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

Claude Mambo Muvunyi[a,b,*], Nathalie Dhont[c,d], Rita Verhelst[c], Tania Crucitti[e], Martin Reijans[f], Brit Mulders[f], Guus Simons[f], Marleen Temmerman[c], Geert Claeys[a], Elizaveta Padalko[a]

[a]Department of Clinical Chemistry, Microbiology and Immunology, Ghent University Hospital, Ghent, Belgium
[b]Department of Clinical Biology, Centre Hospitalier Universitaire-Butare, National University of Rwanda, Butare, Rwanda
[c]International Center for Reproductive Health, Ghent University, Ghent, Belgium
[d]Projet Ubuzima, Kigali, Rwanda
[e]HIV/STI Reference Laboratory, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium
[f]PathoFinder BV, Maastricht, The Netherlands

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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.
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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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* Corresponding author. Tel.: +250-78-849-3814; fax: +250-252530328.
E-mail address: cmuvunyi@nur.ac.rw (C.M. Muvunyi).

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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 RealTime 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.

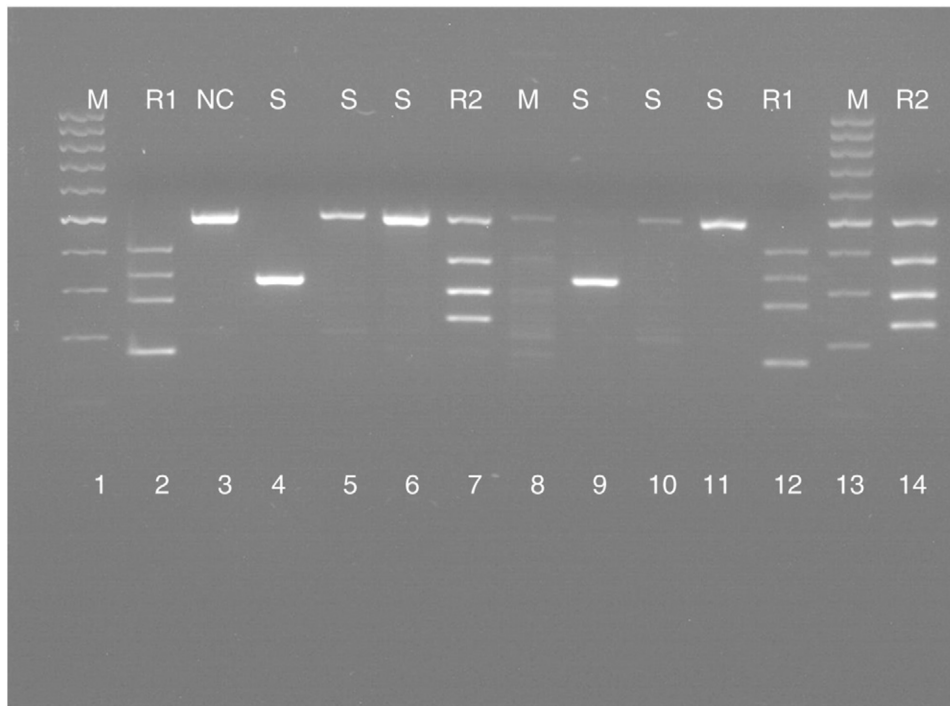

Fig. 1. Agarose gel electrophoresis results of the STD Finder assay. Lanes 1, 8, and 13: molecular size markers (M); lanes 2 and 12: reference sample 1 (containing HSV-2, *C. trachomatis* [CT], *N. gonorrhoeae* [NG], HSV-1); lanes 7 and 14: reference sample 2 (containing IAC, *Trichomonas vaginalis* [TV], *M. genitalium* [MG], *Treponema pallidum* [TP]); lane 3: negative control (water); lanes 4 and 9: specimens positive for CT; lanes 5, 6, 10, and 11: specimens with no pathogen amplified. In lane 10, the specimen showed a weak amplification of the IAC, indicating inhibitory substances in the PCR reaction. In all other negative samples, the IAC was clearly visible.

