Identification of Treponema Pallidum By PCR Assay From Patients And Assessment Of The Assay's Performance By Comparison With Serological Testing

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تشخيص بكتريا Treponema pallidum باستخدام اختبار تفاعل مضاعفة الانزيم (PCR) من المرضى وتقييم كفاءة الاختبار بالمقارنة مع الفحوصات المصلية

الخلاصة:

تم تشخيص السفلس إما باستخدام الفحوصات المصلية أو من خلال تشخيص المسبب المرضي Treponema pallidum وتاريخيا كانت تشخص البكتريا باستخدام المجهر ذو الحقل المعتم والاصبغات المناعية مع الأجسام المضادة المتعددة والوحيدة النسيلة أو باستخدام فحص تلقيح الأرنب والتي تعتبر ذات حساسية ونوعية عالية جدا، ولكنها تكون مختصر للاستخدام البحثي وليس السريري، لأنها تستغرق وقتا لإعطاء النتائج، حيث تم في هذه الدراسة استخدام إختبار تفاعل مضاعفة الإنزيم (TaqMan real-time PCR) للجين polA لبكتريا reponema pallidum، حيث قدر التحليل التحسسي للفحص بـ ١.٧٦ نسخة هدف لكل تفاعل. تم اختبار ٣٠٠ عينة من ٣٠٠ مريضا من مستشفيات محافظة النجف للفترة من بداية شهر شباط ٢٠٠٥ ولغاية نهاية شهر شباط٢٠٠٧ حيث كانت فقط ٢٠(٦.٦%) عينة موجبة لاختبار تفاعل مضاعفة الإنزيم بالمقارنة ١٣(٤.٣%) عينة موجبة بالفحص المصلي للسفلس المبكر في حين كانت ١٢ (٤%) عينة موجبة بكلا الطريقتين الـ TpPCR والمصلية، بينما كانت ٨(٢.٦%) عينات موجبة بالـ TpPCR لكنها سالبة بالفحص المصلي. كذلك كانت عينة واحدة سالبة بفحص TpPCR في حين أنها موجبة بالفحص المصلي. أظهرت لنا هذه الدراسة أن الـTpPCR يعتبر فحصا إضافيا مهما للفحص المصلي في تشخيص السفلس المعدي، وأيضا فحصا حيويا في تشخيص السفلس، خاصة في مستشفيات العراق.

Abstract:

Syphilis is diagnosed by serologic testing or by identification of the causative agent, Treponema pallidum. The bacterium has historically been detected in clinical specimens by dark-field microscopy, immunostaining with polyclonal or monoclonal antibodies, or the rabbit inoculation test (RIT). RIT is considered to be very sensitive and specific, although it is available only in research settings and is not clinically useful due to the length of time required to obtain a result.

A TaqMan real-time PCR assay targeting the polA gene of Treponema pallidum (TpPCR) was used. The analytical sensitivity of the assay was estimated to be 1.76 target copies per reaction. Initially, the assay was used to test a variety of specimens from 300 patients collected from the Hospitals of Najaf Governorate from first February 2005 to end February 2007. Of the 300 tests performed, positive PCR results were obtained for 20 patients. TpPCR results were compared with serology results for 13 patients being investigated for early syphilis. Of these patients, 12 were positive by both TpPCR and

serology, 288 were negative by both TpPCR and serology, 8 were TpPCR positive but negative by serology, and 1 were TpPCR negative but showed evidence of recent or active infection by serology. We found that TpPCR is a important useful addition to serology for the diagnosis of infectious syphilis. T. pallidum PCR concern a vital assay to diagnostic syphilis especially in Iraqi Hospitals

Introduction:

Clinical suspicion, and recognition of the symptoms and signs of syphilis, supported by serology is the mainstay of syphilis diagnosis, as direct detection methods such as dark-field microscopy and direct immunofluorescence are relatively insensitive, require fresh high-quality specimens, and are unsuitable for use on specimens from mucosal sites or if superinfection is present.(4) Serology may be problematic in the early stages of primary syphilis, as rapid plasma reagin assay (RPR) responses may take some time to develop (4), particularly in HIV-infected patients, and few laboratories in Australia routinely use immunoglobulin M (IgM) assays.

