany/1215/73 H2N3 strain and the internal genes of the A/PR/8/34 "master" strain (Webster et al., 2006, Virology, 351: 301-311).

[0060] The vaccines against avian flu usually contain the virus of a single inactivated flu virus strain (monovalent vaccines) but, in certain cases, it may be advantageous to use multivalent vaccines containing several inactivated flu virus strains. This is in particular a vaccine based on H7N1 and H5N9 strains which is a water-in-oil emulsion containing the inactivated vaccinal strains A/chicken/Italy/22A/98 (H5N9) and A/chicken/Italy/1067/1999 (H7N1). The inactivated vaccines may also contain different valences, for example a divalent vaccine against H9N2 avian flu and Newcastle disease. The inactivated vaccines are generally administered parenterally (intramuscularly or subcutaneously). They can also be administered in the form of a spray, as in the case of the Aerovac AI vaccine sold by Investigacion Aplicada, which contains the inactivated vaccinal strain A/chicken/Mexico/232/94/CPA (H5N2). The immunization scheme usually comprises one or two administrations 2 to 4 weeks apart. The vaccinal dose administered varies according to the age of the animals, but usually contains the equivalent of 10 to 200 μl of allantoic fluid having a titer of 10^8 to 10^{10} EID_{50}/ml before inactivation. The vaccinal dose is usually administered in a volume ranging from 0.05 to 1 ml. The preparation of inactivated avian flu vaccines is described by H. Stone (1987, Avian Dis. 31: 483-490). Insofar as the vaccinal strain HA subtype is the same as that of the HA of the strain responsible for pathogenic avian flu, and on the basis of a degree of identity between the protein sequences of the two HAs of the order of 80% to 90%, determined by means of an overall alignment program, the protection rate obtained against the clinical symptoms of avian flu (morbidity and mortality) is generally more than 80%, and preferably more than 90%. This is confirmed by the studies of M. Bublot et al., 2007, Avian Dis. 51: 332-337, which show that a degree of identity of the order of 80% to 90% between the protein sequence of the vaccinal strain HA and the protein sequence of the pathogenic infecting strain HA is sufficient to obtain this protection rate. Furthermore, it is not necessary for the NA subtype of the vaccinal strain to be the same as that of the NA of the pathogenic infecting strain in order to obtain this protection rate. Moreover, the avian flu vaccines greatly reduce the excretion of the virus in immunized animals challenged with infectious virus, demonstrated by a very clear decrease in the viral load observed in oral and cloacal samples (M. Bublot et al., 2007, Avian Dis. 51: 332-337). Another beneficial effect of avian flu vaccines is that of reducing the diffusion of the virus in hen farms.

[0061] According to another embodiment of the method according to the invention, the vaccine used to immunize the hens is in the form of a composition comprising the virus originating from a live attenuated flu virus strain. The vaccinal strain is generally in the form of a reassortant which has been selected subsequent to genetic reassortment between a wild-type strain expressing the HA of interest and secondarily the NA of interest, and a "master" strain which has been cold-adapted and/or which is temperature-sensitive. The reassortant is a viral strain which expresses at its surface the HA and secondarily the NA of interest while at the same time retaining the phenotypic characteristics of the "master" strain which relate to its ability to replicate only within narrow temperature limits, below that of the internal temperature of birds. As a result, the vaccinal strain, after having been administered to hens, replicates in a limited manner and locally. The methods for obtaining these reassortant strains are well known to those skilled in the art and are described in particular in WO 03/091401 and WO 2006/063053 and in 2002, Vaccine 20: 2082-2090. The administration of the vaccine is generally carried out by nebulization. Another means of producing an attenuated strain is to truncate the gene encoding the NS1 protein (Richt J.A. et al., Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine, 2006, J. Virol., 80: 11009-18; Quinlivan M. et al., Attenuation of equine influenza viruses through truncations of the NS1 protein, 2005, J. Virol., 79: 8431-9).

[0062] The vaccinal strains can be produced by any means, using culture techniques on cells such as Vero cells, MDCK cells, PER.C6 cells or chicken embryo cells (CEK, PCJ) and/or using conventional methods of production on embryonated eggs. The methods for harvesting, purifying and, as appropriate, inactivating the virus are also well known to those skilled in the art.

[0063] According to another embodiment of the method according to the invention, the vaccine is in the form of proteins produced in an in vitro expression system. For example, the HA can be produced in an expression system using a recombined baculovirus in insect cells (Crawford J. et al., Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes, 1999, Vaccine, 17: 2265-74). The hemagglutinin can also be expressed in vitro in the form of "virus-like particles" (VLPs) (Prel A. et al., Assessment of the protection afforded by triple baculovirus recombinant coexpressing H5, N3, M1 proteins against a homologous H5N3 low-pathogenicity avian influenza virus challenge in Muscovy ducks, 2007, Avian Dis., 51: 484-9; Pushko P. et al., Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice, 2005, Vaccine, 23: 5751-9) or of a retrovirus-based pseudotype (Szecsi J. et al., 2006, Virol. J., 3: 70). The proteins produced in vitro or the viral particles produced are more or less purified and then adjuvanted with various adjuvants, such as those used in inactivated vaccines.

[0064] According to another embodiment of the method according to the invention, the flu vaccine comprises a vector comprising a nucleic acid fragment encoding the flu virus HA.

[0065] The term "vector" refers to nucleic acid structures which can be propagated in and/or transferred into organisms, cells or cell components. This includes in particular plasmids, viruses, bacteriophages, proviruses, phagemids and artificial chromosomes which are capable of replicating autonomously or which can integrate into the chromosome of a host cell.

