Immunization trials with an avian chlamydial MOMP gene recombinant adenovirus

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Key words: *C. psittaci*, major outer membrane protein gene, live recombinant adenovirus vaccine, immunity, security, chick

Abbreviations: AC, avian chlamydiosis; Ad-MOMP, chlamydial MOMP gene recombinant adenovirus; CPE, cytopathic effect; ELD₅₀, 50% egg lethal dose; HEK293, human embryo kidney cell 293; IHA, indirect hemagglutination assay; MOMP, major outer membrane protein; MTT, 3-[4,5-Dimethylthiazolyl]-2,5-diphenyltetra; PCR, polymerase chain reaction; SPF, specific pathogen free; TCID₅₀, 50% tissue culture infective dose

Chicks were inoculated with a live vector vaccine of avian chlamydial MOMP gene recombinant adenovirus to evaluate efficacy, safety and viability of the vaccine. Five batches of the recombinant adenovirus vaccines, which were prepared using the 22nd generation avian chlamydial MOMP gene recombinant adenovirus cultured in HEK293 cells, were used to vaccinate 7 days-old chicks negative for chlamydial antibody. The recombinant adenovirus vaccine was shown to be both safe and effective in inducing specific immunity in vaccinated chicks.

Introduction

Avian chlamydiosis (AC) is an infectious disease caused by *Chlamydophila psittaci*. Named after psittacosis or ornithosis, the bacterium mainly harms domestic and wild birds. Early in the 20th century, it was fashionable to keep parrots as domestic pets. However, many of these birds were affected with ornithosis, leading to sporadic human outbreaks of AC. Hundreds of people died of AC in America, Europe and Africa.^{1,2} AC exists in many parts of the world and can infect almost all bird species, including domestic poultry.³ When affected with AC, the mortality rate in infant birds is high but decreases as birds reach adulthood.

Between the 1960s to 1970s, the poultry industry in Europe suffered serious losses due to AC.^{4,5} Not restricted to Europe, AC cases involving duck, dove, chicken, turkey, goose and wild birds have also been reported in China. Indeed a mean positive rate of 10.96% AC has been reported in 18 provinces.¹ Furthermore, the incidence of AC cases appears to be increasing in some provinces. However, despite this, no effective vaccine has yet been developed to control the disease.

In the current study, specific pathogen free (SPF) chicks were immunized with an avian chlamydial major outer membrane protein (MOMP) gene recombinant adenovirus vector vaccine developed in our laboratory.⁶ The minimal infection dose of the virulent strain, the most effective inoculation route of the vaccine and the minimal effective immunization dose were determined. Vaccine potency, security, protective period, and the conservation period of the vaccine were also examined.

Results

Chick minimal infective dosage test: the test results are presented in Table 1.

The minimal infective dosage of strain CpL was 0.2ml (5.6×10^{10} ELD₅₀), therefore a 0.3ml (8.4×10^{10} ELD₅₀) dose was used clinically.

Chick minimal immunization dosage test: Three groups were inoculated with different doses of chlamydial MOMP gene recombinant adenovirus vaccine (Table 2) and challenged with 0.3 ml (8.4×10^{10} ELD₅₀) of strain CpL per chick (using the nasal drip approach) 21 days post vaccination. Results are presented in Table 2.

It is evident from Table 2, that the minimal immunization dosage of the vaccine is 0.1ml (3.4×10^9 TCID₅₀), thus a dose of 0.2 ml (6.8×10^9 TCID₅₀) was used clinically.

Comparative tests of different vaccination routes: Chicks immunized subcutaneously and intramuscularly were challenged with 0.3 mL (8.4×10^{10} ELD₅₀) strain CpL via nasal drip 21 days post vaccination. Results are presented in Table 3.

On the basis of the results presented in Table 3, it was established that the subcutaneous and intramuscular immunization route yielded similar results (i.e. 90% protection).

Immunization tests: The potency test, security test, protective period test and conservation period of the MOMP gene recombinant adenovirus vaccine are outlined below.

