Development of -a Basic Membrane Protein Gene- Targeted Nested PCR Assay for the Direct Detection of *T. pallidum* DNA in Specimens from Patients with Suspected Syphilis

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We report the development of a nested-PCR-based assay targeting the basic membrane protein gene for the detection of *T. pallidum* DNA in specimens from patients with genital ulcer disease. The specificity and sensitivity of the test were assessed. The detection limit is down to 28 copies per reaction when analyzed on gel. The technique was then applied to genital ulcer samples from 51 patients with suspected syphilis, who were also tested by serological methods. We obtained positive reactions for 15 specimens out of 16 specimens from patients with primary and secondary syphilis and negative results for all the non-syphilis group. Nested PCR is here compared with other diagnostic methods currently used in diagnosis of venereal syphilis. PCR successfully detected *T. pallidum* from oral, genital, and anal ulcers present during primary or secondary syphilis and the results correlated well with the serology.

INTRODUCTION

Treponema 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 (1).

Current efforts toward the elimination of syphilis depend heavily on early identification of infected persons and prompt treatment to prevent the transmission of the infection (1). The diagnosis of syphilis is based on clinical features, observation of the organisms by dark-field microscopy, and serologic tests (1,2). Detecting syphilis through clinical presentation is highly subjective and depends on factors such as the completeness of the case history, the presence of lesions, and the experience of the physician. Serologic tests for syphilis are relatively insensitive in the early stage of infection.

The Venereal Disease Research Laboratory (VDRL) test and the rapid plasma reagin (RPR) test are routinely used to screen blood samples for potentially infectious pathogens (3). Upon identification of any pathogens, further tests are performed. In the case of *T. pallidum* the most common serological tests include *T. pallidum* heamagglutination assay (TPHA) and fluorescent treponemal antibody-absorption (FTA-ABS) test (4). The FTA-ABS test can accurately detect 70 to 90% of cases (3). Both tests are specific for *T. pallidum* and are used to detect the immunoglobulins IgG and IgM. These tests are not suitable in the diagnosis of a new infection of syphilis, because the antibodies produced after the primary infection remains in the blood. The existing antibodies do not offer any protection against the new infection, as they are present at low levels. In the majority of diagnostic laboratories all or a combination of the tests will be performed in order to provide adequate detection rates. This demonstrates the need for a means of detecting *T. pallidum* directly.

Among methods used for direct detection, dark-field microscopy of the ulcers is easy to perform; however, this method is relatively insensitive (sensitivity is approximately 105 organisms/ml) and requires special equipment (dark-field microscope) and trained and experienced laboratorians (3).

The rabbit infectivity test (RIT), which is still considered the “gold standard” test, has a detection limit of a single organism and can be used to confirm *T. pallidum* infection (2, 5). Performing this test, however, requires access to an animal facility and is extremely time-consuming and expensive.

Molecular biology techniques are being used increasingly in the clinical laboratory. These methodologies, particularly the PCR, have been used in the detection of treponemes. This technique has detected *T. pallidum* in a variety of clinical specimens, including serum (4, 5, 6), cerebrospinal fluid (6, 7, 9, 10, 8), amniotic fluid (4, 5, 6), fixed tissues (4), and lesion exudate (11, 12, 13).

However, its performance relative to other tests, particularly in early disease, remains to be validated by extensive clinical studies. In this study, we describe a method that uses PCR to detect *T. pallidum* in Ulcer samples from patients with suspected syphilis, who were also tested by serological methods.

MATERIALS AND METHODS

Nichol's Strain of *T. pallidum*

Samples utilised in the early PCR assays were extracted from Trepo-Spot IF (bioMerieux, France). These slides contained syphilis seropositive patients' sera, which had underwent the FTA-abs test, previously. They were supplied by the Bacteriology Department at the Royal Victoria Hospital, Belfast, UK.

Selection of primers

The primer sets used, (B and C), were located within the basic membrane protein (*bmp*) gene (table 1). The outer primer set was previously published and validated in the Journal of Clinical Microbiology by a Dutch group (14). The inner primer set for T. pallidum (C) was designed using the standard criteria for producing optimal primers using Lasergene software program, version 5 (DNASTAR, Madison, USA).