[0066] The expression "vector comprising a gene encoding the flu virus HA and/or NA" is intended to mean a vector containing the nucleic acid encoding the HA and/or NA of interest and which, after introduction into an avian cell, expresses the HA and/or NA in this cell. It may be a plasmid expressing the HA of interest, but it is generally a viral vector containing, in its genome, the nucleic acid encoding the HA of interest and expressing the HA of interest in the infected cells. The integration of the nucleic acid encoding the HA into the genome of the viral vector is generally carried out by molecular biology techniques, in particular genetic recombination, cloning, reverse genetics, etc. The HA may or may not be expressed at the surface of the viral vector. Preferably, the viral vector has been conventionally attenuated by multiple passages in vitro or by deletion of certain genes so that the replication of the vector virus in avian cells is sufficiently limited and has no effect on the general state of the hens and is thus considered to be nonpathogenic. As an example of viral vectors, mention is made of avian paramyxoviruses (Ge J., et al., Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses, 2007, J Virol., 81: 150-8); the turkey herpesvirus (HVT) or Marek's disease herpesvirus (Sondermeijer et al., 1993, Vaccine, 11, 349-358); the infectious laryngotracheitis virus (ILTV) (Veits J., et al., Deletion of the non-essential ULO gene of infectious laryngotracheitis (ILT) virus leads to attenuation in chickens, and ULO mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague, 2003, J Gen. Virol., 84: 3343-52; Luschow D. et al., Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene, 2001, Vaccine, 19: 4249-59); adenoviruses (Francois A. et al., Avian adenovirus CELO recombinants expressing VP2 of infectious bursal disease virus induce protection against bursal disease in chickens, 2004, Vaccine, 22: 2351-60; Gao W. et al., Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization, 2006, J Virol., 80: 1959-64; Toro H. et al., Protective avian influenza in ovo vaccination with non-replicating human adenovirus vector, 2007, Vaccine, 25: 2886-91); coronaviruses (Cavanagh, 2007, Vet Res. 38: 281-97; Eriksson, 2006, Clin. Dev. Immunol. 13: 353-60), but use is preferably made, for immunizing hens, of poxviruses, in particular the vaccinia virus, NYVAC (deleted vaccinia virus), the vaccinia virus MVA strain, particularly avipoxes, especially canary pox, ALVAC (attenuated canary pox), pigeon pox, quail pox, turkey pox, sparrow pox and most particularly fowl pox, TROVAC (attenuated fowlpox), which are described in particular in AU 701599B and AU 701781B and in U.S. Pat. No. 5,756,103. As appropriate, the attenuated viral vectors express only the HA of interest: the vaccine involved is in particular the vaccine containing a TROVAC fowlpox vector expressing the HA of the H5N8 flu virus strain (A/turkey/Ireland/1378/83). In other cases, the attenuated viral vector expresses both the HA of interest in combination with an NA of interest, such as the recombinant poxvirus described in 2003, Avian Pathology, 32: 25-32, which expresses both the HA and the NA originating from an H5N1 flu virus strain. An NA of interest is an NA which has the same subtype as the NA of the viral strain against which it is sought to immunize and protect the hens. In yet other cases, the attenuated vector expresses several HAs of interest belonging to different subtypes, as in the case of the recombinant poxvirus described in 2006, Vaccine, 24: 4304-4311, which expresses both the H5 and H7 subtypes. The immunogenic capacity of these vectors can be further reinforced by introducing therein the genes encoding cytokines and/or chemokines which exert an immunostimulatory capacity, such as IL-1, IFN, CSF, GM-CSF, IL-2, IL-12, IL-18 or TNF 5 (2006, Vaccine, 24: 4304-4311). The vaccines based on vectors encoding the flu virus HA can also be adjuvanted in order to increase their immunogenicity.

[0067] The vaccinal compositions containing viral vectors can be administered by various routes, which depend in particular on the vector: for example, transfixion of the alar membrane (poxvirus vector), via the intramuscular, subcutaneous or transdermal route with or without needle (any vector), via the in ovo route (in the 17- to 19-day embryonated egg; for example, HVT/Marek and adenovirus vector), via the ocular or oronasal route, by spray, or in the drinking water (paramyxovirus, coronavirus, adenovirus vector), in one or two injections at least 15 days apart. The vaccinal dose(s) administered is (are) of the order of 1 to 7 $\log_{10}$ 50% infectious unit with a preference for a dose of 2 to 4 $\log_{10}$ for fowlpox vectors. The advantage of an immunization based on a viral vector compared with a conventional immunization using inactivated or attenuated whole flu virus lies in the fact that the immunized animals can be distinguished from the infected animals. Furthermore, immunization with a viral vector promotes the development of a cellular immunity that can reinforce the protection of the animals. As is illustrated in 2007, Avian Dis., 51: 325-331 and 2007, Avian Dis., 51: 498-500, the degree of protection obtained in the hens and the decrease in diffusion of the virus in poultry farms are of the same order as that which is observed with a conventional vaccine containing inactivated flu virus.

[0068] When the immunization of the hens comprises several injections, the vaccine used in the first administration may be different than that used in the second injection or the subsequent injections. Two different attenuated viral vectors may be used, for instance using a recombinant ALVAC vector expressing the HA of interest in the first immunization and a recombinant TROVAC or NYVAC vector expressing the same HA of interest in subsequent immunizations such that the antibody response directed against the ALVAC vector does not prevent the infection of the hen cells by the recombinant TROVAC or NYVAC vector and, consequently, the expression of the HA in the infected cells. It is also possible to use the "prime boost" method, which consists in using, in the first injection, an attenuated viral vector expressing an HA and in using, in the boost injection(s), a vaccine containing for example one or more inactivated vaccinal strains belonging to the same subtype as the HA used in the first immunization, or alternatively carrying out the process in reverse order. Finally, it is possible to carry out a DNA immunization in the first injection, using a plasmid expressing the HA of interest, followed by boost injections using a vaccine containing an inactivated vaccinal strain and/or an attenuated viral vector which express an HA belonging to the same subtype as the HA used in the first immunization or which is identical to the HA used in the first immunization.

[0069] Whatever the type of vaccine administered or the immunization scheme adopted, the protection of the hens against avian flu is provided quite rapidly, generally within a period of 7 to 18 days after the administration of a vaccinal dose. However, in order to ensure protection of the hens against avian flu throughout their egg-laying activity, which lasts approximately one year, one or two booster immunizations, which are carried out within a period of 3 to 16 weeks after the first vaccinal administration, are recommended. Several immunization schemes with inactivated vaccines can be used in egg-laying hens: for example, 2 injections, the first at 3 to 6 weeks old and the second at 16 to 19 weeks old, just before beginning egg laying, or 3 injections, the first around 2 to 4 weeks, the second 3 to 4 weeks later and the third at 16 to 19 weeks old just before beginning egg laying. A booster can also be administered during egg laying. In the "prime-boost" scheme using 2 different vaccines, the chicks can be immunized at 1 day old with a fowlpox vector-based vaccine; they subsequently receive one (at 16 to 19 weeks old just before beginning egg laying) or two (at 3 to 6 weeks old and at 16 to 19 weeks old just before beginning egg laying) immunizations with a vaccine containing inactivated flu virus.

[0070] In the implementation of the method according to the invention, the eggs are preferably taken from the hens once the protection of the hens against avian flu is ensured, which usually occurs within a period of 7 to 18 days after the administration of the vaccine (Bublot M. et al. (2006, Annals of the New York Academy of Sciences 1081: 193-201); Van der Goot et al. (2005, Proc. Natl. Acad. Sc., 102: 18141-6); Ellis et al. (Avian Pathol. 2004, 33, 405-412)).

[0071] Despite the presence of flu antibodies in the eggs of immunized hens, especially of antibodies directed against HA, and in particular of antibodies inhibiting hemagglutination (IHA) which block the penetration of the flu virus into sensitive cells, the method used for producing flu virus from embryonated eggs originating from hens immunized against the flu is conventional. 9- to 14-day embryonated eggs, originating from immunized hens which have been reared preferably in a controlled environment, are used. The embryogenesis process is controlled in the following way: eggs which have been conserved at a temperature between 10 and 20° C., preferably between 16 and 18° C., for a period which does not in general exceed one week after laying, are used. The embryogenesis process is triggered by incubating the eggs at a temperature of 37.5° C.±1° C. in a humid chamber having a relative humidity of 70±10% for a period of between 9 and 14 days. The embryonated eggs are selected by candling, and their allantoic cavities are infected with a dose of virus generally between 2 and 7 $\log_{10}$ $TCID_{50}$ in a small volume (approximately 0.1 to 0.2 ml). The virus is allowed to multiply for a period generally ranging from 1 to 4 days depending on the virulence of the viral strain, and at a temperature which can also vary according to the phenotype of the virus strain and its degree of cold- or hot-adaptation. The temperature for multiplication of the flu virus is generally in a range of from 28 to 39° C. and normally in a temperature range of from 33 to 39° C. The infectious allantoic fluids are then harvested and processed according to the uses intended to be made thereof.

