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Original Article

Development of Nano-ELISA Method for Serological Diagnosis of Toxoplasmosis in Mice

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Abstract

Toxoplasmosis is a widespread parasitic disease caused by a protozoan parasite *Toxoplasma gondii*. Currently, nanotechnology has been used for the diagnosis of many infectious diseases. It could be due to the fact that nanoparticles play an important role in accurate and fast diagnosis. The purpose of this study was to design a Nano-enzyme linked immunosorbent assay (Nano-ELISA) kit using excreted/secreted (E/S) antigens to have higher sensitivity and specificity than those reported for the designed enzyme-linked immunosorbent assay (ELISA) kit for the diagnosis of Toxoplasmosis in mice. Firstly, the serum samples were collected from 15 infected mice with *T. gondii* and 15 healthy ones. Then, E/S antigens were separated from parasite tachyzoites and used for designing an ELISA kit. In addition, the mice sera were evaluated using the designed ELISA kit. Finally, the serum samples were assessed by Nano-ELISA kits designed with E/S antigen and conjugate of gold nanoparticles. The obtained results of the present study showed that the sensitivity and specificity of the designed ELISA kit were reported as 80% and 86.66%, respectively, that both improved to 93.33% in these sera with the designed Nano-ELISA kit. This finding revealed the significant improvement of sensitivity and specificity using gold nanoparticles in designing the ELISA kit. Furthermore, according to the literature, the use of E/S antigens in designing recognizable ELISA kits has been always highlighted considering the presence of numerous antigens in *T. gondii*. The results of this study revealed that the use of E/S antigens in the preparation of an ELISA kit was very effective. This is very important, especially in the lower titers of antibody requiring a more accurate diagnosis. On the other hand, the Nano-ELISA method designed with E/S antigens can be more sensitive and specific than ELISA for the diagnosis of Toxoplasmosis and can be the basis for further studies in this regard.

Keywords: Nano-ELISA, Toxoplasmosis, Mouse

Développement d'un Nano-ELISA Pour le Diagnostic Sérologique de la Toxoplasmose Chez les Souris

Résumé: La toxoplasmose est une maladie parasitaire répandue causée par le parasite protozoaire *Toxoplasma gondii*. Actuellement, la nanotechnologie est utilisée pour le diagnostic de nombreuses maladies infectieuses. Cela peut être dû au fait que l'utilisation des nanoparticules peut aboutir à un diagnostic plus précis et rapide. Le but de cette étude était de concevoir un kit de test d'immunoadsorbant lié aux nano-enzymes (Nano-ELISA) utilisant des antigènes excrétés/sécrétés (E/S) pour obtenir une sensibilité et une spécificité plus élevées que celles rapportées pour le test d'immunoadsorbant lié à des enzymes (ELISA) conçu pour le diagnostic de la toxoplasmose chez la souris. Tout d'abord, les échantillons de sérum ont été collectés sur 15 souris infectées par

T. gondii et 15 souris saines. Ensuite, les antigènes E/S ont été séparés des tachyzoïtes parasites et utilisés pour concevoir un kit ELISA.Ensuite, les sérums de souris ont été évalués à l'aide du kit ELISA conçu. Enfin, les échantillons de sérum ont été évalués par des kits Nano-ELISA conçus avec un antigène E/S et un conjugué de nanoparticules d'or. Les résultats de cette étude ont montré que la sensibilité et la spécificité du kit ELISA conçu étaient respectivement de 80% et 86,66%. L'utilisation du kit Nano-ELISA sur les mêmes sérums permettait d'améliorer ces taux jusqu'à 93,33%. Ces résultats montrent que l'utilisation des nanoparticules d'or dans la conception du kit ELISA améliore de façon significative la sensibilité et la spécificité du diagnostic e. De plus, conformément à la littérature, l'intérêt de l'utilisation d'antigènes E/S dans la conception de kits ELISA est à souligner, compte tenu de la présence de nombreux antigènes dans *T. gondii*. Les résultats de cette étude ont également révélé que l'utilisation d'antigènes E/S dans la préparation d'un kit ELISA était très efficace. Ce point estd'une importance majeure, en particulier dans les titres inférieurs d'anticorps nécessitant un diagnostic plus précis.De plus, la méthode Nano-ELISA conçue avec des antigènes E/S peut être plus sensible et spécifique que l'ELISA pour le diagnostic de la toxoplasmose et peut être le sujet d'études complémentaires.
Mots-clés: Nano-ELISA, Toxoplasmose, Souris