The Victorian Infectious Diseases Reference Laboratory (VIDRL) acts as the state reference laboratory for syphilis serology, confirming positive results from other laboratories and offering less commonly used tests, such as IgM assays. The Hospitals of Najaf Governorate also provides primary diagnostic of sexually transmitted infection (STI) and receives numbers of specimens for VDRL or culture for other agents of STI, allowing easy access to specimens for the development and assessment of novel STI detection assays. Our study was to use a robust, sensitive, and specific real-time PCR assay for the first time to directly detect the presence of pathogenic Treponema pallidum in swabs and biopsy specimens from genital and mucosal ulcers, placental specimens, and cerebrospinal fluid. No comparable assay for the direct detection of T. pallidum was available; an initial assessment of assay performance was done by comparison of PCR results with syphilis serology results where adequate serological follow-up had occurred.

Materials And Methods:

Patients and specimens tested. Specimens tested by a TaqMan real-time PCR assay targeting the polA gene of Treponema pallidum (TpPCR) were referred to the VIDRL between February 2000 and February 2007. 100 genital specimens of various types sourced from The Hospitals of Najaf Governorate. These specimens were sent to laboratory for microscopy and bacterial culture, VDRL and TpPCR assay, specimens were referred specifically for TpPCR, including specimens for the investigation of congenital infections and neurosyphilis. Blood samples were not accepted for testing by TpPCR. Lesion swabs collected by the attending doctor were transported to the laboratory in either bacterial or viral transport medium. Cerebrospinal fluid specimens were sent fresh, and tissue specimens were sent either fresh or paraffin embedded in the case of two placental biopsy specimens. DNA extraction. DNA was extracted using the Roche Diagnostics (Basel, Switzerland) MagNa Pure LC DNA isolation kit III (fungi and bacteria) according to the manufacturer's instructions.

Assay design. A TaqMan real-time PCR assay (minor groove binder [MGB] probe) was designed using the Primer Express software program (Applied Biosystems, Foster

City, CA) targeting a 67-bp sequence within the polA gene of T. pallidum. This sequence (nucleotides 2001 to 2067) (GenBank accession no. TPU57757) is within the region amplified by primers F1 and R1 as described by Liu *et al.*(5). A BLAST search performed using this 67-bp fragment indicated that apart from the T. pallidum polA gene, there were no similar sequences in the GenBank database. Primers were synthesized by GeneWorks (GeneWorks Pty. Ltd., Hindmarsh, Australia), and the TaqMan MGB probe was synthesized by Applied Biosystems. The probe was labeled with the fluorescent dye 6-carboxyfluorescein at the 5' end and a nonfluorescent quencher at the 3' end (Applied Biosystems) (forward primer SyphTF [5'-AGG ATC GCC CAT ATG TCC AA-3'], reverse primer SyphTR [5'-GTG AGC GTC TCA TCA TTC CAA A-3'], and MGB probe SyphTP [6-carboxyfluorescein-ATG CAC CAG CTT CGA-MGB nonfluorescent quencher]).

Real-time PCR mixtures contained template DNA, 0.9 µM concentrations of each primer, a 0.25 µM concentration of the probe, ABsolute QPCR ROX (500 nM) mix (ABgene, Epsom, United Kingdom), and TaqMan exogenous internal positive control (Applied Biosystems) in a total volume of 25 µl in a 96-well plate.

Amplification and detection were performed with the ABI Prism 7000 sequence detection system (Applied Biosystems) using the following program: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

Controls: A positive control was prepared by PCR amplifying a fragment within polA using primers F1 and R1.(5) Five microliters of a 1/108 dilution of this fragment gave a cycle threshold (CT) of approximately 22 for each run. A no-template negative control was also included in each run, as was a reagent-only negative control. The TaqMan exogenous internal positive control DNA and mix present in each well constituted the internal positive control for inhibition. Specimens and controls were tested in duplicate.

