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Simultaneous Identification of 14 Genital Microorganisms in Urine by Use of a Multiplex PCR-Based Reverse Line Blot Assay▽†

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The aim of this study was to develop and evaluate a sensitive method for the simultaneous identification of 14 urogenital potential pathogens. A multiplex PCR-based reverse line blot (mPCR/RLB) assay was developed to detect 14 urogenital pathogens or putative pathogens, namely *Trichomonas vaginalis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma parvum*, *U. urealyticum*, *Gardnerella vaginalis*, *Haemophilus influenzae*, herpes simplex virus type 1 (HSV1) and HSV2, *N. meningitidis*, *Mycoplasma hominis*, *M. genitalium*, and adenovirus, using two species-specific primer pairs and probes for each. The method was validated using a reference strain or a well-characterized clinical isolate of each target organism and was found to be both sensitive and specific. The limits of detection for the mPCR/RLB assay varied among the 14 target organisms from 4.2×10^{-1} to 7.0×10^{-11} ng/μl of genomic DNA. There were no cross-reactions among any of the probes. This method was used to test 529 first-voided urine specimens from male patients with and without urethritis attending two Sydney sexual health clinics. One or more target species were detected in 193 (36%) subjects. Of 233 positive results, overall 216 (93%) were concordant between mPCR/RLB and a comparator method (culture and/or species-specific PCR), 9 were positive only by mPCR/RLB, and 8 were positive only by the comparator method. The mPCR/RLB method was an accurate, convenient, and inexpensive method for the detection of multiple potential pathogens in first-voided urine specimens from men.

Sexually transmitted infections (STIs) are a major global health problem. Worldwide, an estimated 340 million cases of curable STIs, including chlamydial infection, gonorrhea, trichomoniasis, and syphilis, occur annually, and their incidence is increasing in many parts of the world. In developing countries, their complications rank in the top five disease categories for which adults seek health care (www.who.int/mediacentre/factsheets/fs110/en/). Many STIs cause asymptomatic infection; for example, up to 70% of men and women with gonococcal and/or chlamydial infections are symptom free (www.who.int/mediacentre/factsheets/fs110/en/), which creates the potential for unrecognized transmission with significant implications for both individual and population health.

Urethritis is characterized by discharge and dysuria (37) and is broadly classified as nongonococcal (NGU) or gonococcal. It occurs in both men and women but often is unrecognized in women. Acute NGU is one of the commonest STIs affecting heterosexual men, yet a specific pathogen, most commonly *Chlamydia trachomatis*, is identified in only 50 to 70% of cases (7). Pelvic inflammatory disease is an important complication of STI in women; *C. trachomatis* and *N. gonorrhoeae* commonly are implicated, but often the cause is unknown. Bacterial vaginosis is the commonest cause of vaginal discharge and is associated both with recognized STIs and other genital syndromes (3, 18). Additional epidemiological studies are needed to determine the significance of organisms other than recognized genital pathogens in urethral and vaginal syndromes (7, 13–15). In particular, the pathogenic roles, if any, of the two recently defined human *Ureaplasma* species (10), *U. urealyticum* (previously *U. urealyticum* biovar 2) and *U. parvum* (previously *U. urealyticum* biovar 1), and several other genital (32, 43, 44) and respiratory pathogens (20, 30, 33, 42) in NGU are unclear.

The high level of sensitivity of nucleic acid amplification tests, such as PCR, allows the use of less invasive specimen types, including first-voided urine specimens or self-collected vaginal swabs that are unsuitable for less sensitive methods, such as culture and antigen tests (8). This paper describes the development and evaluation of a multiplex PCR-based reverse line blot (mPCR/RLB) assay (19) that can detect any of 14 recognized and potential genital pathogens in urine specimens for use in clinical and epidemiological studies of genital infections.

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MATERIALS AND METHODS

Reference strains. Previously well-characterized clinical isolates of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, herpes simplex virus type 1 (HSV1) and HSV2, adenovirus, *T. vaginalis*, *M. hominis*, *G. vaginalis*, and *N. meningitidis*,

provided by the Centre for Infectious Disease and Microbiology (CIDM) diagnostic laboratory, were used as positive controls. All isolates had been identified according to routine methods (27). In addition, the following organisms were purchased from the American Type Culture Collection (ATCC; Manassas, VA): *U. urealyticum* (ATCC 27813 and ATCC 27814), *U. parvum* (ATCC 27818), *Haemophilus influenzae* (ATCC 10211), and *Streptococcus pneumoniae* (ATCC 27336).

