Molecular Diagnostic of *Treponema pallidum* in Blood Specimens of Donors Using Simple PCR and Real-Time PCR in Thi-qar Province, Iraq

Kamil M. Al-Jobori¹, Amran M. Al-Erjan¹, Yahya, A. Abbas²

1. Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Iraq.
2. Southern Technical University, Nassryia Tech. Institute.

**Abstract**

Syphilis is a perplexing infection; it is a complex multistage disease responsible for a range of severe clinical manifestations when untreated or as a result of treatment failure in its sole human host. The objective of current study was diagnostic of *Treponema pallidum* in blood specimens of donors using molecular methods. A total of 150 samples (25 serums, 25 plasma and 100 whole blood) were used for this purpose. Samples were analyzed using simple PCR and RT-PCR by amplifying 16Sr and polA genes. During the study period, out of 150 samples of serum, plasma and whole blood collected from males and females, whole blood samples of 14 male and 1 female gave positive result for DNA extract, and and only 15 samples were positive for simple PCR and RT-PCR for the 16Sr gene. But polA gene not succeeded to amplify by simple PCR, however, succeeded to amplified 15 samples using RT-PCR. In conclusion, Blood samples, regardless of the compartment (serum, plasma or whole blood) used for testing, appear to be unsuitable for PCR analysis. However, from blood compartment whole blood was the best sample for spirochete detection of syphilis infection than from serum and plasma samples.

**Keywords:** Blood donors, Syphilis, *Treponema pallidum*, 16Sr gene, polA gene, Simple PCR, RT-PCR.

**Introduction**

Blood is one of the main constituents of body, which flows throughout body and becomes a reason for survival. Human blood transfusion is one of the most important components of therapeutic and surgical treatments due to the lack of artificial blood replacements [1]. Syphilis is one of sexually transmitted diseases. It can be transmitted by sexual contact and by mother-infant vertical transmission.

The pathogen is *Treponema pallidum* (T. pallidum) (Tp), which can result in multisystem injury by invading nerve, skeleton, skin, mucous membrane, angiocarpy etc, and can result in abortion, stillbirth and congenital syphilis fetus. Besides, syphilis has synergistic action with HIV and can promote HIV infection [2]. The signs and symptoms of syphilis vary depending in which of the four stages it presents, primary, secondary, latent, and tertiary [3]. *T. pallidum* subsp. *pallidum* is the etiologic agent of venereal syphilis, which presents as distinct clinical stages: the primary chancre or ulcer, the rash of secondary syphilis, the asymptomatic latent stage, and the potentially destructive tertiary stage. The diagnosis of syphilis is made on the basis of a combination of clinical presentation and laboratory tests.

The commonly used laboratory methods include detection of the bacterium and serology; the sensitivity and specificity vary according to the specific method and the stage of the disease. Serologic testing is insensitive for patients with early primary lesions and perhaps for patients with late disease.

It is also difficult to interpret the results for infants with suspected congenital syphilis and for persons with past syphilis. Dark-field microscopy and immunostaining are very useful for new, moist primary and secondary lesions, but sensitivity decreases as the lesions heal, and microscopic identification of *T. pallidum* is not useful in latent or tertiary syphilis [4].
Data obtained from the Ministry of Health, the Republic of Iraq, between 2003 to 2014, show that the incidence of syphilis was fluctuating but it increased dramatically to 1354 in 2014, and the incidence of males was greater than females, reaching 1220 compared to 134[5]. Syphilis increases have been reported in other developing and developed countries including USA [6], Asia [7], Oceania [8] Canada, Australia, and New Zealand [9, 10].

Syphilis remains a challenging and complex infection to diagnose [11,12]. The lack of in vitro culture techniques and genetic systems for T. pallidum has made it extremely difficult to identify virulence or regulatory factors involved in the pathogenesis of syphilis [13].

Forced laboratorians to focus on alternate methods for diagnosing syphilis. Microscopic examination of the fluid from ulcerative lesions, from regional lymph nodes, or from the infected tissue has been used since the early 19th century to presumptively diagnose acute cases.

