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# Rapid One Step Detection of Pathogenic Bacteria in Urine with Sexually Transmitted Disease (STD) and Prostatitis Patient by Multiplex PCR Assay (mPCR)

**Sang Rok Lee, Ji Min Chung, and Young Gon Kim***

*Department of Biology, College of Natural Sciences, Chosun University, Gwangju 501-759, Republic of Korea*

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**We developed a multiplex PCR (mPCR) assay to simultaneously detect *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Ureaplasma urealyticum, Corynebacterium* spp. and *seudomona aeruginosa.* This method employs a single tube and multiple specific primers which yield 200, 281, 346, 423, 542, and 1,427 bp PCR products, respectively. All the PCR products were easily detected by agarose gel electrophoresis and were sequenced to confirm the specificity of the reactions. To test this method, DNA extracted from urine samples was collected from 96 sexually transmitted disease or prostatitis patients at a local hospital clinical center, and were subjected to the mPCR assay. The resulting amplicons were cloned and sequenced to exactly match the sequences of known pathogenic isolates. *N. gonorrhoeae* and *Corynebacterium* spp. were the most frequently observed pathogens found in the STDs and prostatitis patients, respectively. Unexpectedly, *P. aeruginosa* was also detected in some of the STD and prostatitis samples. More than one pathogen species was found in 10% and 80.7% of STD and prostatitis samples, respectively, indicating that STD and prostatitis patients may have other undiagnosed and associates. The sensitivity of the assay was determined by sing purified DNA from six pathogenic laboratory strains and revealed that this technique could detect pathogenic DNA at concentrations ranging from 0.018 to 1.899 pg/μl. Moreover, the specificities of this assay were found to be highly efficient. Thus, this mPCR assay may be useful for the rapid diagnosis of causative infectious STDs and prostatitis. useful for the infectious STDs and prostatitis.**

*Keywords*: STDs, prostatitis, Multiplex PCR, *Chlamydomonas, Neisseria, Mycoplasma, Ureaplasma, Pseudomonas, Corynebacterium*

An estimated 78 to 330 million new cases of genitourinary tract infections due to *Chlamydia trachomatis, Neisseria gonorrhoeae, Ureaplasma urealyiticum* and *Mycoplasma genitalium* are diagnosed each year worldwide. Of these, 5 to 12 million are diagnosed in North America alone (De Schryver *et al.*, 1990; Mahony *et al.*, 1995; World health Organization, 1995; Pepin *et al.*, 2001). Controlling sexually transmitted diseases (STDs) is desirable because it can substantially reduce the transmission of the human immunodeficiency virus (HIV), as well as reducing extensive morbidity in sexually active individuals worldwide. For example, gonococcal infection of the eye is a serious ophthalmic problem that requires urgent medical attention, and while the painful condition cystitis is usually caused by *Escherichia coli*, it can also be caused by chlamydial or gonorrheal infections. In addition, *M. pneumoniae* is responsible for nervous system lesions (Coelho *et al.*, 2004).

Chronic prostatitis is a particularly common disorder in adult men of all ages, and 50% of all men re treated for it at least once in their lives (National Center for Health Statistics, 1993). *Pseudomonas aeruginosa* has been suggested to be frequently responsible for these prostatic and urinary tract infections (Dereviako *et al.*, 2002; Liu *et al.*, 2003). *Corynebacterium* spp. are also found in the prostate fluid of prostatitis patients (Turk *et al.*, 2007). Thus, the easy detection of *P. aeruginosa* and *Corynebacterium* infections may assist in the diagnosis and rapid treatment of bacterial prostatitis. Definitive diagnosis of STD-causing organisms and *P. aeruginosa* and *Corynebacterium* spp. Continue to rely on bacterial cell culture methods. However, attempts are being made to develop less laborious and time-consuming diagnostic methods. For example, DNA hybridization and nucleic acid amplification have been used to detect *N. gonorrhoeae* (Cano *et al.*, 1992). More recently, PCR methods have been developed to detect this and other STD-causing organisms (Jaschek *et al.*, 1993; Crucitti *et al.*, 2003; Chen *et al.*, 2007a; Simpson *et al.*, 2007). One of these methods is mPCR, which is a single step method that employs one tube and permits the simultaneous detection of more than one organism. This method has been used to detect multiple viruses in clinical specimens (Wang *et al.*, 2004; Marshall *et al.* 2007), bacterial STDs (Chen *et al.*, 2007b), prostatitis (Zhou *et al.*, 2003) as well as for the individuals afflicted with cystic fibrosis (Xu *et al.*, 2004).

