Design of Nano-ELISA method for serological detection of Toxoplasmosis in mouse

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Abstract

Toxoplasmosis is a widespread parasitic disorder that caused by protozoan parasite *Toxoplasma gondii*. Nowadays, nanotechnology is used for diagnose of many infectious diseases. It could be because of nanoparticles play an important role in order to accurate and fast diagnose. The purpose of this research is to create a Nano-ELISA (enzyme linked immunosorbent assay) kit with Excerted/Secreted Antigens to have a greater sensitivity and specificity than the designed ELISA kit for detection of Toxoplasmosis in mouse. At first serum was collected from 15 infected mice by *Toxoplasma gondii* and 15 healthy ones. Then E/S antigen is separated from parasite Tachyzoites and used for designing ELISA kit by E/S antigens. Mice sera evaluated by designed ELISA kit. Finally Serum samples are investigated through Nano-ELISA kits that designed with E/S antigen and conjugated containing gold nanoparticles. Results showed that sensitivity and specificity of designed ELISA kit are 80% and 86.66% that both improved to 93.33% with designed Nano-ELISA kit. This results show significant improvement of sensitivity and specificity when use gold nanoparticles in design of ELISA kit. Also In current studies, the operation of E/S antigen for the design of recognizable ELISA kits have always been highlighted. The results of this research show that the use of E/S antigens in the preparation of ELISA kit is very effective. This is very important especially in the lower titers of Antibody that require more accurate diagnosis. In the other hand, Nano-ELISA designed with E/S antigen can be more sensitive and specific than ELISA method for detecting Toxoplasma and also can be the basis for further studies in this field.
Key words: Nano-ELISA, Toxoplasmosis, Mouse

Introduction

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

As AIDS (acquired immune deficiency syndrome) prevalence widespread, Toxoplasma as an opportunistic pathogen has been identified as the leading agent of death among AIDS patients. Toxoplasma induced Encephalitis is the central complications of Toxoplasmosis in AIDS patients. About 20% up to 47% of people who have antibodies against Toxoplasma, face with Toxoplasma Encephalitis (Cox and Wakelin, 2010).

Toxoplasmosis leads to huge damages to both humans and animals. Congenital Toxoplasmosis which is the result of feticide, birth of infants with complications such as Hydrocephalus, Microcephaly, low intelligence, severe abnormalities in organs, blindness, physical difficulties, mental disorders such as Schizophrenia leads to economic damages annually. Patient treatment in the United States cost about $ 0.4 to $ 8.8 and this cost in England estimated to 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 and cats are the final host of this parasite (Mosallanejad et al., 2012). The expansion of these hosts and the close relationship between these animals and humans are a major cause of the prevalence of Toxoplasmosis in human beings. As a result of this fact, 500 to 1 million people, who are about one-third of the world's total population, are faced with this disease (Shirbazou et al., 2013).

Existence of suitable sanitary conditions in the society, especially in high risk communities, prevention of mental disorders which caused by feticide, stillbirth, preterm birth, congenital difficulties, show the importance of constant monitoring of this common disease in urban and rural communities.
Diagnosis of Toxoplasmosis can be done by some methods as well as, molecular method, Immunoblotting techniques, tissue biopsy and serology. These methods include Sabin-Feldman dye test, indirect hemagglutination (IHA), indirect fluorescence antibody (IFA), ELISA (Meganathan et al., 2010). Another test which called the modified agglutination test (MAT) that also has a 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, which has a high sensitivity and specificity (Koloren, 2013). Among the above methods, ELISA method has high sensitivity and specificity. In this case, such a method which has commercial kits can widely use in laboratories all around the world to measure IgM, IgG and IgE antibodies (Montoya, 2002).

Unacceptable positive and negative results can be the most crucial issue in case of these kits. Many studies have been done to identify compounds of Toxoplasma as antigens for use in serologic diagnostic methods to improve their diagnostic significance (Gamble et al., 2005; Glor et al., 2013).

In this regard, high attention has been focused to the E/S antigens of Tachyzoite of Toxoplasma (Suzuki, 2002). Regarding to characteristics which are reported for these antigens, it seems they are suitable for detecting antibodies that act against the parasite in the serum (Nishikawa et al., 2002). Several studies have shown that ELISA method which uses E/S antigens of Toxoplasma, has a high sensitivity and specificity for detecting Toxoplasmosis in rats (Nguyen et al., 1996).

Nowadays, nanotechnology is used to diagnose many infectious diseases (Hauck et al., 2010). In the other hand, gold nanoparticles are one of the particles that are useful for detecting some diseases. The use of gold nanoparticles for protein and DNA analysis is effective due to high absorption and high optical refraction of this particle at certain wavelengths, having the specific fluorescence properties specific to optical detection (spectroscopy) techniques (Ambrosi et al., 2010) and the important characteristics of gold nanoparticles, which have a high surface to volume ratio, as well as having unique properties, that 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 also increase the number of biochemical detection signals (Jia et al., 2009).

Since the serum level 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, and therefore many of the ELISA usual methods has not a favorable result at different levels of the disease (Jia et al., 2009).