Clinical specimens. Five hundred twenty-nine male patients with and without urethral symptoms were enrolled in a study of NGU at Parramatta Sexual Health Clinic (PSHC) and Sydney Sexual Health Centre (SSHC) from November 2006 to September 2007. Men with characteristic gonococcal urethritis, in whom Gram stains of urethral discharge showed gram-negative diplococci, and men who had been treated with antibiotics in the previous 6 weeks were excluded from the study. First-voided urine specimens were collected. Specimens from the SSHC were split, and one portion was sent to the routine diagnostic laboratory serving the clinic for *C. trachomatis* PCR (Roche COBAS Amplicor). Specimens were stored at 4°C at the clinic and transported in weekly batches, in a cool box, to the Centre for Infectious Diseases and Microbiology (CIDM), where they were stored at 4°C until DNA extraction was performed within 24 h of receipt. Specimens from PSHC were stored under the same conditions until being tested for *C. trachomatis* using a Roche COBAS Amplicor at the CIDM diagnostic laboratory.

In addition, to assist in the validation of mPCR/RLB results, urethral swabs were collected from all subjects. A Gram-stained smear was examined at the clinic. The swabs were placed in Stuart's transport medium and transported to the CIDM diagnostic laboratory, where cultures for *N. gonorrhoeae*, aerobic/facultative bacteria (including *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, and *G. vaginalis*), *M. hominis*, and *Ureaplasma* spp. were performed. Swabs were plated on New York City medium, 5% horse blood, chocolate (both in Columbia agar base; Oxoid, Basingstoke, United Kingdom), and A8 mycoplasma (Oxoid, Basingstoke, United Kingdom) agars, incubated for 24 to 48 h in CO_2, and identified by microscopic and colony morphology and biochemical and antigen tests (27).

DNA extraction. The Roche COBAS Amplicor extraction kit (Roche Diagnostics Australia Pty Limited Systems, Australia) was used per the manufacturer's instructions. Briefly, the urine specimens were vortexed thoroughly for 10 s before 500 μl of each specimen was transferred to a tube containing 500 μl of wash buffer. The specimens then were incubated at 37°C for 15 min and centrifuged at 13,000 × *g* for 5 min. The supernatant was discarded, 250 μl of lysis buffer was added, and after incubation at room temperature for 15 min, 250 μl of specimen diluent was added to the lysate. The tubes then were vortexed and centrifuged for 10 min at 13,000 × *g* and stored at −70°C.

Primer and probe design. Two sets of species-specific primers and probes, targeting highly conserved regions, were designed for each organism. The primers and probes used for this assay are shown in Table 1 and in the supplemental material. Primers and probes were designed to have similar physical characteristics to allow simultaneous amplification and hybridization in a multiplex reaction without the loss of sensitivity as follows: melting temperature (T_m), 58 to 65°C; length, 18 to 30 bp; moderate, weak, or no secondary structure; no dimer formation; and amplicon sizes, 80 to 400 bp (19). Some primers were selected from published papers and modified to match the desired characteristics. All probes and primers were checked for specificity against all sequences in GenBank using SeqSearch in the Australian National Genomic Information Services (ANGIS) programs (http://www.angis.org.au). The adenovirus primers used were designed to allow annealing to all 51 known adenovirus types by introducing degenerative base positions (1). Oligonucleotide primers were biotinylated at the 5′ end, and probes had a 5′ amine group and were synthesized by Sigma Aldrich (Sydney, Australia).

mPCR amplification. mPCR amplification was performed using a 25-μl reaction mixture containing 10 μl template DNA, 0.075 μl of each forward (100 pmol μl^{-1}) and reverse (100 pmol μl^{-1}) primer, 1.25 μl deoxynucleoside triphosphates (0.125 mM of each deoxynucleoside triphosphate), 2.5 μl 10× buffer (Qiagen), 3.0 μl 25 mM $MgCl_2$ (final concentration, 3.0 mM), 0.2 μl Qiagen HotStar *Taq* polymerase (5 U μl^{-1}), and water to 25 μl. The thermal profile involved initial denaturation for 15 min at 95°C, 40 cycles of 30 s at 94°C, 55°C for 30 s, and 72°C for 90 s, and a final extension for 10 min at 72°C, followed by a hold at 22°C. Inhibition controls were not included in the assay.