To determine the sensitivity of the assay, a suspension of T. pallidum strain containing 3.5 × 107 organisms per ml was serially diluted in phosphate-buffered saline, and 100-µl aliquots of the 1/10 (3.5 × 105 organisms) to 1/106 (three to four organisms) dilutions were applied to charcoal swabs. DNA was extracted as described above. This showed a detection limit of 35 organisms per swab (1.75 target copies per reaction) at a CT of 38.4. Using the above-mentioned information, and after an initial analysis of TpPCR results versus serology, an assay cutoff at cycle 38 was determined, and duplicate samples with a mean CT of >38 were categorized as negative.

The performance of the TpPCR was assessed against serology by comparing results from a subset of 300 specimens of various types from 300 patients. Only the first TpPCR specimen submitted from each patient was included. Specimen types are shown in Table 2. For this part of the study, all specimens came from patients undergoing investigation for infectious (primary or secondary) syphilis who had a concurrent or subsequent syphilis serology requested. In many cases, results from prior serology testing were also available for comparison.

Serological test: The syphilis serology tests used included T. pallidum particle agglutination (TPPA) (Serodia TPPA; Fujirebio, Tokyo, Japan) and VDRL. All tests were performed according to the manufacturer's instructions and are subject to three regular quality assurance programs per year.

Results:

All samples were tested by PCR assay as shown in figures 1,2 and 3.

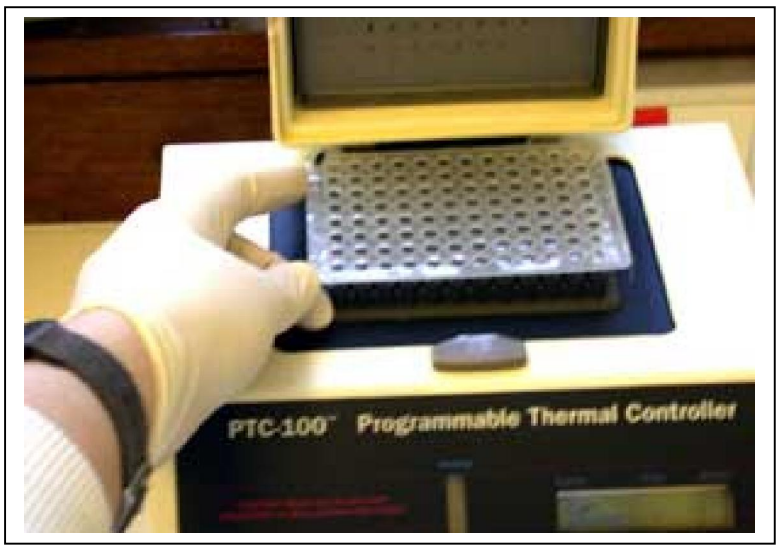

Figure (1): PCR assay kit

Figure (2): PCR machine

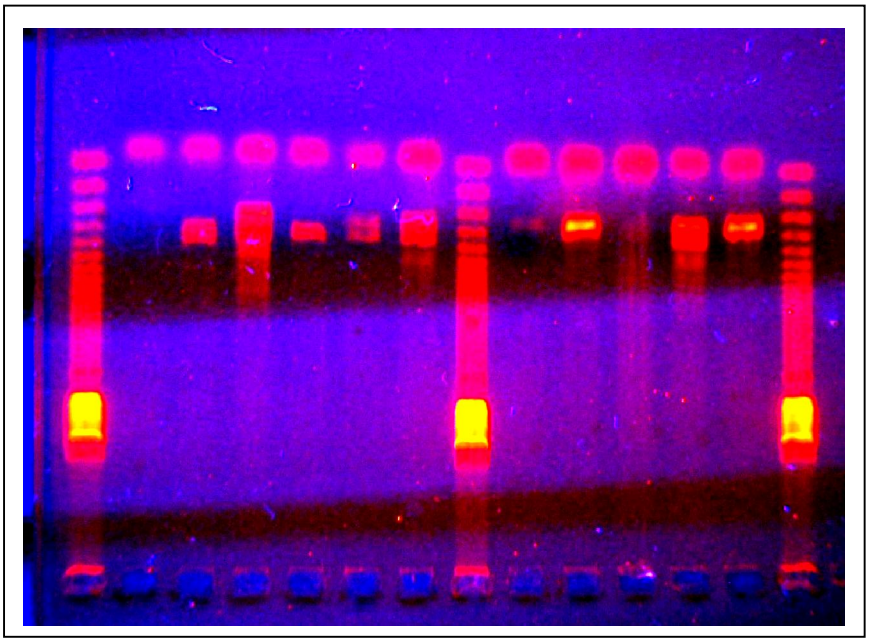

Figure (3): gel electrophoresis of PCR assay; shows13 bands of T. pallidum DNA three of them are control.