Since no research has ever been done to improve the diagnosis of Toxoplasmosis with the design of Nano-ELISA, this study, in case of being successful, will be the first study in the world and lead to create a way to increase and improve the sensitivity and specificity of the methods for detecting Toxoplasmosis.

**Materials and Methods**

Totally 30 serum samples gathered from mice. We divided these mice into positive group (including 15 experimental mice who were weighting from 22 to 25 grams and approximately 7 weeks old) that were experimentally infected and the negative group (including 15 laboratory mice as same as the positive one and free Toxoplasma parasite that prepared 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 detection of Toxoplasmosis in mouse with suitable conjugate was not available, because it was necessary at first, to design ELISA kit for detection of Toxoplasma with anti-rat conjugate and E/S antigens.

Determination of exerted/secreted antigens for ELISA: Studies have shown that E/S antigens of Toxoplasma are well characterized to use in ELISA kits (Nguyen et al., 1996; Nishikawa et al., 2002). To isolate these antigens, the following procedure was performed (Dubey, 2008).

First, six white laboratory mice who were free of toxoplasma with an approximate age of six to eight weeks and a weight of about 22 to 25 grams were selected. Then 0.2 ml of dilution included $1 \times 10^7$ ml of Tachyzoite *Toxoplasma gondii* was injected intra-peritoneally (I.P.) from RH strain. After three days, the animals were inhaled with carbon dioxide gas (with all ethical standards and Animal protection rules) and without bleeding, to prevent peritoneal fluid contamination with blood.

PBS (phosphate buffered saline) injected into the peritoneum and then the exudate was collected. Then the resulting liquid centrifuged five minutes at 500 g. The supernatant was suspended in a Hank's balanced salt solution (HBSS). At this stage, concentration of Tachyzoite and contamination caused by the host cells evaluated using the Neobar counting (Tachyzoite count with a dilution of 1/1000 and an infection with host cells with a dilution ratio of 1.10). Then washing operation was done and last step repeated after each wash, eventually having less
than 0.5% of the infection with the mononuclear host and less than 0.25% infection with blood cell were detected. Until reaching to a final concentration of $1 \times 10^7$ ml, Tachyzoite will be kept in PBS. If there is no evidence of infectious agents, Tachyzoite can be kept with no antibiotics (penicillin or streptomycin).

Production of dilution antigen for ELISA: at first, Centrifuge 5 ml of Tachyzoite for 15 minutes 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. Using a sonication device at 4°C, antigen preparations were continued for 20 seconds. Centrifuging for 30 minutes at same temperature by 10000g leads to 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 2 times for protein content by Loury protein assay and Bradford protein assay.

The protein content was 30 mg/ml. After protein content determination, because that the excereted and secreted antigens of Toxoplasma are proteins with a molecular weight between 6.5 to 200 kilodaltons, SDS-PAGE electrophoresis was used to determine the existing proteins. The samples containing the E/S antigens of toxoplasma was evaluated by electrophoresis.

Design of ELISA method with E/S antigens: In order to design this procedure, the best serum dilution and the best concentration of E/S antigen for coating should be determined in the first step.

After diluting and checking the check board for ELISA, the optimal concentration for ELISA is 2mg/ml and the proper dilution for serum is 1/50. Then, the ELISA design steps were performed with E/S antigen as follows. Connection of antigen to coagulation, blocking empty sites, conjugation of Sigma conjugated Anti Rat, adding a substrate (chromogen-substrate), stopping solution (stop solution), and finally read samples with ELISA reader in 450 nm. All of 30 mice sera samples were evaluated for seroepidemiology that the results of which were described in the results chapter.

Design of Nano-ELISA with E/S Antigen: the procedure was same with design of ELISA kit. Only the conjugation phase was prepared from a gold nanoparticle colloid manufactured by Plasma Chem Company which located in Germany with a 1.500 dilution. Finally samples have been read with ELISA reader in 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 with ELISA kit. The cutoff value was considered as the number one, means that the values above 1 are considered positive and the values lower than 1 are considered negative. The results of the mice positive and negative serum test with ELISA kit are presented in Tables 1 and 2, respectively.

<table>
<thead>
<tr>
<th>Test</th>
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(α): Samples No. 5, 8 and 15 have optical density less than the cutoff point and are negatively evaluated

<table>
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<td>0.157</td>
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</table>

(α): Samples No. 7 and 9 have more optical density than the cutoff point and is positively evaluated

The ability to diagnose all patients correctly is called sensitivity. The ability to test for the correct diagnosis of all those who are not sick is called a specificity.

Using the obtained results contains sensitivity and specificity formulas, can lead to reaching the sensitivity and specificity of the diagnostic kit and it can be calculated as follows.

True positive: Those who are patient and tested positive (TP, True Positive)

True Negative: Those who are not sick and whose tests are negative (TN, True Negative)

False positive: Those who are not sick and whose test is negative (FN, False Positive).

