

Cloning, expression, and protective immunity in mice of a gene encoding the diagnostic antigen P-29 of *Echinococcus granulosus*

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Taeniid tapeworm *Echinococcus granulosus* is the causative agent of Echinococcosis, an important zoonosis with worldwide distribution. In this study, a diagnostic antigen P-29 was cloned from *E. granulosus* and expressed in *Escherichia coli*. Sequence analysis showed that EgP-29 contains 717-bp open reading frame and encodes a protein of 238 amino acid residues with a predicted molecular weight of 27.1 kDa. The recombinant EgP-29 (rEgP-29) could be recognized with anti-mice sera in Western blotting. The specific antibody was detected by enzyme-linked immunosorbent assay. Mice vaccinated with rEgP-29 and challenged intraperitoneally with *E. granulosus* protoscoleces revealed significant protective immunity of 96.6% ($P < 0.05$), compared with the control group. Thus, rEgP-29 protein is a promising candidate for an effective vaccine to prevent secondary echinococcosis.

Keywords *Echinococcus granulosus*; diagnostic antigen P-29; cloning; expression; protective immunity

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Introduction

Echinococcosis is a zoonosis caused by the taeniid tapeworm *Echinococcus granulosus*. The disease has a worldwide distribution, with a considerable impact on both human and animal health and causes important socio-economic consequences in endemic areas [1]. Echinococcosis is preventable. In places where efficient and sustained control campaigns have been implemented, its prevalence has been decreased dramatically [2].

Unfortunately, numerous reports indicated that its incidence was increased in several regions of the world [3–5].

The domestic life cycle of *E. granulosus* is maintained through definitive host and intermediate hosts. The adult *E. granulosus* lives in the small intestine of a carnivore (definitive host). A wide range of mammal species (intermediate host), including humans, may acquire the infection through accidental ingestion of eggs of *E. granulosus*. Therefore, effective immunoprophylaxis for either the definitive host or the intermediate hosts could be an important strategy for interrupting the lifecycle [6].

Vaccination of the intermediate host is a burgeoning area that has moved forward considerably in recent years following the development of a recombinant vaccine against *Taenia ovis* infection in sheep [7]. Recombinant *Echinococcus* proteins and synthetic peptides have been proven to be more reliable for immunodiagnostic purposes than native antigens and their purified subunits/fractions [8]. A number of recombinant proteins are available now as candidate antigens for the immunodetection of *E. granulosus*, mainly in humans [9,10].

P-29, a 29-kDa antigen from *E. granulosus*, is a metacestode-specific component. The immunologic cross-reactivity between P-29 and a major diagnostic antigen of *E. granulosus* (Ag5) [11] indicated that P-29 might be another useful diagnostic antigen of *E. granulosus*.

In this study, we tested whether this putative diagnostic antigen could be used as a vaccine against secondary *E. granulosus*. Secondary echinococcosis occurs in humans when protoscoleces (PSCs) are accidentally released into a body cavity during surgery. It has been studied for many years by injecting PSCs into the peritoneal cavity of mice and other rodents [12].

We found vaccination with recombinant P-29 (rEgP-29) could induce protective immunity against *E. granulosus* PSC challenge in mice effectively. Thus, rEgP-29 is a promising candidate vaccine to prevent secondary echinococcosis and may be useful in immunizing definitive hosts against the intra-intestinal establishment of PSCs.

Materials and Methods

Collection of PSCs

Brood capsules were collected aseptically from fertile *E. granulosus* cysts from the livers and lungs of infected sheep. The collected PSCs were washed in phosphate-buffered saline (PBS-1%) and Hanks' balanced salt solution (Sigma, St. Louis, USA) containing 100 U/ml of penicillin G and 100 µg/ml of streptomycin sulfate. The viability of PSCs was determined by Trypan blue exclusion assay. Only those batches containing more than 90% viable PSCs were used for mice infection.

RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from freshly isolated *E. granulosus* PSCs using reverse transcription–polymerase chain reaction (RT–PCR) kit (Promega, Madison, USA). The *EgP-29* gene was amplified by RT–PCR using two primers according to the nucleotide sequence of *E. granulosus* P-29 gene in GenBank (Accession No. AF078931). I: 5'-ATGGATCCATGTCGGATTTGACGTAC-3' and II: 5'-GTGCGGCCGCTACTCGCCCAGCATCATA-3'. Both primers contained *Bam*HI/*Not*I (Promega) restriction enzyme cleavage sites and were synthesized by Beijing SBS Company (Beijing, China). The RT–PCR thermal profile was programmed: one cycle 48°C for 45 min, one cycle 94°C for 2 min, and 40 cycles 30 s at 94°C, 60 s at 60°C and 2 min at 68°C, with a final extension for 15 min at 68°C. The RT–PCR products purified by using gel cleanup kit (SBS) were ligated into vector pGEMT (Promega). The purified ligation products were transformed into *Escherichia coli* JM109 (Promega) using the routine method [13]. The insert was sequenced by TaKaRa Company (Dalian, China).

