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Evaluation of the Immune Effect of Oral Sustained-Release Vaccine pcDNA3-3-1E-PLGA Microsphere of *Eimeria acervulina* in Chicken

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ABSTRACT

To evaluate the immune effect of oral sustained-release vaccine pcDNA3-3-1E-PLGA microspheres of *Eimeria acervulina* in chicken, seven day-old chickens were randomly divided into three groups, namely the DNA microsphere vaccine of pcDNA3-3-1E-PLGA microspheres of *Eimeria acervulina* oral immunization group, the naked plasmid DNA vaccine pcDNA3-3-1E of *Eimeria acervulina* oral immunization group and control group, with 30 chickens in each group. The chickens were immunized with a dose of 50μg/chicken, respectively, and strengthened immunity one week later. At 7th, 14th, 21st, 28th, 35th day post-injection, respectively, after extracting the total DNA from fresh tissues of immunized chickens, the *3-1E* gene was amplified by PCR, the serum IgG antibody was detected by routine ELISA, peripheral blood lymphocyte proliferation response was evaluated by cell proliferation assay. The results showed that a target band of 583 bp was obtained by PCR in DNA vaccine PLGA microspheres group at 7th, 14th, 21st, 28th, 35th day post-injection. No *3-1E* gene target band was found in the control group and the naked plasmid DNA vaccine group. It indicated that plasmid pcDNA3-1E existed in tissues of immunized chickens with DNA microsphere vaccine, and the plasmid pcDNA3-1E released from microspheres sustained at least 35days. The lymphocyte proliferation of DNA vaccine PLGA microspheres group significantly increased ($P < 0.05$) and the peripheral blood IgG levels were significantly higher ($P<0.05$) than that of the naked plasmid group and the control group, but no difference was found between naked plasmid DNA vaccine group and the control group ($P>0.05$). The results of the present study indicated that the immune response was induced in the chickens after oral delivery of oral sustained-release vaccine pcDNA3-3-1E-PLGA microspheres of *Eimeria acervulina*.

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Key words
***Eimeria acervulina, 3-1E* gene, pcDNA3-3-1E-PLGA microspheres, Immune response**

INTRODUCTION

Coccidiosis was a severe disease in chicken that widely spreads with higher morbidity and mortality (Garbi *et al*., 2015). It was caused by several species of protozoan parasites belonging to the genus *Eimeria* (Abbas *et al*., 2012). *Eimeria acervulina* (*E. acervulina*) was one of the most pathogenic *Eimeria* species in terms of distribution, frequency, and economic losses. The chickens at various ages could infected with it. The *E. acervulina* was not just causing clinical coccidiosis. It was increasing the possibility of bacterial enteritis caused by the secondary bacterial infections, and increasing the mortality (Arczewska, 2015; Lee *et al*., 2012; Lillehoj *et al*., 2007). In present, the prophylactic chemotherapy using ionophores and synthetic drugs and immunoprophylaxis using conventional vaccines were still main method for the control of coccidiosis (Constantinoiu *et al*., 2011; Jenkins *et al*., 2010; Sharma and Shyma, 2015). However, the long-term use of the anti-coccidial drugs was implicated for the development of drug resistance (Tan *et al*., 2017; Yang *et al*., 2015) and the accumulation of drug residues in livestock products (Arczewska, 2015; Ahad *et al*., 2016; Chapman and Jeffers, 2014). Immunoprophylaxis using conventional vaccines including live or attenuated vaccines could cause severe adverse reactions, and had the disadvantage shortcomings of high cost (Zhao *et al*., 2014). Therefore, there is an urgent need for an alternative method of control of coccidiosis in chicken.

The DNA vaccines are plasmids which contains

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specific antigen polypeptide DNA encoding, and are accomplished by injecting purified genes coding for antigens directly into the animal, which is then followed by expression of protein in the host cells resulting in the presentation of normally processed or modified forms of the protein to the immune system of the animal, then stimulating immune response to accomplish the purpose of prevention or treatment (Zhao *et al*., 2014). However, with sole use of naked DNA vaccine can be degraded and cleared rapidly in the body and the immune activity is poor, thus causing repeated use of vaccine, which undoubtedly limits its clinical application. To solve the difficulty, an adjuvant induced immune response and sustained release effect is necessary (Li *et al*., 2015; Spatz *et al*., 2013). Biodegradable polymer microsphere is a kind of controlled vaccine delivery system showed an adjuvant effect, which can wrap plenty of antigens and protect it from rapid degradation under physiological conditions (Li *et al*., 2015). The DNA vaccines encapsulated in microparticles can effectively simulate the traditional vaccine multiple immune process, which can continue to provide the entrapped antigen in a certain period of time, thereby reducing inoculating times and drug costs (Carletti *et al*., 2013; Ma *et al*., 2012). In this study, the effects of oral immunization with oral sustained-release microspheres pcDNA3-3-1E- PLGA vaccine containing major specific antigen gene 3-1E of *Eimeria acervulina* in chicken were studied in order to provide new dosage forms for chicken coccidiosis DNA vaccine.