Introduction

Toxoplasmosis is a zoonotic parasitic disease in animals caused by a parasitic protozoan called *Toxoplasma gondii* (Tavassoli et al., 2013). This parasite has a high potential for the contamination of homoeothermic beings and can be hidden after infecting many species. *T. gondii* is a parasite from the Apicomplexa group that is responsible for the contamination of a wide range of vertebrates. Humans are affected by this pathogen in two ways, namely acquired and congenital (Dubey, 2008).

As acquired immune deficiency syndrome (AIDS) prevalence widespread, *T. gondii* as an opportunistic pathogen has been identified as the leading agent of mortality among AIDS patients. *T. gondii*-induced encephalitis is the central complication of Toxoplasmosis in AIDS patients. About 20% up to 47% of individuals with antibodies against *T. gondii* suffer from Toxoplasmic encephalitis (Cox and Wakelin, 2010).

Toxoplasmosis leads to huge damages to both humans and animals. Congenital Toxoplasmosis which is the result of feticide, the birth of infants with complications, such as hydrocephalus, microcephaly, low intelligence, severe abnormalities in organs, blindness, physical difficulties, and mental disorders, such as Schizophrenia, annually leads to economic damages. The treatment of patients in the United States costs about 0.4-8.8 dollars, and this cost in England is estimated at 1.2 up to 12 million dollars (Markell et al., 2006).

Rodents, ruminants, such as sheep and cattle, birds, and pigs are some of the hosts of this parasite. In addition, cats are the final host of this parasite (Mosallanejad et al., 2012). The expansion of these hosts and close relationship between these animals and humans are the major causes of the prevalence of Toxoplasmosis in humans. Therefore, 500 to 1 million individuals, who are about one-third of the world's total population, suffer from this disease (Shirbazou et al., 2013).

The provision of suitable sanitary conditions in the society, especially in high-risk communities, and prevention of mental disorders caused by feticide, stillbirth, preterm birth, and congenital difficulties, show the importance of constant monitoring of this common disease in urban and rural communities.

The diagnosis of Toxoplasmosis can be performed using molecular methods, Immunoblotting techniques, tissue biopsy, and serology. These methods include the Sabin-Feldman dye test, indirect hemagglutination test, indirect fluorescent antibody test, and enzyme-linked immunosorbent assay (ELISA) (Meganathan et al., 2010). Another test named the modified agglutination

test with high sensitivity and specificity appears on tachyzoite (Gamble et al., 2005).

Polymerase chain reaction (PCR) is also one of the best methods for testing Toxoplasmosis, with high sensitivity and specificity (Koloren, 2013). Among the above-mentioned methods, ELISA is reported with high sensitivity and specificity. In this case, such a method with commercial kits can be widely applied in laboratories all around the world for the measurement of immunoglobulin M, immunoglobulin G, and immunoglobulin E antibodies (Montoya, 2002).

Unacceptable positive and negative results can be the most crucial issues in case of these kits. Multiple studies have been carried out for the identification of *T. gondii* compounds as antigens to be used in serologic diagnostic methods for the improvement of their diagnostic significance (Gamble et al., 2005; Glor et al., 2013). In this regard, high attention has been focused on the excreted/secreted (E/S) antigens of *T. gondii* tachyzoite (Suzuki, 2002). Regarding the reported characteristics of these antigens, it seems that they are suitable for the detection of antibodies acting against the parasite in the serum (Nishikawa et al., 2002). Several studies have shown that the ELISA method using the E/S antigens of *T. gondii* has high sensitivity and specificity for the diagnosis of Toxoplasmosis in rats (Nguyen et al., 1996).

