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Original Article

A Triplex Nested PCR for Diagnosis of *Plasmodium*, *Babesia* and *Toxoplasma* in Blood to Promote Safety of Blood Transfusion Samples

***Abbasali Eskandarian**

Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

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***Correspondence Email:**
aeskandarian@med.mui.ac.ir

Abstract

Background: The risk of transmission of some infectious agents has always been an important life-threatening side effect of blood transfusion. The aim of this study was to develop a triplex nested PCR (tnPCR) to assess the presence of protozoan parasites *Plasmodium*, *Toxoplasma* and *Babesia* in blood samples concurrently. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of developed tnPCR method compared to gold standard methods were determined for all three parasite genera.

Methods: After selecting the genus level specific primers and setting up of tnPCR, blood samples were collected partially from Isfahan Blood Transfusion Organization (IBTO). Some different samples from human and animal were tested by this method in comparison with the gold standard methods (microscopic method for *Plasmodium*, Babesia and ELISA for *Toxoplasma*) in 2021.

Results: This tnPCR works well and the sensitivity, specificity, PPV, NPV and accuracy, of this molecular method were all 100% for *Plasmodium* spp., 93.33%, 99.16%, 93.33%, 99.16% and 99.25% for *Babesia* spp. and 100%,98.5%, 85.72%, 98.5% and 100% for *Toxoplasma gondii* respectively compared to standard methods. In average there were 100%, 99.22%, 95.24%, 99.5% and 99.75% contingency for all three parasites ($\alpha<0.05$). The designed and provided method can detect one, two, or all three potentially dangerous pathogens simultaneously in one tube and one-step, in biological specimens as well as blood.

Conclusion: The developed tnPCR worked well. It could be recommended for facilitating test, saving time, reducing the expense and cross contamination, subsequently the promotion of blood transfusion safety.

Introduction

Blood transfusion is an important and crucial stage of patients' care across almost all medical and surgical disciplines (1). Standard practices should include appropriate testing, selection of donors, blood donation screening, blood compatibility testing, issuance of blood bags in either routine or emergency situations, proper use of blood or return of required units after a problem, and reports of blood transfusion reactions. These are all key aspects that should be used in standard procedures (2).

Screening tests are performed for some known and important microbial agents in blood establishments as a common practice. In Iran, screening tests for hepatitis B and HIV virus, *Treponema pallidum* are performed on all blood donors according to IBTO standards. However, for various reasons, zero risk is not possible (3).

Blood transfusion is susceptible to infection with several dangerous infectious agents, including bacteria, viruses, parasites and fungi. In particular, any factor that may have an intracellular life cycle can be transmitted through blood transfusion.

The transmissibility of an organism depends on several factors ;(i) the presence of the organism in the blood during the infection, (ii) the carrier phase of the infection caused by the infection and (iii) the organism's ability to survive in the stored blood (4).

Many parasitic diseases are transmitted through blood transfusions. *Plasmodium* is a major concern, while toxoplasmosis, babesiosis and visceral leishmaniasis also contribute to the same risk in different geographical areas (1, 3, 5).

Plasmodium is a protozoan infection that is almost transmitted by *Anopheles* mosquito bites. In some cases, infection is also caused by direct injection of infected blood, such as blood transfusions (6). The first case of *Plasmodium* transmission was reported in 1911 (7). People with premonition from endemic areas, with partial immunity to malaria, may have mild fever or even no symptoms which may maintain parasites at low densities. It is generally assumed that asymptomatic infections are frequent in areas of high malaria transmission, where people rapidly receive immunity (8). There are several reasons why *Plasmodium* is a transferable threat. Malaria parasites can survive for up to 20 days at temperatures between 2 and 6 degrees Celsius, which is a good condition for the parasite to survive in the blood bank. Although whole blood and erythrocyte derivatives are the most common sources of this parasite, cases of blood derivatives containing infected red blood cells (RBCs) such as platelets, leukocytes, and fresh frozen plasma as well as frozen RBCs can also contain parasites. Selection of donors or screening of blood donors through interviews in many countries is the only step in preventing blood borne infections such as malaria (9). Malaria symptoms may develop several weeks after blood transfusion, depending on the number of parasites in the freezing. The patient may not have the potential history of malaria exposure and will usually present with nonspecific symptoms without a marked fever. Delay in diagnosis can lead to patient death, especially if *P. falciparum* is involved (6). In 2017, the number of people with malaria is estimated to be 219 million, with deaths from 435,000 to 607,000. Between 2010 and 2017, the highest number reported in Africa (10). In the eastern Mediterranean region including Iran, malaria is one of the most important public health problems that occur mainly in the southeast of Asia (3, 9).