MATERIALS AND METHODS

Vaccines and main reagents

Naked plasmid DNA vaccine pcDNA3-3-1E containing major specific antigen gene 3-1E of *Eimeria acervulina* of chicken, and DNA microsphere vaccine of pcDNA3-3-1E-PLGA of *Eimeria acervulina* of chicken were constructed and conserved by the Laboratory of Parasitology at College of Veterinary Medicine, Agricultural University of Hebei, China. Taq DNA polymerase, DNA marker DL2000, Plasmid large-scale extraction kit, Animal genomic DNA extraction kit were purchased from Sun Biotechnology (Beijing, China) Co., Ltd.

Design and synthesis of primers

A pair of primer of the protective antigen *3-1E* gene of *E. acervulina* was designed on the nucleotide sequence of *E. acervulina* Baoding strain (Lu *et al*., 2009) by Primer 5.0 software, and synthesized by Sangon Biotech. Co., Ltd. (Shanghai, China). Expected amplified fragment was 583pb. Primers sequences are given below:

p1: F 5'-ACTCAGTTAAAATGGGTGA-3';
p2: R 5'– GAAGTTGTTGTTATGAAAGG-3'.

Preparation of DNA vaccine and chickens immunization

Naked plasmid pcDNA3-1E containing merozoite surface antigen *3-1E* gene of *E. acervulina* was extracted using plasmid large-scale extraction kit. The purified plasmid DNA (9.25 μg/μL, $OD_{260/280}$ 1.8) was diluted to 1 μg/μL with PBS to reserve. The DNA microsphere vaccine pcDNA3-3-1E-PLGA containing 50 μg pcDNA3-3-1E-PLGA was prepared into suspension with 1mL sterilized deionized water.

One day old chickens were obtained from a chicken farm in Hebei province, China. Ninety chickens at the age of 7 days were randomly divided into three groups. The group 1 was inoculated with the DNA microsphere vaccine of pcDNA3-3-1E-PLGA of *Eimeria acervulina* at dose of 50μg/chicken by oral immunization, and strengthened immunity one week later. The group 2 was inoculated with the naked plasmid pcDNA3-1E at dose of 50μg/chicken by the same method. The group 3 was the control group, given physiological saline at 50 μL/chicken by the same method.

Detection of 3-1E *gene in various tissues in chicken*

The tissues of stomach, bowel, heart, liver, spleen lung and kidney samples of five chickens in each group were collected on 7^{th}, 14^{th}, 21^{st}, 28^{th}, 35^{th} day after primary inoculation, respectively. After extracting the total DNA from fresh tissues by animal genomic DNA extraction kit, the *3-1E* gene was amplified by PCR with the *3-1E* gene specific primers. 25 μL PCR reaction system was the reaction mixture consisted of 10×buffer 2.5 μL, total DNA template 4 μL, forward primer p1 1μL, reverse primer p2 1μL, dNTP 3 μL, Taq DNA polymerase 0.5 μL and ddH_2O up to 25 μL. PCR was initially denatured at 94 ℃ for 5 min, 30 cycles of denaturation at 94℃ for 45 sec, annealing at 55 ℃ for 45 sec, and extension at 72℃ for 1 min. The reaction was subjected for final extension at 72℃ for 10 min. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under UV light.

Detection of serum IgG antibody and peripheral blood lymphocyte proliferation

Blood samples of five chickens in each group were collected on 7^{th}, 14^{th}, 21^{st}, 28^{th}, 35^{th} day after primary inoculation, respectively. The serum IgG antibodies were detected by routine ELISA, and the peripheral blood lymphocyte proliferation responses were evaluated by cell proliferation assay by MTT method.

RESULTS

Expression of pcDNA3-1E *gene*

The distribution of DNA vaccine in different tissues were detected by 30 copies sensitive grade of PCR detection system. PCR amplification with chicken genomic DNA as template in the DNA microspheres vaccine group showed in *3-1E* gene target band of 583 bp on 7th, 14th, 21st, 28th, 35th day after primary inoculation, respectively (Fig. 1). It showed that that plasmid pcDNA3-1E existed in tissues of immunized chickens with DNA microsphere vaccine, and the plasmid pcDNA3-1E released from microspheres sustained at least 35 days. In the control group and the naked plasmid DNA vaccine group, *3-1E* gene target band was not found.

