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ORIGINAL RESEARCH

Development of an efficient multiplex semi-nested PCR for convenient use in urine samples for diagnosis of toxoplasmosis

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BACKGROUND: Toxoplasmosis is a protozoan parasitic infection. The cerebrospinal, ocular and congenital forms of the disease are complicated and life threatening. Diagnosis of toxoplasmosis is difficult due to invasive sample requirement, complications in pathogenesis, immunology, and interpretation of test results. Using urine as a non invasive sample source for molecular diagnosis of toxoplasmosis is the main object of this research. Attempts were made to diagnose toxoplasmosis using polymerase chain reaction (PCR) technique on the parasite's deoxyribonucleic acid (DNA) in urine.

METHODS: Parasite: The Toxoplasma gondii (T. gondii) RH strain was obtained from peritoneal exudates of small white laboratory mice inoculated with 5x105 tachyzoites three days before aspiration. Urine samples with defined numbers of tachyzoites per ml were used as laboratory samples. The target was a 529 bp segment (AF146527 gene bank) of the T. gondii genome with 200-300 repeats as target in PCR. Selected primers were designed on its sequence.

RESULTS: A multiplex semi-nested PCR technique was developed for obtaining a method for precise diagnosis of toxoplasmosis, time and budget saving and to diminish the DNA cross contamination risk. It was sensitive to detect 2 this tachyzoites DNA per final sample.

CONCLUSION: With further development, nested PCR can be a useful and non-invasive method for diagnosis of cerebrospinal, ocular, and congenital toxoplasmosis.

KEYWORDS: Toxoplasmosis; Toxoplasma gondii; Diagnosis; Urine sample; Multiplex; Nested PCR.

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INTRODUCTION

Toxoplasmosis is a prevalent worldwide infection.[1-3] It is distributed in humans and various animal species in all parts of Iran.[4-7] Toxoplasmosis appears after infestation by a parasitic protozoon, Toxoplasma gondii.[1-6] Practically all infections are chronic, especially in normal immune-competent individuals with only with mild symptoms and with a positive serologic test response for life. Nevertheless, in immune-compromised persons (e.g. AIDS patients), in congenitally infected children, in the ocular form, in cancer patients and organ transplant recipients, toxoplasmosis causes considerable mortality and morbidity.[5-9]

Serologic based methods are recommended for diagnosis of toxoplasmosis, although their results are difficult to interpret for immune compromised patients, fetuses and infants.[10]

The parasites invade almost all body tissues. Parasite invasion of the central nervous system, optic system, and fetuses, render them sometimes risky and life threatening. Laboratory diagnosis of this parasite still depends on invasive specimen collection from infected tissues. Sample collection is associated with hazards and side effects for patients consequently, the investigators prefer to use urine samples because of their safe and easy accessibility.[11-14] At the time of writing, urine sampling for the diagnosis of several parasitic infections includes malaria, toxoplasmosis, amebiasis, trichomoniasis, leishmaniasis, trypanosomiasis and schistosomiasis.[15-23] However, due to different forms of toxoplasmosis, its immunoantigenicity, and its wide range of virulence, its cross reactivity and the various organs involved, many unanswered questions remain concerning new diagnostic methods with the desired sensitivity and specificity.[11,21,23]

The main object of the present research is the diagnosis of toxoplasmosis using the polymerase chain reaction (PCR) technique to detect the parasite's DNA in urine is. Experiments were aimed at DNA extraction from urine samples and the development of a Multiplex semi nested PCR. The latter is a high performance molecular diagnostic method with minimal DNA contamination risk and time saving.

MATERIAL AND METHODS

Parasite

The RH strain of T. gondii was obtained from Parasitology Group of Isfahan University of Medical Sciences and

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Table 1 - Comparative performance and minimum limit of DNA extraction from urine samples by different extraction methods, and their responses to PCR

Extraction method	Tachyzoites per μl urine	Absorbance 260/280 nm	Total DNA(μg)	PCR test	Minimal tachyzoite/ μl for a positive PCR
Fuentes[16]	10	1.02	2.55	positive	5
Holman[28]	10	1.15	3.67	positive +	3-5
El-Awardy[10]	10	0.88	1.20	negative	5-10
Priem[27]	10	0.65	0.93	negative	5-10
Cinnagene DNP kit	10	2.00	9.90	positive	2-5

maintained in laboratory Swiss Webster mice by weekly passages. Infected mice were sacrificed and the peritoneal exudates were aspirated three days post-infection. After partial purification and parasite counting with a hemocytometer, this was used as our T. gondii parasite.

