Original Article

Production and Purification of Anti-*Rhombomys opimus* Immunoglobulins

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Abstract

Background: Zoonotic cutaneous leishmaniasis (ZCL) is an increasing public health problem in some endemic regions. Horseradish peroxidase (HRP) conjugated rabbit anti-*Rhombomys opimus* (*R. opimus*) Ig is needed for immunoblotting and ELISA tests used to explore the immune response of the rodents against the sand fly saliva. In this study, the production of HRP conjugated rabbit anti-*R. opimus* Ig was conducted for the first time.

Methods: *Rhombomys opimus* Ig was purified from serum by protein G affinity chromatography column and injected into rabbit to produce anti-*R. opimus* Ig antibody. The titration of antibody against *R. opimus* Ig in rabbit serum was checked using indirect ELISA. Rabbit anti-*R. opimus* Ig was purified by Sepharose-4B-*R. opimus* Ig column. Reactivity of this antibody was assessed by indirect ELISA and was conjugated to HRP by periodate method.

Results: Approximately 3.5 mg Ig was purified from 1 ml *R. opimus* serum using protein G affinity chromatography column. The molecular weight of purified *R. opimus* Ig was estimated about 150 kDa by SDS-PAGE. Nearly 2.3 mg rabbit anti-*R. opimus* Ig was purified from 1 ml immunized rabbit serum. The purified antibody was conjugated to HRP and the optimum titer of HRP conjugated rabbit anti-*R. opimus* Ig was determined as 1:8000 using direct ELISA.

Conclusion: HRP conjugated rabbit anti-Gerbil IgG has been produced by a few companies, but to our knowledge HRP conjugated rabbit anti-*R. opimus* Ig is not commercially available. Production of HRP conjugated rabbit anti-*R. opimus* Ig is considerably helpful for immunological studies of *R. opimus*, the main reservoir host of ZCL in Iran as well as some other countries.

Keywords: *Rhombomys opimus*, Polyclonal antibody, Zoonotic cutaneous leishmaniasis, Immunoglobulin, Iran

Introduction

Zoonotic cutaneous leishmaniasis (ZCL) is still a public health problem in some of the regions of the endemic areas. *Leishmania major*, the causative agent of the ZCL of the old world, is widely distributed in different populations of rodents in arid and savannah areas. The disease is transmitted to rodents and other vertebrate hosts by phelebotominae sand flies (Gramiccia and Gradoni 2005). Rodents of the subfamily of Gerbillinae are the main reservoirs of ZCL in Iran and other countries where the disease is endemic (Dubrovsky 1979, Strelkoova 1996, Yaghoobi-Ershadi et al. 1996). *Rhombomys opimus*

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(Cricetidae: Gerbillinae) is the main reservoir host of the agent over the vast areas of the Turan lowland (west and south Kazakhstan and central Asia with adjacent parts of Afghanistan and Iran), Mongolia, and seemingly, in some provinces of China. Naturally infected *R. opimus* were found in more than 200 places of Turan lowland. This gerbil is also found to be naturally infected with *L. turanica* and *L. gerbilli* (Strelkova 1996, Akhavan et al. 2010a, 2010b, 2010c). Well-described stable ZCL system in central Asia, Afghanistan and Iran (central and north-east) are associated with *R. opimus*, the main reservoir, and *Phlebotomus papatasi*, the main vector (Javadian 1988, Yaghoobi-Ershadi and Javadian 1996, Javadian et al. 1998, Yaghoobi-Ershadi et al. 1996, 2003, Gramiccia and Gradoni 2005, Yaghoobi-Ershadi 2008).

Investigation on immunogenic components of sand fly saliva and the immune response of the host against it, also interaction among the parasite, sand fly and host is necessary to find possible tools to control the disease specially producing anti *Leishmani*a vaccine and/or transmission blocking vaccine (Akhavan 2011). Detecting the immune response of the host to saliva of sand flies can also be used as a marker of transmission risk of the disease (Barral et al. 2000). Study on the immune response of *R. opimus*, the main reservoir host of ZCL in central and northeast of Iran, to infection of *L. major*, the causative agent of the disease, seems to be necessary. Horseradish peroxidase (HRP) conjugated rabbit anti-*R. opimus* Ig is needed for immunoblotting and ELISA tests, used to find the immune response of the rodents against the sand fly saliva.