Another parasite that may be transmitted through blood transfusions is *T. gondii*. Toxoplasmosis is a zoonotic disease caused by this parasite in a variety of warm-blooded vertebrates such as humans, livestock, birds, marine mammals, etc. (11). Almost one-third of the

world's population is infected with these protozoa. Although toxoplasmosis is a mild disease in people with a strong immune system, it is life-threatening in neonate's immune deficient and cancer patients. The infection is usually transmitted through consumption of oocyst-contaminated food and water, consumption of raw meat and vertical transmission during pregnancy. In addition, *Toxoplasma* infection can be transmitted from a positive serum donor to a negative serum recipient through organ transplantation and renal blood transfusion or transfusion of white blood cells (12). This parasite can cause severe acute complications such as encephalitis in immune deficient cases (13).

T. gondii can be transmitted through renal blood or leukocyte transfusions. It can survive in citrate blood at 5 degrees Celsius for more than 50 days; therefore, accumulation of blood packs during storage cannot delay and prevent the transmission of infection (14).

Another parasite that can be transmitted by blood is *Babesia*. In the United States, induced babesiosis has been reported by blood transfusions (15). The geographical distribution of *Babesia* is increasing. In addition, *Babesia* species has been recognized in many parts of the world. Babesiosis is a parasitic infection caused by a number of *Babesia* spp., most of which are *B. microti* and *B. doncani*, which is transmitted by tick (16).

In healthy young people, the parasite usually causes limited infection, often asymptomatic. However, it can be considered carriers of the parasite. However, high-risk patients, such as AIDS patients, splenectomies individuals, hemophilia, sickle cell disease, thrombosis, or the elderly are at risk for the disease and may cause severe infections in these individuals that may even lead to death (16, 17).

The aim of this study was to develop a (tnPCR) to assess the presence of protozoan parasites *Plasmodium*, *Toxoplasma* and *Babesia* in blood samples simultaneously.

Materials and Methods

Parasite sources and preparation

Infected blood samples, which some infected to *Plasmodium* and some to *Babesia* were prepared and counted its parasitemia using microscopy method. Intraperitoneal fluid from mice that inoculated with RH strain of *T. gondii* aspirated 4 days after infection, all as parasite sources. Using normal human blood-which was carefully tested using microscopic methods-was used for preparation of serial number of parasites; 50, 5 and 1 parasite/µL of blood separately for each parasite. After calculation, the parasites were suspended in 0.5 mL of normal blood and placed in a micro tube. Thick and thin blood smears were prepared from each serial in triplicate and stained with Giemsa for counting and calculation of parasitemia.

DNA extraction: DNA from each blood sample containing a determined parasite number was extracted separately according to the instructions provided by the Genetbio company kit (South Korea). The quality and quantity of the purified DNA assessed with a NanoDrop spectrophotometer.

Nested PCR setting up: The nested PCR was set up for each parasite separately with specific parasitemia and thereafter ensuring its good performance, the multiplex nested PCR set up for all three parasites. The genus specific primers are mentioned in Table 1. The PCR with the following thermal program was used for simultaneous detection of 3 protozoa. Five minutes (min) at 95˚C and then 30 cycles (for first step) 1 min at 95˚C, 1 min at 56.5˚C and 1 min at 72˚C, as well as 26 cycles (second phase), the samples were incubated for 45s at 95˚C 70s at 61˚C and 45s at 72˚C, and then the specimen was incubated for 5 min at 72˚C. The PCR products were visualized with gel electrophoresis and green viewer.