Sequence analysis

The prediction of the open reading frame (ORF) of *EgP-29* was performed with the DNASTAR software package (<http://www.dnastar.com>). Homologous proteins were searched at non-redundant database of the NCBI with the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Expression and purification of rEgP-29

Plasmids of *EgP-29/pGEMT* were digested with *Bam*HI and *Not*I. Then the purified fragment with the expected length of *EgP-29* (about 700 bp) was ligated into expression plasmid vector pET28a (Novagen). The resulting expression plasmid *EgP-29/pET28a* was transformed into *E. coli* BL21(DE3)pLysS, provided by Dr. Xiao Wei (Saskatchewan University, Saskatoon, Canada). Protein expression was induced at 37°C for 5 h in the presence of isopropyl-β-D-thiogalactoside (IPTG, Promega) at a final concentration of 0.4 mM. The recombinant His₆-tagged *EgP-29* was purified from the extract of transformed *E. coli* BL21(DE3) by nickel chelate affinity chromatography (Novagen) according to the manufacturer's instructions. The purified His₆-tagged protein was analyzed on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Protein concentration was determined by Bradford method [14].

Vaccination protocols

Twenty male ICR mice (6–8 weeks old; obtained from the Experimental Animal Centre of Ningxia Medical University, Yinchuan, China) were randomly allocated into two groups of 10 mice each. Mice in group 1 was immunized subcutaneously in the back with 10 µg of rEgP-29 in 100-µl PBS emulsified in Freund's adjuvant for three times (first immunization in Freund's complete adjuvant at week 0 and followed by two booster immunizations in Freund's incomplete adjuvant at weeks 2 and 4). The mice in group 2 were injected with adjuvant in PBS as control. The mice were bled via the tail vein at 2-week interval before each immunization. Sera were stored individually at –20°C.

ELISA and western blot analysis

Serum antibody responses after immunization with rEgP-29 and control (PBS) were quantified by ELISA as described previously [15]. Briefly, 96-well microtiter plates (Sino-American Biotechnology Company, Beijing, China) were coated with rEgP-29 (10 µg/100 µl per well). Serum samples were diluted 1:100 in PBST (PBS with 0.05% Tween-20) and tested in duplicate. Bound antibody was detected by using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Novagen) at a 1:1000 dilution in PBST. The antibody titers were expressed with the optical density values at 490 nm (OD490), which was detected in an ELISA microplate reader (Bio-Rad, California, USA). Serum samples

giving OD490 values greater than the mean OD490 plus four standard deviations for normal control mice were considered seropositive. ELISA for the detection of mouse IgG subclasses was carried out as described above, except that the secondary antibodies specific to mouse IgG1, IgG2a, IgG2b, and IgG3 (Sino-American Biotechnology Company, Beijing, China) were used at 1:1000 dilution. SDS-PAGE and western blotting were carried out according to the method of Laemmli [16]. rEgP-29 and PSC were separated electrophoretically in a 12% polyacrylamide gel and transferred onto nitrocellulose membranes (Millipore, Tokyo, Japan) as described by Towbin *et al* [17]. Membranes were cut into strips and blocked in 5% skim milk at 37°C for 2 h. The strips were then washed with PBST and incubated overnight at 4°C with immunized and control mice sera (1:100 dilution). After washing, membranes were further incubated with HRP-conjugated goat anti-mouse IgG (1:1000 dilution) for 2 h at 37°C. Finally, membranes were visualized by the addition of PBS containing 0.5 mg/ml of 4-chloro-1-naphthol (Sino-American Biotechnology Company), 0.015% H₂O₂, and 16% methanol.

Challenge infection and protective immunity

Two weeks after the final vaccination, 10 mice from each group were challenged with 2000 viable PSC intraperitoneally. Five months post-challenge, the carcasses were dressed and examined superficially for the visible of hydatid cysts. The percentage of protection in mice was determined according to the method of Dempster [18]: protective immunity in vaccinated mice (%) = (1 - average of cysts in test group/average of cysts in control group) × 100%.

Statistical analysis

All data comparisons were tested for significance using one-way analysis of variance (ANOVA). A *P*-value of <0.05 was considered significant.

