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Evaluation of Multiplex Polymerase Chain Reaction Assay for the Simultaneous Detection of Sexually Transmitted Infections Using Swab Specimen

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Sexually transmitted infections (STIs) are caused by the spread of pathogens via sexual activity and can cause serious complications if left untreated, regardless of their symptoms. Therefore, early diagnosis of STI is important, and molecular diagnostic methods for rapid detection and monitoring are needed. In this study, we evaluated a multiplex polymerase chain reaction (PCR) kit for simultaneously detecting 13 different bacterial, fungal, and viral microorganisms that cause STIs. The kit performance was evaluated for its sensitivity, lot-to-lot variation, and interference in detecting different pathogens. Additionally, its clinical usefulness was evaluated by estimating its sensitivity and specificity for clinical samples. The limit of detection (LOD) was 0.021-50.104 copies for each pathogen. In the tests of lot-to-lot, 100% of positive samples were detected at low concentrations and negative samples all showed negative results. This result confirms that there is no the variation of lot-to-lot. In the test for interference between pathogens, the efficiency of amplification for each pathogen was not significantly reduced and no nonspecific amplification product was formed. We tested 322 vaginal swab samples using the multiplex PCR kit and confirmed that its clinical sensitivity and specificity were 100% for all pathogens. This multiplex PCR kit can be used widely for rapid diagnosis and monitoring of STIs.

Key Words: sexually transmitted infection, multiplex PCR, evaluation

INTRODUCTION

Sexually transmitted infections (STIs), also known as sexually transmitted diseases (STDs) or venereal diseases, are spread by sexual activity (1). They are one of the most common types of infectious disease and can be caused by bacteria, fungi, viruses, and parasites. Causes of STIs include the bacteria *Chlamydia trachomatis* (CT), *Gardnerella vaginalis* (GV), *Haemophilus ducreyi* (HD), *Mycoplasma genitalium* (MG), *Mycoplasma hominis* (MH), *Neisseria gonorrhoeae* (NG), *Treponema pallidum* (TP), *Ureaplasma parvum* (UP) and *Ureaplasma urealyticum* (UU), the fungus *Candida albicans* (CA) (2-8), the viruses herpes simplex-1 (HSV-1) and herpes simplex-2 (HSV-2), and the parasite *Trichomonas vaginalis* (TV) (6, 8-10).

The World Health Organization estimates that one million new STIs occur per day. In Korea, 24,526 cases of STI were reported in 2016, including 8,438 cases of CT infection, 3,615 cases of NG infection, and 1,569 cases of TP infection, which is an increase of 32.9% compared to the number in 2015 (11, 12). These diseases are rarely cured without treatment. Although 70-80% of STIs are asymptomatic, they can be dangerous. However, most STIs are easy to treat if they are diagnosed early (12).

The pathogens that cause these STIs are very diverse and often cause complex infections. Although microbial culture is typically performed to identify the pathogens, this technique can take a long time and each pathogen requires complex and different incubation conditions (11). Contamination can also occur during culture (12). By comparison, molecular diagnostics using polymerase chain reaction (PCR), which tests directly for the presence of microorganism DNA, can give results for single or multiple infections within a short time. This technique is now widely used in diagnostic laboratories. Additionally, the use of multiplex PCR, which can detect multiple target DNAs, is increasing (13, 14).

In this study, we evaluated the analytical and clinical usefulness of a commercially available multiplex PCR kit for detecting 13 different microorganisms that can cause STIs without the need for culture. Analytic performance was validated by measuring sensitivity, lot-to-lot variation, and competitive interference. Sensitivity was determined by serially diluting the DNA standards of 13 pathogens before performing PCR. The kit identified the lowest amount of the analyte with a 95% positivity rate. The variation of lot-to-lot was verified using three different lots of the same reagents, and all were detected within the range of concentrations prepared by diluting the reference material. We also confirmed the lack of interference between pathogens with a high prevalence of coinfection. The clinical performance of the kit was then evaluated using clinical samples. Seven pathogens that were not included in control kit were used as a control and were detected by sequencing.

MATERIALS AND METHODS

Clinical samples

From July to September 2016, swab specimens were collected from 322 patients suspected as having an STI. These samples were collected by Genetree Research, Inc. from local hospitals. DNA was purified from all swab specimens and deidentified samples were transferred to The Catholic University of Korea. Swab specimens were stored at 4°C prior to analysis. All specimens were tested with the E kit and several samples were subjected to sequencing. The study was approved by the Institutional Review Board Committee for Ethics and Research of the Catholic University of Korea (1040395-201809-17).