As this material is not produced commercially in the world, its production was essential and inescapable. To our knowledge, in the present study production of HRP conjugated rabbit anti-*R. opimus* Ig has been produced for the first time.

Materials and Methods

Rhombomys opimus serum collection

To purify *R. opimus* Ig, sera were obtained from wild great gerbils collected from natural habitat in Sejzi rural district, Esfahan Province, central Iran. Great gerbils were anaesthetized (ketamin hydrochloride 60 mg/kg and xylazine 5 mg/kg, intramuscularly) and then the blood sample was collected. The individual sera was isolated and kept at -20 °C until use.

Purification of *R. opimus* Ig and polyclonal rabbit anti-*R. opimus* Ig

Rhombomys opimus Ig was purified using HiTrap protein G HP affinity chromatography column (GE Healthcare, Uppsala, Sweden). The 1:5 diluted serum in PBS (0.15M, pH= 7.2) was centrifuged, filtered by 0.2 µm filter and passed through the HiTrap protein G HP affinity chromatography column. Then the column was washed with PBS. The attached *R. opimus* Ig to the column was isolated from the column using Gly-HCl (0.2 M, pH= 2.5). Isolated Ig was dialyzed against PBS and finally the purified *R. opimus* Ig was stored at -20 ºC.

For purification of rabbit anti-*R. opimus* Ig from rabbit serum, a Sepharose-4B-*R. opimus* Ig affinity chromatography column was prepared according to the Amersham Biosciences company instructions (71-7086-00 Edition AC). Using this column, the rabbit anti-*R. opimus* Ig was purified from rabbit serum as described for *R. opimus* Ig purification (see above).

Concentration of purified *R. opimus* Ig and rabbit anti-*R. opimus* Ig was determined by reading the optical density (OD) of samples at 280 nm and calculation of the concentrations regarding to extinction coefficient of IgG molecule.

Purity assessment of purified *R. opimus* Ig

Molecular weight and purity of the *R. opimus* Ig were determined using SDS-PAGE

in non-reducing conditions, 8% separating gel and 4% stacking gel (according to Bio-Rad guideline).

Immunization of New Zealand white rabbit with *R. opimus* Ig

A female New Zealand white rabbit aged 6–7 months was immunized with *R. opimus* Ig using Hudson and Hay's method (Hudson and Hay 1991) with minor modifications. Rabbit was injected intramuscularly into the thigh muscle with 250 µg *R. opimus* Ig mixed with Complete Freund's Adjuvant, and 4 booster injections of 125 µg of *R. opimus* Ig mixed with Incomplete Freund's Adjuvant at 4 weeks intervals between the first and second injection and 2 weeks intervals between subsequent injections.

Before each immunization, blood sample was taken from the marginal vein of the rabbit ear, centrifuged and the sera were checked for anti-*R. opimus* Ig antibody using ELISA method.

Animal care and the procedures were conducted according to the guidelines of the animal care and Ethics Committee of Avicenna Research Institute.

Antiserum titration and reactivity assessment of purified polyclonal rabbit anti-*R. opimus* Ig by ELISA

Rabbit anti-*R. opimus* Ig titer was evaluated using ELISA. Briefly, 10 µg/ml of purified *R. opimus* Ig in 100 µl was added to each well of microtiter polystyrene strips (Maxisorp, Nunc, Roskilde, Denmark) and incubated at 37º C for 1.5 hours, 2 wells received only PBS as negative control, then the plate was washed 3 times with PBS-Tween (0.05%) (PBS-T), the wells were then blocked using 3% skim milk for 1.5 hours at 37º C. The plate was washed 3 times and serial dilutions of rabbit anti-*R. opimus* Ig serum from 1:1000 to 1:64000 dilutions were added and incubated for 1.5 hours at 37º C. Two other wells received PBS as primary antibody step negative control. After three times washing, HRP conjugated sheep anti-rabbit Ig (Avicenna Research Institute, Tehran, Iran) was added as secondary antibody and incubated for 1.5 hours at 37 ºC. After the last wash, the reaction was visualized using TMB substrate (USB, Cleveland, USA) and the optical density measured by ELISA reader (Anthos 2020, Cambridge, England) at 450 nm.