[0072] This method can be used to produce a flu virus whose HA subtype is different than the subtype of the HA contained in the vaccine which was used to immunize the hens. This is, for example, the case where the hens are immunized with an inactivated H5N9 virus in order to protect the hens against H5 avian flu, while the eggs from these hens are infected with an H1N1 or H3N2 flu virus, or even a type B flu virus in order to prepare a vaccine against the epidemic forms of current human flu. According to a variant of the method according to the invention, the HA subtype of the flu virus which is produced using the embryonated eggs from immunized hens has a subtype that is different than the HA which was used to immunize the hens, but, on the other hand, the NA of the flu virus produced has the same subtype as the NA of the virus used in the vaccine. This is, for example, the case where the hens are immunized with an inactivated H5N1 virus strain or a recombinant poxvirus expressing H5 and N1 in order to protect the hens against avian flu, while the embryonated eggs from the immunized hens are infected with an H1N1 virus strain.

[0073] According to another mode of the method according to the invention, the HA of the flu virus produced on embryonated eggs from immunized hens has the same subtype as the HA contained in the vaccine which was used to immunize the hens. This is, for example, the case where the hens are immunized with inactivated H5N1 virus or a recombinant poxvirus expressing H5, while the eggs from the immunized hens are infected with an H5N1 or H5N9 flu virus. Despite the presence of "subtype"-specific crossreactive anti-HA antibodies in the egg (this is the case when the HA contained in the vaccinal composition has the same subtype as the HA of the flu virus strain infecting the embryonated eggs from immunized hens), this has no negative effect on the replication of the virus.

[0074] Furthermore, even more surprisingly, this replication is also not affected in the case where there is complete identity between the flu virus which was used to infect the embryonated eggs from immunized hens and the flu virus which was used to immunize these hens. In this case, this is reflected by the presence in the eggs of a panel of anti-HA antibodies which is even broader since both subtype-specific crossreactive anti-HA antibodies and anti-HA antibodies that are highly specific for the strain (also called strain-specific antibodies) are found (see example 3). Contrary to widely established opinion, the presence of flu antibodies in the egg and, in particular, the presence of anti-HA antibodies does not therefore affect the replication of the flu virus. The amounts of virus and/or of hemagglutinating antigen harvested in the infected allantoic fluids originating from eggs from hens immunized against the flu are of the same order as those harvested in infected allantoic fluids originating from eggs from nonimmunized hens (see examples 1 and 2).

[0075] The method according to the invention can also be taken advantage of for manufacturing a reassortant virus strain. In this case, in a first step, embryonated eggs from immunized hens are coinfected with a wild-type virus strain and a master virus strain which replicates well in embryonated eggs, for instance the A/PR/8/34 strain. In a second step, the infected allantoic fluids containing essentially a mixture of reassortants and the master strain, while the wild-type strain which is not as capable of replicating is generally in very small amount, are collected. The reassortant strain expressing at the same time the phenotypic characteristics of the A/PR/8/34 strain (i.e., its good ability to replicate in embryonated eggs) and the HA and also the NA of the wild-type strain is then selected with successive cloning steps by mixing at each cloning step the harvested infectious allantoic fluid with anti-HA and anti-NA antibodies specific for A/PR/8/34 according to methods well known to those skilled in the art. It is also possible to manufacture a cold-adapted and heat-sensitive reassortant strain with a view to a live attenuated virus vaccine. In this case, in a first step, embryonated eggs from immunized hens are coinfected with a wild-type virus strain and a master strain which has the phenotypic characteristic of being cold-adapted and heat-sensitive. In this case, the temperature for incubation of the infected eggs is a temperature that is lower than normal (the temperature is commonly below 35° C., or even below 30° C.). In a second step, the infected allantoic fluids containing essentially a mixture of reassortants and the master strain, since the cold-sensitive wild-type strain has not replicated, are collected. The reassortant expressing at the same time the phenotypic characteristics of the master strain (in particular the cold-adaptation and/or its thermosensitivity) and the HA and also the NA of the wild-type strain is then selected with successive cloning steps by mixing at each cloning step the harvested infectious allantoic fluid with anti-HA and anti-NA antibodies specific for the master strain according to methods well known to those skilled in the art.

[0076] The method according to the invention can be carried out as first line for preparing vaccines intended to protect breeding colonies of hens and more generally breeding colonies of domestic birds (ducks, turkeys, geese, etc.) against avian flu. The viral strains implicated in the asymptomatic or mild forms of avian flu may be of any subtype, and in particular the H9N2, H6N2, H7N2, H7N3 or H7N1 subtypes. They do not cause any substantial mortality in the breeding colonies but may be the cause of a transient decrease in egg production. The high path viral strains implicated in the serious forms of avian flu which cause substantial mortality in breeding colonies belong generally to the H5 and H7 subtypes, and in particular H5N1, H5N2, H5N8, H5N9, H7N1, H7N3, H7N4 or H7N7.

[0077] Generally, the flu virus hemagglutinin contained in the vaccinal composition which is used to immunize the hens against avian flu of the method according to the invention and the hemagglutinin of the virus which is used to infect the embryonated eggs of the method have the same subtype and are selected in particular from the H5, H6, H7 and H9 subtypes, since it is those which are found principally in the strains of virus responsible for avian flu.

[0078] In a specific aspect, the flu virus hemagglutinin contained in the vaccinal composition which is used to immunize the hens against avian flu of the method according to the invention and the hemagglutinin of the virus which is used to infect the embryonated eggs of the method have the same subtype and are selected from the H5 and H7 subtypes, since it is those which are found in the strains of virus responsible for the serious forms of avian flu and of human flu. The strains of virus responsible for the serious forms of avian flu and/or of human flu which have been characterized generally belong to the H5N1, H5N2, H7N1, H7N3 or H7N7 subtypes.

[0079] Thus, very specifically, a subject of the invention is:

[0080] A method for producing the flu virus comprising:

[0081] a) immunizing a hen with an inactivated flu virus, wherein the hemagglutinin of the virus has the H5 or H7 subtype,

[0082] b) triggering embryogenesis in one or more eggs of the immunized hen,

[0083] c) infecting the one or more embryonated eggs by introducing into the allantoic cavity of the embryonated eggs a flu virus identical to that that used for the immunization,

[0084] d) incubating the one or more infected embryonated eggs under temperature and humidity conditions that allow replication of the virus, and

[0085] e) harvesting the allantoic fluid containing the virus of the one or more incubated eggs.

[0086] When the immunization scheme of the hens comprises two injections, the first injection may be made with a vaccine composition wherein the inactivated flu virus is replaced by a vector, preferably a poxvirus comprising a gene encoding the H5 or H7 subtype of hemagglutinin.

[0087] When the infected allantoic fluids are intended for the production of a flu vaccine, the method according to the invention generally comprises an additional step of purifying the virus strain and is optionally followed or preceded by a viral inactivation step using methods well known to those skilled in the art such as those described in FR 2201079 or in FR 1538322.

[0088] The purification may be brief and may be limited to a step of concentrating the virus by centrifugation after having generally clarified the infected allantoic fluids. The purification may be supplemented with a zonal centrifugation step carried out for example by means of sucrose density gradients (EP 0 7760362). Chromatographic methods may also be carried out in order to purify the virus. A suspension of purified whole viruses which go to make up the composition of the inactivated whole vaccines or of attenuated vaccines is thus obtained.