RLB assay. The RLB assay was performed as previously described (19). Briefly, probes were labeled and fixed to the membrane in various concentrations (0.6 to 10.8 pmol/μl) to determine the optimal conditions. Each PCR product was denatured and immediately chilled on ice. Hybridization was performed at 60°C for 60 min. The washed membrane was incubated in peroxidase-labeled streptavidin conjugate (Roche, Germany) at 42°C for 60 min. The membrane then was incubated in chemiluminescence blotting substrate (ECL direct system; Roche) for 2 min and covered with Hyperfilm X-ray film (Amersham). The film was exposed for 5 min.

sPCR. Single PCRs (sPCRs), using different targets from those used in the mPCR/RLB assay, were used as comparator methods for *T. vaginalis*, HSV1 and HSV2, *M. genitalium*, and adenovirus to confirm the specificity of the mPCR targets. The oligonucleotide primers used for sPCR are shown in Table 2. sPCRs for the other nine pathogens, using the same primers as those used in the mPCR, were performed when culture and mPCR/RLB results were discrepant. The same primers were used to confirm results, as sPCR generally is more sensitive than mPCR and the possibility of cross-reactions, which can occur in mPCR, is avoided.

sPCRs were performed using a 25-μl reaction mixture containing 10 μl template DNA, 0.25 μl of each forward (100 pmol μl^{-1}) and reverse (100 pmol μl^{-1}) primer, 1.25 μl deoxynucleoside triphosphates (0.125 mM of each), 2.5 μl 10× buffer (Qiagen), 3.0 μl 25 mM $MgCl_2$ (final concentration, 3.0 mM), 0.2 μl Qiagen HotStar *Taq* polymerase (5 U μl^{-1}), and water to 25 μl. The thermal profile involved initial denaturation for 15 min at 95°C, 40 cycles of 30s at 94°C, 55°C for 30s, and 72°C for 90 s, and a final extension for 10 min at 72°C, followed by a hold at 22°C.

Plasmid construction. Reference strains of all species were amplified using primers (Table 1; also see the supplemental material) targeting species-specific genes. Amplified products were inserted into a pGEM-T Easy vector system (Promega) and transformed into competent *Escherichia coli* cells JM109 according to the manufacturer's instructions. The Luria-Bertani–ampicillin–5-bromo-4-chloro-3-indolyl-â-D-galactopyranoside–isopropyl-β-D-thiogalactopyranoside plates were screened for positive clones and subcultured. The clones then were extracted. DNA concentrations were determined using UV spectrometry.

Analytical sensitivity. The analytical sensitivity of the mPCR/RLB assay was estimated using a series of 10-fold dilutions of plasmid templates of all species to determine the lowest limit of detection, which was expressed as nanograms per microliter of DNA in the last sample positive in the dilution series for each organism.

The results of mPCR/RLB assays were compared to those of the Roche COBAS Amplicor PCR for *C. trachomatis* in the same specimens (performed by routine diagnostic laboratories serving both clinics) and with those of urethral swab cultures for *N. gonorrhoeae*, *Ureaplasma* spp., *M. hominis*, and aerobic/facultative bacteria, including *S. pneumoniae*, *H. influenzae*, and *G. vaginalis.* Final results, after repeating any tests that were discrepant, were accepted as true positives if (i) both RLB probes were positive or one was positive and the result was confirmed by sPCR; (ii) the culture was positive; and/or (iii) sPCR using an alternative target was positive.

Data analysis. Data analysis was carried out using SPSS software (version 15.0; SPSS Inc., Chicago, IL). Univariate analysis was performed using Fisher's exact test and the chi-squared test for categorical variables. A 95% confidence interval was used.

RESULTS

Testing and validation with clinical isolates and reference strains of target organisms showed that each target species was amplified, hybridized, and correctly identified by the mPCR/RLB assay (Fig. 1). Initially nonspecific binding during hybridization was observed, which was eliminated by adjusting reaction conditions and components. Final probe concentrations used to label the RLB membrane were 10.8 pmol/μl for HSV2, 5.4 pmol/μl for *S. pneumoniae* and adenovirus, 0.6 pmol/μl for *N. gonorrhoeae*, and 1.8 pmol/μl for all other species. In all cases both probes gave positive results in the RLB if target DNA was present in the sample.