DNA extraction methods

The swab specimens in the collection tubes were equilibrated to room temperature and mixed by vortexing. The caps from the specimen tubes were removed carefully to avoid contamination and 1-mL specimens were transferred into 1.5-mL microtubes and centrifuged at 1,500x*g* for 15 min, after which the supernatant was discarded. We then resuspended the pellet in 1 mL of phosphate-buffered saline by vortexing. DNA was extracted from the pretreated specimens using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Total DNA was stored at -80°C until analysis. The quality of extracted DNA was measured with a spectrophotometer (BioTek, Winooski, VT, USA) to confirm DNA purity and yield.

Multiplex PCR assay

We evaluated the E kit (Genetree Research, Seoul, Korea) using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Multiplex PCR distinguishes pathogens based on the sizes of the amplified bands. The kit contains Primer Set 1, which can detect TV, UU, MH, MG, NG, and CT pathogens, and Primer Set 2, which can detect HD, HSV-2, GV, HSV-1, TP, CA, and UP pathogens. The target genes amplified from the 13 pathogens are listed in Table 1. The kit also includes internal controls to confirm that clinical specimens are successfully amplified and detected through electrophoresis analysis (820 base pairs). Internal control used B-actin in the human gene. As a negative control, distilled water was used rather than DNA as the PCR template. To verify the integrity of the primers used in the PCR assay, positive DNA controls from the kit were evaluated in the presence of all primer pairs.

Table 1. Pathogen-specific regions amplified

Organism (Accession)	Target Gene	Amplicon Size (bp)	position
CA (MN743895)	internal transcribed spacer	187	40-226
CT (MG733347)	major outer membrane protein	117	5-121
GV (JQ354967)	CPN60	407	4-410
HD (AY603047)	hemoglobin receptor precursor	670	1685-2354
HSV-1 (EF177455)	glycoprotein B (UL27)	330	1279-1608
HSV-2 (KF588415)	glycoprotein B (UL27)	570	1042-1611
MG (KP318824)	adhesion (mgpB)	248	231-478
MH (KU726667)	16S rRNA	335	91-425
NG (AJ010733)	porA pseudogene	175	657-731
TP (M88769)	47-kilodalton antigen gene	252	561-812
TV (L23861)	G7 hypothetical protein	690	349-1038
UP (AF085733)	UreB	119	632-750
UU (AF085721)	UreA	431	302-732

Abbreviations: CA, *Candida albicans*; CT, *Chlamydia trachomatis*; GV, *Gardnerella vaginalis*; HD, *Haemophilus ducreyi*; HSV-1, Herpes simplex 1; HSV-2, Herpes simplex 2; MG, *Mycoplasma genitalium*; MH, *Mycoplasma hominis*; NG, *Neisseria gonorrhoeae*; TP, *Treponema pallidum*; TV, *Trichomonas vaginalis*; UP, *Ureaplasma parvum*; UU, *Ureaplasma urealyticum*.

PCR amplification was performed as described in the kit manual. The PCR conditions were as follows: 5 min at 50°C and 10 min at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 62°C for 1 min 20 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified PCR products were electrophoresed in 2.2% (w/v) agarose gels and stained with ethidium bromide. The band size of each amplification product was checked against the marker DNA (positive DNA) supplied with the kit (Fig. 1).