[0089] The inactivation of the viral suspension can be carried out by conventional means, using β-propiolactone (E. Budowsky et al. 1991, Vaccine, 9: 319-325; 1991, Vaccine, 9: 398-402; 1993, Vaccine, 11: 343-348), ethyleneimine or derivatives (D. King 1991, Avian Dis. 35: 505-514) or formol (EP 0 776 0362).

[0090] The vaccinal composition based on inactivated whole viruses can be formulated with one or more adjuvants. Although conventionally these vaccines may be formulated with aluminum salts or in a water-in-oil or oil-in-water emulsion (in the case of avian flu vaccines), a water-in-oil emulsion composed of liquid paraffin, of a hydrophilic surfactant such as polysorbate 80, polysorbate 83 or polysorbate 85 and of a lipophilic surfactant such as sorbitan oleate, sorbitan sesquioleate or sorbitan trioleate is normally used. Any adjuvant capable of increasing the humoral and/or cellular response against the flu may be used. As an example of nonlimiting adjuvant formulations, mention is made of the MF59® emulsion, the liposome-based formulations, and formulations based on MPL, on *Corynebacterium parvum*, on saponin, on lysolecithin, on pluronic derivatives, or on combinations thereof.

[0091] The purified virus suspension may also undergo subsequent treatments and "flu virus-derived products" produced. For example the viral suspension may be fragmented using detergents or lipid solvents according using methods well known to those skilled in the art in order to manufacture, for example, vaccines based on fragmented or split viruses, virosomes, or subunit vaccines containing the flu virus hemagglutinin. Fragmented or split viruses, virosomes that contain the hemagglutinin of the flu virus, or subunit vaccines containing the flu virus hemagglutinin derived from the purified flu virus suspension are considered as "flu virus-derived products." These flu virus-derived products may similarly be formulated with one or more adjuvants.

[0092] The vaccines obtained by means of the method according to the invention are for use in protecting humans and animals against the flu.

[0093] In the veterinary field, the vaccine can be mainly used in the avian flu prevention field, but it may also be used for preventing or reducing flu symptoms and/or viral secretion in members of the equine family, in particular horses, members of the canine family, in particular dogs, members of the feline family, in particular cats, members of the porcine family, in particular pigs, mustelids, in particular minks and ferrets, and avian species, in particular hen, duck, turkey, quail, guinea-fowl, goose and ostrich. When the vaccinal composition contains an inactivated whole virus strain or a derived product, it is generally administered subcutaneously or intramuscularly, or optionally in the form of nebulized material in poultry breeding colonies. When the vaccine is in the form of a live attenuated virus, it is generally administered oronasally, by spray, in the drinking water or as a drop in the eye. The immunization scheme generally provides for an injection or an injection followed by a booster. The vaccinal dose administered depends on the size and the age of the animal. It usually contains between 20 and 200 μl of allantoic fluid having a titer of 10^8 to 10^{10} EID_{50}/ml before inactivation, injected in a volume of between 0.05 and 1 ml.

[0094] In humans, the vaccine can be used in the field of epidemic flu and pandemic flu prevention. While epidemic flu affects a human population already sensitized by contact (by infection) or by immunization with one (or more) strain(s) of flu virus for which there exists an antigenic relationship with the HA of the virus strain responsible for the epidemic and in which there exists a certain immunity, even if it is only partially effective, pandemic flu affects a human population not sensitized to a new strain of virus because the HA of this new strain has no or too little an antigenic relationship with the prior circulating virus strains.

[0095] The epidemic flu vaccine is intended to protect the human population against seasonal flu forms brought about by circulating seasonal flu virus strains that have an antigenic relationship with prior virus strains that have already circulated. Currently, the flu virus strains responsible for epidemic flu, also called epidemic flu strains are of type A and belong to the H1N1 or H3N2 subtypes or are of type B.

[0096] The pandemic flu vaccine is intended to prevent the infection of the human population against a pandemic flu strain, which is a flu virus strain that has no antigenic relationship in terms of the HA with prior circulating flu virus strains.

[0097] The epidemic or pandemic flu vaccine may be in the form of a live attenuated vaccine or an inactivated vaccine, although an inactivated vaccine is preferred for the prevention of pandemic flu. The vaccine may be in the form of a monovalent vaccine (vaccine prepared from a single flu virus strain) or of a multivalent vaccine (vaccine prepared from several flu virus strains). The composition of the epidemic flu vaccine is currently in the form of a trivalent vaccine prepared from the H3N2 and H1N1 strains and from a type B virus strain. The inactivated vaccine is generally in the form of whole virus, of fragmented virus (split virus) or of virosomes, or in a subunit form containing HA, and optionally contains one or more adjuvants such as those mentioned above. While the live attenuated vaccine is generally administered orally or nasally in order to promote the development of mucosal immunity, the inactivated vaccine can be administered parenterally (intramuscularly or subcutaneously), intradermally or even mucosally (intranasally), or even by combining two different routes of administration as described in WO 01/22992. The immunization scheme generally provides for an injection or an injection followed by a booster. The vaccinal dose administered depends on the age of the individual and on the presence or absence of an adjuvant. Conventionally, the vaccinal dose contains the equivalent of 15 μg of HA of each vaccinal strain contained in the vaccine. This dose may be reduced to approximately 1 to 2 μg of HA when the vaccine is adjuvanted, or increased to 30 μg of HA or even more in elderly individuals or individuals suffering from an immune deficiency.

[0098] Finally, a subject of the invention also comprises:

[0099] The use of eggs from hens immunized against the flu, for the production of flu viruses or for the manufacture of a flu vaccine.

[0100] The following examples illustrate in a nonlimiting manner various embodiments of the invention.

[0101] FIG. **1** represents the complete nucleotide sequence of the donor plasmid pJY1394.1.

[0102] FIG. **2** represents the nucleotide sequence of the insert in the donor plasmid pJY1394.1 comprising the arms flanking the insertion locus F8, and also the H6 vaccinia promoter followed by the synthetic gene modified at the cleavage site encoding the HA of the A/chicken/Indonesia/7/03 strain.

[0103] The various origins of these sequences are the following:

- **[0104]** From 1 to 53: partial sequence of the cloning plasmid comprising the sequence of the M13F primer (underlined).
- **[0105]** From 54 to 1483: sequence of the "left arm" flanking the F8 insertion locus in the genome of the TROVAC fowlpox vector.
- **[0106]** From 1484 to 1568: linker sequence between the left arm and the H6 promoter.
- **[0107]** From 1569 to 1692: sequence of the vaccinia virus H6 promoter.
- **[0108]** From 1693 to 3387: sequence of the modified synthetic HA gene of the A/chicken/Indonesia/7/03 strain; the amino acid sequence is indicated above the nucleotide sequence using the 1-letter code per amino acid.
- **[0109]** From 3388 to 3414: linker sequence between the HA gene and the right arm comprising a TTTTTAT transcription stop sequence of the fowlpox early genes.
- **[0110]** From 3415 to 4790: sequence of the "right arm" flanking the F8 insertion locus in the genome of the TROVAC fowlpox vector.
- **[0111]** From 4791 to 4885: partial sequence of the cloning plasmid comprising the sequence of the M13R primer (underlined).