Results

Sequence analysis

Nucleotide sequence analysis revealed that a putative 717-bp length ORF of RT-PCR amplicon encodes a 238-amino acid peptide with an expected molecular mass of 27.1 kDa. An NCBI BLAST search using the deduced 238-amino acid peptide of cloned *EgP-29* gene fragment revealed an identity of 100, 97, 73.9, 70.5, and 66.2% to those of previous published *E. granulosus* P-29 (Accession No. AF078931), antigen 6 (*E. multilocularis*),

SJCHGC02883 protein (*Schistosoma japonicum*), AGAP010376-PA (*Anopheles gambiae*), and endophilin b (*Aedes aegypti*), respectively.

Expression and purification of rEgP-29

The product of RT-PCR was about 700 bp in length, which was consistent with the theoretical length of *EgP-29* gene. Sequencing of RT-PCR amplicon further confirmed the successful amplification of the nucleotide sequence of *EgP-29*. The recombinant plasmid *EgP-29/pGEMT* was digested by the restriction enzymes *Bam*HI and *Not*I. Agarose gel electrophoresis of the digested products revealed a DNA band at about 700 bp (Fig. 1). We constructed the recombinant expression plasmid *EgP-29/pET28a* and transformed it into *E. coli* BL21(DE3). The plasmids extracted from transformed *E. coli* BL21(DE3) were digested by the restriction enzymes *Bam*HI and *Not*I (Fig. 2), suggesting that the extracted plasmid was *EgP-29/pET28a*. Nucleotide sequencing revealed that both the sequence and the reading frame of the inserted *EgP-29* fragment were accurate, which further confirmed the successful construction of the recombinant expression plasmid *EgP-29/pET28a*. Following IPTG induction, the His₆-labeled recombinant protein was purified with Ni²⁺-chelating column. The SDS-PAGE staining results demonstrated

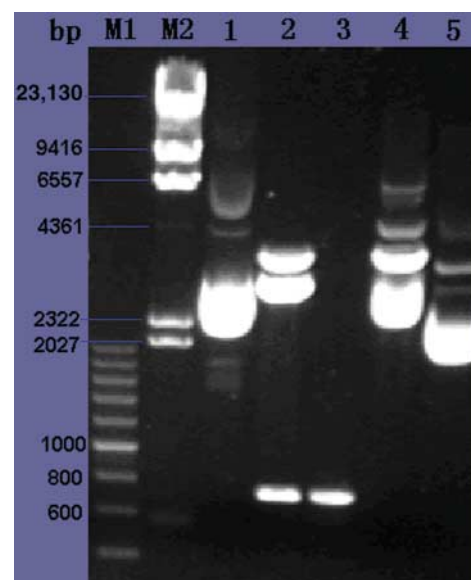


Fig. 1 Restriction enzymes digestion of recombinant vector *EgP-29/pGEMT* using *Bam*HI and *Not*I M1, 200-bp DNA marker; M2, Lander DNA *Hind*III marker; lane 1, recombinant *EgP-29/pGEMT* plasmid; lane 2, *EgP-29/pGEMT* plasmid digested by *Bam*HI and *Not*I; lane 3, RT-PCR product; lane 4, *pGEMT* vector; lane 5, *pGEMT* vector digested by *Bam*HI and *Not*I.

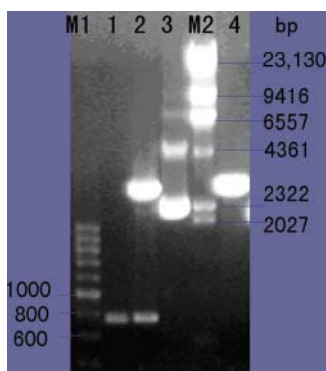


Fig. 2 Restriction enzymes digestion of the recombinant expression vector EgP-29/pET28a using *Bam*HI and *Not*I M1, 200-bp DNA marker; M2, Lander DNA *Hind*III marker; lane 1, RT-PCR product; lane 2, EgP-29/pET28a plasmid digested by *Bam*HI and *Not*I; lane 3, pET28a vector; lane 4, recombinant EgP-29/pET28a plasmid.

that the His₆-tagged EgP-29 protein was successfully expressed in *E. coli* BL21(DE3) (Fig. 3) and purified efficiently from *E. coli* lysate (Fig. 4).