2.3. Primer and probe design

The primers and probes were designed against conserved genes within each pathogen (Table 1). The sequences from these genes were obtained from GenBank and were aligned by using the Clustal X program (version 1.81, NCBI, Bethesda, MD). PCR primers and MLPA probes were designed on the basis of the sequences of these highly conserved regions. The primers were designed with Primer3 software (version 0.2) (http://primer3.sourceforge.net/). The primers flanked the target region of the MLPA probe. The MLPA probes were designed as described earlier (Schouten et al., 2002). Subsequently, all primers and probes were evaluated by performing a BLAST analysis against the sequences in the NCBI database. The primers and probes were approved when no mismatches within the critical regions of the probes (e.g., no mismatch within 5 nucleotides from the ligation site) and primers (e.g., no mismatch at the 3′ end of a primer) were found. To assess the specificity of the primers and the probes, all PCR primers and MLPA probes were tested in either a monoplex or a multiplex MLPA reaction with different samples. No cross-reactivity among the 7 probes and the IAC probe was observed upon amplification of clinical samples that tested positive for any of the 7 pathogens by routine diagnostic analysis.

2.4. MLPA reaction

The STDFinder assay includes primers and probes specific for highly conserved regions in the MgPa adhesion gene of *N. gonorrhoeae*; major outer membrane protein; and open reading frame 8 cryptic plasmid gene of *C. trachomatis*, adhesive protein gene of *Trichomonas vaginalis*, MgPa adhesion gene of *M. genitalium*, 47-kDa membrane immunogen gene of *Treponema pallidum*, glycoprotein G (US4) gene of HSV-1, and glycoprotein D (US6) gene of HSV-2 (Table 1). Preamplification and MLPA analysis were performed essentially as described earlier (Reijans et al., 2008). All reactions were performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, Foster City, CA). All buffers and enzymes for the STDFinder assay were obtained from PathoFinder (Maastricht, The Netherlands).

Initially, amplified products were separated by electrophoresis using 2.5% precast (1.5% agarose gel + 1% metaphor gel) gels containing ethidium bromide with Tris-borate buffer. Gels were electrophoresed at 180 V for 1–1.5 h, photographed, and analyzed using an AlphaImager imaging system (Isogen, De Meern, The Netherlands). In a few cases, the resolution of the agarose gel was insufficient to distinguish all amplified products and was therefore analyzed by capillary electrophoresis on a Mega-BACE DNA analysis system (GE Healthcare Europe, Diegem, Belgium).

Table 1
Nucleotide sequences of preamplification primers and pathogen-specific regions of the MLPA probes

Organism, primer, and probe	Target gene and DNA sequence (5′-3′)	Amplicon size (bp)
C. trachomatis	Major outer membrane protein	336
Forward primer	GGGAATCCTGCTGAACCAA	
Reverse primer	TCAAAACACGGTCGAAAACA	
Probe	GAAGGTTTCGGCGGAGATCCTTGCGATCCTTGCGC// CACTTGGTGTGACGCTATCAGCATGCGT	
C. trachomatis	Open reading frame 8 cryptic plasmid	325
Forward primer	TCGGTCAACGAAGAGGTTTT	
Reverse primer	GTTGCGTGTCCTGTGACCTT	
Probe	CCGCACGTGCTTCGAGCAACCGCTGTGAC// GGAGTACAAACGCCTAGGGTGCTCAGACTCCG	
N. gonorrhoeae	PorA pseudogene	271
Forward primer	CGGCAGCATTCAATTTGTT	
Reverse primer	AAAAAGCCGCCATTTTTGTA	
Probe	CCGAGTCAAAACAGCAAGTCCGCCTATACGCC// TGCTACTTTCACGCTGGAAAGTAATCAGATGAAACCAG	
M. genitalium	MgPa adhesion gene	297
Forward primer	ACCTTGATGGTCAGCAAAACTT	
Reverse primer	CCTTTGATCTCATTCCAATCAGTA	
Probe	GGAAAACCCCTCAACGGTGCAAAGGGGTTTAAATG// GCGAGCCTATCTTTGATCCTTTTAAAGGCTTTGG	
Trichomonas vaginalis	Adhesive protein gene	378
Forward primer	CCAGAAGTGGGCTACACACC	
Reverse primer	ATACCAAGGCCGGAAGCAC	
Probe	CAAGATCAAGGACATCCTCCGCAACTACCCACGCC// AGGACATCCGCTGCATTGTCGTTACAGATGCTGG	
HSV-1	Glycoprotein G (US4) gene	163
Forward primer	CTGTGGTGTTTTTGGCATCA	
Reverse primer	GGTTGTGGAGGAGACGTTG	
Probe	GGCGCCATGCGTGCCGTTGTTCCCAT// TATCCCATTCCTTTTGGTTCTTGTCGGTGTATCGGGGGTTC	
HSV-2	Glycoprotein D (US6) gene	406
Forward primer	CATGGGGCGTTTGACCTC	
Reverse primer	TACACAGTGATCGGGATGCT	
Probe	CAGCTGACCGACCCCCCCGGGGTG// AAGCGTGTTTACCACATTCAGCCGAGCCTGGAGGACCCGTTCC	
T. pallidum	47-kDa membrane immunogen gene	230
Forward primer	GGAGAAGTTTCACTTCGTGGA	
Reverse primer	CTCGCGTCATCACCGTAGTA	
Probe	GGCTGATGGCTCTGAGCTGTCGCAC// CGTGAGTTCATCGACTATGTGATGAACTTCAACACGGTCC	
IAC		498
Forward primer	ACATGTAACCGCCCCCATT	
Reverse primer	TCCACGCACGCACTACTATG	