EXAMPLE 1

Method for Producing Two Vaccinal Strains A/New Caledonia/20/99 (H1N1) and A/New York/55/04 (H3N2) Used to Prepare Vaccines Against Human Epidemic Flu Using Embryonated Eggs from Hens which have been Immunized, Either with Two Injections of an Inactivated Vaccine Containing an Avian Flu Virus Strain A/Chicken/Italy/22A/98 (H5N9), or by an Injection of a Recombinant Avipoxvirus Encoding the HA of an H5N1 Strain Followed by a Second Injection of an Inactivated Vaccine Containing an Avian Flu Virus Strain A/Chicken/Italy/22A/98 (H5N9)

[0112] 1.1) Operating Protocol

[0113] 1.1.1) Construction of the Recombinant Vector vFP2211

[0114] vFP2211 is a recombinant fowlpox virus, into the genome of which has been inserted a synthetic gene encoding the hemagglutinin (HA) of the A/chicken/Indonesia/7/03 H5N1 strain. The HA gene was synthesized so as to obtain an open reading frame which encodes an amino acid sequence identical to the native sequence of the A/chicken/Indonesia/7/03 strain described in the GenBank nucleotide sequence database under the reference EF473080 (or the GenPept protein sequence database under the reference ABO30346), with the exception of the cleavage site. The RERRRKKRG amino acid sequence located between positions 339 and 347 (corresponding to the cleavage site of a high path strain) was replaced with the RE - - - TRG sequence. This cleavage site thus modified corresponds to the cleavage site of the low path strains of subtype H5.

[0115] In a first step, a donor plasmid pJY1394.1 comprising the modified synthetic HA gene under the control of the H6 vaccinia promoter (Taylor J. et al., Vaccine, 1988, 6: 504-508; Guo P. et al., J. Virol., 1989, 63: 4189-4198; Perkus M. et al., J. Virol., 1989, 63: 3829-3836) and bordered by the flanking arms of the F8 insertion locus so as to allow insertion of the HA gene into the F8 insertion locus of the fowlpox vector genome, was constructed. The F8 insertion locus corresponds to the fowlpox gene encoding photolyase described by Srinivasan and Tripathy (2005, Veterinary Microbiology 108: 215-223); this gene is also described under the name FPV158 in the complete sequence of the fowlpox genome (GenBank, reference AF198100). The insertion of the HA gene and of the H6 promoter into the F8 locus results in the deletion of the FPV158 gene from the recombinant fowlpox virus genome. The complete nucleotide sequence of the plasmid pJY1394.1 and also the series of nucleotide sequences bordering the HA gene (which also appear in the complete sequence of the plasmid) are described respectively in FIGS. **1** and **2**.

[0116] The recombinant virus vFP2211 was then obtained by double recombination between the flanking arms of the plasmid pJY1394.1 and the fowlpox genome. For this, primary chicken embryo cells were transfected with the plasmid pJY1394.1 linearized with NotI and infected (multiplicity of infection of 10) with the parental fowlpox strain (TROVAC vector). The TROVAC vector derives from the vaccinal strain of the Diftosec vaccine produced by Merial against fowlpox in chickens. The recombinant viruses were selected by specific hybridization on lysis plaques with a probe for detecting the inserted HA gene. The vFP2211 thus isolated was then produced in rolling bottles on chicken embryo cells.

[0117] 1.1.2) Preparation of the Inactivated H5N9 Vaccine

[0118] The inactivated H5N9 vaccine consisting of the low path A/chicken/Italy/22A/98 H5N9 strain (provided by the laboratory of Ilaria Capua (Istituto Zooprofilattico Sperimentale delle Venezie, Laboratorio Virologia, Padua, Italy)), which was produced on embryonated eggs and inactivated with beta-propiolactone (BPL), and of a water-in-oil emulsion composed of liquid paraffin, of sorbitan oleate and of polysorbate 80, was prepared according to a method equivalent to that described by H. Stone (1987, Avian Dis. 31: 483-490). The HLB (lipophilic hydrophilic balance) of the mixture of surfactants of the emulsion has a value of 5.3. A vaccinal dose is equivalent to 60 μl of allantoic fluid having a titer of $10^{8.9}$ EID_{50}/ml before inactivation.

[0119] 1.1.3) Immunization of Egg-Laying Hens

[0120] Two groups (G1 and G2) of 1-day (D0) Leghorn chicks, having the "specific pathogen free" (SPF) status, were formed. The following immunization scheme was applied to group G1: the 1-day (D0) chicks received subcutaneously, at the base of the neck, using an insulin syringe, 0.2 ml of a viral suspension of vFP2211 having a titer of 3.0 $\log_o$ $CCID_{50}$/0.2 ml. At the age of 8 weeks (W8) the chickens were sexed. Approximately 25 pullets and 16 roosters were conserved and put together. At the age of 17 weeks (W17) they received a booster injection of a vaccinal dose in a volume of 0.5 ml of the monovalent inactivated H5N9 vaccine.

[0121] The following immunization scheme was applied to group G2: the 3-week-old SPF chicks received, by IM injection, in the wishbone, using an insulin syringe, a vaccinal dose in a volume of 0.3 ml of the monovalent inactivated H5N9 vaccine.

[0122] At the age of 8 weeks (W8), the chickens were sexed. Approximately 25 pullets and 16 roosters were conserved and put together. At the age of 17 weeks (W17) they received a booster injection of a vaccinal dose in a volume of 0.5 ml of the monovalent inactivated H5N9 vaccine.

[0123] 1.1.4) Protocol for Infection of Eggs Originating from the Hens of Groups G1 and G2 and from Nonimmunized Hens, with the A/New Caledonia/20/99 IVR-116 (H1N1) or A/New York/55/04 X-157 (H3N2) Virus Strains

[0124] The ability of the reassortant vaccinal strains A/New Caledonia/20/99 (xPR8) called A/New Caledonia/20/99 IVR-116 (H1N1) and A/New York/55/04 (xPR8) called A/New York/55/04 X-157 (H3N2) to replicate was evaluated in the eggs from the hens of group G1 which were laid at 40-41 (test 2) and 49-50 (test 3) weeks of age and in the eggs from the hens of group G2 which were laid at 26-27 (test 1), 40-41 (test 2) and 49-50 (test 3) weeks of age. In parallel, the ability of the virus strains A/New Caledonia/20/99 (xPR8) (H1N1) and A/New York/55/04 (xPR8) (H3N2) to replicate was evaluated in the same manner in the control eggs derived from nonimmunized SPF hens (control group).