ELISA and western blot analysis

The purified protein was coated onto the ELISA plates. ELISA analysis showed that the antiserum collected from immunized mice at week 6 (6 weeks after first immunization) contained high titer of IgG antibody, which reacted strongly with the coated rEgP-29 (Fig. 5). As shown in Fig. 6, four subclasses of IgG against rEgP-29 were induced in ICR mice, and IgG1 and IgG2b isotypes were the prevailing subclasses. The IgG1

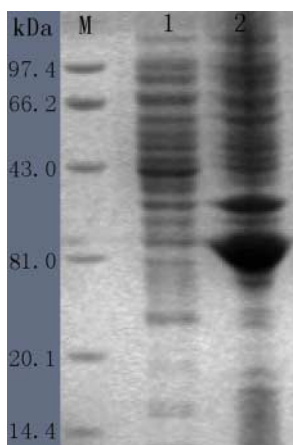


Fig. 3 SDS-PAGE analysis of the rEgP-29 The gel was Coomassie brilliant blue-stained. Given the molecular mass of 6-His tags was about 3 kDa, the expected molecular mass of the His₆-tagged EgP-29 protein was about 30 kDa. M, protein marker; lane 1, *Escherichia coli* lysates with IPTG induction; lane 2, *Escherichia coli* lysates without IPTG induction.

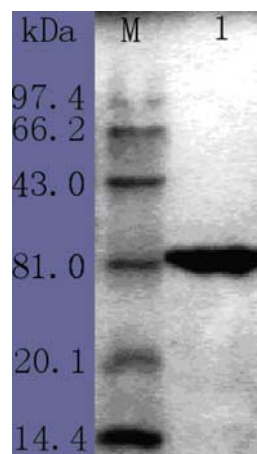


Fig. 4 SDS-PAGE analysis of the rEgP-29 The gel was stained by Coomassie brilliant blue. M, molecular mass markers; lane 1, purified rEgP-29.

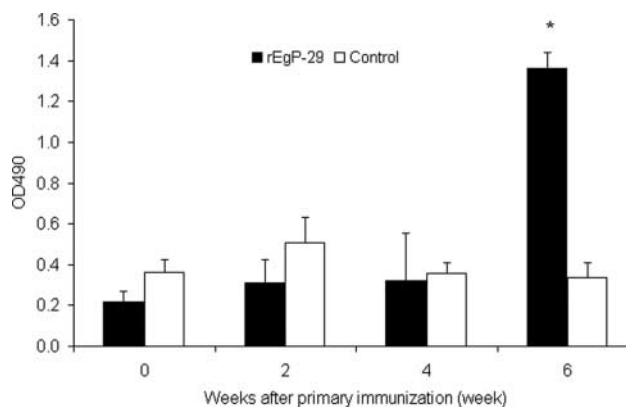


Fig. 5 Total IgG antibody response against rEgP-29 as detected by ELISA analysis of sera taken from mice 0, 2, 4, and 6 weeks after immunization The antiserum collected from immunized mice at week 6 after first immunization contained high titer of IgG antibody. Data are presented as mean \pm SD of 10 mice per group. **P* < 0.05 compared with IgG antibody response of control mice.

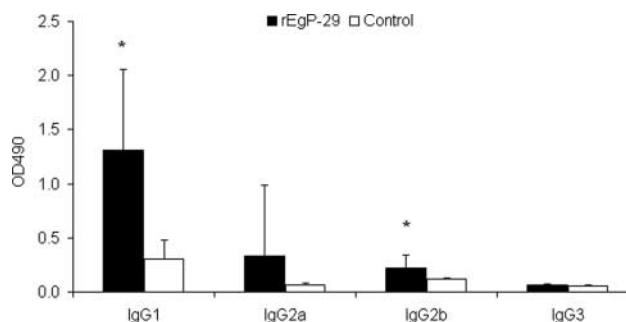


Fig. 6 Isotype distribution of the antibody response against rEgP-29 as detected by ELISA Four subclasses of IgG against rEgP-29 were induced in ICR mice, and IgG1 and IgG2b isotypes were the prevailing subclasses. Data were expressed as mean \pm SD. **P* < 0.05 compared with IgG1 and IgG2b antibody responses of control mice.

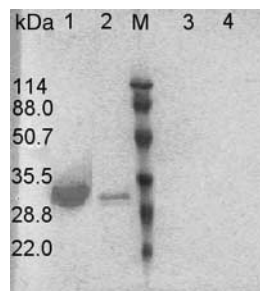


Fig. 7 Western blot analysis of rEgP-29 The proteins rEgP-29 (lane 1) and PSCs (lane 2) were immunoblotted with pooled sera from immunized mice. The proteins rEgP-29 (lane 3) and PSC (lane 4) were probed with pooled sera from control mice. M, protein marker.

and IgG2b titers induced in vaccinated mice were all notably higher than that in controls ($P < 0.05$).

