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A New Multiplex Real-time PCR Test for HSV 1 / 2 and Syphilis- An evaluation of its impact in the laboratory and clinical setting.

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Key messages:

- This manuscript describes the clinical utility and impact of testing for HSV and syphilis in a single sample using a single test.
- Using this single test leads to the detection of unexpected cases of syphilis where the suspected aetiological agent was HSV, and also improves the detection rates of primary syphilis.
- The test is likely to reduce costs and turn around times as testing is performed at a single location for both pathogens and only one sample is needed.

ABSTRACT

Objectives:

To develop, evaluate and implement a new multiplex real-time PCR test for the detection of HSV1, HSV2 and syphilis in a single sample using a single test.

Methods:

A multiplex real-time PCR test detecting HSV1, HSV2 and *T. pallidum* was designed, validated and evaluated for a period of 6 months on patients attending the Sandyford Initiative (a series of GUM clinics in and around Glasgow). A total of 692 samples were tested and *T. Pallidum* PCR positives were confirmed by a second PCR at the Scottish Reference Laboratory (SBSTIRL). All PCR results were aligned with dark ground microscopy (DGM) findings and serological results where available, and compared.

Results:

The laboratory validation of the multiplex assay showed the test to be sensitive, specific, and robust. Of the 692 samples, 139 were positive for HSV1, 136 for HSV2, 15 for syphilis, one for both syphilis and HSV1, and 401 were negative; the reference laboratory confirmed all *T. pallidum* PCR positive samples. The PCR test was more sensitive than both DGM and serological testing for the diagnosis of primary syphilis.

Conclusions:

The introduction of this new test has led to a better turn around time for the diagnosis of genital ulcer disease, better detection of primary syphilis infection and the detection of unexpected cases of syphilis where the aetiological agent suspected was HSV.

Introduction

It is important to be able to diagnose herpes simplex virus (HSV) and *Treponema pallidum* (syphilis) infections as they can be clinically indistinguishable. Primary syphilis can progress to more serious secondary and tertiary sequelae¹, and can be difficult to diagnose, particularly in the early stages. Serological testing can be insensitive during early syphilis infection and therefore may miss cases of primary infection². Dark ground microscopy (DGM) is not available at all clinical locations, is highly user-dependent and is unsuitable for use on rectal and oral sites³. Polymerase Chain Reaction (PCR) is a sensitive and specific test for the diagnosis of primary syphilis^{4,5} and can be used on both oral and rectal sites⁶.

HSV has been routinely diagnosed by PCR at the West of Scotland Specialist Virology Testing Centre (WoSSVC) since 1997, while Syphilis PCR testing became available in Scotland at the Specialist Bacterial STI Reference Laboratory (SBSTIRL) in May 2006. In order to diagnose each infection, separate swabs need to be taken, and to ensure a swab for syphilis is obtained; a high degree of clinical suspicion is required. Sending the swabs to different locations leads to delays in turnaround times and patient treatment because of batching and transport delays. It may also lead to infections not being diagnosed because swabs, particularly for syphilis, are not taken. We decided to develop a multiplex PCR test for the detection of HSV1, HSV2 and syphilis at the WoSSVC to overcome these difficulties.

Methods:

Test Development

A real-time PCR test was developed targeting the 47kDa membrane gene of *T. pallidum*. The primers and probe used were; Forward 5'-GGATAGTTTTTCTGCACGTAAGGTAA, Reverse 5'-ACCCACCGTGTCTACCACAAG and Probe 5' VIC-CAGCATGGAGAGCCCGCACG-tamra at concentrations of 100µM (primers) and 20µM (probe) using standard taqman conditions⁷. This test was then multiplexed with the existing HSV 1/2 real-time PCR test in place at the WoSSVC which uses the fluorophores FAM and Cy5 (previously published⁸).

Prior to the clinical evaluation, the performance of the new multiplex was validated in the laboratory using a number of different panels. Firstly a dilution series of HSV1 and HSV2 was tested using the HSV1 and 2 duplex assay and the newly developed multiplex assay to ensure that the HSV PCR component was not affected by the addition on the syphilis primers and probe. Following this, the specificity of the multiplex assay was assessed by testing a panel containing other viruses and bacteria. A dilution series of a clinical sample containing *T. pallidum* (provided by the SBSTIRL and previously tested by an alternative reference assay) was also tested to ensure that the newly developed multiplex achieved the same end point detection limit for syphilis testing as the reference test already in place in the SBSTIRL. Quality assurance panels were also tested to assess the robustness and reproducibility of the newly designed multiplex assay in the laboratory.