Currently, nanotechnology has been used to diagnose many infectious diseases (Hauck et al., 2010). On the other hand, gold nanoparticles are among the particles that are useful for the diagnosis of some diseases. The use of gold nanoparticles for protein and deoxyribonucleic acid (DNA) analysis is effective due to the high absorption and optic refraction of this particle at certain wavelengths, with fluorescence properties specific to optical detection techniques (e.g., spectroscopy) (Ambrosi et al., 2010). Furthermore, the important characteristics of gold nanoparticles, with a high surface to volume ratio and unique properties, lead to the use of this nanoparticle as the basis of a biomarker (Jia et al., 2009). Finally, it should be noted that gold nanoparticles can easily conjugate with DNA, antibodies, enzymes, and other biomolecules and increase the number of biochemical detection signals (Jia et al., 2009).

Since the serum levels of marker proteins in the early stages of most diseases are very low, and different antibody titers are observed at different stages of the disease, it is not inside the diagnostic range of ELISA; therefore, many of the ELISA usual methods were not reported with favorable results at different levels of the disease (Jia et al., 2009). To date, no studies have been carried out to improve the diagnosis of Toxoplasmosis with designing Nano-enzyme linked immunosorbent assay (Nano-ELISA). With this background in mind, the present study, in case of success, will be the first study in the world leading to the development of a technique to increase and improve the sensitivity and specificity of the methods for the diagnosis of Toxoplasmosis.

Material and Methods

In this study, a total of 30 serum samples were gathered from mice. The mice were divided into two groups. The positive group included 15 experimentally infected mice within the weight range of 22-25 g with approximately 7 weeks of age. The negative group consisted of 15 laboratory mice similar to those of the positive group and free Toxoplasma parasite that provided from Pastor Institute that they were negative after serological tests and isolated for our research. It should be mentioned that the commercial ELISA kit for the diagnosis of Toxoplasmosis in mice with suitable conjugate was not available; accordingly, it was firstly necessary to design an ELISA kit for the diagnosis of *T. gondii* with anti-rat conjugate and E/S antigens.

Determination of excreted/secreted antigens for ELISA. Studies have shown that the E/S antigens of *T. gondii* are well characterized to be used in ELISA kits (Nguyen et al., 1996; Nishikawa et al., 2002). For the isolation of these antigens, a procedure was performed (Dubey, 2008). Firstly, six white laboratory mice free

of *T. gondii* with an approximate age of 6-8 weeks and weight range of about 22-25 g were selected. Then, 0.2 ml of a dilution, including 1×10^7 ml of tachyzoite *T. gondii*, was intraperitoneally injected from the RH strain. After 3 days, the animals were inhaled with carbon dioxide (with the consideration of all the ethical standards and animal protection rules) without bleeding for the prevention of peritoneal fluid contamination with blood.

Phosphate-buffered saline (PBS) was injected into the peritoneum, and then the exudate was collected. Subsequently, the obtained liquid was centrifuged for 5 min at 500 g. The supernatant was suspended in a Hanks' Balanced Salt Solution. At this stage, the concentration of tachyzoite and contamination caused by the host cells were evaluated using the Neubauer counting (tachyzoite count with a dilution of 1/1000 and an infection with host cells with a dilution ratio of 1.10). Afterward, washing operation was performed, and the last step was repeated after each washing. Finally, less than 0.5% of the infection with the mononuclear host and less than 0.25% of the infection with blood cells were detected. Up to reaching a final concentration of 1×10^7 ml, tachyzoite will be kept in PBS. If there is no evidence of infectious agents, tachyzoite can be kept with no antibiotics (i.e., penicillin or streptomycin).

Production of dilution antigen for ELISA. Firstly, 5 ml of tachyzoite was centrifuged for 15 min by 2000 g, and the supernatant was mixed with nine times distilled water. Three times freezing and watering process was performed to break the tachyzoite. Antigen preparations were continued for 20 sec using a sonication device at 4°C. Centrifuging for 30 min at the same temperature by 10000 g leads to the separation of waste components and cellulose residues. The supernatant was stored at a negative temperature of 20°C until use. The prepared antigens were measured two times for the determination of protein content using the Lowry protein assay and Bradford protein assay.