Table1. Primers for T. pallidum nested PCR

	Primer	Sequences (5'-3')	Position*	Product size
Set B	TPA1C	CAG.GTA.ACG.GAT.GCT.GAA.GT	540-560	506 bp
	TPA1D	CGT.GGC.AGT.AAC.CGC.AGT.CT	1046-1027	
Set C	TPA2C2	AGT.TCG.CCA.ATT.ACG.TCA.AG	619-638	126 bp
	TPA2D2	ACG.TAG.CGC.TGC.TGG.CTC.AC	745-726	

* The position of the primers relates to their position within the *bmp* gene of the Nichols strain of *T. pallidum*.

Assay optimization

To optimize the assay, a series of titrations of primer concentrations ranging from (0.2- 0.4 μM) were tested in order to achieve the best results (maximum band intensity, minimal background and non specific staining). The optimal annealing temperature for *T. pallidum* primers in the nested PCR was determined by varying the target temperature in the annealing segment of the amplification program, a range of annealing temperatures (48°C-68°C) were assessed using a PTC-225 DNA Engine Tetrad Petlier Gradient Thermal Cycler (MJ Research Inc., USA). This range was selected because it spans the range at which the T*m* of the primer sets occurs.

PCR amplification

Mastermixes were made in nuclease free water with Promega Taq DNA polymerase, magnesium free reaction buffer (x10) supplied with Taq enzyme, $MgCl_2$ (Promega, Southampton, England, UK) and primers. First round amplification was performed on 2 μl of extract added to 8μl of first round reaction mixture containing 10 mM Tris (pH 9.0), 3.5 mM MgCl2, 200 μM of each deoxynucleoside triphoshate (dATP, dCTP, dGTP, and dTTP), 0.4 μM of *T. pallidum* primers and 0.5 U/μl of *Taq* polymerase.

Second round amplification was performed on 0.2 μl of first round reaction added to 9.8 μl of second round mastermix. Amplification was carried out on a DNA Engine Tetrad PTC 225 (MJ Research, USA). Thermal cycling in the first round consisted of 30 cycles, and in the second round of 35 cycles, of denaturation (94°C-10s), annealing (58 and 54 °C in the first and second rounds respectively -10s) and extension (72°C-30s), preceded by a 3 min denaturation step of 94°C, to facilitate hot transfer from ice, and followed by an extension step of 72°C for 5 min. The first and second round reaction products were separated by agarose gel electrophoresis by adding 1 μl of loading dye containing 0.25% bromophenol blue in 40% sucrose to a 5-μl reaction mixture and loading 5 μl onto a 2% agarose gel (NuSeive 3:1;

FMC). The buffer in the electrophoresis chamber and in the agarose gel was 0.5× Tris-borate-EDTA and contained ethidium bromide (1 µg/ml). One hundred volts and 25 mA were applied across the gel. DNA in the gel was visualized by exposing the gel to UV light and was photographed on Polaroid film.
Positive and negative controls (nuclease free water) were included in each nested PCR.

In order to reduce the possibility of cross-contamination and hence false positives, mastermix preparation, sample manipulation, and addition of samples to mastermixes were performed in class 2 biological safety cabinets within a suite of four purpose-built PCR laboratories. PCRs were performed using eight-strip tubes and lids (Abgene, Surrey, United Kingdom). To avoid cross-contamination, only a single strip was opened at any time during these procedures, and all strip tubes were centrifuged at 6,300 rpm for 10 s in a bench top strip-tube centrifuge (model no. PMC-860; Tomy, Tokyo, Japan) prior to removal of lids.

Construction of synthetic controls and the analytical sensitivity of the assay

To establish the analytical sensitivity of the nested PCR assay, we created a plasmid containing the *T. pallidum* DNA amplicon by using the TOPO TA cloning procedures with pCR4-TOPO vector (Invitrogen, Carlsbad, Calif.). Plasmid DNA was isolated from *E. coli* by the QIAprep spin miniprep kit (QIAgen, Crawly, UK) according to the manufacturer's instructions. Insertion of the correct amplicon was confirmed by nucleotide sequencing using the Thermo Sequenase fluorescent labelled primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Uppsala, Sweden), and the purified recombinant plasmid was quantified by using the Amersham Gene Quant II DNA/RNA spectrophotometer (Amersham Pharmacia Biotech, Piscataway, N.J.).