Such molecular diagnostic tools are set to revolutionize the management of STDs (Morris, 1999). Furthermore, mPCR is a particularly powerful and useful method for diagnosing STDs because it is a one-step procedure that could detect a wide range of organisms. At present, mPCR has been used to simultaneously detect *C. trachomatis, N. gonorrhoeae,*

* To whom correspondence should be addressed.
(Tel) 82-62-230-6656; (Fax) 82-62-234-4326
(E-mail) ygnkim@mail.chosun.ac.kr

*U. urealyiticum* and *M. genitalium* (Mahony *et al.*, 1997). I addition, mPCR was also used to concurrently detect *P. aeruginosa* and *Corynebacterium* spp. infections in cystic fibrosis patients (Da Silva Filho, 2004; Wareham and Curtis, 2007). In this study, we report the development of an mPCR method that simultaneously detects the four STD-causing organisms, *C. trachomatis, N. gonorrhoeae, U. urealyticum,* and *M. genitalium* and the two prostatitis-causing organisms, *P. aeruginosa* and *Corynebacterium* spp. in a one step PCR. We show that our mPCR method is more sensitive than the traditional methodwhich have been used to detect these organisms, including culture, enzyme immunoassay and direct fluorescent antibody staning methods. We believe that this mPCR assay will be a more time- and cost-efficient diagnostic tool than the other current employed techniques.

## Materials and Methods

***Bacterial strains, growth conditions and DNA extraction***

Pure cultures of submitted strains from *C. trachomatis, N. gonorrhoeae, U. urealyiticum, M. genitalium, Corynebacterium* spp. and *P. aeruginosa* were maintained on Luria agar slants at 4°C for working purposes, while stock cultures were stored in 15% glycerol at -80°C. Bacterial cell genomic DNA was extracted as described previously (Orle *et al.*, 1996; Mahony *et al.*, 1997; Kong *et al.*, 2000). Briefly, 10 ml aliquots of cells were centrifuged at 12,000×g for 3 min at 4°C and subjected to DNA extraction via the method described in Pitcher *et al.* (1989). The DNA was then purified by the Ultraclean™ Microbial DNA kit (Mo Bio, USA) according to the manufacturer's instructions. Next, purified DNA was recovered in 200 μl of elution buffer followed by dilution in 1 ml of phosphate-buffered saline (PBS) containing 1 mg/ml chenodeoxycholate (Sigma, USA) and stored at -80°C until further use. Upon thawing, the specimens were divided into aliquots for assessment and comparison to cloned markers and a 3.0 kb pGEM-T easy vector (Promega, USA) to ensure the DNA had originated from the correct bacterial species.

***Preparation of samples from patients***

First-void urine (FVU) specimens (20 ml) were obtained from a total of 96 patients (70 STD patients and 26 putative prostatitis patients) in addition to three normal healthy men at the local Gwangju hospital clinical center. The FVU urine specimens were collected into sterile 50 ml conical tubes on-site and briefly vortexed (Ho *et al.*, 1992). After centrifugation at 3,000 rpm for 20 min, the urine pellets were washed in 5 ml of sterile water and then centrifuged again at 2,000 rpm for 10 min. The DNA in the pellet was then extracted by three rounds of resuspension in 30% polyethylene glycol (PEG 8,000, Sigma) and 3 mM NaCl (Madico *et al.*, 1998), followed by incubation on ice for 30 min, and centrifugation at room temperature for 5 min at 15,000 rpm. The final pellet was frozen at -20°C.