The protein content was reported as 30 mg/ml. After the determination of protein content, because the E/S antigens of *T. gondii* are proteins with a molecular weight range of 6.5-200 kDa, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used for the determination of the existing proteins. The samples containing the E/S antigens of *T. gondii* were evaluated by electrophoresis.

Design of ELISA method with E/S antigens. In order to design this procedure, the best serum dilution and concentration of E/S antigen for coating should be determined in the first step. After diluting and checking the cheek board for ELISA, the optimal concentration for ELISA is 2 mg/ml, and the proper dilution for serum is 1/50. Then, the ELISA designing steps were performed with E/S antigen as follows:

Connecting the antigen to coagulation, blocking empty sites, conjugation of Sigma conjugated Anti Rat, adding a substrate (i.e., chromogenic substrate), stopping solution (stop solution), and reading samples with ELISA reader at 450 nm

All of the sera samples of 30 mice were evaluated for seroepidemiology the results of which are presented in the results section.

Design of Nano-ELISA with E/S Antigen. The procedure was similar to designing an ELISA kit. Only the conjugation phase was prepared from a gold nanoparticle colloid manufactured by PlasmaChem GmbH in Germany with a 1/500 dilution. Finally, the samples were read with an ELISA reader at 450 nm. The results of testing positive and negative samples are presented in the results section in detail.

Results

Positive and negative mice sera were evaluated using an ELISA kit. The cut-off value was considered 1 indicating that the values higher and lower than 1 were regarded as positive and negative, respectively. The results of the mice positive and negative serum test with an ELISA kit are presented in tables 1 and 2, respectively.

Table 1. Results of 15 positive mice sera with designed enzyme-linked immunosorbent assay kit

Test	OD	Test	OD	Test	OD
1	1.364	6	1.250	11	2.185
2	1.902	7	1.508	12	1.054
3	1.190	8	0.579 α	13	1.722
4	2.135	9	1.183	14	1.483
5	0.882 α	10	1.645	15	0.874 α

(α): Samples no. 5, 8, and 15 have an optical density lower than the cut-off point and are negatively evaluated.

Table 2. Results of 15 negative mice sera with designed enzyme-linked immunosorbent assay kit

Test	OD	Test	OD	Test	OD
1	0.480	6	0.459	11	0.245
2	0.539	7	1.132 α	12	0.331
3	0.281	8	0.770	13	0.381
4	0.775	9	1.180 α	14	0.409
5	0.138	10	0.157	15	0.525

(α): Samples no. 7 and 9 have higher optical density than the cut-off point and are positively evaluated.

The ability to correctly diagnose all patients is called sensitivity. The ability to test for the correct diagnosis of all those who are not sick is called specificity. Using the obtained results containing sensitivity and specificity formulas can lead to reaching the sensitivity and specificity of the diagnostic kit calculated as follows:

True positive: those who are sick and tested positive (TP).

True negative: those who are not sick and whose tests are negative (TN).

False positive: those who are not sick and whose tests are positive (FP)

False negative: those who are sick and tested negative (FN).

$$\text{Sensitivity: } 100 \times \frac{TP}{TP+FN}$$

$$\text{Specificity: } 100 \times \frac{TN}{TN+FP}$$

In this study, 15 sera from the infected laboratory mice and 15 sera from the healthy laboratory mice were tested using ELISA as previously described in Table 3.

Table 3. Sensitivity and specificity of enzyme-linked immunosorbent assay kit in mice

True positive	True negative	False positive	False negative	Sensitivity (%)	Specificity (%)
12	13	2	3	80.00	86.66

Results of Nano-ELISA method designed with E/S antigen. The mice positive and negative sera designed with the Nano-ELISA kit were prepared to be evaluated. The cut-off value was considered 1 indicating that the absorbance values higher and lower than 1 were regarded as positive and negative, respectively. The results of the mice positive and negative serum test with the designed Nano-ELISA kit are presented in tables 4 and 5, respectively.

Table 4. Results of 15 mice positive sera with designed Nano-enzyme linked immunosorbent assay kit

Test	OD	Test	OD	Test	OD
1	1.481	6	1.533	11	2.182
2	2.175	7	1.489	12	1.579
3	1.225	8	0.540 α	13	1.919
4	1.884	9	1.646	14	1.510
5	1.249	10	2.039	15	1.043

(α): Sample no. 8 has an optical density lower than the cutoff point and is negatively evaluated.