Log dilutions (typically in the range 10^{-4} to 10^{-11}) were made by serial dilution of *T. pallidum* plasmids with Qiagen Buffer EB (10mM Tris HCl, pH 8.5), and the sensitivity of the assay was determined by carrying out nested PCR on serial log dilutions of plasmid DNA with the aim of determining the detection endpoint copy number.

Assay specificity:

To determine the specificity of the assay, we tested a panel of microorganisms, including pathogens involved in sexually transmitted diseases (such as N. gonorrheae, C. trachomatis, HSV-1 and 2, and T. vaginalis and a variety of spirochetes. The two primer sets amplified DNA from only the three supspecies of *T. pallidum* (subsp. Pallidum, pertenue, and endemicum).

Clinical samples

Ulcer samples from 51 patients with suspected syphilis, who were also tested by serological methods, were submitted for PCR testing. The patients had attended the Genito-Urinary Medicine clinics at the Royal Victoria Hospital, Belfast, UK during a recent outbreak in Northern Ireland. Forty-six patients were male and, of these, 28 were MSM. Of the MSM, 15 were HIV positive.
Swabs of 21 penile, 12 oral, 10 anal, 6 vulvar, 1 rectal, and 1 unspecified ulcer were collected, placed into a dry tube or a tube with transport medium, and sent to the PCR testing laboratory. One mL of saline was added to the samples in the dry tubes. Specimens were vortexed, centrifuged, and the DNA was extracted from the cell pellets using the QIAamp DNA blood Mini Kit (Qiagen Ltd., Crawley, England, UK) for genomic DNA extraction according to manufacturer's instructions.
Clinicians diagnosed the patients as having primary or secondary syphilis, or as not having syphilis, according to the clinical details and their available laboratory test results. The laboratory tests included the serological tests of RPR, VDRL, TPHA, TPPA, an EIA detecting IGM and IgG, and an EIA detecting IgM only. Dark ground microscopy was performed on 34 cases.

Results

Optimisation and development and of *T. pallidum* nested PCR

Optimal results were obtained with primer concentrations of 0.4 (figure 1), The

annealing temperatures (58°C) were chosen empirically as no single temperature was determined as optimal as successful amplification of the second round product was obtained for the range 48.0°C – 68.1°C(figure 3)

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Figure 1. Optimisation of nested PCR by altering the concentration of primers of the first round (set B). Amplification of 506bp first round products of T. pallidum was performed using different primers concentrations. Lane 1: 0.05 μM. Lane 2: 0.1μM. Lane 3: 0.2μM. Lane 4: 0.3μM. Lane 5:0.4 μM. Lanes 6 & 7 represent two separate positive controls. . Lane 8. Nuclease free water. M corresponds to a DNA ladder. Products were separated on a 2% agarose gel.

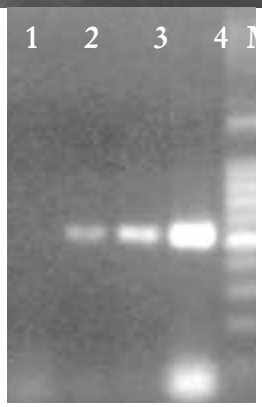

Figure 2. Optimisation of nested PCR by altering the $MgCl_2$ concentration. Lane 1: 1 mM concentration, lane 2: 1.5 mM, lane 3: 2.5 mM, lane 4: 3.5mM. M corresponds to a DNA ladder. DNA was extracted from Nichol's strain of *T. pallidum*l. Products were separated on a 2% gel.

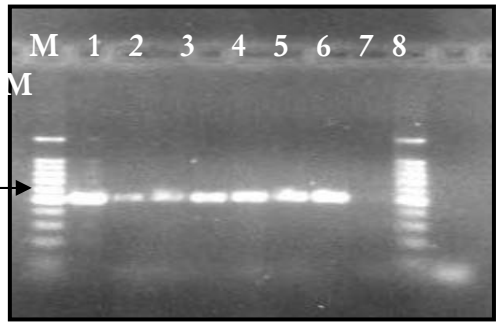

Figure 3. Annealing temperature optimisation of the first round of nested PCR assay. The 506bp target sequence of *T.pallidum* was amplified under different annealing temperatures. The temperatures represented in each of the lanes are lane 1: 48.0°C, lane 2: 51.8°C, lane 3:54.6C, lane 4: 58.0°C, lane 5:62.0°C, lane 6: 65.4°C, lane 7: 68.1°C, lane 8: Nuclease free water. M corresponds to a DNA ladder. Products were separated on a 2% agarose gel.