1) Potency test of the vaccine: All five batches of vaccine groups (n = 10/group) were challenged with strain CpL [using a dose of

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Submitted: 01/21/10; Revised: 03/25/10; Accepted: 03/12/10

Previously published online: www.landesbioscience.com/journals/biobugs/article/12115

1.1 x 10¹¹ELD₅₀ in 0.4ml] via nasal drip 21 days post vaccination. Table 4 shows that only two chicks in group 1 and one chick in groups 2, 3 and 4 were affected, while all of the control birds were ill. Batch 5 offered 100% protection.

The protective rate of the five vaccines for poultry ranged from 80–100%, with an average protective rate of 90% (45/50), while all of the control chicks were infected following challenge.

2) *Security tests of the vaccines*: Super-dose safe tests and a single dose repeated inoculation tests were carried out.

(a) *Super-dose inoculation safe tests of the vaccines*: The clinical dosage of the vaccine was 0.2 ml (6.8 x 10⁹TCID₅₀), and chicks were inoculated at 0.6 ml (14.4 x 10⁹TCID₅₀) i.e. three times the clinical dosage. Results are presented in Table 5.

The super-dose inoculated chicks of the five batch vaccines were observed clinically, and well-being, activity levels, appetite, feces, inoculation site, body weight and biological nature were all normal and the vaccine was therefore safe for chicks.

(b) *Single dose repeated inoculation test results of the vaccines*: Groups of 10 chicks/batch were re-inoculated with the same dose 2 weeks after the initial inoculum of 6.8 x 10⁹TCID₅₀ in a volume of 0.2 ml. Results are presented in Table 6.

The single dose re-inoculated chicks were observed clinically and the inoculated chicks' well-being, activity levels, appetite, feces, inoculation site, body weight and biological nature were all found to be normal, thus suggesting that the vaccine is safe for chicks.

3) *Evaluations of the MOMP gene recombinant adenovirus vaccine security*

(a) *Clinical observations of inoculated chicks*: The chicks appeared healthy, with normal activity levels, appetite, normal feces and fleshy feathers.

(b) *Measurement of inoculated chick body weights*: The inoculated chicks' body weights were determined 1, 4, 20, 60 and 170 days post-vaccination. The test results (mean values) are presented in Table 7.

On the basis of statistics software SPSS, $t = 0.462$, $\text{Sig} = 0.668$, $p > 0.05$, the difference was not significant, suggesting that vaccination did not restrain weight gain in inoculated chicks.

(c) *Detection of recombinant adenovirus in feces*: Fresh feces were sampled from the chicks on days 15, 60, 120 and 180 post-vaccination and the recombinant adenovirus DNA was extracted and subjected to PCR to confirm whether or not the recombinant adenoviruses were excreted in the chicks' feces. Results are presented in Fig 1.

No recombinant adenovirus DNAs were detected in feces sampled from the immunized chicks over a six month period, suggesting that the recombinant virus was not shed.

4) *The protective period test of the vaccine*

(a) *Anti-MOMP antibody levels of the immunized chicks*: Blood specimens were sampled from chicks inoculated with the recombinant adenovirus vaccine 15, 21, 60, 120 and 180 days post-vaccination. The anti-MOMP antibody levels of the immunized chicks were then measured using a chlamydial IHA kit as outlined in Table 8.

The anti-MOMP antibody titers rapidly increased to 1:64 21 days post vaccination and remained at 1:32 four months

after vaccination, falling to levels of 1:16 by six months post-immunization.

Lymphocyte transformation test Ten blood samples, taken from chicks in the immunized group before both vaccination and challenge, respectively, were measured for lymphocyte transformation rates using the MTT assay As outlined in Table 9.

On the basis of statistics software SPSS, $t = 23.583$, $\text{Sig} = 0$, $P < 0.05$, the difference was significant.

The vaccine protective period test. The immunized chicks were challenge with virulent strain CpL at various times to measure the protective period of the vaccine. The results are presented in Table 10.

When three groups of chicks vaccinated for 6 months were challenged, the protective rates of the three groups were 9/10, 9/10 and 8/10, respectively, with an average protective rate of 86.7% (26/30). After 8 months the protective rates of the three groups were 9/10, 8/10 and 8/10, respectively, with an average protective rate of 83.3% (25/30). While after 12 months, the protective rates of the three groups were 8/10, 7/10 and 6/10, respectively, with an average protective rate of 70.0% (21/30). Thus, suggesting that the optimum protective period of the vaccine is between 6 to 8 months.