Table 5. Results of 15 mice negative sera with designed Nano-enzyme linked immunosorbent assay kit

Test	OD	Test	OD	Test	OD
1	0.423	6	0.629	11	0.284
2	0.486	7	0.859	12	0.214
3	0.362	8	0.770	13	0.467
4	0.560	9	1.125 α	14	0.338
5	0.125	10	0.123	15	0.418

(α): Sample no. 9 has an optical density higher than the cutoff point and is positively evaluated.

According to the obtained results, the sensitivity and specificity of the Nano-ELISA designed with E/S antigen and gold nanoparticles are presented in Table 6.

Table 6. Sensitivity and specificity of designed Nano-enzyme linked immunosorbent assay kit in mice

True positive	True negative	False positive	False negative	Sensitivity (%)	Specificity (%)
14	14	1	1	93.33	93.33

The results of the Nano-ELISA kit designed with the E/S antigens of T. gondii showed that the sensitivity and specificity of this test was higher than those reported for the ELISA designed kit with E/S antigens.

Discussion

Toxoplasmosis is a common parasitic disease caused by a protozoan organism parasite called T. gondii. The prevalence of Toxoplasmosis is different according to location, cultural level, and health information. Accordingly, the prevalence of Toxoplasmosis in North America and United Kingdom is reported within the range of 16-40%, and it is estimated within the range of 50-80% in Central and South America and Europe (Habibi et al., 2012; Glor et al., 2013).

According to the results of studies focusing on mice, the infection rate of T. gondii was reported as up to 70% in Montaña, Italy, in 1991 (Genchi et al., 1991). Childs and Seegar showed the infection rate of 49.5% in their studies in Baltimore, Maryland, in 1986 (Childs and Seegar, 1986). According to studies carried out in England, it was reported that 42.2% of the infection was associated with T. gondii in rats according to PCR (Hughes et al., 2006).

The detection of antibodies produced against T. gondii in the sera of individuals is a common method of diagnosis in Toxoplasmosis (Fuentes et al., 1996). The use of the ELISA method seems more appropriate among the available techniques for the diagnosis of Toxoplasmosis. The sensitivity of ELISA is relatively high; therefore, the reaction will also occur with 1-5 ng of antigen (Hassan et al., 1997).

The present study aimed to evaluate the Nano procedure in increasing the sensitivity and specificity of the serological methods of ELISA. As previously mentioned, a commercial ELISA kit was not available for the diagnosis of Toxoplasmosis in mice; therefore, it was first necessary to design an ELISA kit for mice with suitable conjugate and E/S antigens and then compare it to the newly designed Nano-ELISA kit with gold nanoparticles.

The obtained results showed that the sensitivity and specificity of the designed ELISA kit were 80.00% and 86.66%, respectively; however, the sensitivity and specificity of the designed Nano-ELISA kit in the same serum samples were 93.33% indicating a significant improvement in the use of nanotechnology in the

ELISA method. The reason for the increased sensitivity and specificity of ELISA with nanoparticles in the current study is probably the high level of improvement in the volume of gold nanoparticles that causes more antibodies to enter the antigen-antibody complex with the help of nanoparticles leading to a better dyeing.

One of the advantages of this study was that in all of the ELISA methods, the serum dilution was much less studied than other methods. It should also be noted that the use of gold nanoparticles resulted in a lower dilution of serum. Considering the emphasis of all government officials and authorities on using national products, another advantage of this study was designing a native Nano-ELISA with gold nanoparticles. Although this method has high sensitivity and specificity, it requires a low cost of production of the masses. The present study also demonstrated that the use of nanoparticles in the design of screening kits is useful and could be very important, especially in some diseases with fatal side effects.

Authors' Contribution

Study concept and design: R. M.
Acquisition of data: A. Kh.
Analysis and interpretation of data: N. H. R.
Drafting of the manuscript: N. A.
Critical revision of the manuscript for important intellectual content: R. M.
Statistical analysis: A. Kh.
Administrative, technical, and material support: RVSRI

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that there is no conflict of interest.

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