[0125] All the eggs laid during the week were conserved in a temperature-controlled chamber (12 to 15° C.). The eggs of the same test and originating from the same group of animals were grouped together and then the embryogenesis process was triggered by incubating the eggs for 10 days at 37-38° C. in a chamber in which the relative humidity was approximately 70-80%. After incubation for 10 days, the vitality of the embryo was verified in each egg by means of a candling device, and the allantoic cavity was pinpointed with a cross. The embryonated eggs of the same test and originating from the same group of animals were grouped together in pools of 8 eggs. The embryonated eggs of the same pool were infected with the same infectious dose of flu virus injected in a volume of 200 μl at the level of the cross after having disinfected and then pierced the shell of the egg. Infectious doses of 10^2, 10^3 and 10^4 EID_{50} (egg infectious dose 50%) of A/New Caledonia/20/99 (xPR8) (H1N1) and A/New York/55/04 (xPR8) (H3N2) virus were tested. Infectious doses of 10^3 and 10^4 EID_{50} per egg were tested in the eggs of tests 1 and 2. Infectious doses of 10^2 and 10^3 EID_{50} per egg were tested in the eggs of test 3. The infected embryonated eggs were then incubated at 34° C. for 48 hours in a chamber in which the relative humidity was 80%, and then placed at +4° C. overnight. The infected allantoic fluid was then taken from each egg. The infecting titer was evaluated on each allantoic fluid taken by measuring the HAU (hemagglutinating unit) titer. Turkey red blood cells were used to measure the HAU titers of the allantoic fluids infected with the A/New York/55/04 (xPR8) (H3N2) strain. Hen red blood cells were used to measure the HAU titers of the allantoic fluids infected with A/New Caledonia/20/99 (xPR8) (H1N1). The HAU titer was expressed by the inverse of the last dilution of the infected allantoic fluid which showed visible hemagglutination in the presence of a suspension of hen or turkey red blood cells at 0.5% in phosphate buffer.

[0126] 1.2) Results

[0127] The means of the HAU titers obtained in the eggs originating from the various groups of immunized and nonimmunized hens as a function of the infectious dose of virus injected into the eggs and as a function of the time at which the eggs were sampled are given in tables I and II.

TABLE I

Hemagglutinating titers obtained in the allantoic fluids as a function of the infecting dose of A/New Caledonia/20/99 (xPR8) (H1N1) used, of the origin of the eggs (G1, G2, nonimmunized control) and of the moment at which the eggs were laid (test 1, test 2, test 3).

Infecting dose (EID_{50}/egg)	100	1000			10 000	
Group	test 3	test 1	test 2	test 3	test 1	test 2
Nonimmunized control	640*	1050	1660	1280	861	1522
G1 (vFP2211/ Vac. Inact. H5N9)	1140	NA	1974	830	NA	1974
G2 (Vac. Inact. H5N9/Vac. Inact. H5N9)	806	905	1660	1159	905	1974

*Hemagglutinating titer (HAU) expressed in the form of the geometric mean of 8 HAU titers/50 µl obtained on the allantoic fluids of the 8 eggs included in each condition.
NA: Not applicable

TABLE II

Hemagglutinating titers obtained in the allantoic fluids as a function of the infecting dose of A/New York/55/04 (xPR8) (H3N2) used, of the origin of the eggs (G1, G2, nonimmunized control) and of the time at which the eggs were laid (test 1, test 2, test 3)

	Infecting dose (EID_{50}/egg)			
	100	1000		10 000
Group	test 3	test 1	test 3	test 1
Nonimmunized control	211*	861	349	830
G1 (vFP2211/Vac. Inact. H5N9)	88	NA	254	NA
G2 (Vac. Inact. H5N9/Vac. Inact. H5N9)	254	987	557	844

*Hemagglutinating titer (HAU) expressed in the form of the geometric mean of 8 HAU titers/50 µl obtained on the allantoic fluids of the 8 eggs included in each condition.
NA: Not applicable

[0128] The results obtained in tables I and II show that the means of the HAU titers of the allantoic fluids of the eggs originating from immunized hens (G1 and G2) are equivalent to the means of the HAU titers of the allantoic fluids of the eggs originating from nonimmunized hens.

[0129] A statistical analysis was carried out in order to study the immunization and dose factors. The individual values of the HAUs were converted to $\log_2$ and then analyzed using a model of variance. The variance heterogeneity was tested using the test for reduced size, performed with the residues of the model (SAS v8.2 software).

[0130] The statistical analysis carried out on the individual values of the HAU titers showed that there was no immunization effect. Neither was there any effect related to the dose of virus which was used to infect the eggs. The HAU titers did not substantially fluctuate during the egg-laying period studied (ranging from 26 weeks (test 1) to 50 weeks (test 3)).

EXAMPLE 2

Method for Producing Two Pandemic Vaccinal Strains A/Chicken/Italy/22A/98 (H5N9) and A/Vietnam/1194/04 NIBRG14 (H5N1) Using Embryonated Eggs from Hens which have been Immunized, Either with Two Injections of an Inactivated Vaccine Containing an Avian Flu Virus Strain A/Chicken/Italy/22A/98 (H5N9), or with an Injection of a Recombinant Avipoxvirus Encoding the HA of an H5N1 Strain Followed by a Second Injection of an Inactivated H5N9 Vaccine Containing an Avian Flu Virus Strain

[0131] 2.1.1) Immunization of Hens

[0132] The protocol for immunizing the hens was identical to that which was used in example 1.

[0133] 2.1.2) Protocol for Infection of Eggs Originating from the Hens of Groups G1 and G2 and from Nonimmunized Hens, with the A/Chicken/Italy/22A/98 (H5N9) and A/Vietnam/1194/04 NIBRG14 (H5N1) Virus Strains

[0134] The infection protocol was the same as that which was used in example 1, except for the following modifications:

[0135] The low path A/chicken/Italy/22A/98 (H5N9) avian strain originating from the Laboratoire Istituto Zooprofilattico Sperimentale delle Venezie, Laboratorio Virologia, Padua, Italy and an attenuated pandemic reassortant vaccinal strain A/Vietnam/1194/04 NIBRG14 (H5N1) were used to infect the eggs. The latter strain was provided by the National Institute for Biological Standards and Control (NIBSC) laboratory, South Mimms, Potters Bar, Herts EN6 3QG, UK and was obtained by reverse genetics, as is described by C. Nicolson et al., Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system, 2005, Vaccine, 23: 2943-2952.

[0136] Infectious doses of 10^2, 10^3, 10^4 and 10^5 EID_{50} (egg infectious dose 50%) were tested for these two strains. Infectious doses of 10^3, 10^4 and 10^5 EID_{50} per egg were tested in the eggs of test 1. Infectious doses of 10^4 and 10^5 EID_{50} per egg were tested in the eggs of test 2. Infectious doses of 10^2 and 10^3 EID_{50} per egg were tested in the eggs of test 3.

[0137] Hen red blood cells were used to measure the HAU titers of the allantoic fluids infected with A/chicken/Italy/22A/98 (H5N9) or A/Vietnam/1194/04 NIBRG14 (H5N1).

[0138] 2.2) Results

[0139] The HAU titers obtained in the eggs originating from the various groups of immunized and nonimmunized hens, as a function of the infectious dose of virus injected into the eggs and as a function of the time at which the eggs were taken, are given in tables III and IV.