Urine samples

The samples were made artificially by taking some urine from normal individuals and adding a determined number of tachyzoites.

DNA Extraction

DNA extraction experiments were done by different methods so that we could compare and select the best one. Five different methods have been reported for extraction of pathogenic agent DNA from human urine.[9,11,15,25-30] A kit produced by an Iranian company (Cinnagene © extraction kit) is also available. The Cinnagene © DNP extraction kit was finally selected, as shown in Table 1.

DNA amplification

A multiplex semi-nested PCR was developed with 3 primers, two forward and a common reverse primer on the basis of a 529 bp fragment of T. gondii (AF146527 gene bank) with 200-300 repetitive frequency,[16] as shown in Table 2.

PCR reaction

PCR was done in a volume of 25 μl containing:

Tris HCl pH(8.3) 10 mM, KCl 50 mM, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM; Primers 0.2 μM of each, Taq DNA polymerase 1 IU/25 μl, template DNA 3-5 ng; and deionized water was added to bring thefinal volume to 25 μl.

PCR conditioning

This was obtained by primary denaturation at 94 °C for 7 min, followed by 94 °C for a 30 sec denaturation, 55 °C for 40 sec annealing, 72 °C for 50 sec extension for 35 cycles, and 72 °C for 10 min as a final extension.

Table 2 - Primers used and their roles and sequences

Primer name	Role	Sequence	From
Tox4	forward	5'- CGCTGCAGGGAGGAAGACGA AAGT-'3	Homan[16]
ToxIF	forward	5' GGCAAGCTCGCCTGTGCTTGG AG-'3	Eskandarian[6]
Tox	reverse	5'-CGCTGCAGACACAGTGCATCT GGA-'3	Homan[16]

RESULTS

Due to the very small number of organisms and the presence of a large amount of undesired factors in urine, a good method for DNA extraction is critical. After many experiments, the DNA was finally extracted from urine samples using the Sinnagene DNP® extraction kit according to the manufacturer's instructions. By performing a large number of tests, we obtained a sensitivity of 2-5 tachyzoites DNA per PCR, the best positive result, as shown in Table 1.

In this study, a multiplex semi-nested PCR was designed. On the basis of T. gondii genome, 3 primers were used, 2 as forwards (Tox4 and Tox IF) and the third, Tox5, as a common reverse primer. Two primers, namely Tox4 and Tox5, were derived from Homan.[15] The 3rd primer, ToxIF, was designed specifically and for the first time for this research project. It worked well with the other primers, and allowed us to develop a new multiplex semi-nested PCR, as shown in Table 2.

For setting up the multiplex semi-nested PCR the samples were made up of a serial dilution of tachyzoites in normal urine, so that the DNA of a sample contained about 2 parasites per micro liter. This was extracted with the Sinnagene extraction kit. PCR was run concurrently and compared with a multiplex semi-nested PCR under the same conditions. PCR products were electrophoresed against 2% agarose gel. The result of a run is presented here in Figure 1.

Figure 1 shows an electrophoregram of PCR products under UV transillumination after running in a 2% agarose gel and stained with ethydium bromide. The sharp band in line 3 is due to the good performance of the Multiplex semi-nested PCR.

DISCUSSION

There are many complicated clinical conditions in toxoplasmosis, namely congenital, ocular, and acute toxoplasmosis, as well as toxoplasmosis in immunocompromised (HIV/AIDS) patients. These conditions are hazardous. The presence of toxoplasmosis poses serious risks, and may actually be life-threatening. Additionally, there are some difficulties in routine (mostly serologic) methods of infection diagnosis.[1-3] In fact, molecular methods, especially PCR, have become the methods of choice, although there are some limitations associated with them.[9,11,29-31]

Invasive sampling in toxoplasmosis is a serious problem, especially in cerebrospinal, congenital, and ocular toxoplasmosis.[4,9-12] In contrast, urine is a very simple and accessible biological source for sampling, although it requires a considerable amount of research in order to eliminate the problems which occur in the extraction of its DNA.[20,30,32,33]