Reactivity of purified polyclonal rabbit anti-*R. opimus* Ig with related antigen was assessed by indirect ELISA test. ELISA procedures including coating antigen and secondary antibody were performed as described above. However, the serial dilutions of purified rabbit anti-*R. opimus* Ig (by two fold dilutions from 10000 to 1.2 ng/ml) were added as primary antibody.

Conjugation of polyclonal rabbit anti-*R. opimus* Ig with HRP

Conjugation of polyclonal rabbit anti-*R. opimus* Ig with HRP was performed by periodate method (Wilson and Nakane 1978). Briefly, HRP was oxidized by sodium periodate. Oxidized HRP was mixed by rabbit anti-*R. opimus* Ig. Then, sodium borohydride was added to this mixture and finally the conjugate was purified from excess reagents by dialysis.

Titration of HRP conjugated rabbit anti-*R. opimus* Ig by ELISA

The optimum titer of HRP conjugated rabbit anti-*R. opimus* Ig was assessed by direct ELISA according to the procedure described for the antiserum titration ELISA. The wells were coated by *R. opimus* Ig (10 µg/ml) as coating antigen and two wells were coated with PBS only as negative control. Titration of HRP conjugated rabbit anti-*R. opimus* Ig was performed by two fold dilutions from 1:250 to 1:128000.

Results

From 1 ml *R. opimus* serum 3.5 mg *R. opimus* Ig was purified. The purified *R. opimus*

Ig related SDS-PAGE shows high purity of the purified Ig. Regarding to the molecular weight of mouse IgG as a molecular weight marker, the molecular weight of purified *R. opimus* Ig is about 150 kDa (Fig.1).

The results showed that the specific antibodies were detectable following the first injection in rabbit. After the second injection, the antibody titer reached near the maximum level and there was no remarkable increase following subsequent injections. However, booster injections resulted in gradual and stepwise increase in the specific antibody titer against *R. opimus* Ig (Fig. 2).

From 5 ml immunized rabbit serum 11.5 mg rabbit anti-*R. opimus* Ig was purified. It means that there was 2.3 mg rabbit anti-*R. opimus* Ig in each ml of the immunized rabbit serum.

The results showed strong reactivity of this antibody with related antigen and this antibody can detect its antigen in very low concentrations of about 1.2 ng/ml (Fig. 3).

After the HRP conjugation, it was necessary to find the optimum dilution of HRP conjugated rabbit anti-*R. opimus* Ig by direct ELISA. Optimum dilution for a specific conjugate is the highest dilution of conjugate which can produces the highest signal.

According to the HRP conjugated rabbit anti-*R. opimus* Ig titration curve, the optimum dilution of this conjugate is 1:8000 and it shows that the efficiency of conjugation is high (Fig. 4).