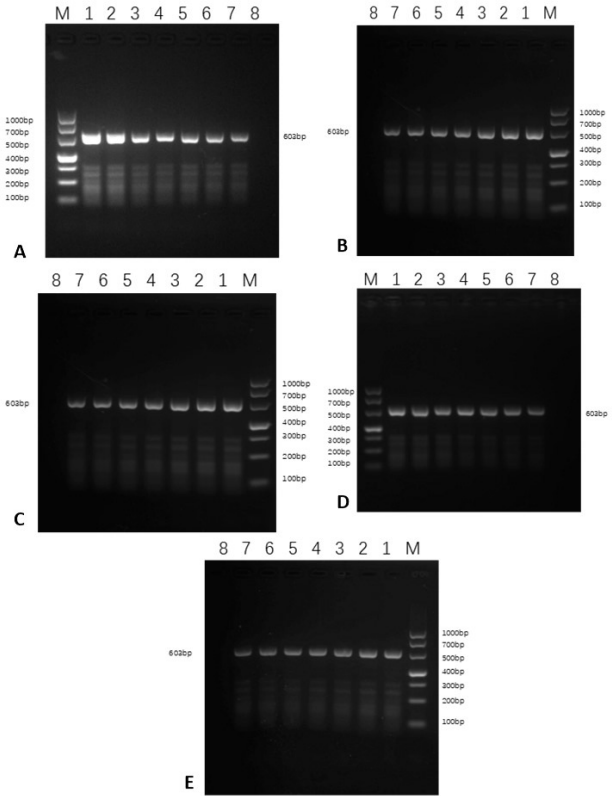

Fig. 1. The tissue distribution of *3-1E* gene in chicken in DNA microspheres vaccine group.
A-E represent PCR amplification of the *3-1E* gene in chicken at 7th, 14th, 21st, 28th, 35th day after primary inoculation, respectively. Lane M, DL1000 DNA Marker; Lanes 1-8, show products of the gastric, duodenal, splenic, lung, heart, liver, intestinal sample and blank control, respectively.

Serum IgG

The results of specific IgG antibody levels of each group were shown in Table I. The level of group 1 was significanly higher than the other groups ($p<0.05$) at 7th day after primary inoculation, increased progressively at 14th day and 21st, and reached the peak value at 21st day, then was still higher than other at 35th day. The results showed that the DNA microsphere vaccine group (Group 1) antibody level was significantly higher than that in control group (group 3) and naked plasmid DNA vaccine group (group 2) ($P<0.05$). It was not significantly different between the control group (group 3) and naked plasmid DNA vaccine group (group 2) ($P>0.05$).

Peripheral blood lymphocyte proliferation

The results of peripheral blood lymphocyte proliferation of chicken in each group were shown in Table II. The peripheral blood lymphocyte proliferation response was no remarkable difference in each groups at 7th day after primary inoculation ($p>0.05$). Increased progressively at 14th day, and reached the peak value at 21st day, then was still higher than other at 35th day in group 1. The results showed that peripheral blood lymphocyte proliferation response in the DNA microsphere vaccine group (group 1) was significantly higher than that in control group (group 3) and naked plasmid DNA vaccine group (group 2) ($P<0.05$). It was not significantly different between the control group (group 3) and naked plasmid DNA vaccine group (group 2) ($P>0.05$).

DISCUSSION

A prominent priority of DNA vaccine lies in that it can induce abroad cell immunoreaction and form long perdured immunity memory. But injecting methods of administration of naked plasmid DNA vaccine can result in high production cost and intense irritant effect on tissue. By oral immunization, naked DNA vaccine can be digested by gastric acid and pepsin, result in lower immune response owing to inactivation of plasmid vaccine. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled DNA delivery carrier can protect the DNA against the intestinal pH, enzyme inactivation, degradation, and activate antigen-presenting cells (APCs) and antigen-specific T cell (Pavot *et al*., 2014; Jia *et al*., 2017) . The oral administration of DNA microsphere vaccine induced both mucosal immune response and systemic immune response effectively (Brekke *et al*., 2014; Neuhaus *et al*., 2014), led to a protective immunologic effect, and had effective adjuvant effect (Hatzifoti *et al*., 2010; Basu *et al*., 2005). It has proved that the microspheres by oral administration were transported and absorbed through the intestinal mucosa (Zastre *et al*., 2013; Li *et al*., 2000; Matsunaga *et al*., 2001). Generally, absorption site of PLGA microspheres is

Table I. The serum IgG antibody levels in chicken ($\bar{x}$±s) (g/L).

Group	Post-immunization days				
	7	14	21	28	35
DNA microsphere vaccine	17.56±1.83^{b}	27.55±1.43^{c}	30.86±1.52^{c}	31.20±1.63^{c}	31.15±1.33^{c}
Naked plasmid DNA vaccine	11.40±1.44^{a}	11.37±1.69^{a}	12.48±1.38^{a}	12.39±1.72^{a}	12.42±1.62^{a}
Control	11.22±1.42^{a}	11.45±1.85^{a}	12.50±1.46^{a}	12.25±1.65^{a}	12.35±1.45^{a}

Note: Data marked with different letters represent significant difference (P<0.05). Data marked with the same letters represent no significant difference (P>0.05) (the same as below).