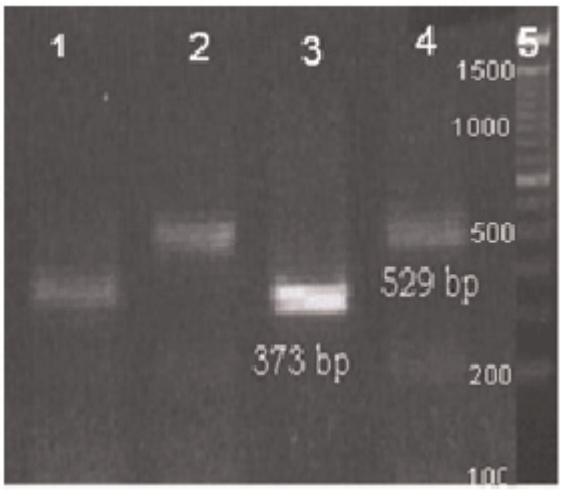

Figure 1 - Electrophoresis of PCR product after running against 2% agarose gel and staining using ethydiumbromide under UV transillumination. Line 1: current PCR with Tox IF and Tox 5 (internal fragment 373 bp; Line 2: current PCR with Tox4 and Tox 5 (external fragment 529 bp); Line 3: Multiplex semi nested PCR with Tox 4, Tox IF and Tox5; Line 4: current PCR with Tox 4 and Tox 5 repeated; Line 5: DNA size marker (100 bp ladder); There is a sharp band in the line 3 due to good performance of Multiplex semi nested PCR.

Several researchers have tried to solve the problems and develop simple and reliable method using urine as a biological sample.[9,11,12,18,23,34] We have also attempted to do this as described here.

Several genes and genome fragments were used in these studies, such as B1, P30, TGR1E, or a segment of the 18S rRNA gene, all of which showed different results. A 529 bp segment of T. gondii genome with 300 copies and which worked well in PCR was reported by Homan, as well as in three different reports.[9,14,23] It revealed satisfactory results with a 10-fold sensitivity in most studies.[15,16,23]

A good working multiplex semi-nested PCR was set up, using urine samples and the AF146527, a 529 bp fragment as gene target; this resulted in maximum performance and sensitivity and a reduction in undesired factors.

The results of most studies state that the AF146527 is a valuable target in molecular diagnosis of T. gondii in all biological samples,[34] although there is one study with opposite results.[35]

CONCLUSION

We obtained accurate and satisfactory results. We anticipate that they will become useful for the diagnosis of all forms of toxoplasmosis. Further experiments on accuracy and specificity are being conducted in our laboratory.

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RESUMO

OBJETIVOS: A toxoplasmose é uma infecção parasitária a protozoário. As formas cerebrospinal, ocular e congênita da doença são graves e apresentam risco de morte. O diagnóstico da toxoplasmose é difícil devido à necessidade de amostragem invasiva, a complicações patogenéticas, imunológicas e relativas à interpretação dos resultados dos testes. O uso de urina como fonte de amostra não invasiva para o diagnóstico molecular da toxoplasmose é o principal objetivo desta pesquisa. Foram feitas tentativas para diagnosticar toxoplasmose utilizando a reação em cadeia de polimerase (PCR) sobre ácido desoxirribonucleico do parasita (ADN) na urina.

MÉTODOS: Parasita: a cepa RH de Toxoplasma gondii (T. gondii) foi obtida a partir de exsudato peritoneal de camundongos brancos de laboratório inoculados com 5x105 taquizoítos três dias antes da coleta de aspiração. As amostras de urina com números definidos de taquizoítos por ml foram usados como amostras de laboratório. Um segmento bp 529 (banco de genes AF146527) do genoma do T. gondii com 200-300 repetições foi usado como alvo em PCR. As sequências de ''primers'' selecionados foram analisadas.

RESULTADOS: A técnica ''semi-nested'' de PCR multiplex foi desenvolvida para a obtenção de um método para o diagnóstico preciso da toxoplasmose, com economia de tempo e de orçamento e reduzindo o risco de contaminação cruzada de DNA. O método mostrou-se sensível para detectar 2 taquizoítos de DNA por amostra final.

CONCLUSÃO: Com o desenvolvimento, a técnica de ''semi-nested'' PCR pode ser um método útil e não-invasivo para o diagnóstico de toxoplasmose cerebrospinal, ocular e congênita.

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