Plasmid quantification and analytical sensitivity:

The molecular weight of both the 506 bp sequences and plasmid sequence was calculated by multiplying the number of nucleotides present in the sequences (4456 bp= pCRII TOPO vector 3950 bp + 506 bp *T. pallidum*) by 660 (average weight of one nucleotide in μg). This value was then used in accordance with Avogadro's number to determine the copy number of *T. pallidum* DNA in a 1 ml volume. This was found to be $14x10^{14}$copies/ ml using the following mathematical formula

$$\frac{6x10^{23}\ \text{(copies/mol)} \times \text{concentration (g/ml)}}{\text{MW (g/mol)}}$$

The sensitivity of the real-time PCR was determined using serial dilutions of *T. pallidum* plasmid DNA. The dilution series ranged from 10^{-7} to 10^{-14}.The detection end point was10^{-11} (figure 4). End point copy number was calculated using the following mathematical formula

End point copy number

= Concentration (copies/ml) x input in PCR (2 x 10^{-3}) ml x detection end point in PCR

T.pallidum DNA end point copy number = $14x10^{14}$ (copies/ml) x [$2x\ 10^{-3}$] x 10^{-11}
=28/2 µl (reaction).

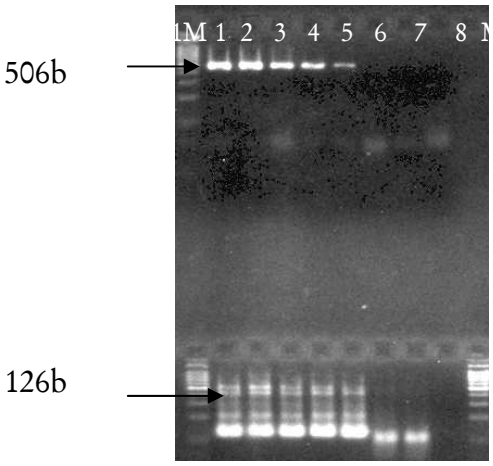

Figure 4. Determination of sensitivity of the nested PCR assay using plasmid DNA. Nested PCR was used to amplify 10^{-7} down to 10^{-13} dilution series of T. pallidum plasmids in order to determine the detection end point copy numbers; M corresponds to a 100bp DNA ladder. Products were analyzed on a 2 % agarose gel.

Detection of *T. pallidum* DNA in clinical samples isolated from a recent outbreak in Northern Ireland

The results of the PCR, RPR, and TPHA or TPPA tests on specimens from clinician-diagnosed cases of primary, secondary, or no syphilis are shown in table 2, figure 5.

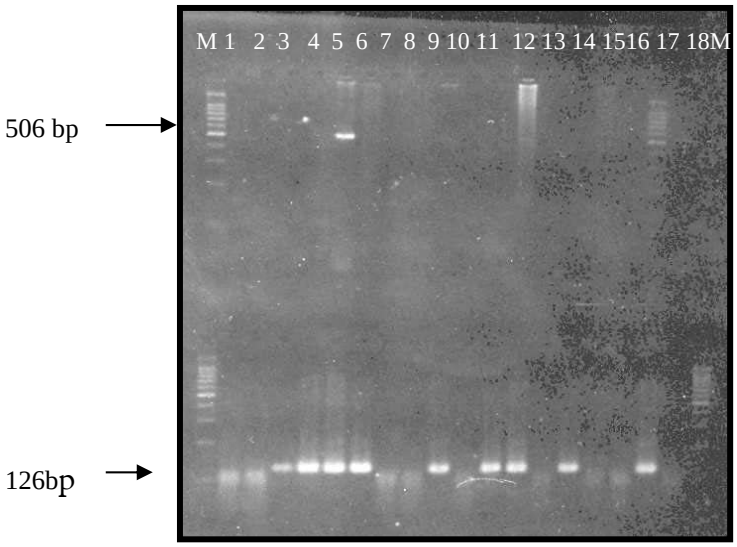

Figure 5. Detection of *T. pallidum* DNA in clinical specimens by nested PCR. Lanes 1 to 16 contained samples amplified by nested PCR using primer sets B and C. DNA of the correct size was amplified in samples 5 and 12 in the first round. However, in the second round of the assay *T. pallidum* DNA of the correct size was detected in lanes 3, 4, 5, 6, 9, 11, 12 and 14. NFW was used as a negative control, lane 18. Nichol's strain of T. pallidum was used as a positive control: Lane 17 M corresponds to a DNA ladder. Samples were analysed on a 2% agarose gel.