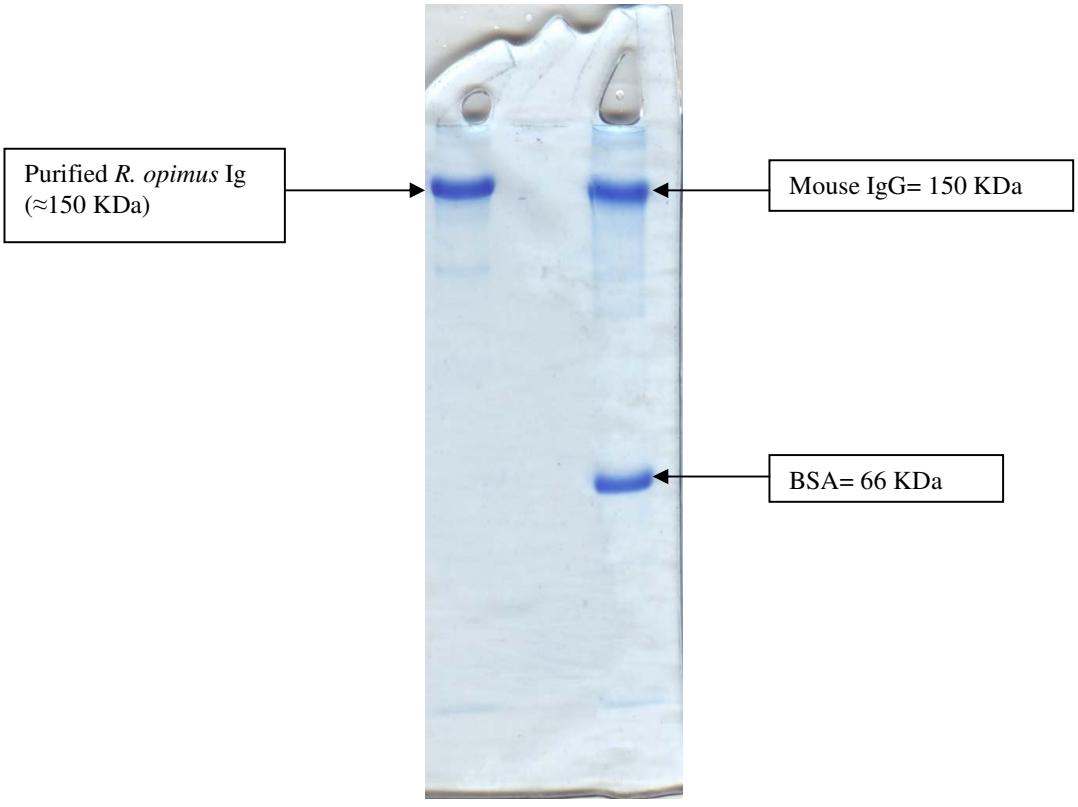

Fig. 1. The SDS-PAGE analysis of purified *R. opimus* Ig by HiTrap Protein G HP affinity chromatography column