Table 1: Genus specific primers, which were used in tnPCR

Parasite	*Stage*	*Primers assigns and sequences*	*Reference*
Plasmodium	Nested one	Rplu1(tcaaagattaagccatgcaagtga) Rplu5 (cctgttgttgccttaaacttc)	(18)
	Nested two	Rplu3 (tttttataaggataactacggaaaagctgt) Rplu4 (tacccgtcatagccatgttaggccaatacc	(18)
Babesia	Nested one	Bab5 (5'-aattacccaatcctgacacagg-3') Bab8 (5'-tttggcagtagt tcgtctttaaca-3')	(19)
	Nested two	Bab6 (5'-gacacaggg aggtagtgacaaga-3') Bab7(5'-cccaactgctcctattaaccattac-3')	(19)
Toxoplasma gondii	Nested one	F1 (ggaggactggcaacctggtgtcg) R1 (ttgtttcacccggaccgtttagcag)	(20)
	Nested two	Tox1(cggaaatagaaagccatgaggcactcc) Tox2(acgggcgagtagcacctgaggagat)	(20)

Sample collection

Due to the type of this project which was the designing and developing an applicable method, the artificial samples by adding some determined number of each parasite to define volume of normal human blood to make the 50, 5 and 1 parasites per microliter.

More than 150 samples were used altogether for setting up the PCR, especially tnPCR.

After setting up the tnPCR, 133 blood samples belonging to different origins were used for comparing the results of this method against gold standard tests (Table 2).

Table 2: Comparative results of developed nested PCR against gold standards (microscopy for *Plasmodium* and *Babesia* and the ELISA for *Toxoplasma gondii*) on 133 samples

Row	*Sample type*	*Sample size*	*Parasites were diagnosed*		
			Plasmodium	*Babesia*	*Toxoplasma gondii*
1	Donors' blood	60	-	-	2
2	Birds' blood	3	1	-	-
3	Cows' blood	4	-	2	-
4	Human patients' blood/serum	24	-	-	12
5	Animal patients' serum	22	10	-	-
6	Disease sheep, horse, goats' blood	20	-	14	-
Sum		133	11	16	14

Analysis method

The data were analyzed using a contingency table for comparing the accuracy of the new method with the gold standards method ($\alpha \leq 0.05$).

Results

Due to the format and object of this study, most of the time I focused on the set up and upgrading the nested PCR thereafter it is multiplexing. In the first step, the PCR was set up for each parasite specifically. At the second step, the tnPCR was set up. The results are shown in Fig.1 and 2.

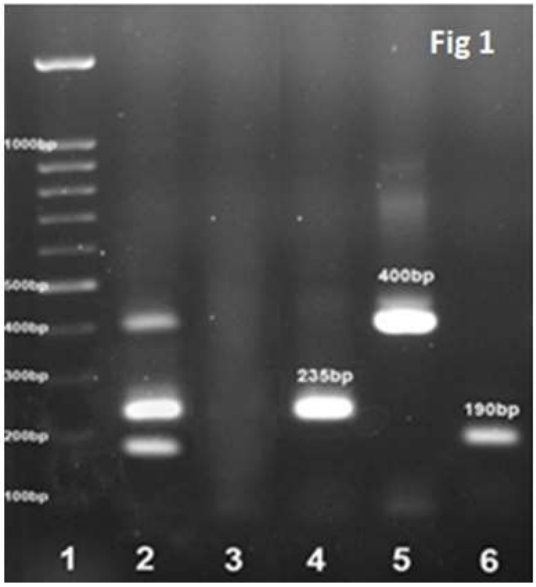

Fig. 1: agarose gel electrophoresis showing PCR products obtained by triplex nested PCR (tnPCR). Nested PCR products electrophoresed on 2% agarose gel shows the specific band for each parasite beside the positive and negative controls: 1. 100bp DNA size marker ladder 2. positive control (three bands of parasitic agents at the same line), 3. Negative control, 4. *Plasmodium*, 5. *Babesia*, 6. *Toxoplasma* specific bands separately