To assay the immunoreactivity of the prepared antiserum to rEgP-29 and native antigen, PSC from human hydatid cysts were prepared. Western blot analysis showed that the antiserum collected from mice vaccinated with rEgP-29 reacted with both rEgP-29 and PSC, with the specific band migrating to 30 kDa. In contrast, there was no observable reaction between control mice sera and neither of those two antigens (**Fig. 7**).

Protective immunity in mice

All of the mice were killed 5 months after the challenge of PSC of *E. granulosus* and their internal organs were examined carefully for the visible hydatid cysts. After careful examination of internal organs of dead immunized mice, we confirmed the deaths of two immunized mice were not caused by PSC challenge. It appeared that the mice vaccinated with rEgP-29 had low numbers of visible cysts compared with the mice in control group. The numbers of cysts in vaccinated mice were significantly lower than that in the control group (**Table 1**; $P < 0.05$). Based on the numbers of visible cysts, protective immunity induced by rEgP-29 was 96.6%. Additionally, the average size of hydatid cysts in vaccinated mice was 0.9 mm, much smaller than that of control mice (8.1 mm).

Table 1 Numbers of hydatid cysts and protective immunity in vaccinated and control mice

Groups	No. of mice	No. of cysts	Mean \pm SD
Control	8	8.13 \pm 17.21	—
rEgP-29 group	8	0.88 \pm 2.10*	96.6%*

* $P < 0.05$ compared with control group.

Discussion

In China, cystic echinococcosis is a serious problem in human and animals. In this study, we have identified the P-29 gene as a potent immunogen. It was previously reported that the secreted protein 14-3-3 [19], the fatty-acid-binding protein EgDf1 [20], and the fibrillar protein EgA31 [21], derived from the *E. granulosus* adult worm, exhibit strong immunogenic properties in dogs experimentally infected with the parasite. A recombinant antigen vaccine was developed for use in ruminant intermediate hosts of *E. granulosus* [22]. The recombinant antigen, termed EG95, provides significant protection (mean, 96–98%) against the development of hydatid cysts in immunized sheep, with almost complete immunity persisting for more than a year after vaccination [23]. Interestingly, an *E. multilocularis* protein homologous with EG95 (designated EM95) could induce significant levels of protection (78.5–82.9%) against challenge infection with *E. multilocularis* eggs [24]. Additionally, Kouguchi *et al* [25] found EMY162, another *E. multilocularis* protein that shares structural features with the EM95 antigen, could also induce a significant level of host protection (74.3%) in mice infected with *E. multilocularis* eggs.

The EgP-29 identified by this study has the same amino-acid sequence as that of *E. granulosus* isolates from Uruguay [11], suggesting that EgP-29 may be well conserved among geographically different isolates of *E. granulosus*. Thus, the vaccination of the rEgP-29 may induce comparable immunity protection against different geographical isolates of *E. granulosus*.

Numerous studies have demonstrated that humoral immunity plays a crucial role in the protection against *E. granulosus* [26,27]. Specific antibodies against rEgP-29 can mediate the protective immunity. As shown in this study, rEgP-29 induced high levels of specific antibodies in mice after the third immunization. The protective efficacy of humoral immunity in *E. granulosus* was not only correlated with the level of IgG, but also associated with the isotype of IgG. In this study, the prevailing isotypes of IgG induced by rEgP-29 in mice were IgG1 and IgG2b, implying that rEgP-29 antigen can induce protective immunity.

Suppression of cestode development has also been observed recently [28] following the injection of a vaccine composed of recombinant EgM proteins, which induced a very high level of protection (97–100%) in dogs. Hashemitabar *et al* [29] observed that the

protective immunity induced in sheep against protoscolices and gravid worms of *E. granulosus* by application of whole-body antigen was 90.9%. Petavy *et al* [30] demonstrated that the vaccine EgA31-EgTrp had a significant decrease of parasite burden in vaccinated dogs (70–80%) and a slower development rate in all remaining worms. Our results also demonstrate that 96.6% protection can be induced by rEgP-29 in a murine model of Echinococcosis. Efforts are being directed towards finding out how protection levels are influenced by the profile of the resulting immune response. Although our data provide information on the profile of the protective response, little can be advanced on the mechanism of the rEgP-29-induced protection. In particular, the examination of the protective immunity of various antigen combinations in sheep needs to be undertaken. If successful, large-scale production of the respective protein(s) can be attempted.

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