Analytical sensitivity of mPCR/RLB and comparison to alternative methods. The limit of detection by mPCR/RLB ranged from 4.2×10^{-1} to 7.0×10^{-11} ng/μl for different species. Results for mPCR/RLB and the comparator method for each target species (except HSV2, which was not detected in any specimens) are shown in Table 3. Of a total of 233 positive results, 211 (90%) were concordant in mPCR/RLB and comparator methods on initial testing; 14 were positive in

TABLE 1. Oligonucleotide primers and probes developed or modified for the mPCR/RLB assay used in this study[a]

Primer/probe name[b]	Specificity[c] (target)	GenBank accession no.	Primer-probe sequence[d] (5′–3′)	T_m[e] (°C)	Reference or source[g]
TV-Ap	*T. vaginalis* (*btuB*)		904TGT TGT GAG CTT GAG TGT ACG G883	65.1084	This study
TV-Sp			916CGA TCT TAA CCA CCT TGT TTC C945	63.31923	14 (mod)
NGpSb	*N. gonorrhoeae* (CP)	M10316	3249TGC TGT TTC AAG TCG TCC AG3268	64.06359	This study
NGpAp			3317GAT AGT CAT AGC AGG GCT GTT C3296	61.5549	This study
NGpSp			3452CCG TAA CGT CTC TAA GTC TGC TT3474	62.51156	This study
NGpAb			3503CGA AGC CGC CAG CAT AGA GC3484	71.22094	This study
NG16Sb	*N. gonorrhoeae* (ITS)	AF223396	404CCA AAA CTT AAC AAA TGA AAG CAA G428	63.41	This study
NG1S			453TGA TTT GCG AAG TAG AAT AAC G474	60.64	This study
NG2A2			456ATC AAA ATA AGC TGC TAA AAA CAG433	59.41	This study
NGITSAb			490TGT TAA AGA TCG ATG CGT CGT472	64.33	This study
CT24b	*C. trachomatis* (CP)	X06707	840GGG ATT CCT GTA ACA ACA AGT CAG G864	67.33045	This study
CTS1p			865TTG CGC ATA ATT TTA GGC TTG885	63.59178	This study
CTA2p			1021ACA CTT TGT CTC GAT GAA AGA CA999	62.57137	This study
CT27b			1047CCT CTT CCC CAG AAC AAT AAG AAC AC1022	67.37814	This study
UP-Sb	*U. parvum* (*ureB*)	AF085731	637GAT CAC ATT TTC ACT TGT TTG AAG TG662	64.37767	23 (mod)
UP-Ap			702CTT CAT TTC CTT TTT CAT CAA AAA ATA C675	63.43446	This study
UP-Sp			688AAA AAG GAA ATG AAG ATA AAG AAC G712	61.40322	23 (mod)
UP-Ab			735AAC GTC GTC CAT AAG CAA CTT TG713	65.8875	23 (mod)
UU-Ap	*U. urealyticum* (*ureB*)		705CTT CAT TTC CTT TTT CAT CAA AAA ATA C678	63.43446	This study
UU-Sp			691AAA AAG GAA ATG AAG ATA AAG AAC G715	61.40322	23 (mod)
UU-Ab			739AAA CGA CGT CCA TAA GCA ACT TTA716	64.34317	23 (mod)
GV-Ap	*G. vaginalis* (ITS)		416TCC TGT CTA CCA AGG CAT CC397	63.97078	This study
GV-Sp			632CGT GTG ATA ACC GTC AGG TG651	64.06355	This study
HSV1-Sp	HSV1 (gD)		545CGT TTG AGA CCG CCG GCA562	73.04224	This study
HSV2-Sp			572CCT TCG AGA CCG CGG GTA589	68.53278	This study
NM-Sb	*N. meningiditis* (*porA*)	AY319969	929GCT TCG GTA ATG CAG TTC CA948	64.94242	17 (mod)
NM-Ap			1010CTG GTA TTT TCG CCT TTT TTA C989		17 (mod)
NM-Sp			953TCA GCT ATG CCC ATG GTT970		This study
MH-Ap	*M. hominis* (*gap*)		770CTG AAT AAA CAA CTG TTT TAA CAC CTT CGCT740	68.7016	2 (mod)
MH-Sp			702CAG GTG CTA AAA AGG TGT TTA TTA CTG CT730	66.55416	This study
MgPAa-Ap	*M. genitalium* (*mgpA*)		1463TAT CAT ACC TTC TGA TTG CAA AGT1445	60.39573	This study
MgPa-Sp			1473CGG TAG AGC TTT ATA TGA TAT TAA CTT AGC1502	61.46476	This study
AdVdeSb	Adenovirus (hexon)		GCC SCA RTG GKC WTA CAT GCA CAT C[f]	69.2	1 (mod)
AdVdeAp			CCY ACR GCC AGI GTR WAI CGM RCY TTG TA	68.16371	1 (mod)
AdVdeSp			GCC CGY GCM ACI GAI ACS TAC TTC	63.76665	1 (mod)
AdVdeAb			CAG CAC SCC ICG RAT GTC AAA	62.80243	1 (mod)