TABLE III

Hemagglutinating titers obtained in the allantoic fluids as a function of the infecting dose of A/chicken/Italy/22A/98 (H5N9) used, of the origin of the eggs (G1, G2, nonimmunized control) and of the time at which the eggs were laid (test 1, test 2, test 3)

Infecting dose (EID_{50}/egg)	100	1000		10 000		100 000	
Group	test 3	test 1	test 3	test 1	test 2	test 1	test 2
Nonimmunized control	35*	53	32	53	64	54	53

TABLE III-continued

Hemagglutinating titers obtained in the allantoic fluids as a function of the infecting dose of A/chicken/Italy/22A/98 (H5N9) used, of the origin of the eggs (G1, G2, nonimmunized control) and of the time at which the eggs were laid (test 1, test 2, test 3)

Infecting dose (EID_{50}/egg)	100	1000		10 000		100 000	
Group	test 3	test 1	test 3	test 1	test 2	test 1	test 2
G1 (vFP2211/ Vac. Inact. H5N9)	64	NA	54	NA	59	NA	86
G2 (Vac. Inact. H5N9/Vac. Inact. H5N9)	102	64	57	59	70	102	59

*Hemagglutinating titer (HAU) expressed in the form of the geometric mean of 8 HAU titers/50 μl obtained on the allantoic fluids of the 8 eggs included in each condition.
NA: Not applicable

TABLE IV

Hemagglutinating titers obtained in the allantoic fluids as a function of the infecting dose of A/Vietnam/1194/04 (H5N1) RG14 used, of the origin of the eggs (G1, G2, nonimmunized control) and of the time at which the eggs were laid (test 1, test 2, test 3)

	Infecting dose (EID_{50}/egg)				
	1000	10 000		100 000	
Group	test 1	test 1	test 2	test 1	test 2
Nonimmunized control	194*	232	197	172	181
G1 (vFP2211/Vac. Inact. H5N9)	NA	NA	197	NA	166
G2 (Vac. Inact. H5N9/Vac. Inact. H5N9)	152	279	215	181	235

*Hemagglutinating titer (HAU) expressed in the form of the geometric mean of 8 HAU titers/50 μl obtained on the allantoic fluids of the 8 eggs included in each condition.
NA: Not applicable

[0140] The results given in tables III and IV and also the statistical analysis show that the HAU titers of the allantoic fluids originating from the eggs from the immunized hens (G1 and G2) are similar to the HAU titers of the allantoic fluids of the eggs from the nonimmunized hens, even in the situation where the hens were immunized with a strain identical to that which was used to infect the embryonated eggs originating from these hens (in the case of groups G1 and G2 immunized with the A/chicken/Italy/22A/98 (H5N9) strain). There is no effect, either, related to the dose of virus which is used to infect the eggs. The HAU titers did not substantially fluctuate during the egg-laying period studied (ranging from 26 weeks (test 1) to 50 weeks (test 3)).

[0141] The statistical analysis was carried out as in example 1.

EXAMPLE 3

Analysis of the Anti-H5 Maternal Antibody Response in the Eggs from Hens Which Have Been Immunized with the A/Chicken/Italy/22A/98 (H5N9) Vaccinal Strain

[0142] 3.1) Operating Protocol

[0143] The presence of anti-H5 antibodies in the eggs from the hens of groups G1 and G2 which were immunized with the A/chicken/Italy/22A/98 (H5N9) strain was measured according to the protocol described in paragraph 1.1.1). In parallel, the anti-H5 response was also analyzed in the yolks of eggs from nonimmunized hens (control group).

[0144] The anti-H5 response was analyzed in the yolks of eggs (or vitelline fluids) which were taken at the time of tests 1 (W26-27), 2 (W40-41) and 3 (W49-50). The analysis of the anti-H5 response in the yolks of eggs of test 1 was carried out before embryogenesis (D0) and after embryogenesis (D10) (i.e. after the phase of incubation for 10 days at 37° C.). The analysis of the anti-H5 responses in the yolks of eggs of tests 2 and 3 was carried out only after embryogenesis (D10). The same analysis was also carried out with eggs originating from nonimmunized hens (see paragraph 3.2.2)). The anti-H5 response was also studied in the allantoic fluids of the eggs of test 2 after embryogenesis (D10). The egg yolks were removed by suction using a pipette with a disposable tip. With the exception of test 1, where the egg yolks were kept frozen without having been prediluted, the egg yolks of tests 2 and 3 and the allantoic fluids were prediluted to ⅕th in phosphate buffer before freezing. The diluted egg yolks and the allantoic fluids were conserved frozen until the time of the anti-H5 antibody assay which was carried out using the method of assaying by inhibition of hemagglutination (IHA) of hen red blood cells which takes place in the presence of the A/chicken/Italy/22A/98 (H5N9) strain. The assay was based on the ability of the neutralizing antibodies directed specifically against the HA of the virus to inhibit the "hemagglutinating" activity of the virus. In this test, anti-A/Vietnam/1194/04 sheep serum provided by the NISBC was used as positive control and naive mouse serum was used as negative control. Successive two-fold dilutions of the samples (diluted egg yolks or allantoic fluids) were carried out in a conical-bottomed microplate in order to obtain 50 μl of each of the dilutions per well. 50 μl of a viral suspension having a titer of 4 hemagglutinating units (4HAU) and originating from a clarified allantoic fluid which was infected with the A/chicken/Italy/22A/98 (H5N9) strain provided by the laboratory of Ilaria Capua (Istituto Zooprofilattico Sperimentale delle Venezie, Laboratorio Virologia, Padua, Italy), were added to each well. This was left to incubate for 1 hour at laboratory temperature before adding 50 μl of a solution of hen red blood cells at 0.5% in phosphate buffer. After leaving to stand for 1 hour at +4° C., the test was read. The presence of inhibition of hemagglutination was reflected by the presence of a red spot at the bottom of the microwell, while the presence of hemagglutination was reflected by the presence of a pinkish halo in the microwell. The IHA antibody titer was represented by the inverse of the last dilution where no hemagglutination is observed in the microwell.

[0145] 3.2) Results

[0146] 3.2.1) Analysis of the Anti-H5 Response in the Egg Yolks of Test 1

[0147] At D0, 8 of the 8 egg yolks analyzed of the nonimmunized group were negative in the IHA test, whereas 6 of the 8 egg yolks originating from group 2 were positive in the IHA test, which indicated the presence of anti-H5 antibodies.

[0148] At D10, 4 of the 4 egg yolks analyzed of the nonimmunized group were negative in the IHA test, whereas 4 of the 4 egg yolks originating from group 2 were positive in the IHA test.

[0149] 3.2.2) Analysis of the Anti-H5 Response in the Egg Yolks and the Allantoic Fluids of Test 2

[0150] At D10, 5 of the 5 egg yolks analyzed of the non-immunized group were negative in the IHA test, whereas 4 of the 5 egg yolks originating from group 1, and 4 of the 5 egg yolks originating from group 2, were positive in the IHA test, which indicated the existence of anti-H5 antibodies in these groups. The individual values of the IHA titers obtained are given in table V.

TABLE V

IHA titers for the yolks of eggs taken after embryogenesis (D 10)

Egg yolks	D 10*
Group 1	160
(vFR2211/Vac. Inact. H5N9)	80
	320
	80
	<5
Group 2	80
(Vac. Inact. H5N9/Vac.	160
Inact. H5N9)	40
	40
	<5
Nonimmunized group	<5
	<5
	<5
	<5
	<5

*The titers at D 10 originate from different egg yolks

[0151] On the other hand, all the allantoic fluids originating from groups 1 and 2 were negative in the IHA test, which means that there were no antibodies inhibiting the hemagglutination of the H5N9 flu virus that were detectable in the allantoic fluids.

[0152] 3.2.3) Analysis of the Anti-H5 Response in the Egg Yolks of Test 3

[0153] At D10, 5 of the 5 egg yolks analyzed of the non-immunized group were negative in the IHA test, whereas 5 of the 5 egg yolks originating from group 1 were positive in the IHA test.