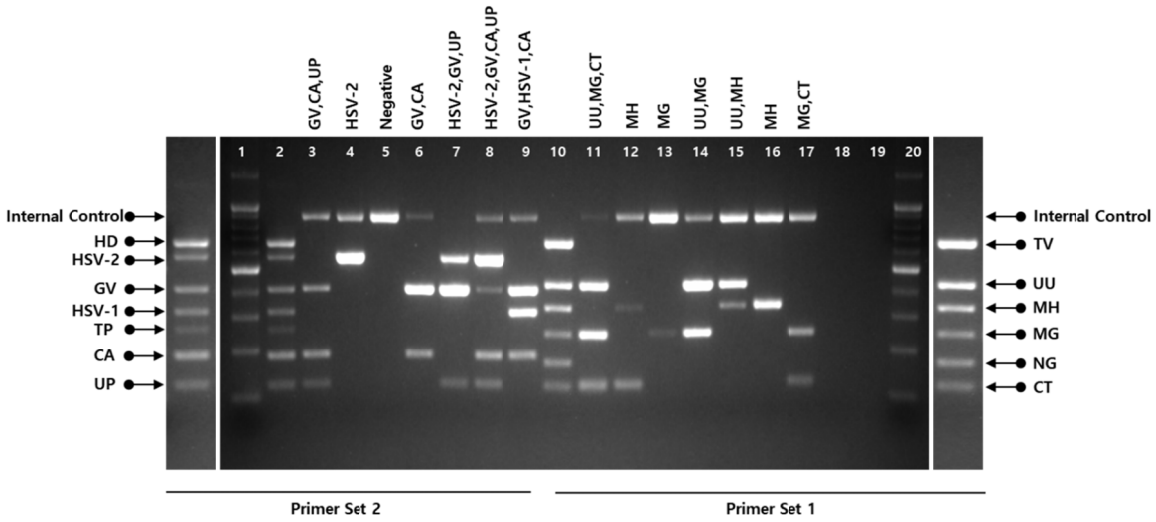

Fig. 1. DNA amplification by multiplex PCR (E kit) in clinical specimens

The band size of each amplification product was checked against the positive control. Lanes #1 and 20: Ladder to distinguish amplification bands. Lane #2: positive control for 7 pathogens amplified by Primer Set 2, Lane #10: positive control for 6 pathogens amplified by Primer Set 1, Lanes #3-9 and #11-17: PCR results from clinical samples, Lanes #18 and 19: negative control (distilled water).

Abbreviations: HD, *Haemophilus ducreyi*; HSV-2, Herpes simplex 2; GV, *Gardnerella vaginalis*; HSV-1, Herpes simplex 1; TP, *Treponema pallidum*; CA, *Candida albicans*; UP, *Ureaplasma parvum*; TV, *Trichomonas vaginalis*; UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; NG, *Neisseria gonorrhoeae*; CT, *Chlamydia trachomatis*.

Wise STD 6 detection Kit

The Wise STD 6 detection kit (Wisemeditec, Anyang, Korea) as control consists only of Primer Set 1 (unrevealed), which can detect TV, UU, MH, MG, NG and CT pathogens. Amplification reactions were performed in a volume of 16 µL consisting of 8 µL STD6 mixture, 2 µL of primer mix, 2 µL of Internal control primer mix, 4 µL of DNA template. The PCR conditions were as follows: 5 min at 50˚C and 10 min at 94˚C, followed by 40 cycles of denaturation at 94˚C for 20 s, annealing at 62˚C for 1 min 20 s, and extension at 72˚C for 1 min, and a final extension at 72 ˚C for 5 min. The amplified PCR products were electrophoresed in 2.2% (w/v) agarose gels and stained with ethidium bromide.

Sequencing

Amplicons were purified using an exonuclease/shrimp alkaline phosphatase mixture and sequenced using ABI Prism BigDye Terminator version 3.1 (Applied Biosystems) with both forward and reverse sequence-specific primers. Twenty nanograms of purified PCR products were used in a 10-µL sequencing reaction solution containing 1 µL of BigDye Terminator v3.1 and 0.1 µM of the same PCR primer. Sequencing reactions were performed as follows: 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Sequence data were generated using the ABI Prism 3730 DNA Analyzer (Applied Biosystems). Sequences were analyzed using the Basic local alignment search tool (BLAST) from The National Center for Biotechnology Information.

Statistical analyses

The limit of detection (LOD) was calculated using 95% probit analysis and IBS SPSS Statistics (v. 20; SPSS, Inc., Chicago, IL, USA), and clinical data were calculated using MedCalc software version 14.8.1 (MedCalc software, Ostend, Belgium).

RESULTS

Determination of sensitivity

To verify the sensitivity of the E kit, we performed sensitivity testing using 24 replicates with four concentrations of kit standards at 0.01-1,000 copies per pathogen. Viral DNA (Vircell S.L., Granada, Spain) or cloned compounds were used in a tenth serial dilutions with TE buffer to determine sensitivity. See Table 2 for diluent concentrations for each pathogen. The sensitivities determined from the 95% confidence level in the probit analysis using repeated measures data were 0.021-50.104 copies for each pathogen. The sensitivities of the E kit were estimated as 1.212 copies for CT, 1.708 copies for NG, 4.3 copies for MG, 2.636 copies for MH, 33.577 copies for UU, 0.021 copies for TV, 3.071 copies for UP, 4.918 copies for CA, 2.636 copies for TP, 17.077 copies for HSV-1, 4.918 copies for GV, 16.231 copies for HSV-2, and 50.104 copies for HD (Table 2).