Table 2. Detection of *T. pallidum* DNA in clinical samples isolated from a recent outbreak in Northern Ireland

Clinical Diagnosis	Number Of cases	Number of specimens with test results					
		PCR		PRP		TPHA/TPPA	
		Positive	Negative	Positive	Negative	Positive	Negative
Primary syphilis	10	9	1	8	2	9	1
Secondary syphilis	6	6	0	6	0	6	0
No syphilis	35	0	35	2	33	3	32

DISCUSSION

PCR has had a major impact upon molecular biology. Its basic principle of amplifying a specific DNA sequence has been modified and developed into a wide range of PCR-based procedures. One of these applications is the diagnosis of bacterial infections through direct detection of the causative bacteria. PCR has many advantages in the diagnosis of bacterial infections over serology-based techniques, such as the capacity to detect pathogens before an immune response has been initiated when pathogens are present at low titres. However the major advantage of PCR is that it allows direct detection of the bacterial DNA. Furthermore it does not require tissue culture of the bacteria, which is important in the diagnosis of the fastidious spirochete *T. pallidum*.

There have been many target genes used for primer selection in the detection of *T. pallidum* DNA by PCR. The first of these genes were the *tmpA* and *tmpB* genes (15 and more recently the 47-kDa protein gene (16). This 47-kDa protein is the most frequently reported target gene for PCR but non-specific reactions have been reported from its use (17) and a DNA hybridisation step is commonly carried out to confirm the specificity. PCR has been used to detect *T. pallidum* in genital ulcer specimens using unique regions of the DNA polymerase I gene with a sensitivity of 95.8% and specificity of 95.7% (18). DNA polymerase I is important in DNA replication and repair and is consequently highly conserved between organisms, which explains the high specificity associated with it.

In this study a nested-PCR assay was developed for the detection of *T. pallidum* DNA. The two primer sets used for amplification were selected from the basic membrane protein (*bmp*) gene of Nichol's strain of *T. pallidum*, which is highly conserved between *T. pallidum* subtypes. Although the function of this target gene is poorly characterised the outer set of primers were validated by a Dutch group and have been used in routine diagnostic PCR for the detection of *T. pallidum* (14). And the inner primer set was designed in house considering the primer design parameters such as homology of primers with their target nucleic acid sequences, their length and their GC content.

The optimisation of *T. pallidum* PCR assay aimed at minimizing or reducing non-specific products, which may amplify more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Empirical testing and a trial-and error approach was used, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design. The first of these experiments was investigating the effect of different concentrations of primers and $MgCl_2$ on the nested-PCR assay. The concentration of primers and Mg^{2+} ions is thought to increase the rate of PCR amplification. A range of both primer and $MgCl_2$ concentrations were explored and a primer concentration of 0.4 μM and an $MgCl_2$ concentration of 3.5 mM were found to be optimal. A range of annealing temperatures was investigated in order to determine the optimal temperature. No single temperature was determined as optimal as successful amplification of the second round product was obtained for the range 48.0°C – 68.1°C. So annealing temperature of 58°C was chosen to be as high as possible, taking care not to reduce the sensitivity of the assay.

In order to further improve the sensitivity and specificity of the assay, two

variations in the methodology were carried out, the first was hot start PCR which eliminated non-specific reactions (particularly production of primer dimmers) caused by primer annealing at low temperature (4 to 25°C) before commencement of thermo-cycling. The second was nested PCR, which increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets; we found that by performing the test without nested PCR amplification, the sensitivity was approximately one order of magnitude less.

The sensitivity of this PCR assay was determined using a dilution series of quantified Plasmid DNA. This along with a calculation of the copy numbers indicated that the PCR assay was capable of detecting down to 14-copies/μl.

DNA samples isolated from patients during a recent outbreak were tested by the nested PCR assay. The nested PCR was capable of detecting *T. pallidum* DNA in the confirmed group . The clinical sensitivity of the assay was determined by comparing the nested PCR results with those obtained from serology-based techniques performed on the same patients. This comparison showed that identical results were obtained in both these methods.