Of the 300 specimens from 300 patients tested, positive TpPCR results were obtained for 20 specimens from 20 patients (Table 1).. Results by specimen site tested are shown in Table 2,3 and 4 .

A number of other organisms, including Staphylococcus aureus, beta-hemolytic streptococci, and HSV types 1 and 2, were detected in specimens that were both positive and negative by TpPCR and they are excluded from this study.

Table (1): Breakdown of TpPCR results by sex.

Sex	Specimens	Patients		
	Total	PCR positive	Total	PCR positive
Male	**100**	8	**100**	8
Female	**200**	12	**200**	12
Total	**300**	**20**	**300**	**20**

Table (2): TpPCR results by type of specimen.

Specimen	TpPCR positive	TpPCR negative	Total
Cerebrospinal fluid	0	10	**10**
Urethral	4	16	**20**
Oropharyngeal, tongue and lip	0	20	**20**
Other superficial body site	0	8	**8**
Genital swab, not further specified	5	25	**30**
Groin, scrotum, pubis, perineum	0	10	**10**
Anorectal, perianal, etc.	2	28	**30**
Vulvovaginal, cervix	7	125	**132**
Penile	2	30	**32**
Total	**20**	**280**	**300**

Of the 300 patients with adequate PCR follow-up, the assay compared with serology for the detection of early syphilis (Table 5).

Table (3): VDRL results by type of specimen:

Specimen	VDRL positive	VDRL negative	Total
Cerebrospinal fluid	0	10	**10**
Urethral	2	18	**20**
Oropharyngeal, tongue and lip	0	20	**20**
Other superficial body site	0	8	**8**
Genital swab, not further specified	3	27	**30**
Groin, scrotum, pubis, perineum	0	10	**10**
Anorectal, perianal, etc.	1	29	**30**
Vulvovaginal, cervix	4	128	**132**
Penile	3	29	**32**
Total	**13**	**288**	**300**

Table (4): TPPA results by type of specimen

Specimen	TPPA positive	TPPA negative	Total
Cerebrospinal fluid	0	10	**10**
Urethral	2	18	**20**
Oropharyngeal, tongue and lip	0	20	**20**
Other superficial body site	0	8	**8**
Genital swab, not further specified	3	27	**30**
Groin, scrotum, pubis, perineum	0	10	**10**
Anorectal, perianal, etc.	1	29	**30**
Vulvovaginal, cervix	4	128	**132**
Penile	3	29	**32**
Total	**13**	**288**	**300**

Table (5): Comparison of PCR results against serology for 300 patients with adequate serological testing.

Result	PCR result	Serology result
Positive	20	13
Negative	280	287
Total	**300**	**300**

CT values of >35

Discrepant results included two patients with positive PCR results from penile lesions but one of them serological evidence of recent infection: all of these patients had mean CT values of >35.

Excluding patients with reinfections or clinical notes indicating nonprimary syphilis, 5 patients with a positive PCR result had all available serology tests performed on a concurrent serum sample. Of the 12 patients with more than one positive serology test result, TPPA was positive for all 12 of these patients.

Discussion:

Studies of this type have several problems, the major one being the lack of a "gold standard" for the direct detection of T. pallidum with which to compare the PCR assay. Serological confirmation may be delayed, absent, or difficult to interpret in cases of potential reinfection or reactivation of disease, and some patients, such as HBs-infected patients with low CD4 counts, may have atypical responses.(4) Discrepant results were obtained for 5 patients; those that were TpPCR positive but negative by serology all had CT values close to the cutoff at cycle 38. These results may represent the detection of low levels of T. pallidum or nonviable organisms that failed to initiate an invasive infection in the patient or may represent false-positive results due to low-level assay contamination. It was not known if any of these patients were receiving antibiotics at the time of sampling.