Table 2. Limit of detection of E kit multiplex PCR

Pathogen	DNA Concentrations (copies/μL)	LOD
CA	0.01-10	4.918
CT	0.01-10	1.212
GV	0.01-10	4.918
HD	1-1000	50.104
HSV-1	0.1-100	17.077
HSV-2	0.1-100	16.231
MG	0.1-100	4.3
MH	0.1-100	2.636
NG	0.01-10	1.708
TP	0.1-100	2.636
TV	0.001-1	0.021
UP	0.1-100	3.071
UU	0.1-100	33.577

Abbreviations: LOD, limit of detection.

Determination of lot-to-lot variation

Three different lots of the same reagent showed a 100% detection rate for both medium (1,000 × LOD) and low (10 × LOD) concentration reference materials over 20 iterations (Table 3). No visible changes in band strength were observed. All negative samples (distilled water) produced negative results. These results confirm the within-run, between-run, and lot variation of the assay for all positive samples.

Table 3. Lot-to-lot variation for each pathogen

Pathogen	Concentration	No. of Positive reaction		
		Lot 1	Lot 2	Lot 3
CA	10 × LOD	20	20	20
	1000 × LOD	20	20	20
CT	10 × LOD	20	20	20
	1000 × LOD	20	20	20
GV	10 × LOD	20	20	20
	1000 × LOD	20	20	20
HD	10 × LOD	20	20	20
	1000 × LOD	20	20	20
HSV-1	10 × LOD	20	20	20
	1000 × LOD	20	20	20
HSV-2	10 × LOD	20	20	20
	1000 × LOD	20	20	20
MG	10 × LOD	20	20	20
	1000 × LOD	20	20	20
MH	10 × LOD	20	20	20
	1000 × LOD	20	20	20
NG	10 × LOD	20	20	20
	1000 × LOD	20	20	20
TP	10 × LOD	20	20	20
	1000 × LOD	20	20	20
TV	10 × LOD	20	20	20
	1000 × LOD	20	20	20
UP	10 × LOD	20	20	20
	1000 × LOD	20	20	20
UU	10 × LOD	20	20	20
	1000 × LOD	20	20	20
Distilled water		0	0	0

Abbreviations: LOD, limit of detection.

Determination of interference

The interference test was conducted for cases of NG and CT co-infections as well as cases of HSV-1 and HSV-2 co-infections. The competition interference test was conducted by defining each concentration as 10 × LOD, 100 × LOD, and 1,000 × LOD. The interference assays confirmed that the pathogens could be detected at all concentrations without a loss in sensitivity (Table 4).

Table 4. Test for interference between CT and NG, HSV-1 and HSV-2

Bacterial species (concentration)	NG (10 × LOD)	NG (100 × LOD)	NG (1000 × LOD)
CT (10 × LOD)	NG-positive	NG-positive	NG-positive
	CT-positive	CT-positive	CT-weakly positive
CT (100 × LOD)	NG-positive	NG-positive	NG-positive
	CT-positive	CT-positive	CT-positive
CT (1000 × LOD)	NG-weakly positive	NG-positive	NG-positive
	CT-positive	CT-positive	CT-positive
virus species (concentration)	**HSV-1 (10 × LOD)**	**HSV-1 (100 × LOD)**	**HSV-1 (1000 × LOD)**
HSV-2 (10 × LOD)	HSV-1-positive	HSV-1-positive	HSV-1-positive
	HSV-2-positive	HSV-2-positive	HSV-2-positive
HSV-2 (100 × LOD)	HSV-1-positive	HSV-1-positive	HSV-1-positive
	HSV-2-positive	HSV-2-positive	HSV-2-positive
HSV-2 (1000 × LOD)	HSV-1-positive	HSV-1-positive	HSV-1-positive
	HSV-2-positive	HSV-2-positive	HSV-2-positive