To confirm the pathogenic bacterial infection, an initial 16S rDNA consensus primer from the causative bacteria in all specimens were investigated via PCR and automated sequencing analysis. Biefly, DNA sequencing of amplified products was performed by the GenoTech Corp. (Korea), which used individual synthetic primers on an Applied Biosystems 3730 xi DNA analyzer according to the manufacturer's protocol. The resulting sequences were compared with reported sequences in the NIH-GeneBank database using the BLAST search engine (http://ncbi.nlm.nhi.gov/BLAST).

***Uniplex PCR and mPCR reaction assays***

The specific primers used to amplify the six target organisms were synthesized by Geno Tech Corp (Korea) (Table 1) and are described as follows: 1) the CT primer amplifies a 200 bp fragment from the *orf 8* gene of *C. trachomatis*, 2) the NG primer amplifies a 281 bp fragment from the 16S rRNA gene of *N. gonorrhoeae*, 3) the UU primer amplifies a 423 bp fragment from the *ureA-B* gene of *U. urealyticum*, 4) the MG primer amplifies a 346 bp fragment from the *mgpa* gene of *M. genitalium*, 5) the CS primer amplifies a 1,427 bp fragment from the 16S rRNA gene of *Corynebacterium* spp., and 6) the PA primer amplifies a 542 bp fragment from the *groEL* gene of *P. aeruginosa.*

Initially, to establish the conditions for the mPCR, we performed uniplex PCRs using the DNA from the laboratory bacterial strains. All amplification reactions were performed in a volume of 25 μl containing 2 μl of extracted DNA

**Table 1.** Sequence of PCR primers

| Pathogenic bacteria | Target gene | Primers | Size (bp) / Reference |
|---|---|---|---|
| *C. trachomatis* | *Orf8* | f5'-CTAGGCGTTTGTACTCCGTCA | 200 / This work |
| | | r5'-TCCTCAGGAGTTTATGCACT | |
| *N. gonorrhoeae* | 16S rRNA | f5'-ACTGCGTTCTGAACTGGGTG | 281 / This work |
| | | r5'-GGCGGTCAATTTCACGCG | |
| *U. urealyiticum* | *ureA-B* | f5'-GAAACGACGTCCATAAGCAACT | 423 / This work |
| | | r5'-GCAATCTGCTCGTGAAGTATTAC | |
| *M. genitalium* | *mgpa* | f5'-AGTTGATGAAACCTTAACCCCTTG | 346 / This work |
| | | r5'-CATTACCAGTTAAACCAAAGCCT | |
| *Corynebacterium* spp. | 16S rRNA | f5'-GAACGCTGSCGGCGTCGTGCTTAAC(S=G or C) | 1,427 / Tanner *et al.* (1999) |
| | | r5'-TTGTTACRRCTTCGTCCCAATCGCC(R=A or G) | |
| *P. aeruginosa* | *groEL* | f5'-TGAAGCTTCGTCCTCTGCAT | 542 / This work |
| | | r5'-CGAACTTGTCTTTCAGCTCGAT | |

**Table 2.** Outcome of mPCR assays from 96 FVU samples from 70 STD and 26 prostatitis syndrome patients

| Disease | Organism | No. of positive-mPCR | | | |
|---|---|---|---|---|---|
| | | Single organism | More than one organism | subtotal | % |
| STD (n=70) | *C. trachomatis* | 15 | 1 (with *N. gonorrhoeae*) | 16 | 2.9 |
| | *N. gonorrhoeae* | 20 | 3 (with *M. genitalium*) | 23 | 32.9 |
| | *M. genitalium* | 19 | 0 | 19 | 27.1 |
| | *U. urealyticium* | 9 | 3 (with *P. aeruginosa*) | 12 | 7.1 |
| Prostatitis (n=26) | *P. aeruginosa* | 1 | 0 | 1 | 3.9 |
| | | | 12 (with *Corynebacterium*) | 12 | 46.1 |
| | | | 9 (with *M. genitalium* and *Corynebacterium*) | 9 | 34.6 |
| | *Corynebacterium* | 4 | 0 | 4 | 15.4 |

%: represents the positive ratio (%) of detected pathogens compared with the total number of STD (n=70) and prostatitis (n=26) patients, respectively. The details were described in the results. In one example, 4.2% (3/70) of the STD patients (n=70) had mixed infections with *P. aeruginosa*, whereas 34.6% (9/26) of the prostatitis patients showed mixed infections with *M. genitalium*. Although other pathogens were detected in the STD and prostatitis patients, as well as normal healthy positive patients, the data were compared with reported sequences from the NIH-GeneBank database using the BLAST search engine (http://ncbi.nlm.nhi.gov/BLAST/) (not shown here).