2.5. Confirmation of the STDFinder results

Previously published species-specific single or duplex PCRs, amplifying targets different from the targets of the STDFinder assay, were performed on all samples to confirm the specificity of the MLPA targets. For *C. trachomatis* and *N. gonorrhoeae*, the STDFinder results were first compared to the Abbott Real-Time CT/NG assay, and Gene-Probe Aptima Combo 2 assay (Gen-Probe, San Diego, CA, USA) was carried out as a confirmatory test to resolve discrepant results. The Abbott Real-Time CT/NG assay targets the cryptic plasmid of *C. trachomatis* and the opacity gene of *N. gonorrhoeae* (Marshall et al., 2007), while the Gen-Probe Aptima Combo 2 detects targets in the 23S rRNA of *C. trachomatis* and the 16S rRNA of *N. gonorrhoeae* (Gaydos et al., 2003). For *M. genitalium*, an in-house real-time PCR targeting the MgPa gene was performed according to the method of Jensen et al. (2003) and was used as a comparator assay.

For *Trichomonas vaginalis*, the result of the STDFinder assay was compared with microscopic examination and confirmed by 2 additional species-specific PCRs using primers designed by Kengne et al. (1994) (TVK3/TVK7) and Shaio et al. (1997) (IP1/IP2). For HSV-1 and -2, the STDFinder results were compared with results obtained with the Argene real-time PCR HSV1/2 using an IQ5 real time detection (Biorad, Nazareth, Belgium) and an Abbott real-time HSV1/2 PCR kit in the ABI PRISM® 7000 and 7900HT Sequence Detection System (Applied Biosystems) was used on discrepant results as a confirmatory test. All

commercial assays were performed according to the manufacturers' instructions.

Determination of true-positive cases for the microorganisms studied was based on an expanded gold standard, which was defined by a positive result by at least 2 PCR assays. Determination of true-positive cases for the microorganisms studied and prevalence of infection were determined by the total number of positive samples after discrepant analysis.

3. Results

The analytical detection limit of the STDFinder assay was evaluated for *C. trachomatis*, *N. gonorrhoeae*, and HSV-1 and HSV-2 using external quality control samples (Quality Control for Molecular Diagnostics, Glasgow, Scotland, UK) and was found to be 1 copy/MLPA assay for *C. trachomatis*, 20 copies/MLPA assay for *N. gonorrhoeae*, 3 copies/MLPA assay for HSV-1, and 5 copies/MLPA assay for HSV-2. Previous evaluations of multiplex MLPA assays which detect respiratory viruses and viruses causing CNS infections showed similar detection limits (Reijans et al., 2008; Wolffs et al., 2009). There were no cross-reactions between any of the 7 target species or with related species (data not shown). The incorporation of an IAC in all reactions allows discrimination between a true-negative and a false-negative result due to a PCR/MLPA failure as illustrated in Fig. 1.

The STDFinder assay was evaluated on 242 vaginal specimens collected consecutively from infertile women seen at an infertility research clinic at the Kigali Teaching Hospital between November 2007 and March 2010. The specimens were tested blind with species-specific single PCR. Of the 242 examined vaginal specimens, 28.9% (70/242) were positive for 1 or more pathogens in the STDFinder assay (Table 2). Altogether, 2.9% (7/242) of samples were positive for *C. trachomatis*, 4.1% (10/242) for *N. gonorrhoeae*, 1.2% (3/242) for *M. genitalium*, 19.4% (47/242) for *Trichomonas vaginalis*, and 6.2% (15/242) for HSV-2, and all 7 pathogens were undetectable in the remaining 69.4% of specimens. No specimen was positive for either *Treponema pallidum* or HSV-1.

