

Short Communication

Designing of enzyme linked immunosorbent assay (ELISA) kit for diagnosis copro-antigens of *Giardia lamblia*

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The sensitivity of microscopic examination of fecal samples to recognize *Giardia* parasites is low. In the methods based on antigen scanning of parasites such as enzyme linked immunosorbent assay (ELISA), copro-antigens of parasite will be traced and diagnosed even if the live parasite is absent in the fecal samples. To design this method, a pure antibody against parasite as well as an antibody conjugated to a proper enzyme is needed. In this study, an anti-*Giardia* IgG extracted from serum of contaminated rabbit was purified by ion-exchange chromatography and conjugated to the enzyme horse radish peroxidase (HRP). This antibody was used to design direct and indirect ELISA kits to measure conjugation titer. In both direct and indirect ELISA methods, optical densities (ODs) were 1 by using dilution of 1/4000 of conjugation. According to the results of both tests and the success in produced conjugate, it could be proceeded to prepare ELISA kits to diagnose giardiasis infections in various samples.

Key words: Enzyme linked immunosorbent assay (ELISA), antibody, copro-antigen, *Giardia lamblia*.

INTRODUCTION

Giardiasis is an intestinal infection with a worldwide spread caused by *Giardia lamblia* (Adam, 2001). Common method to diagnose giardiasis is microscopic examination of fecal samples (Rosoff and Stibbs, 1986). Disappearing parasite cyst in fecal samples will not always mean uprooting *Giardia* from the intestine of infected persons because this parasite has periodic expulsion in alternative days or various hours of day and also has short latent time in some patients and there is always a probability of hiding it by bile pigments (Gharavi et al., 2005). So the

sensitivity of microscopic examination of fecal samples even with multiple examinations is low (50 to 70%) (Janoff et al., 1989; Burke, 1977). In patients who show clinical symptoms of giardiasis, ELISA is a rapid, sensitive, and economic method to confirm infection and copro-antigens of that parasite which could be traced and diagnosed even if the live parasite is absent in the fecal samples (Aldeen et al., 1995; Goldin et al., 1990).

For finding copro-antigens of parasite, anti-parasite antibodies could be used in a sandwich ELISA method. Comparing other diagnostic methods, sensitivity and specificity of this method is very high (about 100%) and could help in cases where the results of microscopic test are negative (Braga and Catapani, 2005; Duque et al., 2002; Fallahi et al., 2008). Nevertheless, the methods based on antibody search have ignorable uses in clinical diagnosis and the presence of antibodies especially in endemic places is not always a reason of infection because the results of these approaches would be positive even after months of

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Abbreviations: HRP, Horse radish peroxidase; TMB, tetra methyl benzidine; PBS, phosphate-buffered saline; ELISA, enzyme linked immunosorbent assay.

Table 1. Conjugation titer levels using the designed direct ELISA kit. (parasite was coated to each well in constant quantity of $10^5/100 \mu\text{l}$ of PBS).

Conjugate Dilution	1/1000	1/2000	1/4000	1/5000	1/10000	1/20000
Optical Density	3.2	2.4	1.1	0.8	0.32	0.1

Table 2. Conjugation titer levels using the designed indirect ELISA kit.

Parasite quantity in 100 μl of PBS	Conjugate dilution						
	1/100	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000
10^3	3	1	0.6	0.41	0.32	0.27	0.12
10^4	3.2	1.6	0.9	0.63	0.5	0.42	0.24
10^5	3.4	2.2	1.3	1	0.9	0.7	0.4

full recovery. In the method of parasite antigen finding, there is a need to pure antibody against parasite along with an antibody conjugated to a proper enzyme. So we had to conjugate the gained pure antibody against the parasite *G. lamblia* with a proper enzyme and by designing ELISA kits, we measured its acceptable titers by direct and indirect ELISA methods.

MATERIALS AND METHODS

In this fundamental-practical study, we purified parasite from faeces of patients and injected it into rabbit and extracted polyclonal antibody from serum of rabbit (Barazesh et al., 2007). Then the produced rabbit anti-*Giardia* IgG was purified by ion-exchange chromatography and its identity was confirmed by SDS-Page electrophoresis (Barazesh et al., 2007).

Conjugation

The purified antibody was conjugated by periodate method (Nakane and Kawaoi, 1974) that was slightly modified (Baradaran et al., 2006; Majidi et al., 2007). In this method, 5 mg of horse radish peroxidase (HRP) enzyme was mixed with 0.3 ml of 0.1 M sodium periodate and incubated at room temperature for 20 min. The mixture was then dialyzed by sodium acetate buffer (1 mM) at 4°C for a night and added to 0.5 ml of the mixture of 10 mg/ml of *G. lamblia* in sodium carbonate. After incubation for 2 h at room temperature, 100 μl sodium borohydride (4 mg/ml) was added to the mixture and incubated again at 4°C for 2 h. Finally, it was dialyzed several times by exchange against PBS overnight at 4°C.