The negative result obtained from one patient in the primary stage of syphilis can be explained. Most patients with primary syphilis had received antibiotic treatment and as the lesions were ten weeks old in some cases the negative results are acceptable. The high percentage of positives from specimens from patients with primary and secondary syphilis indicate the superiority of this molecular assay for the direct detection of T. pallidum as compared with conventional techniques.

The nested-PCR assay was found to be capable of detecting *T. pallidum* DNA in clinical samples. This study used a small region of the *T. pallidum* genome, believed to be highly conserved, but poorly characterised and although the genome of *T. pallidum* is comparatively conserved among strains, genes of unknown function may contain insertions or deletions. Recent studies have indicated heterogeneities among *T. pallidum* species in genes such as *arp* and *trp* (19). In order to determine the sensitivity of the nested PCR assay with respect to these heterogeneities it would be necessary to test a wide range of clinical samples. These samples would also need to be sequenced to allow comparisons them and the Nichol's strain of *T. pallidum*. Ideally the samples should be obtained from different countries to increase the chance of identifying new emerging strains of *T. pallidum.*

In conclusion the nested PCR assay appears fully optimised and capable of the detection of *T. pallidum* DNA in clinical samples. It is thought in the near future that this nested PCR assay will be used routinely in the diagnosis of syphilis. It offers reliable testing in a short period of time. Furthermore the assay has the capacity to detect *T. pallidum* DNA one day after infection (data not shown). This is of major importance as rapid detection and treatment reduces the transmission of the disease. The PCR results correlated well with the serology. PCR provided an earlier diagnosis than serology in some cases, and offered a confirmatory diagnosis or a differential diagnosis between *T. pallidum* and HSV in other cases

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تطوير الجين البروتينى الجدارى الاساسى بهدف تقييمه بتفاعل البلمره المتسلسل العشى للكشف النووى المباشر للتريبونيما باليدم من عينات لمرضى مشتبه اصابتهم بمرض الزهرى

لبنى متولى ، ماك برايد

معمل الفيروسات الاقليمى ،مستشفى فيكتوريا الملكيه . بلفاست ،المملكه المتحده.

تهدف الجهود الحالية للتخلص من مرض الزهرى إلى التشخيص المبكر للمرضى المصابين و سرعة معالجتهم و ذلك للحد من إنتشار المرض. و يعتمد تشخيص المرض على التعرف على الصورة الإكلينيكية للمريض و ملاحظة ميكروب التريبونيما باليدم فى عينات الأشخاص المصابين بإستخدام الميكروسكوب المعتم و كذلك بعض الإختبارات المصلية التى قد تفتقد الحساسية خاصة فى المراحل الأولى للمرض ولذلك كانت الحاجة إلى الإختبارات المباشرة وذلك للكشف عن الدى إن إيه لميكروب التريبونيما باليدم فى عينات المرضى.

تهدف هذه الدراسة إلى تصميم تحليل للكشف السريع عن بكتيريا التريبونيما باليدم بواسطة تفاعل البلمرة المتسلسل. و لتقييم مدى حساسية هذا التحليل فقد تم إستخدام البلازميد المستنسخ الذى يحتوى على نواتج تضخيم الدى إن إيه من بكتيريا التريبونيما باليدم بالدم. وقد تم التأكد من إخنصاصية هذه النواتج بإستخدام إختبار تسلسل الدى إن إيه و كذلك تم الحساب الكمى لهذا البلازميد المستنسخ و ذلك لحساب مدى حساسية الإختبار.

ولقد تم حساب مدى حساسية الإختبار لتكون ٢٤ نسخة من الدى إن إيه فى التفاعل الواحد و للتأكد من إختصاصية التحليل لبكتيريا التريبونيما باليدم تم فحص عدد من الميكروبات التى قد تسبب القرح التناسلية مثل ميكروب النيسيريا المسبب للسيلان و كذلك فيروس الهيربس سمبلكس و كذلك ميكروب الترايكوموناس و جميعها كانت سالبة. ولإختبار كفاءة التحليل قد تم تحليل عدد من العينات من أشخاص مشتبه إصابتهم بالزهرى ولقد توافقت نتائج التحليل مع تشخيص الأطباء و كذلك الإختبارات المعملية.