Table 2
Comparison of the results obtained by the STDFinder assay with comparator assays in 242 clinical specimens

STDFinder assay ($N = 242$)		No. of positive/negative by comparator assays[a]		
		Positive	Negative	Total (%)[b]
C. trachomatis	Positive	6	1	7 (2.9)
	Negative	0	235	235
N. gonorrhoeae	Positive	10	0	10 (4.1)
	Negative	3	229	232
M. genitalium	Positive	3	0	3 (1.2)
	Negative	0	239	239
Trichomonas vaginalis	Positive	7	40	47 (19.4)
	Negative	0	195	195
HSV-2	Positive	5	10	15 (6.2)
	Negative	0	227	227

[a] Alternative single PCR for most organisms, wet mount microscopy for *Trichomonas vaginalis* as described under the Materials and Methods section.
[b] Positive rates by STDFinder results.

Duplex PCRs for *C. trachomatis/N. gonorrhoeae* and single real-time PCRs for the other pathogens included in the STDFinder assay were performed on all 242 specimens, and the results were compared with the STDFinder assay results (Table 2).

The STDFinder assay and the Abbott real-time *C. trachomatis/N. gonorrhoeae* PCR results were the same for both *C. trachomatis* in 241 of 242 specimens and for *N. gonorrhoeae* in 239 of 242 specimens. The STDFinder assay detected 1 more positive specimen for *C. trachomatis* which was found to be negative by Abbott real-time CT/NG assay. All discrepant results for *N. gonorrhoeae* were positive by the Abbott m2000 PCR assay, but negative by the STDFinder assay. The Gen-Probe APTIMA Combo 2 Assay confirmed the STDFinder result in all discrepant cases.

All 3 specimens that were positive for *M. genitalium* with the STDFinder assay were also positive with the real-time single PCR, and no additional positive specimen was detected.

Only 7 vaginal specimens tested positive for *Trichomonas vaginalis* by wet mount examination and the STDFinder assay confirmed all these 7 positive specimens. However, the assay detected an additional 40 positive specimens for this protozoon. The Kengne et al. primer set (TVK3/TVK 7) confirmed all positive and negative STDFinder results, but only 19 of the positive specimens were found positive by the Shaio et al. primer set.

For 5 HSV-2-positive and 227 HSV-2-negative specimens, concordant results were obtained by STDFinder assay and real-time PCR assay. The STDFinder assay detected an additional 9 positive specimens. A second real-time PCR was performed on the 9 discrepant results and only 1 specimen could be confirmed as being positive for HSV-2. Neither the STDFinder assay nor the real-time PCR found any specimen positive for HSV-1.

After the discrepant results were resolved, the prevalence rates of *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* remained unchanged, whereas those of *Trichomonas vaginalis* and HSV-2 were 10.7% and 2.9%, respectively. Multiple pathogens were detected in 7 specimens (3 pathogens in 1 specimen and 2 in 6 specimens). The overall sensitivity and specificity of the STDFinder assay for the detection of *C. trachomatis* and *N. gonorrhoeae* were determined to be 100%. The STDFinder assay was both 100% sensitive and specific for the detection of *M. genitalium*. For *Trichomonas vaginalis*, the sensitivity of the STDFinder assay was 100% and the specificity was 90.2%. For HSV-2, the sensitivity and specificity were, respectively, 100% and 96.1% (Table 3).

To estimate the diagnostic sensitivity and specificity of the evaluated STDFinder assay, an additional 80 samples

Table 3
Sensitivities and specificities of STDFinder assay in relation to the study's expanded gold standard (i.e., positive by 2 PCRs)

STDFinder assay	No. of results with the following results (N = 242)[a]				Sensitivity (%)	Specificity (%)
	True positive	False positive	True negative	False negative		
C. trachomatis	7	0	235	0	100	100
N. gonorrhoeae	10	0	232	0	100	100
M. genitalium	3	0	239	0	100	100
Trichomonas vaginalis	26	21	195	0	100	90.2
HSV-2	6	9	227	0	100	96.1

[a] No target pathogens were detected in 190 of 242; 1 pathogen in 37; 2 pathogens in 6; and 3 pathogens in 1 specimen (total 52).