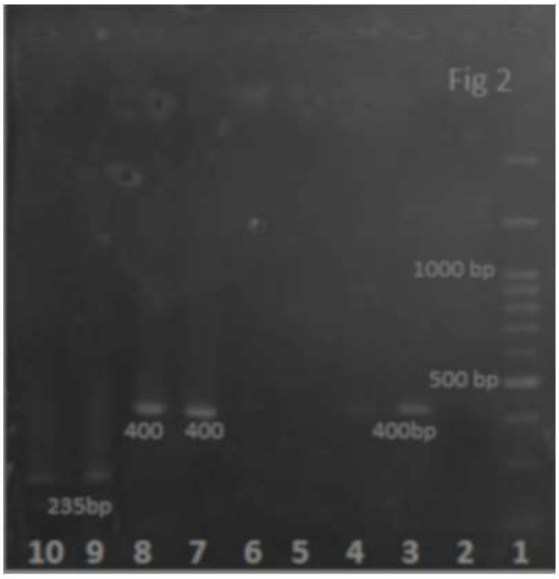

Fig. 2: Agarose gel electrophoresis showing triplex nested PCR (tnPCR) products in some human and animals' samples.
Electrophoresis of tnPCR products of some human and animals' samples on 2% agarose gel 1. 100bp DNA size marker ladder 2. Bird blood sample (Negative), 3-6: cow blood samples from slaughterhouses (3 and 4 positives for *Babesia*), 7 and 8: sheep blood samples (positive for *Babesia*), 9 and 10: human blood samples (positive for malaria) on some real blood samples

Because the primers were genus specific for each parasite, 133 blood samples from humans and animal's origins were tested using my developed method as well as by standard methods, double blindly (Table 2). The sensitivity, specificity, PPV, NPV and accuracy were determined by contingency table analysis. These samples were used only for testing the performance of developed tnPCR not as a descriptive or clinical diagnostic project. The analysis of data was done accordance with contingency table formula for each parasite (Fig. 3).

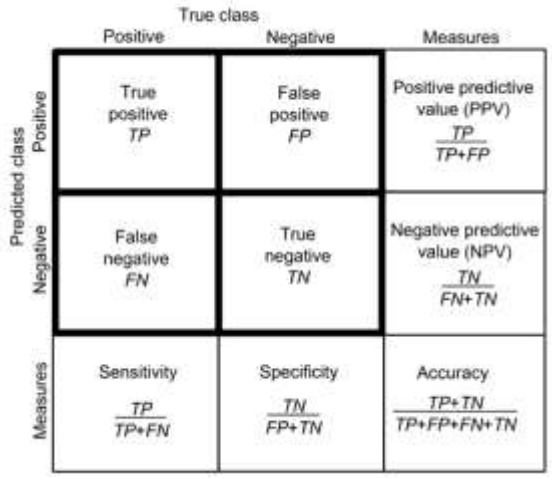

Fig. 3: contingency table and each character formula (Vihinen BMC Genomics2012,13 (Suppl 4): S2 http://www.biomedcentral.com/1471-2164/13/S4/S2)

The data of this study showed that this tnPCR worked well. The sensitivity, specificity, PPV, NPV, and accuracy, of this molecular method compare gold standard methods determined using contingency are shown in Tables 3-5. The designed and provided method can detect one, two, or all three potentially dangerous pathogens simultaneously in one tube and one-step, in biological specimens as well as blood.

Table 3: Contingency table of tnPCR method against microscopy as gold standard for *Plasmodium* spp. gold standard method (microscopy)

tnPCR test	***Positive***	***Negative***	***Positive predictive value***
Positive	15	0	15/15=100%
Negative	0	118	neg. predictive value 118/118=100%
Parameters	Sensitivity 15/15=100%	Specificity 118/118=100%	Accuracy 133/133=100%

Table 4: Contingency table of tnPCR method against microscopy as gold standard for *Babesia* spp. gold standard method (microscopy)

tnPCR	***Positive***	***Negative***	***Parameters***
Positive	14	1	Pos. predictive value 14/15=93.33%
Negative	1	118	Neg. predictive value 118/119=99.16%
Parameters	Sensitivity 14/15=93.33%	Specificity 118/119=99.16%	Accuracy 132/133=99.25%

Table 5: Contingency table of tnPCR method against ELISA as gold standard for *Toxoplasma gondii* gold standard method (ELISA)

tnPCR	*Positive*	*Negative*	*Parameters*
Positive	12	2	Pos. predictive value 12/14=85.72%
Negative	0	131	Neg. predictive value 131/133=98.50%
Parameters	Sensitivity 12/12=100%	Specificity 131/133=98.50%	Accuracy 145/145=100%

There was a considerable agreement between our developed tnPCR and gold standard methods. As a matter of fact, the newly developed tnPCR showed the same or higher accuracy in sensitivity, specificity, positive and negative predictive values and accuracy compared to the current gold standards. It worked with a high performance on human and different animals' blood and/or serum samples.