(equivalent to 300 ng of genomic DNA), 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween 20, 2.5 mM dNTP (each), 2.5 $MgCl_2$, 2.5 U Taq polymerase, and the primer concentrations indicated Table 2. The reactions involved one cycle at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 54°C or 56°C for 45 sec, and extension at 72°C for 80 sec. Finally. The reaction was followed by one last cycle at 72°C for 5 min. The reactions were performed on a PCR system (Takara Ltd., Japan) using a microAmp base.

Next, the mPCR conditions were established by using a mixture of DNA from all six laboratory bacterial strains. The 25 μl mPCR reaction mixture consisted of 2 μl from each extracted DNA (containing 34 ng of genomic DNA), 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl; pH 8.8, 0.01% Tween 20, 3 mM $MgCl_2$, 2.5 mM dNTP (each), 3 U Taq DNA polymerase (Biron), 1 U uracil-N-glycosylase to prevent carryover contamination and 10 μM of each specific oligonucleotide primer. The reaction for the uniplex PCR was as just described except that annealing was performed at 54°C.

Once the mPCR conditions had been optimized, the 96 FVU samples were subjected to mPCR. Next, 20 samples were run in each PCR. In addition, negative controls lacking DNA and positive controls consisting 300 ng of genomic DNA from all six laboratory strains were tested. The amplified PCR products from the uniplex PCRs and mPCR were electrophoresed on 1.5% agarose gels containing ethidium bromide in buffer and were photographed with an Image Master (Pharmacia Ltd, UK). Each gel of mPCR products contained one normal control and seven randomly selected patient samples (five STD and two prostatitis).

***Determining the sensitivity and specificity of mPCR patients***

To determine the sensitivity of the PCR reaction, stock concentrations from the genomic DNA of the six laboratory specimens were 1.844, 1.757, 1.897, 1.802, and 1.987 pg/μl for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *U. urealyticum*, *P. aeruginosa* and *Corynebacterium* spp., respectively. These were then subjected to uniplex PCRs as described above along with 10, 50, and 100 fold dilutions of stock concentrations. The specificity of the mPCR assay was determined by sequence analysis of the primer products from random samples, as previously described (Werle *et al.*, 1994).

***Data analysis***

mPCR was compared to the uniplex PCR assays after resolving discordant data. The specificity as well as the positive and negative predictive values was calculated as described by Fleiss (1981).

## Results

***PCR assays***

To establish the PCR conditions which permit the amplification of sequences from *C. trachomatis, N. gonorrhoeae, U. urealyiticum, M. genitalium, Corynebacterium* spp. and *P. aeruginosa*, the laboratory strains of these organisms were cultured, followed by extracting their DNA and performing PCR assays. The primers, amplification targets and PCR product sizes are listed in Table 1 and the optimal amplification conditions were described in the materials and methods. The optimal results were obtained at an annealing temperature of 54°C or 56°C. These temperatures allowed for the amplification of the six specific bands along with reduced levels of non-specific amplification products. Fig. 1 demonstrates the results form the agarose gel electrophoresis of the mPCR products and indicate that these conditions resulted in clear bands whose different sizes facilitate their identification.

Next, the mPCR assay which aimed to simultaneously amplify the DNA from each of the six organisms was established by carrying out further optimization assays using the DNA from cultured laboratory strains. These assays revealed that the 54°C annealing temperature efficiently generated all six products. The optimal amplification conditions used for the mPCR assay are described in materials and methods. Fig. 1 shows the results of agarose gel electrophoresis in the mPCR, and indicate that each of the six bands is clearly produced.