[a] In addition to primers specifically designed or modified for this study, published primers were used without modification for several targets. Details are shown in the supplemental material.
[b] The suffix b indicates a biotin-labeled primer, and p indicates an amine-labeled probe. An A indicates antisense, and S indicates sense.
[c] Abbreviations: CP, cryptic plasmid; ITS region, intergenic spacer region; gD, glycoprotein D.
[d] Numbers represent the base positions at which the primer/probe sequence starts and finishes (starting at point 1 of the corresponding gene sequence in GenBank).
[e] Melting temperatures were provided by the primer synthesizer (Sigma-Aldrich).
[f] S = G+C; R = A+G; K = G+T; W = A+T; Y = C+T; M = A+C; I = inosine.
[g] Some primers were modified (mod) from the published primers.

mPCR/RLB only (of which 10 were resolved by repeating the comparator method); and 7 were positive in the comparator method only.

Of the 55 specimens in which *C. trachomatis* was detected using the mPCR/RLB method, 5 (10%) initially were negative using the Roche COBAS Amplicor PCR. However, on retesting, *C. trachomatis* was detected in all five specimens by the Amplicor PCR. All specimens that initially were positive with the Amplicor PCR also were positive with mPCR/RLB. Although subjects with clinical or microscopic evidence of gonorrhea on presentation were excluded, seven had positive tests for gonorrhea in the mPCR/RLB test and were culture positive. Only two of these specimens were positive for *N. gonorrhoeae* using the Roche COBAS Amplicor PCR when tested initially, but all were positive on retesting.

Ureaplasma spp. were identified in 86 urethral swab specimens by culture but in only 84 urine specimens by mPCR/RLB (*U. parvum*, 31; *U. urealyticum*, 53). All mPCR/RLB results were confirmed by species-specific sPCR.

Clinical specimens. The age of the 529 subjects ranged from 19 to 76 years (mean, 37 years; median, 35 years). One or more pathogens or putative pathogens were identified in 193 (36%) men, including 136 of 277 (49%) men with urethritis symptoms and 57 of 252 (23%) asymptomatic men. Two or more target

TABLE 2. Oligonucleotide primers used for sPCR

Primer name	Specificity (target)	GenBank accession no.	Primer sequence[a] (5′-3′)	T_m[b] (°C)	Reference
Tv1	*T. vaginalis* 18S rRNA gene	U17510	874TAA TGG CAG AAT CTT TGG AG894	59.2	25
Tv2			1185GAA CTT TAA CCG AAG GAC TTC1165	58.3	
HSVPolA1	HSV DNA polymerase		ATC ATC TAC CGC GAC ACG GACT	68.8	49
HSVPolA2			TCC ACG CCC TTG ATG AGC ATC T	72.0	
MG16-45F	*M. genitalium* 16S rRNA gene	X77334	45TAC ATG CAA GTC GAT CGG AAG TAG C69	68.8	16
MG16-447R			469AAA CTC CAG CCA TTG CCT GCT AG447	69.2	
AD1	Adenovirus hexon gene	U20821	CTG ATG TAC TAC AAC AGC ACT GGC AAC ATG GG	76.1	36
AD2			GCG TTG CGG TGG TGG TTA AAT GGG TTT ACG TTG TCC AT	83.4	
GV1	*G. vaginalis* 16S-23S rRNA gene	LO8167	364TTA CTG GTG TAT CAC TGT AAG G385	55.8	51
GV2	23S		695CCG TCA CAG GCT GAA CAG T677	64.1	

[a] Numbers represent the base positions at which the primer/probe sequence starts and finishes (starting at point 1 of the corresponding gene sequence in GenBank).
[b] Melting temperatures were provided by the primer synthesizer (Sigma-Aldrich).

organisms were identified in 30 men (16 symptomatic and 14 asymptomatic) (Table 4). Figure 2 shows the numbers and percentages of specimens in which each organism was detected by symptom status. A simple comparison of results in symptomatic and asymptomatic men showed that *C. trachomatis* (48/277 and 7/252, respectively; $P < 0.001$), *N. gonorrhoeae* (7/277 and 0/252, respectively; $P = 0.01$), and *M. genitalium* (12/277 and 3/252, respectively; $P = 0.03$) were detected significantly more frequently in men with symptoms. There were no significant differences in detection rates between symptomatic and asymptomatic men for any other pathogens. A detailed analysis of clinical and epidemiological data will be reported separately (D. Couldwell, unpublished data).

