

# STUDIES ON THE DEVELOPMENT OF DNA VACCINE AGAINST *CYSTICERCUS CELLULOSAE* INFECTION AND ITS EFFICACY

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**Abstract.** DNA vaccine against *Cysticercus cellulosae* infection was developed and its efficacy was tested. A pair of primers specific to antigen B gene of *C. cellulosae* was designed which amplified the gene successfully with RT-PCR. The gene was ligated to PV93 vector, and the recombinant of antigen B gene and PV93 was transformed to JM83 cells. The transformed JM 83 cells were cultured in a large scale and the plasmid purified. Based on the recombinant plasmid, a DNA vaccine was developed and used to vaccinate two groups of experimental pigs. In each group, there was a routine vaccine, an enhanced vaccine and a control group. Groups 1 and 2 were challenged at 4 months and at 14 days post vaccination respectively with eggs of *Taenia solium*. The antibody response was also tested with ELISA. The results suggested that all animals vaccinated AgB gene DNA vaccine, no matter by routine or enhanced vaccine, their antibodies reached maximum peak 23 days post vaccination and decreased gradually. When the animals were challenged 4 months after vaccination, they had strong immunity and the parasites decrease rates were 91.2% and 93.1% respectively. When pigs vaccinated with AgB gene DNA vaccine were challenged 14 days post vaccination with 18,000 eggs/pig. The animals showed strong immunity and the parasite decrease rates were 99.5% and 84.9% respectively. However at that time, the antibodies did not reach the peak. While in the control group, the number of *C. cellulosae* was as many as 2,500. It was concluded that the pigs vaccinated with DNA vaccine had strong immunity against infection of eggs of *T. solium*.

## INTRODUCTION

Cysticercosis caused by *Cysticercus cellulosae* infection is a parasitic zoonosis. The disease is widely distributed in the world especially in the developing countries in central and south Africa, Asia and south America. Investigations in recent years suggested there were more cases of *Cysticercus cellulosae* infection in humans than in swine in developed countries, eg USA, UK, Japan, Australia. It is estimated that about 4 million people were infected by *Taenia solium*, 5 million people suffered from cysticercosis throughout the world. Cysticercosis is one of the most important constraints to animal industries and a serious disease in human being.

The reason for the high prevalence of cysticercosis in the world is that the control methods are not satisfactory. Although chemotherapy has been administrated, the residue and resistance of the parasites limits its application. The development of vaccine against *C. cellulosae* DNA vaccine with its advantages has received much attention. Many studies

suggested that antigen B of *C. cellulosae* resulted in a positive sera against the organism. The antigen B, which is a protein similar to mucin and interferes with the function of compliment and induces the hosts to produce protective antibody. It is a potential candidate antigen for vaccine development (Landa *et al.*, 1993).

This paper described the isolation, purification and ligation of antigen B gene by PCR with a pair of primers designed by us for the development of DNA vaccine.

## MATERIALS AND METHODS

### Isolation and collection of parasite

The fresh pork infected by *C. cellulosae* was collected from a slaughter house. The parasites were isolated and washed with normal saline for 3-4 times, and then stored at -70°C.

### Extraction of RNA

RNA was extracted by APGC method of Chomzyski and Such (1987) described briefly as follow: 200 µl of buffer were added to 80 mg of pork infected with *C. cellulosae*. The tissue was cut into small pieces in a 2 ml tube, normal saline was added and then washed, and centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded. The pellet was homogenized on ice. The homogenate was transferred to a 2 ml tube and 80 µl of NaAC was added

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and mixed. Then 800 µl of phenol: chloroform was added, the tube turned gently upside down several times for 10 seconds, on ice for 15 minutes, centrifuged at 12,000 rpm for 20 minutes, and the water phase was transferred to another 2 ml tube.

### Precipitation of RNA

An equal volume of isopropanol was added to the water phase, stored at -20°C and the mixture centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded and pellet was suspended in 400 µl buffer. An equal volume of isopropanol was added and centrifuged as mentioned above, then 75% cold ethanol was added and centrifuged, the supernatant was discarded and pellet was vacuum dried and dissolved with Rnase free solution and the concentration of RNA was determined.

### Designing of primers and synthesis

According to the published sequence of *C. cellulosa*, one pair of primers which were located at conservative region of antigen B, was designed and synthesized using OLIGO program. Forward primer: 5'-ATC GAA TTC AGG CAC ACA ACC-3'. Reverse primer: 5'-CAC GAA TTC TGC CCT TTT GAG-3'. In the sequence of the primer, GAA TTC is the digest site of EcoRI.