All samples were extracted using the virus kit on the MDX extractor (Qiagen) and tested using the new triplex real-time PCR assay using UGD platinum supermix (Invitrogen) on the ABI 7500 (Applied Biosystems).

Patients and samples

Clinical samples were obtained from patients presenting to the Sandyford Initiative with symptoms of genital ulceration or suspected early syphilis, in cases where an ulcer swab would usually have been taken. During initial clinical use (6 months), 692 samples (including genital, oral and anorectal swabs) were received.

All samples were processed as outlined above. All positive syphilis PCR samples were sent to the SBSTIRL for confirmatory PCR before reporting. The *T. pallidum* PCR test used at the reference laboratory targets the *polA* gene and has been previously published¹⁰. The PCR results were then aligned with DGM and serological results and analysed. Serological screening was performed using a combined IgG/IgM kit (Newmarket).

Results:

The initial validation showed that the HSV components of the newly developed multiplex assay had the same endpoint detection limits as the HSV 1 and 2 duplex assay already in

place in the WoSSVC. The syphilis component was also shown to have the same endpoint detection limit as the syphilis reference test in place at the SBSTIRL. No cross reactions were observed when the sensitivity panel was tested and the multiplex produced reproducible results in the ongoing EQA.

The results of the 692 clinical samples are shown in table 1

Table 1: PCR Results for GUM samples

PCR RESULT	TOTAL	%
negative	401	57.95
HSV1	139	20.09
HSV2	136	19.65
SYPHILIS	15	2.17
HSV1 AND SYPHILIS	1	0.14
GRAND TOTAL	692	

The 16 syphilis PCR positive samples were from 15 patients and were all confirmed by the SBSTIRL. Of the 15 positive patients; 12 had a blood sample taken for serological testing on their first visit; of which 10 were positive (sensitivity 83.3% compared to PCR). The remainder, who were either sero-negative on their first visit or had no blood taken, all had positive syphilis serology on follow-up serological testing.

Only six of the 15 PCR positive patients were tested by DGM yielding three positives (50% sensitivity). During the study period, all cases of primary syphilis, diagnosed by the non-PCR tests (DGM/ serology) were reviewed to check if any of them had been missed by the PCR. The reviews showed that the PCR did not miss any cases.

Discussion:

The HSV1/HSV2/*T. pallidum* PCR had better detection rates for primary syphilis than both DGM and serology. In addition, the *T. pallidum* component of the new multiplex test was shown to perform well in comparison to the PCR already in place at the SBSTIRL. Less than half of the patients with PCR-proven primary syphilis had DGM testing performed, illustrating the difficulty of relying on this alone. DGM at the Sandyford is performed by a

highly-experienced full-time laboratory biomedical scientist, but even here sensitivity was just 50% compared to PCR.

The multiplex PCR test is targeted at symptomatic patients so can be used as a valuable front line test to ensure primary syphilis infection is not missed or misdiagnosed; and that serological follow-up and patient review is carried out. In two of the syphilis positive patients during this study period, follow up and blood sampling was only initiated due to the positive PCR result. In one case, a young heterosexual woman with documented previous HSV2 was unexpectedly found to be *T. pallidum* positive when an HSV2 recurrence had been suspected.

Previously, separate swabs had to be taken and transported to the reference laboratory for syphilis testing. As syphilis and HSV testing is now performed using a single sample at a local laboratory, it is likely that the turnaround time and costs have been reduced as well as the number of misdiagnoses.

The multiplex assay covers the huge majority of diagnosable causes of genital ulceration in the UK. Additional targets might eventually include *C. trachomatis* (LGV serovars) which now generally presents as severe proctitis, although some classical genital ulceration is seen¹¹, and *H ducreyi*¹², which remains very rare in the UK. However, care should be taken to ensure that there is no loss of sensitivity when increasing PCR targets¹³.

Conclusions:

Multiplex PCR testing for HSV1, HSV2 and *T. pallidum* on ulcer swabs can be incorporated into routine laboratory workflows, improving detection rates and reducing turnaround times and costs, ultimately reducing the risk of onwards transmission of primary syphilis and development of sequelae.

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