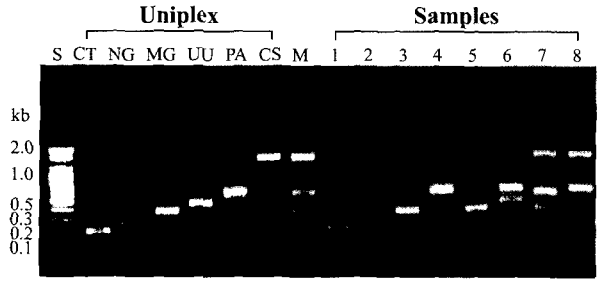


**Fig. 1.** The capacity of mPCR to co-amplify six selected bacterial species. Lanes; S, standard marker; M, mPCR; CT, *C. trachomatis*; NG, *N. gonorrhoeae*; MG, *M. genitalium*; UU, *U. urealyticum*; PA, *P. aeruginosa*; CS, *Corynebacterium* spp.; M, mPCR. In the FVU, samples, (numbers 1 and 3-6 are from male STD patients, while 7 and 8 are from men with prostatitis syndrome. Number 2 is FVU from a healthy male).

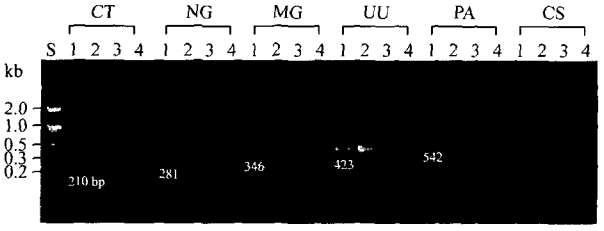


**Fig. 2.** Sensitivity of the uniplex PCR assays used to detect *C. trachomatis* (CT), *N. gonorrhoeae* (NG), *M. genitalium* (MG), *U. urealyticum* (UU), *C.* spp. (CS) and *P. aeruginosa* (PA) in STD and prostatitis patients. DNA from the laboratory strains were subjected to uniplex assays using the stock concentration (lane 1) diluted 10- (lanes 2), 50- (lanes 3) and 100-fold (lanes 4). The stock concentrations were 1.844, 1.757, 1.709, 1.897, 1.802, and 1.899 pg/μl for CT, NG, MG, UU, PA and CS. S; 100 bp DNA ladder marker. Each 25 μl PCR reaction was run for 35 cycles, and a 10 μl aliquot of the reaction was analyzed by agarose gel electrophoresis with ethidium bromide staining. This sizes (bp) of the specific PCR products are indicated on the left.

When the resulting sequences were compared with reported sequences from the NIH-GeneBank databases, all the sequences were confirmed as their target sequences (data not shown). Furthermore, the results revealed that all the patients were positive for the indicated causative bacteria for both the STD and prostatitis cases, respectively, whereas the normal healthy samples were negative. Despite this, all the patients, including normal healthy samples were positive for the presence of *Escherichia coli, nterococcus fecalis, Lactobacillus iners, Ochromonas danica chrosoplast, Mycoplasma hominis* as well as other unknown bacteria.

***Sensitivity and specificity of the mPCR products***

The results indicate that the minimum concentration detected by the species-specific uniplex PCR varied, with detection limits ranging from 0.017 to 0.184 pg/μl (Fig. 2). Thus, this technique has different detection limits for the six pathogenic bacterial species. Notably, *U. ureayliticum* and *Corynebacterium* spp. were detected at a higher sensitivity than the other species (0.017 and 0.38 pg/μl, respectively). The analytical specifities for the mPCR assay detection of *C. trachomatis*, *N. gonorrhoeae, M. genitalium* and *U. urealyticum* were 100% (16/16), 100% (23/23), 100% (19/19) and 100% (12/120 for the STD analysis, respectively and 100% (22/22) and 100% (4/4), respectively for the prostatitis analysis of *P. aeruginosa* and *Corynebacterium* spp. (Table 2)

***Detection of pathogens from STD and prostatitis patients***

We subjected 96 clinical FVU isolated from 70 STD and 26 suspected prostatitis patients to an mPCR analysis. This method detected one or more of the six bacterial strains tested in every FVU sample tested. Monoinfections of *C. trachomatis, N. gonorrhoeae, M. genitalium,* and *U. urealyticum* were detected in the urine specimens of 15, 20, 19, and nine STD patients, respectively, while seven STD patients (10%) were infected by more than one bacterium (including three with *P. aeruginosa* and *U. urealyticum*) (Table 2). When the 26 prostatitis samples were tested by this method, four *Corynebacterium* spp. and one *P. aeruginosa* were detected, while the other 21 patients (80.7%) had mixed infections of *Corynebacterium* spp. (46.1%) and *M. genitalium* (34.6%). These were commonly occurring pathogens in the prostatitis syndrome patients and suggest that *M. genitalium* and *Corynebacterium* spp. have the potential to cause urinary tract infections as well as acute bacterial prostatitis.