[0154] The individual values of the IHA titers are given in table VI.

TABLE VI

IHA titers of the yolks of eggs taken after embryogenesis (D 10) as a function of the origin of the eggs

Egg yolks	D 10
Group 1	80
(vFP2211/Vac. Inact. H5N9)	80
	160
	5
	320
Nonimmunized group	<5
	<5
	<5
	<5
	<5

[0155] In conclusion, on the basis of the results of examples 2 and 3, the presence of maternal anti-H5N9 antibodies, which was revealed in the egg yolks of the 3 tests, has no effect on the viral productivity of the allantoic fluids infected with an H5N1 or H5N9 strain.

EXAMPLE 4

Analysis of the Serological Response of Hens Immunized with the A/Chicken/Italy/22A/98 (H5N9) Vaccinal Strain and of the Maternal Anti-H5 Antibodies Present in the Eggs from These Immunized Hens

[0156] Blood samples were taken, at 28 and 36 weeks of age, from the immunized hens of groups 1 and 2. The eggs laid by these hens were collected at weeks 27 and 37 in order to evaluate the importance of the transfer of the maternal antibodies into the egg yolks. The anti-H5 antibody response was evaluated by means of the hemagglutination inhibition test using as antigen the A/chicken/Italy/22A/98 H5N9 strain homologous to the inactivated vaccine strain. The results are expressed as $\log_{10}$ in table VII.

TABLE VII

Hemagglutination-inhibiting antibody titers (A/chicken/Italy/22A/98 H5N9 antigen) in the serum of immunized egg-laying hens and in the vitellus of the eggs laid by these hens

Weeks of age	Serum 28	Vitellus 27	Serum 36	Vitellus 37
G1	2.10 ± 0.61 (15)*	1.96 ± 0.94 (21)**	1.92 ± 0. 91 (15)	1.63 ± 0.89 (10)
G2	2.20 ± 0.62 (15)	1.81 ± 0.88 (13)	2.26 ± 0.65 (15)	1.43 ± 0.89 (14)

*geometric mean of the hemagglutination-inhibiting antibody titers, expressed as $\log_{10}$ ± standard deviation (number of samples tested per group)
**the lowest dilution tested on the vitelli is 1/10 (1 $\log_{10}$); for the calculation of the means and standard deviation, the values of the eggs that were negative at the 1/10 dilution were placed at 0.7 $\log_{10}$.

TABLE VIII

Hemagglutination-inhibiting antibody titers (A/turkey/Wisconsin/68 H5N9 antigen) in the vitellus of the eggs laid by the immunized hens of groups 1 and 2

Weeks of age	Vitellus 27
G1	1.67 ± 0.79 (21)*
G2	1.39 ± 0.80 (13)

*geometric mean of the hemagglutination-inhibiting antibody titers, expressed as $\log_{10}$ ± standard deviation (number of samples tested per group); the lowest dilution tested is 1/10 (1 $\log_{10}$); for the calculation of the means and standard deviation, the values of the eggs that were negative at the 1/10 dilution were placed at 0.7 $\log_{10}$.

[0157] These results confirm the transmission of the maternal antibodies into the egg yolks of the eggs laid by the immunized hens of groups 1 and 2.

[0158] The homologous anti-H5N9 antibody titers (table VII) in the vitellus are greater than the heterologous anti-H5N9 titers (table VIII), although this difference is not statistically significant.

EXAMPLE 5

Production of the A/Turkey/Wisconsin/68 H5N9 Strain on Eggs Laid by Hens Immunized with an Inactivated H5N9 Vaccine (A/Chicken/Italy/22A/98 Strain) of Group 2

[0159] 5.1. Operating Protocol

[0160] The eggs (approximately 220-230) from the hens of group 2 immunized twice at 3 and 17 weeks of age with the inactivated H5N9 vaccine (A/chicken/Italy/22A/98 strain) were taken during weeks 28 and 29. These eggs, after storage at controlled temperature (between 12 and 15° C.), were incubated at 37° C. for 11 days. After candling in order to verify that the embryos had good viability, the eggs were inoculated in the allantoic cavity with 0.2 ml of a dilution of a stock viral solution of the A/turkey/Wisconsin/68 H5N9 strain having a titer of 9.68 $\log_{10}$ EID_{50}/ml (inoculum). Seven groups of approximately 30 eggs were formed. Four groups (G1 to G4) were inoculated with the following dilutions of the inoculum: 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2}. Three other groups (G5 to G7) were inoculated with the 10^{-3} dilution of the inoculum. After inoculation, the eggs were incubated at 37° C.±1.5° C. in 70%±10% humidity. After incubation for 20 h, the eggs were candled in order to eliminate the dead eggs derived from the inoculation and they were then reincubated under the same conditions. Forty-two hours after the inoculation, the eggs were placed in the cold before harvesting the allantoic fluid. The allantoic fluids of the eggs of groups 1 to 4 were pooled (one pool of fluid per group). The allantoic fluids of the 4 groups of eggs were stored at −70° C. before being titered in terms of hemagglutinating units and of egg infectious dose 50% (EID_{50}).

[0161] 5.2. Results

[0162] The results are given in table IX.

TABLE IX

Hemagglutinating and infectious titers obtained after production of the A/turkey/Wisconsin/68 H5N9 strain on eggs originating from hens immunized with an inactivated vaccine containing the A/chicken/Italy/22A/98 H5N9 strain

Group	Inoculum dilution	Titer in hemagglutinating units ($\log_{10}$)	Infectious titer EID_{50} ($\log_{10}$/ml)
1	10^{-5}	2.25	9.50
2	10^{-4}	2.40	9.33
3	10^{-3}	2.25	9.67
4	10^{-2}	2.40	9.88

[0163] These results show very similar titers between the groups and there is therefore no effect of the dilution of the inoculum. Furthermore, the titers obtained are very high and are comparable to those obtained on eggs having no maternal anti-H5N9 antibodies (historical laboratory data). These results therefore confirm that, under the conditions tested, the presence of maternal antibodies in the eggs does not interfere with the production of a flu virus of the same subtype.

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Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65                  70                  75                  80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
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Glu Lys Ala Asn Pro Ala Asn Asp Leu Cys Tyr Pro Gly Asn Leu Asn
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Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
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Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
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16. (canceled)
17. (canceled)
18. (canceled)
19. (canceled)

20. A flu vaccine composition which comprises a flu virus or a flu virus-derived product obtained by

a) immunizing a hen by administering a flu vaccine to the hen,

b) triggering embryogenesis in one or more eggs of the immunized hen,

c) infecting the one or more embryonated eggs by inoculating a flu virus into the allantoic cavity of the eggs,

d) incubating the one or more infected embryonated eggs under temperature and humidity conditions that allow replication of the virus,

e) harvesting the allantoic fluid of the one or more incubated eggs containing the virus,

f) purifying the virus, and

g) inactivating the virus.

21. A method for preventing infection against an epidemic or a pandemic flu strain in a human subject, which method comprises administering to the human subject an effective amount of a flu vaccine composition as claimed in claim **20**.

22. A method for preventing flu in an animal selected from the group of equine family, porcine family, canine family, feline family, mustelids and avian species, which method comprises administering to the animal an effective amount of a flu vaccine composition as claimed in claim **20**.

* * * * *