For the PCR-negative but serology-positive patients, the most likely explanation for the lack of correlation is incorrect timing or site of sampling. One patient with seroconversion and a positive PCR from a penile ulcer with a mean CT of 34.6 showed a weaker positive result (CT of 37.7) when the lesion was resampled 7 days later. This suggests that only small amounts of T. pallidum DNA may remain in healing chancres, nearing or exceeding the detection limit of the assay.

To date, there have a been few other published studies that compared syphilis PCR assays with serology results. A study by Orle *et al.*(6) in 1995 used a T. pallidum 47-kDa integral membrane lipoprotein gene target in a multiplex AMPLICOR PCR format (Roche Molecular Systems) and confirmed results with a second PCR assay targeting the basic membrane protein (bmp) gene. Correlation with TPPA or VDRL serology in Iraqi population of STDs clinic patients; however, no T. pallidum-specific serology was performed. In 2001, Bruisten *et al.*(1) used a bmp gene target in a nested gel-based PCR format and compared results against RPR, TPHA, and fluorescent treponemal antibody data in a population of 364 Dutch STI clinic patients. They found a correlation of 96%; however, syphilis infection was uncommon in the population studied, with 12 cases detected by PCR, 7 cases detected by serology, and only 3 cases detected by both PCR

and serology. In 2003, Palmer *et al.*(7) used a gel-based PCR assay to detect the T. pallidum 47-kDa integral membrane lipoprotein gene in a population of 98 STI clinic patients in the United Kingdom and compared the results with serology using a combination of RPR, TPPA/TPHA, and IgG and IgM EIAs. Those authors estimated the sensitivity and specificity of the PCR for the detection of primary syphilis to be 94.7% and 98.6%, respectively, and estimated the sensitivity and specificity of the PCR for the detection of secondary syphilis to be 80% and 98.6% compared with serology. Dark-ground microscopy was positive for only 2 of 10 patients with primary syphilis and none of 3 patients with secondary syphilis. Those authors described three PCR-negative but serology-positive patients and proposed that those results were due to sampling error or inadequate sensitivity of the PCR assay, and those authors also described one PCR-positive but serology-negative result for an HBs patient with a low CD4 count who also received antibiotics 6 days after the positive PCR swab was taken and postulated that the combination of a blunted immune response and early treatment was the likely explanation for the lack of a serological response.

More targeted studies including more detailed clinical information, repeat sampling, and direct comparison of other PCR techniques will be required before a more precise assessment of sensitivity and specificity can be estimated. However, overall, there was 95% agreement between PCR and serology results in the investigation of early syphilis. This study also highlights the importance of laboratories performing adequate serological testing guided by clinical information, as laboratories that screen with RPR alone will fail to detect a small number of cases of infectious syphilis. Our study also demonstrates the high sensitivity of modern T. pallidum-specific assays (TPPA and VDRL) in early syphilis, and the widely quoted(4) 1- to 4-week "window period" after the appearance of the chancre, but before the development of antibodies, may be reduced in laboratories using a range of modern commercial syphilis serology assays. Whether the use of the single-copy polA gene target will provide adequate sensitivity for the use of this assay in the later stages of syphilis, where low levels of T. pallidum are widely distributed throughout the body, will be difficult to assess, as late syphilis is a rare disease in Iraq at present.

In initial and ongoing studies, a few positive PCR results were obtained for specimens submitted only for other tests such as herpesvirus PCR or bacterial culture, and syphilis serology was not concurrently requested for these patients. This would imply that the clinical diagnosis of syphilitic lesions is not always a simple matter, particularly in HBs-infected patients and in patients with syphilitic lesions superinfected with other organisms. We believe that the T. pallidum PCR will be a valuable addition to serology for the diagnosis of early syphilis and will be useful for the confirmation of other diagnostic methods such as histopathology in late and congenital syphilis.

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