Designing of direct ELISA kit

To measure the produced conjugation titer, both direct and indirect ELISA kits were designed. In direct ELISA method, 1×10^5 parasite cysts/100 μl to each well were used. Then they were incubated 45 min at 37°C and washed twice by PBS buffer and dried completely. The blocking procedure was done by adding 100 μl per well of 0.5% Tween and microplates were incubated at 37°C for 1 h. After washing, HRP-conjugated anti-*Giardia* IgG was added to the wells at dilutions of 1/1000, 1/2000, 1/4000, 1/5000, 1/10000, and 1/20000 and incubated at 37°C for 45 min and then were washed 5 times. 100 μl tetra methyl benzidine (TMB) was added to each well and

incubated at room temperature for 20 minutes. Finally, block solution (sulfuric acid 5%) was added to wells and their optical densities (OD) were read at 450 nm by ELISA reader (State fax-USA).

Designing of indirect ELISA kit

100 μl of pure un-conjugated anti-*Giardia* antibody with dilution 1/100 was coated on each well and was incubated, washed and blocked using 10^3 - 10^5 parasite cysts/100 μl for wells. After incubation and washing, pure HRP-conjugated antibody in dilutions 1/1000 to 1/32000 was added to wells. The rest of the processes were like that in designing of direct ELISA kit; after incubation and washing, TMB was added and incubated. Block solution was added and their OD was read by ELISA reader.

RESULTS AND DISCUSSION

Direct and indirect ELISA kits were designed to measure the titer of produced conjugation and as shown in Table 1, OD of conjugation was above 1 for 10^5 parasites in 100 μl and 1/4000 dilution of conjugation (Table 1).

Table 2 shows that OD was above 1 for 10^3 parasites in 100 μl of PBS and 1/1000 dilution of conjugation as well as 10^5 parasites in 100 μl of PBS and 1/4000 dilution of conjugation (Table 2).

In the patients with giardiasis, *Giardia* has periodic expulsion in alternative days or various hours of day and its quantity will be decreased when the disease becomes chronic. Because of its short latent period, triggering clinical symptoms could be faster than beginning of cyst expulsion in some cases, and sometimes hiding the parasite by bile pigments could cause the parasite not to be seen in microscopic examination of fecal samples and subsequently result to false negative clinical report of the disease (Gharavi et al., 2005). Nowadays antigen scanning introduces better detection methods because it is believed that increase in antigen level of individual/sample shows that there is an increase in parasite level of infected individual/sample (Winięcka - Krusnell, 1995). In ELISA method, copro-antigens of parasite could

be traced and diagnosed even if the live parasite is absent in the fecal samples (Aldeen et al., 1995; Goldin et al., 1990). To design such method, along with a pure antibody against parasite, there is a need for an antibody to be conjugated to a proper enzyme.

To conjugate antibody to a proper enzyme, the usage instances should be considered. An enzyme that is proper to design ELISA kit could be unsuitable in some other objectives like immunochemistry. Furthermore, conditions of examination, pH, ionic strength, buffer composition and so on, could be incompatible with examination of sample and/or antigen-antibody reaction. In this study, the enzyme HRP was used to conjugate the pure antibody against *G. lamblia*. HRP is one of the most known enzymes in conjugation in serologic studies. Its molecular weight is about 44000 Da and there are various proper substrates for it in which their end product could be either soluble or insoluble. HRP is used in similar studies and the results of these approaches shows that a suitable enzyme is chosen (Majidi et al., 2007; Abdolalizade et al., 2008).

There are various methods to link an enzyme to an antibody (Nakane and Kawaoi, 1974; Yoshitake et al., 1982). To select the best method, it is important that the enzyme should be linked to antibody completely without deactivation of antibody and/or enzyme, the complex should be constant and whole for a long time and the method should be cheap and easy to use. HRP could conjugate to antibody by using each of periodate or CHM-NHS methods (Nakane and Kawaoi, 1974; Yoshitake et al., 1982). In this study, the antibody was purified by ion-exchange chromatography and then was conjugated by periodate method using previous (Baradaran et al., 2006; Majidi et al., 2007) approaches. In periodate method, the conjugation was done at carbohydrate end of each molecule and because its place has usually no effect on active site of enzyme and/or antibody, it is better than CHM-NHS method. Periodate also causes unfolding of carbohydrate ring that could then conjugate to free amino groups (Wilson and Nakane, 1987). On the other hand, CHM-NHS is a method with two separate action that its maleimide links enzyme to thiolated antibody.

In this study, periodate was chosen as conjugation method and the results has shown that it was a correct choice. In direct ELISA test, in constant quantities of parasite and dilution of 1/4000, optical density of conjugation was above 1. In indirect ELISA test, OD was about 1 of dilution 1/4000 of conjugate and quantity of 10^5 parasite/100 μ l of PBS. Both results show that the conjugation is correct and accurate. Referring to the gained conjugation, manufacturing ELISA kits to scan giardiasis infection in various samples should be worked on. This kind of test could be used as a proper screening test to detect asymptomatic carriers that expulse cyst and have main role in spreading of infection.

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