Discussion

Blood transfusion has been always one of the important issues in medicine however, some parasitic agents such as *Plasmodium* spp., *Babesia*, *Toxoplasma*, *Borrelia*, *Trypanosoma*, *Leishmania,* may transfer from donors to recipients via blood transfusion, and so investigating of donated bloods or donors is a vital requirement.

There are a few routine tests for healthy checking of donated blood in many countries as well as Iran but these do not cover the above-mentioned pathogenic parasites completely.

Malaria after hepatitis is the most important organism transmitted by blood transfusion, *P. vivax* does not survive in the blood at 4°C for more than 96 hours while *P. malariae* and *P. falciparum* are able to survive up to 20 days. Malaria transmitted through blood transfusion can cause death of the blood recipient if not timely diagnosed and treated (21, 22). Severe malaria-related anemia is one of the factors that can occur in hospital blood recipients, especially children, which can be a serious risk (6). *Babesia* is also a parasite that is transmitted through white blood cells and platelets containing intracellular parasites. The role of blood and blood products is important regarding this parasite. It is a protozoan parasite of human and animal red blood cells that *B. divergens*, a bovine parasite that can infect humans as well. Human infections have been predominantly seen in those without spleen, and parasites can also be transmitted through blood transfusion (7, 8). Toxoplasmosis is one of the most common parasitic diseases in the world that can survive for up to 50 days at 4°C in refrigerated blood bank conditions. So, one of the risk factors for seemingly healthy carriers is *Toxoplasma* (9, 23, 24). Since donated blood is not monitored for *T. gondii* infection in IBTO and on the other hand, blood recipients are sometimes immune compromised, so it is necessary to check the blood.

The sensitivity of PCR for detection was higher than that of microscopically. The accuracy and efficiency of this method is also much higher than that of the microscopic method, which increases the diagnostic value of this method (25). On the other hand, it is very important to design a method that can detect all three parasites simultaneously with high accuracy and sensitivity at the same time and save time and cost (26).

In a study on 130 blood samples from Thai people, they examined the presence of *P. falciparum* using microscopy and nested PCR

methods, which revealed higher sensitivity for molecular methods than microscopy (27).

Accordingly, I developed a tnPCR to simultaneously detect all three parasitic agents *Plasmodium*, *Babesia*, and *Toxoplasma* in blood samples (the sensitivity was 100%, specificity 99.22%, PPV, NPV 95.24%, 99.50% and accuracy 99.75% in average for all three parasites molecular and microscopy methods respectively).

IgG and IgM anti- *T. gondii* antibody titers were determined by ELISA, 132 (52.8%) had IgG antibody and 3 (3.6%) had IgM antibody against *T. gondii*, indicating the presence of this organism in donated blood (28). In a study, 70 samples of patients suspected of having severe toxoplasmosis and demonstrated the high prevalence of toxoplasmosis (28, 29).

A study on blood donors referring to Hamadan blood transfusion was conducted, indicating the necessity of detecting this parasite in donated blood. Also, in a study on urine sample containing *Toxoplasma*, highlighted the value of the multiplex method in saving money, time, and efficiency and its high sensitivity compared to simple methods (30-32).

The results of the first molecular investigation on *P. falciparum* using the nested PCR and microscopy methods on some isolates from southern Iran was reported (33). Considering that even minor cases of this parasite can cause acute complications, especially in people with weakened immune systems, it is therefore necessary to investigate this parasite.

On the other hand, *Babesia* parasites have been widely reported in livestock, for example a study on 372 cattle, sheep and camel blood samples showed that 19 cases were positive by PCR in Isfahan (34).

Conclusion

Two *T. gondii* infected samples were noticed between about 60 out of 133 samples from transfusion blood samples, so special attention should be paid to methods that can correctly identify the causative agent of infection. In addition, according to standard tests compared to molecular methods, this method has higher sensitivity and specificity than gold standard methods (microscopic and ELISA) and also has higher PPV and NPV compared to those methods.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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