previously tested by Abbott Real-Time CT/NG PCR were analyzed by the STDFinder assay. These specimens, selected from the same population of women, were enriched in positive samples for *C. trachomatis* and *N. gonorrhoeae* (31 positive samples by Abbott Real-Time CT/NG PCR). These 31 positive samples (11 *C. trachomatis*, 17 *N. gonorrhoeae*, and 3 mixed infections) were correctly detected positive with the STDFinder assay and all 49 specimens that were negative by Abbott Real-Time CT/NG PCR for the 2 organisms were also found negative by the STDFinder assay (Table 4). Thus, the STDFinder assay exhibited a high sensitivity and an excellent specificity when using the Abbott m2000 PCR assay test as the gold standard, with 100% agreement on the 80 specimens retrospectively studied.

4. Discussion

The current study describes the evaluation of a new multiplex PCR assay for the simultaneous detection in clinical specimens of 7 potential STD pathogens including *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *Trichomonas vaginalis*, *Treponema pallidum*, HSV-1 and HSV-2 which are difficult to identify by other methods. Laboratory diagnosis of most STD pathogens based on culture, for example, is costly because it requires stringent specimen collection and special media suitable for a fastidious organism. The design of the STDFinder assay evaluated in this study is methodologically based on those previously described for the detection of viruses in a routine diagnostic laboratory utilizing identical reagent and cycling conditions (Reijans et al., 2008; Wolffs et al., 2009). The assay uses the advantage of multiplex PCR in which more than 1 set of primers leads to the amplification of several target sequences under a single set of reaction conditions (Markoulatos et al., 2002). Clinical specimens from patients with STDs (including symptomatic and asymptomatic cases) frequently contain multiple pathogens (Stellrecht et al., 2004). Therefore, assays that detect multiple pathogens in a single assay with sufficient sensitivity to detect low levels of a pathogen and a high specificity have considerable potential to diagnose STDs accurately and rapidly. The analytical sensitivity of this assay is very good with the detection limits of DNA corresponding to less than 20 copies/MLPA assay for most of the 7 organisms. Moreover, this assay demonstrated excellent analytical specificity. The multiplex PCR assay did not cross-react with either other closely related target species or with a variety of other common urogenital organisms.

We further evaluated the sensitivity and specificity of the STDFinder assay using clinical specimens from infertile women seen at an infertility research clinic, and we compared the results with previously described species-specific PCRs. The performance, in terms of sensitivity and specificity, of the STDFinder was very good since there was an almost perfect agreement with the comparative tests.

The STDFinder assay, when tested on 242 vaginal specimens, correctly detected 7 of 7 *C. trachomatis*-positive, 10 of 10 *N. gonorrhoeae*-positive, and 3 of 3 *M. genitalium*-positive samples An agreement of 100% was found when comparing the STDFinder method for the detection of *C. trachomatis* and *N. gonorrhoeae* and the Abbott Real-Time CT/NG PCR test using an additional 80 vaginal specimens (with retrospectively known results). The performance of the STDFinder assay for *C. trachomatis* and *N. gonorrhoeae* was comparable to, or higher than, that reported for other molecular techniques in both vaginal and urine specimens (Gaydos et al., 2003; Levett et al., 2008; Lowe et al., 2006). Good sensitivity for genital *Mycoplasma* detection with the STDFinder assay is consistent with the literature (Diaz et al., 2010; Luki et al., 1998).

The STDFinder assay is highly sensitive for the detection of *Trichomonas vaginalis*. All positive specimens by wet mount examination were detected by the STDFinder assay. Furthermore, all the additional specimens found positive (40/47) by the STDFinder assay but not detected by the wet mount examination were confirmed positive by the Kengne et al. (TVK3/TVK7) primer set. However, the Shaio et al. (IP1/IP2) primer set could only confirm half of the discrepant

Table 4
Performance of STDFinder assay on 80 specimens previously analyzed by the Abbott m2000 system