The vaccine conservation period test. Three batches of the vaccines (050418, 050425, 050512) were stored for 12 months and 15 months at -20 to -30°C, and the recombinant adenovirus titers were subsequently measured. Chicks were vaccinated with the three batches and challenged 21 days post-vaccination to establish the vaccine conservation period.

1) *Detection of recombinant adenovirus titers*: Recombinant adenovirus titers of vaccines held for 12 months at -20 to -30°C were measured by means of limited dilution method and the results are presented in Table 11. The number of wells exhibiting CPE are shown in Table 12.

CPE occurred in all four wells in rows 1, 2, 3 and 4 (dilutions of 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶, respectively). Only one well in row 5 (dilution of 10⁻⁷) exhibited CPE while no CPEs were observed evident in rows 6, 7, 8 and 9 (i.e. for dilutions 10⁻⁸–10⁻¹¹)

On the basis of Karber, TCID₅₀ was calculated.

Formula: $\lg \text{TCID}_{50} = L - d(s - 0.5)$,

L: logarithm of maximal dilution; d: difference between dilution logarithm; s: summation of positive tubes proportionment Therefore, in this test, the recombinant adenovirus titer:

$\lg \text{TCID}_{50} = -3 - 1(4.25 - 0.5) = -6.75$

So $\text{TCID}_{50} = 1.8 \times 10^{-7} / 0.2$. Ad-MOMP contents per ml reached 9.0×10^7 . The test indicated that while the vaccine was stored for 12 months at -20 to -30°C, the recombinant adenovirus titers fell from 3.4×10^{10} to 9.0×10^6 .

2) *Challenge test to determine vaccine conservation period*: Three batches of the vaccine (050418, 050425, 050512) were kept for 12 months and 15 months at -20 to -30°C, respectively, and chicks were vaccinated with the three batches of vaccines and subsequently challenged 21 days post-vaccination, to establish the vaccine conservation period. Results are presented in Table 13.

As presented in Table 13, it was shown that the vaccines were kept at -20 to -30°C for 12 months and the recombinant

Table 1. Chick minimal infective dosage test result

Groups	Tested chicks	Infective dosages	Infection route	Affected chicks
A	5	0.2ml (5.60×10^{10} ELD ₅₀)	Nasal drip	3
B	5	0.3ml (8.4×10^{10} ELD ₅₀)	Nasal drip	5
C	5	0.4ml (1.1×10^{11} ELD ₅₀)	Nasal drip	5
control	5	0.4ml normal saline	Nasal drip	0

Table 2. Chick minimal immunization dosage test results

Groups	Tested chicks	Vaccination dosage (recomb.adenovirus)	Strain CpL challenge	Prot. rate (protected chicks/tested chicks)
Immunized A	10	0.1ml (3.4×10^9 TCID ₅₀)	0.3mL	60% (6/10)
Immunized B	10	0.2ml (6.8×10^9 TCID ₅₀)	0.3mL	90% (9/10)
Immunized C	10	0.3ml (1.0×10^{10} TCID ₅₀)	0.3mL	90% (9/10)
HEK293 control	10	0.2ml (0)	0.3mL	0 (9/10)

Table 3. Comparative tests of different (s.c and i.m.) vaccination routes

Groups	Tested chicks	Immunized dose (recomb. adenovirus)	CpL dose	Protective rate (protected chicks/tested chicks)
Subc. vaccination	10	0.2mL (6.8×10^9 TCID ₅₀)	0.3ml	90% (9/10)
Subc. inoc. wild type adenovirus control	10	0.2mL	0.3ml	0 (0/10)
Subc. inoc. 293 cell control	10	0.2mL	0.3ml	0 (0/10)
Muscle vaccination	10	0.2mL (6.8×10^9 TCID ₅₀)	0.3ml	90% (9/10)
Mus. inoc. wild type adenovirus control	10	0.2mL	0.3ml	0(0/10)
Mus. inoc. 293 cell control	10	0.2mL	0.3ml	0(0/10)