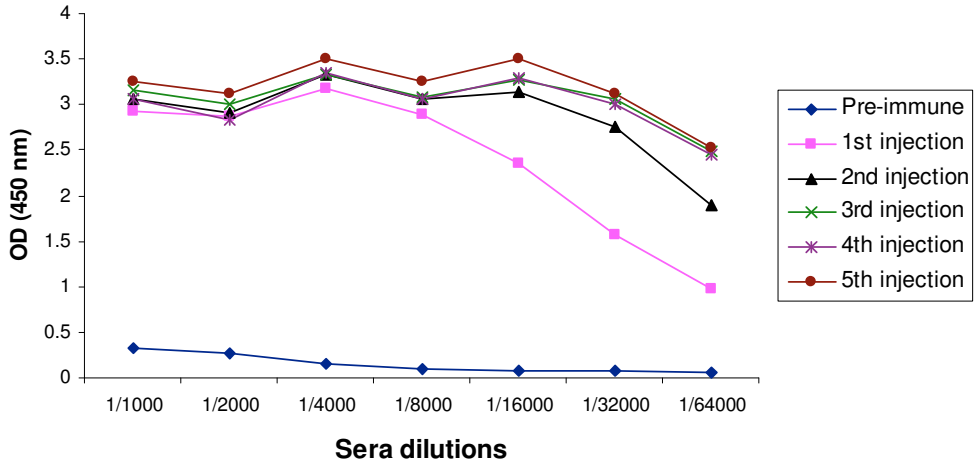

Fig. 2. Titration curve of rabbit anti-*R. opimus* Ig by indirect ELISA

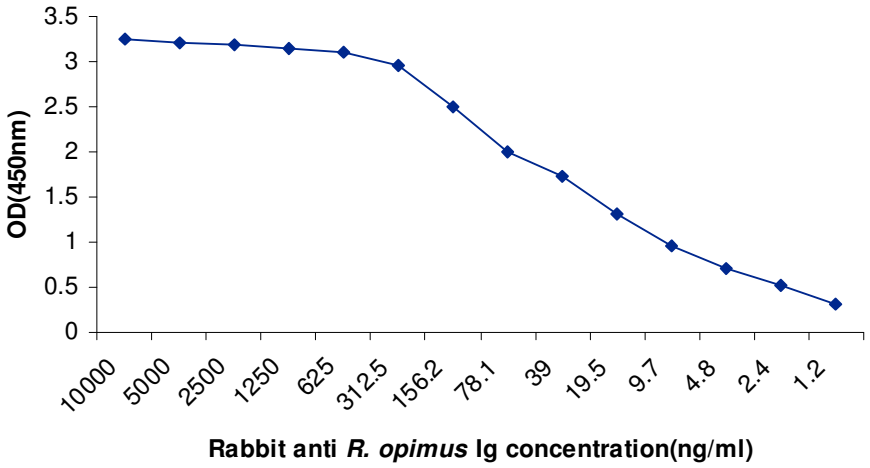

Fig. 3. Reactivity test of purified rabbit anti-*R. opimus* Ig by indirect ELISA

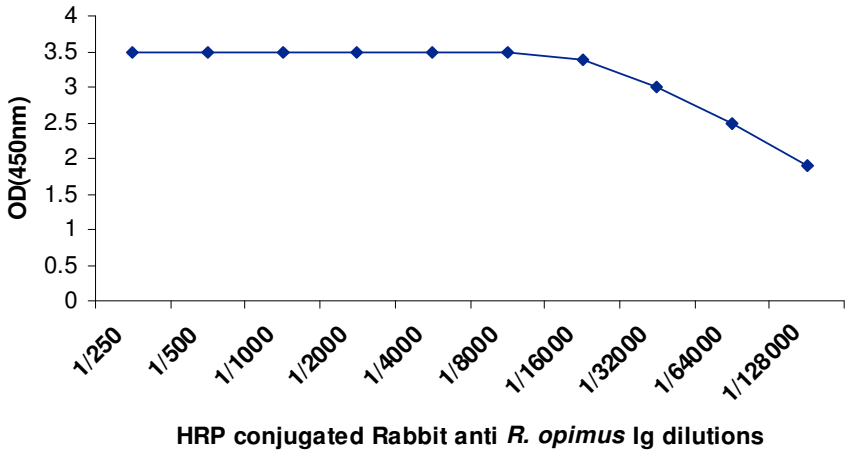

Fig. 4. Titration of HRP conjugated rabbit anti-*R. opimus* Ig by direct ELISA

Discussion

HRP conjugated rabbit anti-Gerbil IgG is produced by a few companies; however, to our knowledge there is no record of production of HRP conjugated rabbit anti-*R. opimus* Ig. Production of HRP Conjugated Rabbit anti-*R. opimus* Ig is needed for serological and immunological studies of *R. opimus*, the main reservoir host of ZCL in Iran as well as some other countries, which may lead to plan immunological control of the disease (Akhavan 2011).

Phlebotomus papatasi, vector of ZCL, co-injects *Leishmani*a parasites along with saliva into the skin of *R. opimus* while feeding. Saliva from the sand fly is known to have pharmacological as well as immunomodulatory activities such as vasodilatory and anticlotting effects which exacerbate the infectivity of the parasite (Kamhawi 2000). Investigation on the immune response of the host against salivary gland antigens of the vector, also interaction among the parasite, sand fly and host are necessary to find possible tools to control the disease, especially in developing anti *Leishmani*a vaccine and/or transmission blocking vaccine. Detecting the immune response of the host to saliva of the sand fly may also be used as a marker of transmission risk of the disease (Barral et al. 2000). The HRP conjugated rabbit anti-*R. opimus* Ig produced in this study helps the investigators to study on the immune response of *R. opimus*, to *L. major* exposure and infection. The HRP conjugated antibody is needed for immunoblotting and ELISA tests, which are used to detect the immune response of the rodents against the sand fly saliva.

Purification of *R. opimus* Ig is a necessary step for production of polyclonal antibody. No established protocol was recorded for purification of *R. opimus* Ig. In this regard, considering the fact that *R. opimus* is a mammal belonging to the order Rodentia and according to previous studies (Kemp 2005), protein G could adsorb Ig molecules from many mammals and rodents such as human, mouse and rabbit. Thus it seems reasonable that protein G is a suitable candidate for purification of IgG molecules from *R. opimus* serum. Using this method, 3.5 mg *R. opimus* Ig was obtained from 1 ml serum.

Moreover, due to the lack of knowledge about the molecular size of *R. opimus* Ig, another rodent IgG (mouse) was used as a standard for molecular weight determination in SDS-PAGE. The very similar migration of *R. opimus* Ig in the polyacrylamide gel to that of murine IgG allowed us to estimate it as around 150 kDa.

A high titer of rabbit anti-*R. opimus* Ig production after the first injection of *R. opimus* Ig showed that *R. opimus* Ig mixed with adjuvant was immunogenic in rabbit, so that after the second injection, the antiserum titer in the rabbit hardly increased. However, we continued the immunizations to increase the avidity or affinity of the produced antibodies.

In conclusion, this study describes the successful purification of *R. opimus* Ig and production of polyclonal antibodies against it in rabbit.

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