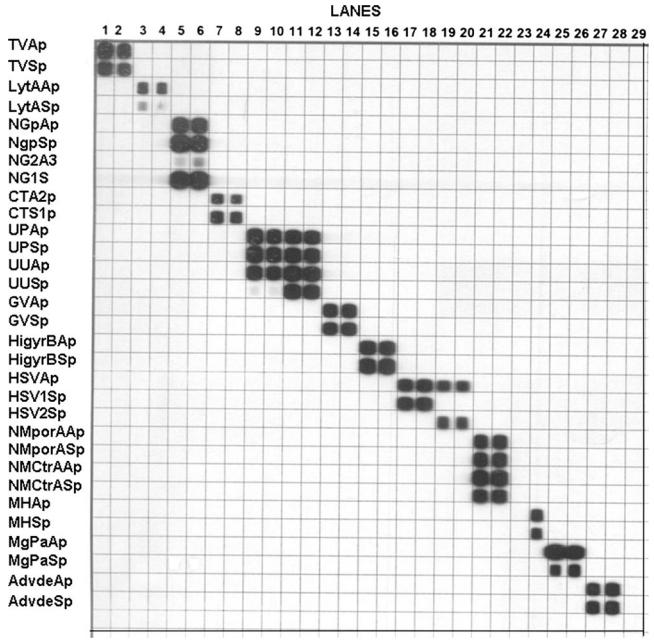

FIG. 1. mPCR/RLB results using reference strains. Lanes: 1 and 2, *T. vaginalis*; 3 and 4, *S. pneumoniae*; 5 and 6, *N. gonorrhoeae*; 7 and 8, *C. trachomatis*; 9 and 10, *U. parvum*; 11 and 12, *U. urealyticum*; 13 and 14, *G. vaginalis*; 15 and 16, *H. influenzae*; 17 and 18, HSV1; 19 and 20, HSV2; 21 and 22, *N. meningitidis*; 23, blank; 24, *M. hominis*; 25 and 26, *M. genitalium*; and 27 and 28, adenovirus type 1.

DISCUSSION

We have developed an mPCR/RLB hybridization assay that permits the reliable, simultaneous detection of 14 known or potential urogenital pathogens, many of which are difficult to identify by other methods. We have used mPCR/RLB previously to identify multiple pathogens in respiratory specimens and blood cultures (45, 46). Others (40) have used gel-based mPCR to identify *Ureaplasma* spp., *M. genitalium*, and *M. hominis* in first-voided urine samples, and there is a recent report of an mPCR to detect 16 pathogens using a microplate assay (25). mPCR/RLB potentially is applicable to routine diagnosis, can be modified to add or delete targets, and is particularly suitable for epidemiological studies to examine the roles of putative pathogens in genital syndromes. It is more practicable and less expensive than microarray technology.

Overall, 75 target organisms were detected in 57 of 252 asymptomatic men, and 158 were detected in 136 of 277 men with symptoms; multiple organisms were identified in approximately equal numbers of men with and without symptoms. They included two mixed infections with *N. gonorrhoeae* and *C. trachomatis*, which is not uncommon and probably results from simultaneous transmission (25, 31). Both are well-established genital pathogens, whether or not they cause symptoms, and *M. genitalium* also has been implicated in NGU (7, 24). In this study, all three were significantly associated with the presence of urethral symptoms ($P < 0.05$). The rate of the detection of *M. genitalium* was similar to that reported by others (5, 25, 40), and more widespread testing for it in patients with NGU has been advocated (6).

The roles of the other organisms or combinations of organisms targeted in this study are uncertain, since many commonly are found among the normal genital flora. Providing further evidence for their roles in urethritis was the aim of the clinical component of this study (of which the results will be reported separately). There were differences between symptomatic and asymptomatic men in the rates of the detection of HSV1, adenovirus, and *U. urealyticum* as in other studies (7, 50), but the numbers were small and overall differences did not reach statistical significance.