Table II. The peripheral blood lymphocyte proliferation response in chicken ($\bar{x}$±s).

Group	Post-immunization days				
	7	14	21	28	35
DNA microsphere vaccine	0.276±0.034^{a}	0.620±0.031^{b}	0.627±0.029^{b}	0.625±0.026^{b}	0.623±0.021^{b}
Naked plasmid DNA vaccine	0.241±0.029^{a}	0.243±0.036^{a}	0.249±0.033^{a}	0.252±0.029^{a}	0.242±0.023^{a}
Control	0.245±0.036^{a}	0.234±0.032^{a}	0.250±0.023^{a}	0.253±0.032^{a}	0.243±0.025^{a}

Note: Data marked with different letters represent significant difference (P<0.05). Data marked with the same letters represent no significant difference (P>0.05) (the same as below).

mainly the Payer's junction in intestinal mucosa, which is accomplished by non-specific binding with M cells, followed by phagocytosis and transport by M cells into gut-associated lymphoid tissue (GALTs) and peyer's patches, lymph node, spleen, and other immune organs to induce a systemic immune response and local mucosal immune responses. DNA vaccine microspheres with specific targeting APCs could specifically activate macrophages, dendritc cells and other APCs and increase the uptake of APCs, to improve the transport efficiency and targeting ability, thus improve antigen expression, enhance the immune effect. In the process of local degradation, the DNA vaccine microspheres can continue to slow release the entrapped vaccine antigen in a certain period of time, constantly stimulate immune cells to produce immune response, consequently be used for controlled drug delivery systems (Li *et al*., 2017; Seok *et al*., 2017).

In this study, chickens were inoculated with the DNA microsphere vaccine of pcDNA3-3-1E-PLGA of *Eimeria acervulina* and the naked plasmid pcDNA3-1E by oral immunization, and whether the recombinant plasmids were expressed in chickens was detected using RT-PCR technology. No *3-1E* gene target band was found by PCR in the naked plasmid DNA vaccine group. It may be due to a variety enzyme in the digestive system of chickens and the imfavourable pH condition lead to hydrolysis or degeneration of naked plasmid DNA. In DNA vaccine PLGA microspheres group, *3-1E* gene was obtained by PCR on 7th, 14th, 21st, 28th, and 35th day post-injection. It showed that plasmid pcDNA3-1E existed in tissues, and the plasmid pcDNA3-1E released from microspheres sustained at least 35 days. This shows that the microspheres DNA vaccine had controlled release of *3-1E* gene from microspheres in a long period, provided storage for tissue cell uptake and prolonged antigen absorption and antigen expression time, ultimately leading to prolonged duration of immunity.

By oral administration, transmission and absorption of vaccine antigen mainly occurs in intestinal parts. After oral administration in chicken, naked plasmid vaccine was degraded or denatured by avariety of nucleic acid enzymes in the digestive tract, and thus can not enter the body to express antigen. Absorption and transformation mechanism of oral DNA vaccine PLGA microspheres was that DNA vaccine PLGA microspheres was transported from intestinal to the epithelial macrophages, Subsequently, plasmid DNA was sustained released from microspheres, which was then followed by expression of protein in the host cells (Seok *et al*., 2017; Briones *et al*., 2001). In this study, compared with the oral administration of naked plasmid DNA vaccine, level expression of DNA microsphere vaccine of pcDNA3-3-1E-PLGA microspheres with administrating by oral route in chickens was the highest. Antigen was transferred via intestinal, led to an increase in leukocyte numbers. Meanwhile, induced mucosal antibody production and induced a protective immune response. In this study, in the case of same dose, the level of serum IgG antibody and the eripheral blood lymphocyte proliferation response in the DNA microsphere vaccine group was significantly higher than that in other groups, which indicated that the *3-1E* gene recombinant plasmid PLGA microspheres can efficiently

induce the production of cellular and humoral immune responses in oral immunized chicken. New dosage forms of recombinant plasmid of *3-1E* gene PLGA microspheres could avoid the problem that the PLGA-coated antigen was destroyed by gastric acid and protease, and had the best effect on enhancing specific humoral and cellular immune responses and showed adjuvant efficiency and slow-release effect. The microspheres of DNA vaccine play an important role in local positioning function of releasing antigen, delaying releasing antigen, has become a main direction of current oral DNA vaccine research (Cazorla *et al*., 2015; Xu *et al*., 2012) .

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Statement of conflict of interest

The authors have declared no conflict of interest.

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