Table 4. Potency test results of the 5 batches of vaccine

Groups (vaccine batch number)	Tested chicks	Immunized dose (recomb. adenovirus)	CpL dose	Protective rate (protected chicks/tested chicks)
Immu. group 1 (050418)	10	0.2mL (6.8×10^9 TCID ₅₀)	0.4ml	80% (8/10)
Immu. group 2 (050425)	10	0.2mL (6.8×10^9 TCID ₅₀)	0.4ml	90% (9/10)
Immu. group 3 (050512)	10	0.2mL (6.8×10^9 TCID ₅₀)	0.4ml	90% (9/10)
Immu. group 4 (050519)	10	0.2mL (6.8×10^9 TCID ₅₀)	0.4ml	90% (9/10)
Immu. group 5 (050528)	10	0.2mL (6.8×10^9 TCID ₅₀)	0.4ml	100% (10/10)
HEK293 control	10	0.2mL (0)	0.4ml	0 (0/10)

adenovirus titer was reduced, but in the poultry potency tests, the protective rate of the three vaccine batches was 8/10, 8/10 and 9/10, respectively, with an average protective rate of 83.3%, (25/30). However, extending the storage period to 15 months reduced the protective rate to 7/10, 6/10 and 6/10, respectively, (the mean protective 63.3 percent (19/30). Thus, the conservation period of the vaccines at -20to -30°C is 12 months.

Discussion

Chlamydia sp represent a group of strict intracellular parasitic microorganisms. AC occurs worldwide and has resulted in significant economic loss to the poultry industry. However, as yet there has been no effective method for controlling AC, apart from the administration of tetracycline and other similar antimicrobial

Table 5. Super-dose inoculation safe test results of the vaccines

Groups (vaccine no.)	Tested chicks	Vaccination dose	Safe rates
Safe test group 1 (050418)	10	0.6ml (14.4 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 2 (050425)	10	0.6ml (14.4 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 3 (050512)	10	0.6ml (14.4 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 4 (050519)	10	0.6ml (14.4 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 5(050528)	10	0.6ml (14.4 x 10 ⁹ TCID ₅₀)	10/10
HEK293 control	10	0.6mL (0)	10/10

Table 6. Single dose repeated inoculation test results of the vaccines

Groups (vaccine no.)	Tested chicks	First vaccination dose	Repeated vaccination dose 14 days later	Safe rate
Safe test group 1 (050418)	10	0.2ml	0.2ml (4.8 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 2 (050425)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.2ml (4.8 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 3 (050512)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.2ml (4.8 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 4 (050519)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.2ml (4.8 x 10 ⁹ TCID ₅₀)	10/10
Safe test group 5 (050528)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.2ml (4.8 x 10 ⁹ TCID ₅₀)	10/10
HEK293 control	10	0.2ml	0.2ml	10/10

Table 7. Measurement of inoculated chicks body weights

Groups	1st d	4th d	20th d	60th d	170th d
Immun. group	45.2g	54.2g	87.2g	1130g	3480g
Control group	44.4g	54.4g	94.2g	1230g	3430g

Table 8. Anti-MOMP antibody levels of the immunized chicks

Time post vaccination	15 d	21 d	60 d	120 d	180 d
Mean anti-MOMP antibody titers	1 : 8	1 : 64	1 : 32	1 : 32	1 : 16

agents. Although these measures have some benefit in controlling AC, this is nonetheless an expensive and often hazardous approach, with high drug residues being detected in poultry products (meat and eggs) which can directly threaten human health. While an inactivated vaccine has been developed, it was found to be ineffective; a finding that is perhaps related to the fact that inactivated Chlamydia antigens are unable to induce cell mediated immunity responses in inoculated poultry, thus preventing effective immune protection. Because AC can infect humans as well as birds, it is essential that a safe and stable attenuated live vaccine

strain be made available.^{3,12} Indeed, reports on attenuated live vaccines have to date been relatively infrequent.^{9,10} However, recent work in our laboratory described the construction of a recombinant adenovirus expressing the avian Chlamydia MOMP gene as a novel live attenuated vaccine for AC.⁶ The 22nd generation recombinant adenovirus was inoculated in HEK293 monolayer cells to propagate and produce CPE and its virulence titer reached 3.4 x 10¹⁰TCID₅₀/ml.⁶ In order to prove that the recombinant adenovirus could propagate and express chlamydial target protein MOMP, blood samples from the vaccine inoculated chicks were