### RT-PCR amplification of the target gene

**Amplification:** 10 µl of RNA suspension was added to 40 pmol reverse primer solution, pre-denatured at 85°C for 5 minutes and ice bathed for 5 minutes, 10 U Rnase, 10 µl 5x reverse transcript buffer, 5 µl 0.1M dNTP, 10 U M-MLV reverse transcriptase were added, water bathed at 37°C for 90 minutes, and denatured the reversed transcript at 90°C for 5 minutes.

With 10 µl reverse transcript product, 5 µl 10x Taq DNA buffer, 5 µl 2.5mM dNTP, 40 pmol of both of the primer was added, and the final volume was added to 50 µl with water, mixed and 1 U Taq DNA ligase was added, amplified with PTC-100 Gene thermocycler at program: 94°C 1 minute, 50°C 1.5 minutes, 72°C 3 minutes for 35 cycles and extended at 72°C for 10 minutes. The final solution was used for electrophoresis on 0.1% agarose to examine the purification of product.

**Recovery of PCR product:** The final solution from PCR amplification was extracted with phenol: chloroform, digested with EcoRI, electrophoresed on 0.1% agarose, and the target gene was recovered as described in the manual from Promega.

### Cloning and identification of antigen B

**Establishment of recombinant of antigen B:** The recovered product was digested with EcoRI, ligated

to PV93 eukaryotic express vector (PV93 vector was provided by Harbin Veterinary Research Institute, CAAS). The ligation reaction (PV93 2 µl, recovered target gene solution 6 µl, 10 x T4 ligase buffer 2 µl and T4DNA ligase enzyme 1 U) was performed at 22°C for 3 hours and at 4°C overnight.

### The transformation of recombinant involves 2 steps:

**Preparation of competent cells.** Fifty microliters of the seed solution of JM83 cells was spread on a LB agar plate in line, cultured at 37°C for 16-20 hours. The clone of JM 83 cells was selected and inoculated into 50 ml SOC medium, 37°C for 3 hours. The cells were transferred into a 50 ml tube on ice for 10 minutes, and then centrifuged at 4°C at 6,000 rpm for 10 minutes; the supernatant was discarded. The pellet was suspended with 25 ml 0.1 M cold CaCl<sub>2</sub> on ice for 45 minutes, centrifuged at 6,000 rpm for 10 minutes and the supernatant discarded. The pellet was suspended with 25 ml 0.1 M cold CaCl<sub>2</sub>, aliquoted into tubes (200 µl each tube), and stored at 4°C for 12-24 hours for next step.

**Transformation of ligation product.** Five microliters of the ligation product solution were pipetted directly into the competent cells and mixed by stirring gently with the pipet tip. This was incubated in a tube on ice for 30 minutes, heat shocked for exactly 90 seconds in 42°C water bath and placed on ice for 3 minutes, 800 µl LB medium was added at 37°C. The tube was shaken on a rocker at 37°C for one hour and centrifuged at 7,000 rpm for 5 minutes and the supernatant discarded. The pellet was spread on LB agar plates with 16 µl Amp+, 16 µ X-Gal and 4 µl IPTG, 37°C for 12-16 hours. The white clones were selected and cultured at 37°C overnight. Plasmids were abstracted and digested for analysis and identification.

**Identification of positive clones:** Plasmids from positive clone were diluted at 1:10. One µl of the diluted solution was added into 20 µl PCR reaction solution. The PCR products were electrophoresed and sequenced (only 600 bp at 5' in cDNA of the external gene in plasmid).

### Development of vaccine

#### Production of plasmid containing antigen B gene:

The PV93 plasmid containing the antigen B gene was transformed to JM 83 cells as the methods mentioned above, cultured on a large scale, purified, and analyzed by electrophoresis to determine the concentration of plasmids.

**Preparation of vaccine:** Each dose consisted of 0.2 mg antigen B-PV93 plasmid, which was mixed with diluent.

**Routine vaccine.** Each dose consisted of 0.1 mg antigen B-PV93 plasmid + 0.4 ml diluent.

**Enhanced vaccine.** Each dose consisted of 0.1 mg antigen B-PV93 plasmid + 0.4 ml diluent + 0.5 ml transfer factor.

**Animal test and efficacy observation**

**Animal test:** Thirty pigs, 2-3 months of age, free of *C. cellulosa* were divided into two batches of 15 pigs. There were three groups in each batch: the routine vaccine group, the enhanced vaccine group and control group. Each group had 5 pigs.