The comparison of mPCR/RLB results to those of alternative detection methods showed very good correlation. Several

TABLE 3. Comparison of results of mPCR/RLB and comparator methods in detection of genital infection/colonization with 14 recognized or putative genital pathogens

Organism	Limit of detection[a] (ng/μl)	No. positively detected by mPCR/RLB[b]	Comparator method[c] (target)	No. positively detected by comparator method
T. vaginalis	1.5×10^{-9}	1	sPCR (18S rRNA gene)	1
S. pneumoniae	7.4×10^{-1}	1	Culture	1
N. gonorrhoeae	4.3×10^{-2}	7	Culture and Amplicor PCR	2 (+5)[d]
C. trachomatis	7.0×10^{-11}	55	Amplicor PCR	50 (+5)[d]
Ureaplasma spp.[e]	7.8×10^{-9}	84	Culture	86
G. vaginalis	7.8×10^{-3}	3	sPCR (16-23S rRNA gene)	3
H. influenzae	7.0×10^{-8}	31	Culture	35
HSV1	4.3×10^{-8}	8	sPCR (*pol*)	5
N. meningitides	8.2×10^{-8}	2	Culture	1
M. hominis	5.5×10^{-1}	15	Culture	16
M. genitalium	2.1×10^{-9}	15	sPCR (16S rRNA gene)	15
Adenovirus	6.3×10^{-7}	4	sPCR (hexon gene)	4

[a] For mPCR/RLB.
[b] sPCR, using the same primers as those for mPCR, was performed on specimens with discrepant mPCR/RLB and culture results. In all cases the mPCR/RLB and sPCR results were concordant.
[c] Comparator methods were either the culture of urethral swab collected at the same time as first-voided urine specimen or sPCR on the same urine DNA extract as that used for mPCR/RLB, using a different, species-specific target (except for adenoviruses, for which the same hexon gene target was used).
[d] Of the 7 and 55 specimens positive by mPCR/RLB for *N. gonorrhoeae* and *C. trachomatis*, respectively, only 2 and 50 were positive initially in the Roche Amplicor PCR; all were positive on retesting.
[e] Urethral specimens were cultured for ureaplasmas, but isolates were not speciated. *Ureaplasma* spp. identified in the mPCR/RLB-positive specimens are shown in Fig. 2.

organisms were detected in very few (<5) specimens (*T. vaginalis*, *S. pneumoniae*, *G. vaginalis*, *N. meningitidis*, and adenovirus), but results agreed in all but one (one culture negative and mPCR/RLB positive for *N. meningitidis*). mPCR/RLB identified *N. gonorrhoeae*, HSV1, and *M. genitalium* in all specimens that were positive by comparator methods and HSV1 in three additional specimens. It did not detect *M. hominis* in one and *Ureaplasma* spp. in two urine specimens from men whose urethral swabs were culture positive. These specimens were from men attending SSHC, where urine specimens were stored at 4°C for several days before being processed, which may have reduced the sensitivity of mPCR/RLB compared to that of the culture of urethral swabs, which were stored at room temperature in Stuart's transport medium. The refrigeration of specimens for several days had no apparent effect on the detection of other pathogens. Stellrecht et al. (40), using urine and swabs for PCR, recorded sensitivities similar to those of culture for *Ureaplasma* spp., *M. genitalium*, and *M. hominis*, and similar sensitivities have been reported by others for other species (2, 12, 23, 40, 50). Nevertheless, these results suggest that specimens should be processed for PCR as soon as possible after collection and, if they cannot be tested immediately, stored as DNA extracts.

TABLE 4. Mixed genital infections/colonization with target organisms detected by mPCR/RLB assay from 30 men with and without urethral symptoms

Symptom(s)[a]	Organisms detected[b]	No. of cases
Yes	NG, HI	1
	CT, NG	2
	CT, MG	1
	CT, UU	1
	CT, HSV1, UP	1
	CT, NM	1
	CT, HI	1
	MG, UU	3
	MG, UP, GV	1
	MG, HI	1
	UU, MH	1
	UP, ADV	1
	UP, MH	1
Total		16
No	CT, MH	2
	UU, MG, MH	1
	UU, MG, HI, GV	2
	UU, GV	1
	UU, HI	1
	UU, MH	1
	HSV1, HI	1
	HSV1, UP	1
	UP, MH	4
Total		14

[a] A symptomatic man was a patient who had urethral symptoms (dysuria, urethral discomfort, or urethral discharge), and an asymptomatic man was a patient who presented with no symptoms.
[b] Abbreviations: CT, *C. trachomatis*; NG, *N. gonorrhoeae*; HI, *H. influenzae*; UU, *U. urealyticum*; GV, *G. vaginalis*; UP, *U. parvum*; MH, *M. hominis*; MG, *M. genitalium*; ADV, adenovirus; and NM, *N. meningitidis*.