Evaluation of the method using clinical samples

We used 322 human vaginal swab samples to evaluate the accuracy of the E kit (test) compared to the Wise STD 6 detection kit (control). As described above, the E kit can detect 13 pathogens (TV, UU, MH, MG, NG, CT, HD, HSV-2, GV, HSV-1, TP, CA, and UP), whereas the Wise STD 6 detection kit can detect 6 pathogens (NG, CT, TV, MG, MH, and UU). Therefore, the presence of the 7 microorganisms (HSV-1, HSV-2, GV, TP, CA, HD, and UP) detected by the E kit but not by the Wise STD 6 detection kit was confirmed by sequencing. Additionally, if the results of the two kits were discordant, sequencing was conducted to verify the clinical sensitivity and specificity. All results from the E kit showed that clinical sensitivity and specificity were 100% (Table 5). However, 4 samples showed different results in PCR with the Wise STD 6 kit as control kit and E kit as experimental kit. These results were confirmed by sequencing. The BLAST match rate was consistently >90%.

Table 5. Sensitivities and specificities of E kit in 322 swab specimens

Control PCR & sequencing	Results		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%)	NPV (%)
Organism	Positive	Negative				
CA	44	278	100 (96.61-100)	100 (99.36-100)	100	100
CT	38	284	100.0 (95.01-100)	100.0 (99.39-100)	100	100
GV	138	184	100 (98.87-100)	100 (99.00-100)	100	100
HD	0	322	NA	NA	100	100
HSV-1	17	305	100 (94.31-100)	100 (99.40-100)	100	100
HSV-2	24	298	100 (94.22-100)	100 (99.40-100)	100	100
MH	65	257	100 (97.75-100)	100 (99.29-100)	100	100
MG	33	289	100 (94.72-100)	100 (99.40-100)	100	100
NG	28	294	100.0 (94.40-100)	100.0 (99.40-100)	100	100
TP	1	321	100 (93.02-100)	100 (99.41-100)	100	100
TV	16	306	100 (94.22-100)	100 (99.40-100)	100	100
UP	74	248	100 (98.68-100)	100 (99.09-100)	100	100
UU	60	262	100 (96.79-100)	100 (99.35-100)	100	100

Abbreviations: PCR, polymerase chain reaction; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

DISCUSSION

The standard methods for diagnosing STIs are culture and serologic tests. However, for TP, samples showing positive results following culture were reported as negative in serologic testing, and antibody detection was found to be less sensitive than the culture method (15, 16). Although the most appropriate test for viruses such as HSV-2 is cell culture, this assay shows low sensitivity (17). HD is also difficult to cultivate experimentally (18, 19). PCR methods that are rapid and highly sensitive are used when low-sensitivity test methods and culture are not possible (20, 21). Therefore, PCR can improve the ability to diagnose infectious diseases (16, 20-22, 23). It has been suggested that diagnosis methods involving amplification of nucleic acid are 20-30% more sensitive than other methods (24, 25). Additionally, multiplex PCR allows for the rapid identification of several pathogens in the same specimen. Because STIs have a high incidence of

coinfections, multiplex PCR rather than single PCR is suitable for their diagnosis and treatment: its ability to detect multiple pathogens simultaneously is a major advantage (13, 14). Therefore, several multiplex diagnostic kits have been developed for detecting STI pathogens.

In this study, we evaluated the E kit, which can detect 13 STI pathogens simultaneously. The usefulness of molecular diagnostics is often evaluated by analytical evaluation and clinical performance testing. The analytical and clinical performance for E kit were assessed to determine whether it is appropriate for STI diagnosis. The analytical sensitivity (LOD) is the lowest measurable concentration of analyte that gives reliable results; the sensitivity of this assay ranged from 0.021 to 50.104 DNA copies for each pathogen. The within-run, lot-to-lot assay of the kit were stable and excellent.

For combinations of pathogens with a high prevalence of coinfection (NG and CT, HSV-1 and HSV-2), we confirmed that there was no interference in the assay by varying their concentrations. We also evaluated the clinical performance of the E kit in 322 clinical samples. Four discrepancies were observed between the results of the test (E kit) and control (Wise STD 6 kit). However, sequencing confirmed the results of the test kit. Therefore, we confirmed that the E kit has high sensitivity and specificity for 13 pathogens.

Early diagnosis may reduce the complications or sequelae of STIs and reduce the risk of transmission, and thus this multiplex PCR test for STI is expected to be very useful in the clinical field. Although the clinical performance of the kit for HD could not be confirmed because no positive specimens were isolated during the collection period, our future analysis will apply the test to patients with suspected HD infection. In addition, this study used only one commercial kit for comparison. Therefore, additional study is needed to establish the clinical efficacy of E kit.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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