Table 9. Lymphocyte transformation results of tested chicks

time	OD570										Average
Before vaccination	0.391	0.435	0.423	0.416	0.384	0.456	0.439	0.446	0.455	0.378	0.418 ± 0.029
Post vaccination	0.649	0.656	0.656	0.673	0.666	0.673	0.657	0.659	0.647	0.652	0.672 ± 0.031

Table 10. The vaccine protective period test results

Groups (vaccinated months)	Tested chicks	Vaccin. dose (Ad-MOMP titer)	Challen. dose (CpL titer)	Protective rate
Immun.1 (6 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	90% (9/10)
Immun.2 (6 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	90% (9/10)
Immun.3 (6 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	80% (8/10)
HEK293 control	10	0.2ml (0)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	0 (0/10)
Immun.1 (8 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	90% (9/10)
Immun.2 (8 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	80% (8/10)
Immun.3 (8 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	80% (8/10)
HEK293 control	10	0.2ml (0)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	0 (0/10)
Immun.1 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	80% (8/10)
Immun.2 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	70% (7/10)
Immun.3 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	60% (6/10)
HEK293 control	10	0.2ml (0)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	0 (0/10)

Table 11. Results of the recombinant adenovirus titers

dilution	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	10 ⁻¹¹
A	*+	+	+	+	+	+	**-	-	-	-	-
B	+	+	+	+	+	+	-	-	-	-	-
C	+	+	+	+	+	+	+	-	-	-	-
D	+	+	+	+	+	+	-	-	-	-	-
E	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-

*+: CPE took place in HEK 293 cells; ** -: no CPE produced in HEK 293 cells

measured using a chlamydial IHA kit and lymphocyte transformation test. All showed positive reactions, which indicated that both cell mediated immune responses and humoral immunity were evoked in the vaccinated chicks. However, propagation of the recombinant adenovirus in the host was very limited, and the protective stage of the vaccine was short (6 months).

In the current study, five batches of the vaccine were prepared using the 22nd generation recombinant adenoviruses to complete an extensive series of trials. It was observed that the vaccines were safe and the protective rate reached 90% in vaccine potency tests. The protection period of the vaccine was six months. Because growth periods of meat-used chicks were

Figure 1. Detection of the recombinant adenovirus in feces by PCR M: DL2000 marker; lane 1: positive control (1170bp in size); lane 2, 3, 4, 5: PCR products of chicks' fecal specimens sampled on days 15, 60, 120 and 180 post-vaccination

five to six months, each batch of chicks were inoculated only once with the vaccine and protected for the required period of time i.e. until entry to market. However, egg-laying chicks must be vaccinated twice, new born chicks were inoculated initially, and then a second time on entering the egg production stage. Thus, the vaccinated hens would exhibit resistance against AC in the peak egg-laying stage. In tests, the 22nd generation recombinant adenoviruses displayed good genetic stability and immunogenicity.

In the vaccine conservation period test, although the live vaccine titer decreased following storage for 12 months at -20 to -30°C, the results of the chick potency test were encouraging. Ad-MOMP, being both safe and effective in inducing specific immunity in vaccinated chicks, shows promise as the first effective live recombinant vaccine against AC.

Materials and Methods

Preparation of chlamydial MOMP gene recombinant adenovirus vaccine. Generation 22 of chlamydial MOMP gene recombinant adenovirus (Ad-MOMP),⁶ containing 3.4×10^{10} TCID₅₀/ml of virulence value was inoculated into HEK293 cells. Five batches (batches no. 050418, 050425, 050512, 050519, 050528) of Ad-MOMP vaccines were produced for chick immunization tests. Control chicks were inoculated with HEK293 cell suspension.

***C. psittaci* virulent strain CpL.** The virulent strain CpL was originally isolated from an AC infected chick and fully characterized in our laboratory. The CpL virulent value was 2.8×10^{11} ELD₅₀/0.4ml, as determined using the chicken embryo method. In potency tests, birds were challenged with the CpL strain through the nasal drip route.

Dosage. One hundred, 7-day-old SPF chicks (50 each of male and female) were transported from Shandong province poultry research institute and used in the following tests:

1) *Minimal infective dosage test:* The four groups (each consisting of 5 chicks, n = 5) included Group 1: 0.2ml of

strain CpL; Group2: 0.3ml of strain CpL; Group 3: 0.4ml of strain CpL and Group 4: the control group (not infected with CpL).