However; the utility of this test is limited by the inability of even experienced observers to distinguish the organism from other, nonpathogenic treponemes in some specimens [11]. Isolation of T. pallidum must be performed by the inoculation of the sample into susceptible rabbits, known as the rabbit infectivity test (RIT). In recent years, new diagnostic assays based on the amplification of T. pallidum DNA have been developed. PCR has increasingly replaced culture for the identification of pathogens. Several PCR methods have been reported, each test uses a different target gene [4].

A fast and reliable PCR is therefore of great potential value for the diagnosis of primary syphilis, specifically in settings in which it is not possible to perform dark-field microscopy [14]. The advantages of a real-time PCR are the ability to detect the pathogen directly, the short turnaround time, and the ease of performance [15]. Therefore, this study aimed to investigate the presence of T. pallidum by conventional PCR and RT-PCR in blood donors (serum, plasma or whole blood).

Materials and Methods

Study Population

During the beginning of April / 2016 to the end of March / 2017 a study was undertaken to assess the prevalence of syphilis in Thi-qar province, Iraq, a total of 28287 subjects who visited the main blood bank AL-Nassiriya and blood banks of Suq AL- Shiok , Refaei and AL-Shatra to donated blood aged from 20-75 years of were of both sexes.

Diagnostic Tests

Treponema serologic tests were performed for 28287 blood volunteer using a specific serological T. Pallidum hem agglutination assay (TPHA), the nonspecific treponemal assay Venereal Disease Research Laboratory (VDRL), fluorescent Treponemal absorption test (FTA-ABS) and enzyme immunoassay (EIA).

PCR Primers

The two primer sets (F1-R1 and F2-R2) chosen for evaluation with clinical specimens give rise to amplicons of 203 and 209 bp from the 16Sr and polA genes of T. allidum . The sequences of the two primers are as follows: for primer 16Sr: F (forward primer), 5'- AGCGATACGCCTCTTGACAC- 3' ; R (reverse primer), 5'- TG TAGCCCGGACATAAG- 3' ; for primer polA: F (forward primer), 5'- AG ACG GCTGCACATCTTTCTCCA - 3' ; R (reverse primer), 5'-AG CAG ACG TT ACA TC GAGCGGA - 3'. The primers were supplied by Integrated DNA technologies /USA.

Detection of Amplicons using Agarose Gel Electrophoresis

The gels were run with a 100-bp ladder (Kapa /USA) at 5-8 V/cm² for 1.5 h. Bands were visualized on a UV transilluminator after staining with ethidium bromide (BDH/England).

DNA Preparation and Amplification Conditions

DNA was extracted from 200μl of whole blood or body fluids using a commercially available DNA extraction and purification kit [G-Spin total DNA extraction (Intron/Korea)] following the manufacturer’s instructions. All PCR assays were performed in 50-ml PCR vials. The samples were analyzed by simple PCR targeting the polymerase 16Sr polA genes of T. pallidum. The reaction mixture contained 5 μl of Taq PCR Pre Mix, 2 μl of each of the primers (1 μl f and1 μl R), 1.5 μl of template DNA, and 16.5 μl distill water.
The following conditions were used for 16Sr gene: 95°C for 3min, 95°C for 30s, 60°C for 30s, and 72°C for 45s (45 cycles). After the final cycle, the mixture was incubated at 72°C for 10 min to complete the reaction, and the following conditions were used for polA gene: 95°C for 3min, 95°C for 30s, 57°C for 35s, and 72°C for 45s (45 cycles). After the final cycle, the mixture was incubated at 72°C for 10 min to complete the reaction. The PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide (0.5 g/ml for 15 min, rinsed, and photographed under UV light illumination.

**T. pallidum Real-time PCR**

A RT-PCR assay targeting the 16Sr and polA genes of *T. pallidum* was performed [16]. The reaction mixture contained 25 µl of GoTaq® qPCR Master Mix, 2X, 13 µl of nuclease-free water, 1 µl upstream PCR primers and 1 µl downstream primers, and 10 µl of template DNA. After a denaturation cycle of 2 min at 95°C, the amplification profile consisted of 40 cycles of 15 s at 95°C, and 1 min at 60°C, the Melting Curve analysis 1 s at 60-65°C. The cutoff positive cycle threshold (CT) was _36. CT values of 36 to 40 were considered to be in the gray zone. These samples were retested and were considered positive if the CT value was _40.