	Abbott m2000 assay	STDFinder assay
C. trachomatis	11	11
N. gonorrhoeae	17	17
C. trachomatis + *N. gonorrhoeae*	3	3
Negative for both organisms	49	49
Total	80	80

results (19/40), leading to a final sensitivity and specificity of 100% and 90.5%, respectively. Other investigators reported sensitivities between 59% and 100% and specificities between 94.6% and 100% of PCR assays for the detection of *Trichomonas vaginalis* in vaginal specimens (Crucitti et al., 2003; Diaz et al., 2010; Lawing et al., 2000; Mayta et al., 2000; Smith et al., 2005; van Der Schee et al., 1999). While these studies are similar in their use of nucleic acid amplification technology, the important differences in PCR primers, reference standard, and detection methods make direct comparisons between assays difficult. In a prior comparative study, the primer set used in this study (TVK3/TVK7) performed better than other previously published primer pairs for *Trichomonas vaginalis* detection in vaginal specimens (Crucitti et al., 2003). The high sensitivity of the STDFinder assay and the Kengne et al. primer set may permit the detection of small numbers of *Trichomonas vaginalis* organisms, which could be missed on wet mount examination. Moreover, the STDFinder assay could possibly detect a larger number of nonviable organisms. Nonviable organisms could be present in specimens from women previously treated. In such a case, the STDFinder result would be analytically positive, but clinically false positive (Burkardt, 2000). However, as we do not have clinical information on specimens included in our study, this scenario cannot be confirmed.

The sensitivity of the STDFinder assay for the detection of HSV-2 is 100%, a value comparable to the sensitivity of other real-time PCR assays evaluated on specimens other than vaginal (Kawada et al., 2004). However, in resolving the STDFinder positive/real-time negative specimens, a second real-time PCR confirmed only 1 specimen (1/10) as truly positive, resulting in a final specificity of 96.1%. It is possible that the confirmatory PCR used to resolve the discordant results in this study was not as sensitive as the STDFinder assay and that the unconfirmed positive results were also actually true positives. To resolve this, further studies using genetic typing of the virus for discrepancy resolution will be needed. HSV-1 was neither detected by STDFinder assay nor by the alternative real-time PCR used in this evaluation; both assays are comparable in terms of their specificities for the detection of HSV-1. Consistent with our observation, previous studies have suggested that most genital HSV infections are caused by HSV-2 (Duran, 2003; Suntoke et al., 2009; Tang, 1993). Similarly, the sensitivity for *Treponema pallidum* could not be calculated due to the absence of any *Treponema pallidum*-positive samples. For the diagnosis of syphilis infection, dark field and fluorescent microscopy are the most commonly used methods for the direct detection of treponemes in material from lesions during the very early stage of the infection. However, since the organism is not easily accessible in the early stage of infection, serologic tests are still the main and essential tools in the diagnosis of syphilis (Sam Ratnam, 2005).

Although some species targeted in this study were found infrequently, the study was not designed to confirm the roles of these organisms in lower genital tract infections, but to develop a tool for laboratory diagnostic use.

The evaluation of any new testing method requires a careful selection of the reference test. Although culture is generally considered to be the gold standard for detecting pathogens in specimens, previous reports (Cosentino et al., 2003; Shattock et al., 1998; Stellrecht et al., 2004) have shown that nucleic acid amplification tests have a higher detection rate for sexually transmitted pathogens such as *C. trachomatis*, *N. gonorrhoeae*, and *Mycoplasmas* in clinical specimens compared to culture. In this study, previously described PCR assays were used as reference methods because of their high sensitivity and specificity. Thus, the STDFinder assay and the comparator PCR assays were different in their target genes. Amplification of an IAC is included to avoid false-negative results in each individual sample, due to handling error, inadequate extract, or the presence of PCR inhibitors. Analysis of the specimens investigated here shows that the IAC works well in practice. Weak or no amplification of the IAC in a few of the specimens indicated the presence of PCR inhibitors; these were reanalyzed to validate negative test results. Of particular observation, the resolution of this system was not fully sufficient on agarose gel for the identification of all individual products amplified by the STDFinder assay. On high-resolution electrophoresis systems such as the MegaBACE DNA analysis system, all individual probes could be clearly distinguished. Another advantage of the multiplex PCR, as demonstrated in this study, is the ability to detect co-infections, although the overall improvement in positive rate was not substantial due to the relatively few numbers of co-infection in our study population.

In conclusion, we have described and evaluated the clinical performance of a new multiparameter assay for the simultaneous detection of 7 clinically relevant pathogens of STD. The results show that the STDFinder assay has comparable clinical sensitivity to the conventional single and duplex real-time PCR assay and is suitable for the routine detection of a broad spectrum of these STDs at relatively low cost due to multiplexing.

Further testing of a larger population, or a population with a higher incidence of certain STDs, and using different specimens would be required to achieve the power necessary to adequately assess this assay.

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