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Sequence analysis of Bs-Ag2 gene from *Baylisascaris schroederi* of giant panda and evaluation of the efficacy of a recombinant Bs-Ag2 antigen in mice

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ABSTRACT

The gene of Bs-Ag2 from *Baylisascaris schroederi* was amplified, cloned and sequenced. Sequence analysis indicated that the nucleotide sequences of the Bs-Ag2 from adult, L2 and L3 of *B. schroederi* were completely identical. A homology search performed by BLAST revealed that Bs-Ag2 shared the highest amino acid sequence identity with As16 protein from *Ascaris suum* (94%). The recombinant Bs-Ag2 proteins can be successfully expressed in *Escherichia coli* BL21 (DE3). The rBs-Ag2 was used to evaluate their ability to induce immune protective responses in BALB/c mice against L3-challenge infection in a mouse-*B. schroederi* model. There was a 63.66% reduction ($P < 0.001$) of recovery of larvae compared with that in the control group. Specific anti-Bs-Ag2 antibodies from immune protected mice had significantly higher levels of immunoglobulin G (IgG) ($P < 0.0001$). Our data supported the use of Bs-Ag2 as a potential candidate for vaccination against *B. schroederi* infection.

1. Introduction

The giant panda (*Ailuropoda melanoleuca*) is one of the world's most recognized and threatened animals in the world [1,2]. Unfortunately, the low rates of reproductive success and infectious disease resistance have severely hampered the development of captive and wild populations of the giant panda [3,4].

Among all the diseases in the giant panda, parasitosis is the most serious one. While *Baylisascaris schroederi* (roundworm), represents a significant threat to both wild and captive populations [5]. *B. schroederi* is the only endoparasite that appears to be consistently recognized in the giant panda. The cause of infection of panda with *B. schroederi* is highly pathogenic. Larvae of this ascarid hatch in the intestine and penetrate the intestinal wall to enter the portal circulation to the liver and lungs where their migration may cause extensive inflammation and scarring. In addition, adult ascarid may enter some of the pipelines linked to the intestines, including the pancreatic tube and bile duct. This may cause intestinal obstruction, inflammation, and even death [6–8]. Its infection rate among wild pandas may reach over 50% or even 100%, making it one of the leading causes of death for primary and secondary infection in wild populations. It has been previously reported that the probability of death of wild pandas being caused by this disease increased significantly between 1971 and 2006. *B. schroederi* represented the most important cause of death during the most recent period (2001–2005), responsible for 12 out of 24 of the deaths reported [4].

Because *B. schroederi* eggs are highly resistant to environmental degradation, it is difficult for an animal to avoid exposure once a physical area is contaminated. Treatment of individuals with antiparasitic drugs may require multiple doses until the animal ceases to expel worm or shed eggs in faeces. The ascarids remain a major health problem worldwide and the feasible strategies for treatment are limited [9–11]. Methods available for the control of the ascarids infection are mainly based on chemotherapy and immune modulation [12,13]. However, the effective and long-lasting control strategies are hampered by the persistent exposure of host animals to environmental stages of parasites, the incomplete protective response of the host. Therefore there is an urgent need for novel control strategies such as vaccines against the ascarids infection that can be an effective adjunct to the existing methods in the control of ascarids.

Immunization with non-infective material would be more practical [14–17]. Using recombinant proteins to prevent parasite invasion is one of the most straightforward approaches in developing a preventive vaccine. In the study, we studied L3-challenge infection in a mouse-*B. schroederi* model, mice immunized with *Escherichia coli*-expressed recombinant Bs-Ag2 coupled with Freund's complete adjuvant (FCA) showed protection against challenge

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infection with *B. schroederi* L3. We examined whether vaccination with rBs-Ag2 induced protection in a mouse-*B. schroederi* model in order to evaluate the use of rBs-Ag2 as a vaccine candidate for parasitic diseases caused by *B. schroederi*. Our aim in this study was to develop a recombinant vaccine that reduces *B. schroederi* levels below the disease-causing threshold.

2. Materials and methods

2.1. Animals

Four to 6-week-old female specific pathogen-free (SPF) BALB/c mice were obtained from Chengdu Institute of Biology Laboratory Animal Center, Chinese Academy of Sciences (Chengdu, China). New Zealand white rabbits were purchased from Laboratory Animal Center of Sichuan Agricultural University (China). Institutional Ethical and Animal Care guidelines were adhered to during the sampling exercise and all procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. Parasites

B. schroederi used in the present study were originally derived from infected giant panda provided by Department of Parasitology, College of Veterinary Medicine, Sichuan Agricultural University. Unembryonated and embryonated eggs were obtained essentially as described elsewhere [18]. L2 and L3 were obtained as previously described [19].