**Challenge test:** In the routine vaccine group, each pig was given 0.5 ml vaccine intramuscularly. In the enhanced vaccine group, each pig was injected intramuscularly with 1 ml vaccine but pigs in the control group were not vaccinated. In group 1, the animals were vaccinated on 11 February 1999 and challenged on 15 June 1999 by oral administration of 10,000 eggs of *T. solium*. In group 2, the animals were vaccinated on 9 June 1999 and challenged on 23 June 1999 by oral administration of 10,000 eggs of *T. solium*. The animals were bled at intervals of 10 days, killed and examined for the parasite.

**Determination of efficacy of immunity:** Sera were tested with ELISA for antibodies. All the animals were slaughtered 3 months post-challenge and examined for *C. cellulosa*. The parasite rate decreased and the protection rate were calculated as follows:

$$\text{Parasites decrease rate} = \frac{\text{Mean number of C.c* in control group} - \text{mean number of C.c in vaccinated group}}{\text{Mean number of C.c in control group}} \times 100\%$$

\*C.c = *Cysticercus cellulosa*

**RESULTS AND DISCUSSION**

**Extraction of RNA**

The total RNA is seen in Fig 1. The purification and concentration of RNA: A260=1.746, A280=0.826, A260/A280 = 2.114; the concentration of RNA was 69.85 µg/ml. It can be seen from result of electrophoresis and measurement with spectrophotometer that purification of RNA abstracted by Chomczynski and Soch (1987) method was good and concentration was high. The 28S, 18S ribosomal bands are seen clearly. The RNA was not damaged. The Chomczynski and Soch method (1987) was thus suitable for abstraction of total RNA of *C. cellulosa*.

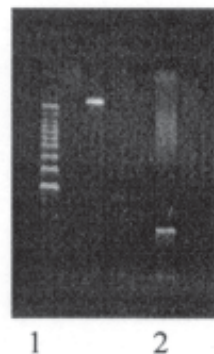


Fig 1- The result of electrophoresis of total RNA. 1.Marker, 2.Total RNA.



Fig 2- Agar gel electrophoresis of PCR product. 1. PCR product, 2. Marker, 3. pCB/EcoRI.

**Electrophoresis of RT-PCR**

The electrophoresis of RT-PCR is shown in Fig 2. It can be seen that the length of target gene from RT-PCR was 2.6Kb which was the expected outcome.

**Clone and identification of PCR**

Figs 3 and 4 show 6 positive clones that had been selected from the white clones. When the plasmid digested with EcoRI, two bands at length of 2.6Kb and 3.2Kb were observed. When digested with PvuII, three bands at 2.9Kb, 1.7Kb and 0.9Kb were shown. All these results coincided with target gene.

**Verification test by sequencing of antigen B gene**

Fig 5 suggesting the likelihood of 540 bp sequence from 5' side of isolated gene CPMY with TPMY was 99% and the reading frame was the same. It was concluded that antigen B gene had been cloned and ligated into PV93 vector.



Fig 3- Restriction analysis of recombinant plasmid.  
1 Marker, 2. Recombinant plasmid/PvuII.

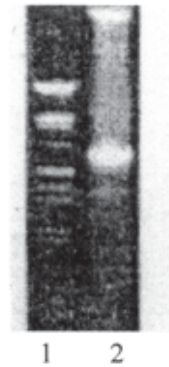


Fig 4- PCR of recombinant plasmid.  
1. Marker, 2. PCR product of recombinant plasmid.

CPMY	ATCCATTGCG	CACGTGCCGT	GATTCACCAC	AAAACGGGAC	CTATCAGAGA	CGAAGACATG	(60)
TPMY	-----CC-----	---TC-----	-----G---T---	-----C---	--C-----T-	-----	
CPMY	TCTGAATCAC	ACGTCAAAT	TTCTCGTACC	ATCATACGCG	GGACTTCCCC	AAGTACTGTT	(120)
TPMY	-----	-----	-----	-----	-----	-----	
CPMY	CGACTTGAGA	GTCGCGTACG	AGAACTGGAG	GACCTGCTCG	ATCTTGAGCG	AGATGCTCGC	(180)
TPMY	-----	-----	-----	-----	-----	-----	
CPMY	GTCCGGGCTG	AACGCAATGC	CAACGAGATG	AGCATTACGC	TGGACACCAT	GGCTGAACGT	(240)
TPMY	-----	-----	-----	-----	-----	-----	
CPMY	CTTGACGAAT	TGAGTGGTAC	TTCCTCTCAG	ACTCACGATG	CTATTCGCCG	TAAGGATATG	(300)
TPMY	-----	-----	-----	---A-----	-----	-----	
CPMY	GAGATCTCGA	AGCTGCGCAA	GGATCTGGAA	AATGCCAACG	CTGCTTTCGA	AACTGCCGAG	(360)
TPMY	-----	-----	-----	-----	-----	-----	
CPMY	GCCACTCTGC	TCCTCAAACA	CAACACCATG	ATCTCCGAGA	TCTCCAGCGA	GGTTGAGAAT	(420)
TPMY	-----	-----	-----	-----	-----	-----	
CPMY	TTGCAGAAGC	AGAAGGGCAG	GGCAGAGAAG	GACAAGAGCC	AGCTCATGCT	TGAAATCGAT	(480)
TPMY	-----	-----	-----	---T-----	-----	-----	
CPMY	AACGTTCTTG	GTCAACTTGA	TGGCGCTTTA	AAGCCAAGG	CCTCAGCGGA	GAGCAAACGT	(540)
TPMY	-----	-----	-----	-----	-----	-----	