## Discussion

This study describes a rapid one-step method for the detection of pathogenic bacteria in the urine of STD and prostatitis patients by mPCR. We have tested the 96 FVU specimens by uniplex PCR assays and found 100% agreement with the mPCR results. Moreover, mPCR had equivalent specificity and sensitivity to the uniplex PCR assays. In particular, we found that 4.25 (3/70) of the STD patients had mixed infections with *P. aeruginosa*, whereas 34.6% (6/26) of the prostatitis patients showed mixed infections with *M. genitalium*. This suggests that either these patients have been misdiagnosed (they were all diagnosed on the basis of their clinical symptoms) or they were infected with both diseases. These observations strongly suggest that the testing of FVU samples by mPCR could improve our understanding of the epidemiology of STDs and the prostatitis syndrome.

In summary, we have extended the work of Mahony *et al.* (1997), who had developed an mPCR method for the simultaneous detection of *C. trachomatis, N. gonorrhoeae, U. urealyticum* and *M. genitalium.* Our mPCR assay not only detected these STD strains simultaneously, but also two or more bacterial strains associated with prostatitis, namely *Corynebacterium* spp. and *P. aeruginosa* (Table 2). When the 96 FVU specimens from STD and prostatitis patients were tested by our mPCR method, We found 100% agreement with the samples tested for the uniplex PCR assays. Moreover, we found the detection limit for specific bacterial DNA by our mPCR method to be 0.017 and 0.38 pg/μl for *U. urealyticum* and *Corynebacterium spp., respectively.* The most frequently detected infections in STD patients were *N. gonorrhoeae* and *M. genitalium* (30% and 31%, respectively), while the most frequently occurring pathogen in the prostatitis patients was *Corynebacterium* spp. (>61.5%).

Interestingly, many mixed infections were detected (80.7%

of prostatitis cases had mixed infections). Moreover, even the moderate levels of mixed infections in STD patients (10%) confound the usual expectation that particular syndromes are due to one type of organism. It is possible that such complex microbial superinfections may actually be typical for some pathological states: however, this has not been previously realized due to cultivation limitations. In addition, these patients may have additional undiagnosed, syndromes which are caused by other pathogens. We found that 17.1% of the STD patients infected with *U. urealyticum* were also infected with *P. aeruginosa*. It is possible that these patients have cystic fibrosis and suffer from these opportunistic co-infections as a result of their compromised immune systems (Govan and Deretic, 1996; Da Sliver Filho *et al.*, 2004). As for *P. aeruginosa*, we found that 3.9% of the prostatitis cases were attributed to sole infections with *P. aeruginosa*, which is consistent with a previous report that found only 0.2% of prostatitis syndrome is caused by this pathogen (Skerk *et al.*, 2004). This also suggests that prostatitis is rarely caused by *P. aeruginosa* alone. Instead, *P. aeruginosa* infection in prostatitis ptients was frequently detected in conjunction with *M. genitalium* or *Corynebacterium* spp. co-infections. The most likely cause of prostatitis is a chronic infection with *Corynebacterium* spp. since 80.7% of our prostatitis patients showed evidence of infection with these pathogens.

The diagnosis of STDs and prostatitis has traditionally been largely dependent on methods such as culture, enzyme immunoassay, DNA hybridization, antibody staining as well as the examination of rectal and prostatic fluids for the signs of inflammation and infection. The previous methods did not suffice as a standard diagnostic studies have recognized mPCR as being a more sensitive detector of pathogenic bacteria compared to traditional tests involved in the detection process (Takahashi, 2005; Denks *et al.*, 2007).