False-negative mPCR/RLB results also may have resulted from the prolonged storage of DNA extracts (up to 18 months at −20°C) prior to testing, which can affect DNA quality and PCR efficiency (28), or from PCR inhibitors in urine (9, 34).

Initial false-negative results for Roche COBAS Amplicor PCR for *N. gonorrhoeae* and *C. trachomatis* (some from both diagnostic laboratories performing routine testing for the two clinics) were positive on retesting. These results reflect the real-life pitfalls of diagnostic testing, even by reputable laboratories using generally reliable assays.

A significant limitation of this study, in common with other studies of new, potentially more sensitive tests, was that there was no single gold standard for the analysis of mPCR/RLB. We chose to culture, where practicable, a different specimen (urethral swab) for target bacteria, for which urine would have been inappropriate, to identify men with urogenital colonization/infection. To optimize the reliability of the mPCR/RLB, we measured limits of detection, quantitatively, using cloned targets; confirmed all positive results using culture or sPCRs; designed primers and probes based on targets used in well-

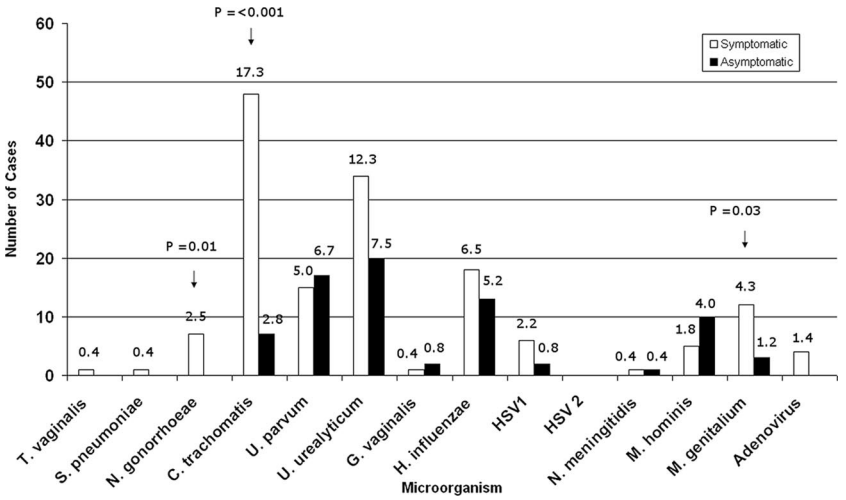

FIG. 2. Results of mPCR/RLB on first-voided urine specimens for 14 target urogenital organisms from men with and without symptoms of urethritis. Numbers on bars indicate the percentages of subjects with positive results for each target organism. Denominators are 277 for symptomatic men and 252 for asymptomatic men; the *y* axis shows the number of positive specimens. The arrows indicate organisms detected significantly more frequently in men with urethral symptoms than in men without symptoms ($P \leq 0.05$).

established sPCR methods, which had been shown to be specific; and avoided contamination by the use of appropriate negative and no-DNA controls. There were relatively few discrepancies between methods, and we believe that the decision to regard any confirmed positive result as a true positive for the purposes of comparison was justified. Nevertheless, we cannot exclude the possibility that a small number of mPCR/RLB results were false positives. Even in this relatively large number of subjects, most target organisms were identified too infrequently to calculate accurate sensitivities and predictive values.

This study confirmed the sensitivity and specificity of the mPCR/RLB assay for the detection of a wide range of potential urogenital pathogens in first-voided urine specimens. However, cross-reactivity can occur if primers and probes are not designed correctly, and the optimization of reaction components and conditions is required to produce a stable system without nonspecific reactions. The advantages of mPCR/RLB are that it can simultaneously test up to ~40 specimens for up to ~40 target genes in a single reaction, and it could be used for a variety of specimens other than urine, including cervical smears collected for cytology (48) or self-collected vaginal swabs. Given the high frequency of the multiple species identified, we believe that such an approach could contribute to an effective public health response to STIs.

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