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Evaluation of a new multiplex polymerase chain reaction assay STDFinder for the simultaneous detection of 7 sexually transmitted disease pathogens ☆

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Abstract

We evaluated a new multiplex polymerase chain reaction (mPCR), "STDFinder assay", a novel multiplex ligation-dependent probe amplification (MLPA) assay for the simultaneous detection of 7 clinically relevant pathogens of STDs, i.e., *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Mycoplasma genitalium*, *Treponema pallidum*, and herpes simplex virus type 1 and 2 (HSV-1 and HSV-2). An internal amplification control was included in the mPCR reaction. The limits of detection for the STDFinder assay varied among the 7 target organisms from 1 to 20 copies per MLPA assay. There were no cross-reactions among any of the probes. Two hundred and forty-two vaginal swabs and an additional 80 specimens with known results for *N. gonorrhoeae and C. trachomatis*, obtained from infertile women seen at an infertility research clinic at the Kigali Teaching Hospital in Rwanda, were tested by STDFinder assay and the results were confirmed by single real-time PCR using different species-specific targets. Compared to the reference standard, the STDFinder assay showed specificities and sensitivities of 100% and 100%, respectively, for *N. gonorrhoeae*, *C. trachomatis*, and *M. genitalium*; 90.2% and 100%, respectively, for *Trichomonas vaginalis*; and 96.1% and 100%, respectively, for HSV-2. No specimen was found to be positive for HSV-1 by either the STDFinder assay or the comparator method. Similarly, the sensitivity for *Treponema pallidum* could not be calculated due to the absence of any *Treponema pallidum*-positive samples. In conclusion, the STDFinder assays have comparable clinical sensitivity to the conventional mono and duplex real-time PCR assay and are suitable for the routine detection of a broad spectrum of these STDs at relatively low cost due to multiplexing.

Keywords: STDFinder; Multiplex PCR; *Neisseria gonorrhoeae*; *Chlamydia trachomatis*; *Trichomonas vaginalis*; *Mycoplasma genitalium*; *Treponema pallidum*; *Herpes simplex virus type 1/2*

1. Introduction

Sexually transmitted diseases (STDs) are a major cause of morbidity in sexually active individuals and continue to pose major medical, social, and economic burden worldwide. According to the World Health Organization, 340 million new cases of gonorrhoea, chlamydia, syphilis, and trichomoniasis occurred throughout the world in 1999 in men and women aged 15–49 years (http://www.who.int/mediacentre/factsheets/fs110/en/). Untreated STDs, particularly gonorrhoea and chlamydial infection which cause pelvic inflammatory disease in women, can lead to infertility in both men and women. Other sequelae of some STDs include ectopic pregnancy and risk of developing genital cancers, which cost the individuals and health care systems billions of dollars annually. Apart from causing serious sequelae, STDs

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increase the risk of both transmission and acquisition of HIV (http://www.who.int/mediacentre/factsheets/fs110/en/). The development of fast laboratory diagnostic screening method for STDs is therefore an imperative tool to minimize damage to the reproductive tract and to simultaneously improve women's health worldwide.

Several methods are available for detecting *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, and *Mycoplasma genitalium*, including bacterial culture, enzyme-linked immunosorbent assay for antigen or antibody detection (Clad et al., 2000), strand displacement amplification (Chan et al., 2000), and polymerase chain reaction (PCR), performed in monoplex or multiplex (Chernesky et al., 2005; Eastick et al., 2003; Geraats-Peters et al., 2005; Kawada et al., 2004; Ryu et al., 1999). Of these, PCR has been found to be a highly sensitive method for detecting these sexually transmitted pathogens. A multiplex assay has an additional advantage in screening since it involves the simultaneous detection of multiple pathogens. Moreover, the incorporation of an internal control reaction in a multiplex assay identifies the possible presence of PCR inhibition. A multiplex test is a prerequisite to reduce the costs of an assay as well as hands-on time. However, simultaneous molecular amplification and detection of multiple targets are technologically challenging as they may result in the reduction of the ability to amplify by competition between the amplification of the different mix reactions or to detect individual targets through nonspecific interactions between primers and probes (Markoulatos et al., 2002).