**Results & Discussion**

During the three past decades, several target genes of *T. pallidum* have been used for detection by PCR, and a number of researchers have described PCR procedures for the diagnosis of syphilis based on the detection of different *T. pallidum* gene targets [17, 18].

*T. pallidum* is a highly invasive organism that is able to penetrate endothelial cell monolayers and to enter the bloodstream *in vivo* [19]. Serological tests revealed that out of 28287 blood volunteer 200 were infected with syphilis (unpublished data). From 150 samples of serum, plasma and whole blood collected from males and females, whole blood samples of 14 male and 1 female only gave positive result for DNA extract (Table 1). Previous studies on syphilis infection reported that whole blood was the best sample from blood compartment for spirochete detection.

<table>
<thead>
<tr>
<th>Gender contains</th>
<th>DNA from serum, plasma and whole blood</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male</td>
<td>female</td>
</tr>
<tr>
<td>Serum</td>
<td>20(0)</td>
<td>5(0)</td>
</tr>
<tr>
<td>Plasma</td>
<td>20(0)</td>
<td>5(0)</td>
</tr>
<tr>
<td>Whole blood</td>
<td>90 (14)</td>
<td>10(1)</td>
</tr>
<tr>
<td>Total</td>
<td>130(14)</td>
<td>20(1)</td>
</tr>
</tbody>
</table>

In Iraq, PCR is not used for laboratory investigation of syphilis. Therefore, in this study, simple PCR and RT-PCR was used to diagnose syphilis in various specimens (serum, plasma and whole blood) using PCR primers that target three different *T. pallidum* genes (16sr and polA and). Optimization of primer concentration is an important step in any PCR reaction. DNA was extracted from 150 samples (25 serum, 25 plasma and 100 whole blood) and only 15 samples were positive for simple PCR for the 16Sr gene (Figure 1). The sensitivity of PCR detection assays has been found to vary depending on the specimen types and the stage of disease [20]. The optimal concentration is that which gives the lowest Ct value [21]. Real time PCR quantification applied in the present experiment utilized the SYBR green, a fluorescent dye which recognizes any double stranded DNA, in terms of gene expression, high Ct values indicate low gene expression and low Ct value indicates a high gene expression[22].
Samples that were positive for simple PCR for 16sr gene were also detected using RT-PCR for confirming and confirming the results, and those entire samples found positive also (Table 2). Samples exhibited Ct ranged between 27.25 – 38.11 these high values are associated with the low treponemal levels in blood (low quantities of DNA).

Table 2: Diagnosis of syphilis infections using simple PCR and RT-PCR

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>Simple PCR</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16Sr gene</td>
<td>PolA gene</td>
</tr>
<tr>
<td>Serum</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plasma</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Whole blood</td>
<td>25</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

The sequence of polA gene is well described and is conserved between organisms, especially in the functional domains. The sequence comparison clearly clarifies the uniqueness of the regions that are rich in cysteine and the four insertions [23]. polA gene is considered a fingerprinting for T. Pallidum bacteria. In this study it has been working on amplifying the gene using simple PCR at different conditions and temperatures but without success and the result was every time negative, and then try the amplification using RT-PCR. The work was done on 100 samples of whole blood and the results were positive for the same samples that were positive for the 16Sr gene (Table 2). Marfin et al.[24] detected the polA gene in whole blood samples of 64% (9/14) of individuals, which are higher than results of this study 10%(15/150) of individuals. In conclusion Blood samples, regardless of the compartment (serum, plasma or whole blood) used for testing, appear to be unsuitable for PCR analysis. However, from blood compartment whole blood was the best sample for spirochete detection of syphilis infection than from serum and plasma samples [25-26].

References

3. CDC (2015)"Syphilis - CDC Fact Sheet (Detailed)“. 2.