2.3. RNA extraction and amplification of Bs-Ag2

Total RNA was extracted from adult, L2 and L3 of *B. schroederi* using an RNA isolation kit (Waston), respectively, and first-strand cDNA synthesis was performed using a cDNA synthesis kit (Fermenmtas) and an oligo $(dT)_{18}$ primer (Fermenmtas). The resulting cDNA was amplified by PCR using sense primer (5′-GCACGAGGCTCATCGTGTGT-3′) and an antisense primer (5′-TCGTACGATAGGCGATATGAGCT-3′). PCR products were separated by agarose gel electrophoresis, purified using a QIAquick Gel Extraction Kit (Waston) according to the manufacturer's instructions and cloned into a pMD18-T vector (TaKaRa) as described in the manufacturer's protocol. The resultant plasmid was transferred into *E. coli* strain DH5α (Invitrogen) and sequenced.

2.4. DNA sequence analysis

An Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the BLAST network server of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyze Open Reading Frame of the Bs-Ag2, the nucleotide sequence and deduced the amino acid sequences in determining similarities with previously reported sequences in the current database. Analysis of the signal sequence was performed using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

2.5. Expression and purification of recombinant Bs-Ag2 fusion protein

The coding region of Bs-Ag2, except the signal peptide was amplified by PCR using a sense primer (5′-CCGGAATTCCAAACACC-ATCACGCGTACCACCCT-3′) which contains an *EcoRI* site and an antisense primer (5′-CCCAAGCTTCTTACGCACCGCCAGCGATTGCCTT-3′) which contains a HindIII site were used. The PCR fragments were digested with EcoRI (Fermenmtas) and HindIII (Fermenmtas) and ligated into plasmid expression vector Pet32a(+) (TaKaRa), which had also been digested with the same enzymes as described in the manufacturer's protocol. BL21 *E. coli* cells were transformed with the recombinant constructs using standard methods [20]. Transformant colonies were evaluated by DNA restriction analysis of the plasmid. *E. coli* BL21 (DE3) (Invitrogen) harboring the recombinant plasmid was propagated in LB (50 mg of ampicillin, 5 g of Bacto yeast extract, and 10 g of NaCl per liter of distilled water [pH 7.0]) and expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Invitrogen) at 37 °C for 3 h. Purification of the recombinant Bs-Ag2 were carried out as described by Chen et al. [21].

2.6. Rabbit immune serum against B. schroederi

The rabbit immune serum was obtained from inoculating rabbits by gastric irrigation with *B. schroederi* embryonated eggs. Each rabbit was inoculated with 3600 eggs, followed by repeated inoculation every 2 weeks for a total of four inoculations. The rabbits were bled 2 weeks after the final inoculation, and the serum was stored at −20 °C until use.

2.7. Immunoblot analysis

SDS-PAGE analysis was performed in 14% polyacrylamide gels with the recombinant protein electrophoresis system. Electrophoresis was carried out at constant voltage of 160 V for 180 min. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (PerkinElmer). The membrane was blocked with 1% bovine serum albumin-PBS-Tween (0.05%) for 2 h at room temperature. Sera from vaccinated rabbit at a dilution of 1:500 were used for detection of the antigenicity of rBs-Ag2. After the membrane were washed five times with PBS-T, Horseradish peroxidase-conjugated goat anti-rabbit IgG (BLS) was used as a secondary antibody at a 1:8000 dilution. After the membranes were washed five times with PBS-T, the signal was visualized with 3,3′,5,5′-tetramethylbenzidine (TMB) (Invitrogen) according to the manufacturer's instructions.

2.8. Challenge infection and sampling

Mice were divided into three groups with 10 animals each. For preparation of conjugation, rBs-Ag2 was coupled with Freund's complete adjuvant (FCA) (Sigma) in darkness at 4 °C for 16 h. A group of 10 mice was injected i.p. with 30 μg rBs-Ag2 mixed with FCA in a total volume of 200 μl on 0th, 14th, and 28th days. As a control, a group of 10 mice was immunized i.p. with adjuvant alone; another group of 10 mice was immunized i.p. with phosphate buffer solution (PBS). Two weeks after the final immunization, all animals were inoculated orally with 3200 *B. schroederi* infective embryonated eggs. The mice were euthanatized on day 35. Mice were bled before autopsy and sera were collected and frozen at −20 °C. Their lungs and livers were removed and minced with a surgical knife, and larvae were recovered by the method of Baermann and counted under a light microscope [22–24]. Data from representative experiments are presented in figures. Percent reduction was calculated as follows: % reduction = [(average recovered larvae in control mice − average recovered larvae in immunized mice)/average recovered larvae in control mice] × 100.

2.9. ELISA

Serum levels of antigen-specific IgG antibody were measured by enzyme-linked immunosorbent assay (ELISA). The recombinant proteins were bound to 96-well microtitre plates (Invitrogen) by incubating 2 μg/ml of 0.1 M carbonate buffer (10 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) for 16 h at 4 °C, and washed five times