The detection limit of the six pathogens for our mPCR method varied by 10% and this variation was likely attributed to the species-specific primers, whose amplification efficacy depends on their structures and the environment of the target gene on the genomic DNA. These levels of sensitivity are adequate, since they at least match the sensitivity of the traditional methods used to detect these infections.

Following the optimization of the mPCR conditions, we found that the best cysle number, annealing temperature, $MgCl_2$ concentaration, and target DNA concentration was 35 cycles, 3 mM, 54°C and 10 pg/μl, respectively. Following the uniplex PCRs, we found that the amplification of the 16S RNA from *Corynebacterium* spp. to be optimal at 56°C while, the optimal temperature for the other five species was 54°C. This difference in the optimal temperature between 16S RNA of *Corynebacterium* spp. and the other five species is likely due the GC concentration of the 5' primers, since we found that primers with higher GC concentrations require higher temperatures (data not shown). In addition, we used 3 mM $MgCl_2$ in the mPCR when 2.5 mM $MgCl_2$ sufficed in the uniplex PCRs. As a result, we used a higher $MgCl_2$ concentration than was needed for our mPCR and this likely resulted in $Mg^{2+}$ ions to intercalate with the primer mixture at a higher degree than observed in the PCRs. The optimal concentration of the primer in the six bacterial strains ranged from 0.4 to 0.6 μM. In the mPCR method described by Mahony *et al.* (Da Silver Filho *et al.*, 2004), the optimal primer concentrations were 0.1 μM for *C. trachomatis* and *M. geneitalum*, and 0.5 μM for *N. gonorrhoeae* and *U. urealyticum* (our primer differed from those used in this study).

We found that the mPCR technique can be hindered due to the possible homology between the various genes being targeted. Consequently, special attention must be paid in the primer design. Moreover the amplification conditions must be assiduously optimized since the specificity of the reactions can be affected by several factors, including temperature and duration of DNA denaturation, primer annealing, extension as well as the concentrations of the polymerase, $MgCl_2$ and primers. Moreover, the mPCR products should initially be sequenced to verify the specificity of the assay. It has also been recommended that Southern blot assays be used to check for specific PCR products; however, these assays are more cumbersome and time consuming (Mansy *et al.*, 1999). At present, the universal features of mPCRs lead to highly reliable, sensitive and specific multiple target gene detection is not known. Nevertheless, our sequencing data showed that our mPCR method yields highly reliable, pathogen-specific information (data not shown). Moreover, this method is also capable of detecting as little as 17 fg/μl of pathogen DNA. Thus, our method was clearly sensitive enough to detect the infecting pathogens in FVU samples, despite the fact that it is potentially complicated by the need to detect six pathogens rather than just four. This method has the potential to be suitable as a single test for the detection of pathogens which cause STDs and prostatitis syndrome. However, it should be noted that we cannot rule out the other possible candidates for STD's and prostatitis patients because the other pathogens are detected in the NIH-GeneBank database using the BLAST search engine (http://ncbi.nlm.nhi.gov/BLAST/).

A person infected with an STD has been known to have a higher likelihood to become infected with HIV (Brotman *et al.*, 2007), genital herpes (HSV) (Aggarwal and Kaur, 2004) and human papillomavirus virus (HPV) (Denks *et al.*, 2007). The infected person cannot be cured; however, the symptoms can be managed with medication. On the other hand, bacterial STDs, such as gonorrhea and chlamydia, can be cured with antibiotics upon diagnosis (Pettifor *et al.*, 2000). The other fungal agents are also linked to the infected STD (Raska *et al.*, 2007). Bacterial prostatitis, on the other hand, can be caused by the growth of bacteria such as *Escherichia coli* and *Klebsiella*, which are normally found in prostatic fluid (Skerk *et al.*, 2004). Urine that flows back into the urethra (urine reflux) and enters the prostate can also cause the prostatitis. There is no known cause for nonbacterial prostatitis or prostatodynia; however, atypical organisms (e.g., viruses, chlamydial organisms) have recently been suggested as a possible cause (Santillo and Lowe, 2006). To improve the diagnosis of STDs and the prostatitis syndrome, it may be possible to design an mPCR method which could also apply for testing the presence of other unknown pathogens.

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