The multiplex ligation-dependent probe amplification (MLPA) technology, which uses a single easy-to-perform assay, is able to amplify up to 45 different targets simultaneously using one universal primer set in the final amplification (Schouten et al., 2002). Previously developed MLPA applications include the detection of changes in the copy numbers of specific chromosomal regions (Schouten et al., 2002), detection of CpG methylation of genes (Nygren et al., 2005), detection of recombination events (Langerak et al., 2005), and expression profiling studies (Eldering et al., 2003). Most recently, MLPA has successfully evaluated the identification of a wide range of viruses causing respiratory tract and central nervous system (CNS) infections (Reijans et al., 2008; Wolffs et al., 2009). Our aim was to evaluate and demonstrate the utility of the STDFinder assay (Fig. 1), a novel MLPA technology for the simultaneous detection of 7 clinically relevant pathogens of STDs, i.e., *N. gonorrhoeae*, *C. trachomatis*, *Trichomonas vaginalis*, *M. genitalium*, *Treponema pallidum*, and herpes simplex virus type 1 and 2 (HSV-1 and HSV-2).

2. Materials and methods

2.1. Study population and clinical specimens

A total of 242 vaginal swabs taken from a case group consisting of infertile women, seen at an infertility research clinic at the Kigali Teaching Hospital in Rwanda (the largest public hospital in Rwanda) between November 2007 and March 2010, who were all eligible for enrollment in a case-control study investigating the aetiology and risk factors of infertility and its link with HIV/STDs were tested in this evaluation (Dhont et al., 2010). During speculum examination, 2 swabs of upper vaginal secretions were collected by standard practice. The first swab was collected using the conventional rayon swab of the Amies gel Transystem (Copan Italia, Brescia, Italy); the second was collected using the Copan flocked swab of the ESwab system (Copan Italia). The first swab was rolled and smeared directly onto a glass slide for wet mount preparation. The second swab was placed immediately into the ESwab transport tube containing 3 mL of liquid transport medium (Copan Italia). The ESwab specimen tube was briefly vortexed and the swab was removed according to the manufacturer's instructions. The ESwab liquid was split into 2 portions and stored at −80 °C until shipment on dry ice to the Ghent University Hospital laboratory for STDFinder assay evaluation and testing by the assays as described below. In order to increase the ability of the study to evaluate the performance of the multiplex PCR assay, an additional 80 specimens (31 positive for either *C. trachomatis* or *N. gonorrhoeae* and 49 negative for both pathogens) previously tested by a duplex PCR Abbott Real-Time CT/NG (Abbott, Des Plaines, IL, USA) were added to the 242 clinical specimens. This approach allowed the determination of sensitivity and specificity but precludes the determination of positive and negative predictive values, which are dependent upon the prevalence in the population studied.

2.2. Nucleic acid extraction

The DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, specimens were vortexed thoroughly for 10 s before 200 μL of each specimen was transferred and mixed together with 20 μL of proteinase K and 200 μL of buffer AL in a 1.7-mL microcentrifuge tube and incubated at 56 °C for 10 min. After incubation, 200 μL of ethanol was added to the tubes and mixed briefly. This mixture was placed in a QIAamp spin column and centrifuged at 14,000 × *g* for 1 min, after which the filtrate was discarded and 500 μl of buffers AW1 and AW1 was added to each spin column and centrifuged each time again at 14,000 × *g* for 1 min. Finally, 100 μl of buffer AE was used to elute the DNA. Before the start of the extraction, 5 μl of the internal amplification control (IAC) containing an encephalomyocarditis virus RNA transcript was added to the lysed sample. IAC was constructed as described previously (Reijans et al., 2008). Whenever possible, the extracts were analyzed immediately after extraction. If this was not possible, they were divided into 3 aliquots and kept frozen at −20 °C. Each aliquot was used only once to avoid the degradation of genomic material during repetitive freezing and thawing.