Fig 5- 5' nucleotide sequence of AgB gene (CPMY) of *Cysticercus cellulosae* and comparison.

### Efficacy of vaccination of animals

Antibodies detected by ELISA test are presented in Tables 1 and 2 and the anatomic examination of experimental animals is shown in Table 3. Tables 1 and 3 suggest that all animals vaccinated with AgB gene DNA vaccine, no matter by routine or enhanced vaccine, their antibodies reached maximum peak 23 days post-vaccination, and decreased gradually. When the animals were challenged 4 months after vaccination, they had strong immunity and the parasites

decrease rates were 91.2% and 93.1% respectively. Furthermore, the *C. cellulosae* found in anatomic examination were calcified and lost their activity. Some of the experimental animals were totally protected.

Table 2 and 3 suggested that when pigs vaccinated with AgB gene DNA vaccine were challenged 14 days post-vaccination with 18,000 eggs/pig, the animals showed strong immunity with parasite decrease rates were 99.5% and 84.9% respectively. At that time,

however, the antibodies did not peak. In the control group, the number of *C. cellulosa* was as many as 2,500 and the cyst was like bean. The outer membrane was semi-transparent and the whitish head section was seen inside the parasites, suggesting that *C. cellulosa* was very active.

Animal experiments did not showed that transfer factor could enhance the immunity of animals which might be because of recipe of vaccination or emulsification and needed to be further studied.

It could be concluded that the pigs vaccinated with

DNA vaccine, which were prepared from recombinant plasmid containing antigen B gene and PV93 eukaryotic expressing vector, had strong immunity against infection of eggs of *T. solium*. This result agreed with that by antigen B of other tapeworms (Molinari, 1993); the immunity and reactions were similar to whole *C. cellulosa* antigen (Xu and Liew, 1984), tissue culture cell antigen (Zhang *et al*, 2000); better than other engineering vaccines (Manutecharian, 1996; Agustin *et al*, 1999; Tang *et al*, 2000), and DNA vaccine (Xu and Liew, 1994; Rothel *et al*, 1997; Wu *et al*, 1999).

Table 1  
Antibody response of animal vaccinated and challenged in 1999 (measured by ELISA).

Time(day) \ Group	0	12	23	38	52	65	78	92	106	121	140	159	180
	11/2	23/2	4/3	19/3	2/4	15/4	28/4	12/5	26/5	11/6	30/6	19/7	10/8
Routine vaccine	0.45	0.55	1.40	1.34	1.28	1.02	0.97	0.87	0.67	0.46	0.59	1.27	1.10
Enhanced vaccine	0.36	0.72	1.41	1.23	1.13	1.00	1.26	0.96	0.97	0.93	0.77	1.20	1.07
Control	0.48	0.51	0.62	0.78	0.51	0.59	0.48	0.40	0.59	0.40	0.70	1.06	1.18

Table 2  
Antibody response of animal vaccinated and challenged in 2000 (measured by ELISA).

Time ( day) \ Group	0	7	14	31	41	52	82
	9/6	16/6	23/6	10/7	20/7	1/8	1/9
Routine vaccine	0.48	0.62	0.62	1.15	0.93	1.05	1.03
Enhanced vaccine	0.48	0.58	0.63	1.12	1.16	0.99	1.14
Control	0.44	0.49	0.51	0.94	0.91	0.85	1.13

Table 3  
Anatomic examination of experimental animals vaccinated and challenged in 1999 and 2000.

Group	Number of <i>C. cellulosa</i>						Parasite decrease rate		Complete protection rate	
	1999		2000				1999	2000	1999	2000
Routine vaccine	38	41	0	24	14	1	91.2%	99.5%	20%	25%
Enhanced vaccine	0	7	14	304	359	44	93.1%	84.9%	20%	0
Control	174	255	275	2,100	2,460					
	373	112		600	2,546					

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