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

Sensitivity: \(100 \times \frac{TP}{TP+FN}\)

Specificity: \(100 \times \frac{TN}{TN+FP}\)
In this study, 15 sera from infected laboratory mice and 15 sera from healthy laboratory mice were performed by using the ELISA as described above in Table 3.

Table 3: Sensitivity and Specificity of the ELISA kit in mice

<table>
<thead>
<tr>
<th>TP</th>
<th>TN</th>
<th>FP</th>
<th>FN</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<tbody>
<tr>
<td>12</td>
<td>13</td>
<td>2</td>
<td>3</td>
<td>80.00</td>
<td>86.66</td>
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</tbody>
</table>

Results of the Nano-ELISA method prepared with E/S antigen: mice positive and negative sera designed with Nano-ELISA kit, prepared to be evaluated. Cut Off was considered as the number one, means that the absorbance values above 1 are considered as positive and the values lower than 1 are considered negative. 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 a designed Nano-ELISA kit

<table>
<thead>
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<td>1.249</td>
<td>10</td>
<td>2.039</td>
<td>15</td>
<td>1.043</td>
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</tbody>
</table>

(α): Sample No. 8 optical density is less than the cutoff point and evaluates negatively

Table 5: Results of 15 Mice Negative Sera with Designed Nano-ELISA Kit

<table>
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<tr>
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<th>OD</th>
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<tr>
<td>2</td>
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<td>0.859</td>
<td>12</td>
<td>0.214</td>
</tr>
<tr>
<td>3</td>
<td>0.362</td>
<td>8</td>
<td>0.770</td>
<td>13</td>
<td>0.467</td>
</tr>
<tr>
<td>4</td>
<td>0.560</td>
<td>9</td>
<td>1.125</td>
<td>14</td>
<td>0.338</td>
</tr>
<tr>
<td>5</td>
<td>0.125</td>
<td>10</td>
<td>0.123</td>
<td>15</td>
<td>0.418</td>
</tr>
</tbody>
</table>

(α): Sample No. 9 optical density is over than the cutoff point and evaluates positively

According to the 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 the designed Nano-ELISA kit in mice

<table>
<thead>
<tr>
<th>TP</th>
<th>TN</th>
<th>FP</th>
<th>FN</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<tr>
<td>14</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>93.33</td>
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</table>

The results of the Nano-ELISA designed with E/S antigens of Toxoplasma showed that the sensitivity and specificity of this test is greater to those of ELISA designed with Excreted/Secreted antigens.

Discussion:

Toxoplasmosis is a common parasitic disorder that caused by a protozoan organism parasite which called *Toxoplasma gondii*. The prevalence of Toxoplasmosis is different according to the location, differences in the cultural level, and health information, so that in North America and the United Kingdom the prevalence of Toxoplasmosis is 16-40% and in Central and South America and the Europe is estimated to be 50-80% (Habibi et al., 2012; Glor et al., 2013). According to studies which focused on mice, the infection rate to Toxoplasma was mentioned up to 70%, that was reported in 1991 in Montaña, Italy (Genchi et al., 1991). Childs and his colleagues in Baltimore, Maryland, in 1986, pointed 49.5% of the infection in their studies (Childs and Seegar, 1986). Researchers in England, reported that according to PCR method, 42.2% of the infection revealed with Toxoplasma in Rat (Hughes et al., 2006).

The detection of antibodies produced against Toxoplasma in the serum of individuals is a common method of diagnosis in Toxoplasmosis (Fuentes et al., 1996). The use of ELISA method seems more suitable among the techniques available to diagnose Toxoplasmosis. The sensitivity of ELISA is relatively high; so the reaction will be occur with 1 to 5 Nano grams of antigen too (Hassan et al., 1997).

In this study, the aim of the research was to evaluate the Nano procedure in increasing the sensitivity and specificity of serological methods of ELISA. As mentioned, commercial ELISA kit for detecting Toxoplasmosis in mouse was not available and this was necessary to design ELISA kit for mouse with suitable conjugate and E/S antigens at first, and then compare it with new designed Nano-ELISA kit with gold nanoparticles.

The results showed that the sensitivity and specificity of the designed ELISA kit were 80.00% and 86.66%, respectively, whereas the sensitivity and specificity of the Nano-ELISA kit designed, in the same serum samples were 93.33% that indicating a significant improvement in the use of nanotechnology in the ELISA method.
The reason for the increased sensitivity and specificity of the ELISA with nanoparticles in this research is probably the high level of improvement in the volume of gold nanoparticles, which causes more antibodies to enter the antigen-antibody complex with the help of nanoparticles which leads to a better dyeing.

It should mention that in all of the ELISA methods, the serum dilution was much less studied than other researches. It should also be noted that the use of gold nanoparticles led to lower dilution of serum.

Considering the emphasis of all government officials and authorities in using national products, another benefit of this study is the design of a native Nano-Elisa with gold nanoparticles, although this method has a high sensitivity and specificity, but a low cost of production the masses are needed.

Our research also determined that use of nanoparticles in design of screening kits is useful and could be very important especially in some disease with fatal side effects.

References:


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