2) *Minimal immunization dosage test via the muscle route:* The four groups (n = 5) were group 1: immunization with 0.1ml of Ad-MOMP; group 2: immunization with 0.2ml of Ad-MOMP; group 3: immunization with 0.3ml of Ad-MOMP; group 4: non-immunized.

3) *Ad-MOMP vaccination test via the muscle route:* The three groups (n = 10, inoculum 0.2 ml/chick; vaccination site at the left or right side of the bird's breast) included group 1: vaccine immune group; group2: wild type adenovirus control, and group 3: HEK293 cell control group.

4) *Ad-MOMP vaccination test via the subcutaneous route:* The three groups (n = 10, inoculum 0.2 ml /chick; vaccination site at the subcutaneous area of bird's breast or thigh) included group 1: vaccine immune group; group 2: wild type adenovirus control group, and group 3: HEK293 cell control group.

Chick Ad-MOMP vaccine immune tests. For these tests, 300 (150 each of male and female) 7-day old crossbred meat chicks were transported from Gansu province breeding poultry farm. For all 300 animals, serum antibodies to *C. psittaci* were found to be negative (lower than 1:4). The chicks were grouped randomly into vaccine potency test groups, vaccine security test groups and vaccine conservation period test groups.

Measurement of antibody levels to MOMP in chicks. The serum antibody levels against MOMP of the chicks inoculated with Ad-MOMP vaccine in the vaccine conservation period tests were measured on the days 15, 21, 60, 120 and 180 post vaccination, using a chlamydial IHA kit produced by the Lanzhou Veterinary Research Institute.

Lymphocyte transformation test. The lymphocyte transformation rate in the immunized chicks was determined by the MTT method.⁷ Lymphocytes were separated from peripheral blood sampled from the immunized chicks as follows:

1) 2 ml of fresh blood sampled from immunized chicks was added to a 10 ml test tube containing 2 ml balanced salt solution for dilution.

2) The blood was mixed carefully with a transfer pipette.

3) 4 ml of the diluted chick blood was added (carefully, along the tube wall) to a centrifuge tube containing 30 ml of lymphocyte separation liquid, and the mixed liquid was centrifuged for 30 to 40 minutes at 400g at 18 to 20°C.

4) The lymphocyte layer was carefully removed and transferred to a centrifuge tube.

5) Three times the volumes of balance salt solution was added to the centrifuge tube containing the lymphocyte layer and mixed carefully. The mixed liquid was centrifuged for 10 minutes at 60 to 100g under 18 to 20°C condition and then supernatant fluid was removed. This step was then repeated.

6) The lymphocytes were suspended in RPMI 1640 medium and counted under a microscope. A suspension of 1×10^7 lymphocytes/ml was then prepared.

Testing the security of the MOMP recombinant adenovirus. For vaccine safe tests, well-being, activity level, appetite, feces,

Table 12. Ad-MOMP caused HEK 293 CPE in each of four wells

dilution	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	10 ⁻¹¹
Normal wells	0	0	0	0	3	4	4	4	4
CPE wells	4	4	4	4	1	0	0	0	0

Table 13. Potency tests to determine vaccine conservation period

Vaccine no. (kept months)	Tested chicks	Vaccinated dose (Ad-MOMP titer)	Challenging dose (CpL titer)	Protective rate
No. 050418 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	80% (8/10)
No. 050425 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	80% (8/10)
No. 050512 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	90% (9/10)
HEK293 control	10	0.2ml (0)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	0 (0/10)
No. 050418 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	70% (7/10)
No. 050425 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	60% (6/10)
No. 050512 (12 months)	10	0.2ml (6.8 x 10 ⁹ TCID ₅₀)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	60% (6/10)
HEK293 control	10	0.2ml (0)	0.4ml (1.1 x 10 ¹¹ ELD ₅₀)	0 (0/10)

body weight and biological nature of the vaccinated chicks were observed from the second day post-vaccination. PCR was used to assay for recombinant adenovirus in the inoculated chick's feces in order to determine whether or not the recombinant adenoviruses were discharged with the feces.

Acknowledgements

The tests were supported by the national “863 program” funds (project No. 2003AA241110) and the national technology-science support program funds (project No 2007BAD40B02).

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