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Multiplex PCR Testing Detection of Higher-than-Expected Rates of Cervical *Mycoplasma*, *Ureaplasma*, and *Trichomonas* and Viral Agent Infections in Sexually Active Australian Women▽

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Knowing the prevalence of potential etiologic agents of nongonococcal and nonchlamydial cervicitis is important for improving the efficacy of empirical treatments for this commonly encountered condition. We describe four multiplex PCRs (mPCRs), designated VDL05, VDL06, VDL07, and VDL09, which facilitate the detection of a wide range of agents either known to be or putatively associated with cervicitis, including cytomegalovirus (CMV), enterovirus (EV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), herpes simplex virus type 1 (HSV-1), and herpes simplex virus type 2 (HSV-2) (VDL05); *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma genitalium*, and *Mycoplasma hominis* (VDL06); *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, and group B streptococci (VDL07); and adenovirus species A to E (VDL09). The mPCRs were used to test 233 cervical swabs from 175 women attending a sexual-health clinic in Sydney, Australia, during 2006 and 2007. The agents detected alone or in combination in all cervical swabs (percentage of total swabs) included CMV (6.0), EV (2.1), EBV (2.6), VZV (4.7), HSV-1 (2.6), HSV-2 (0.8), HSV-2 and VZV (0.4), *U. parvum* (57.0), *U. urealyticum* (6.1), *M. genitalium* (1.3), *M. hominis* (13.7), *C. trachomatis* (0.4), *T. vaginalis* (3.4), and group B streptococci (0.4). Adenovirus species A to E and *T. pallidum* were not detected. These assays are adaptable for routine diagnostic laboratories and provide an opportunity to measure the true prevalence of microorganisms potentially associated with cervicitis and other genital infections.

Cervicitis, an acute or chronic inflammation of the uterine cervix, is generally viewed as a consequence of infection with sexually transmissible agents. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are the most commonly reported pathogens, possibly because they are most frequently screened for. However, the etiology of most cases is undetermined and could be multifactorial in nature (11, 34, 35, 40). Studies undertaken in other epidemiologic settings indicate significant differences in the prevalences of other cervical infectious agents (1, 41, 44, 45, 58). An underappreciation of the prevalences of and roles played by these nongonococcal and nonchlamydial agents potentially jeopardizes the effectiveness of empirical treatments for cervicitis. Unresolved cervicitis can result in ascending infection, endometritis, pelvic inflammatory disease, and salpingitis (11, 23, 46). Furthermore, cervicitis may enhance human immunodeficiency virus susceptibility by the disruption of mucosa, allowing increased viral replication within recruited inflammatory cells (30). The development of molecular methods, such as PCR and DNA hybridization, has allowed the detection of a range of agents whose etiologic roles in genital infections need to be further investigated, including the viruses cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1) and HSV-2 (4, 43), adenovirus (6, 10, 50), and the *Mollicutes* *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Mycoplasma genitalium* (1, 28, 59). There have also been reports of genital infections caused by Epstein-Barr virus (EBV) (4, 55), varicella-zoster virus (VZV) (27), and enterovirus (EV) (24). We report here the use of four multiplex PCR (mPCR) assays, designated VDL05, VDL06, VDL07, and VDL09, based on a conventional platform, for the detection of 19 microorganisms in cervical swabs, including *Treponema pallidum* and *C. trachomatis*, *Trichomonas vaginalis*, group B streptococci, and five adenovirus species, in addition to those mentioned above. The assays were developed using cervical swabs from different women taken on one or more occasions during different visits to a sexual-health clinic.

MATERIALS AND METHODS

Patients. Cervical swabs (n = 233) were taken from 175 women consecutively attending a sexual-health clinic in Sydney, Australia (between one and three visits), during 2006 and 2007 who were all eligible for recruitment to an as-yet-unpublished case-control study investigating cervicitis. They included women with and without cervicitis. All of the women were aged ≥18 years, had been sexually active in the preceding 3 months, required an internal examination regardless of symptoms, had not been treated with antibiotics or received gynecologic intervention in the preceding month, did not have an intrauterine contraceptive device in situ, and were not currently menstruating or pregnant. Women with pelvic inflammatory disease were excluded. Written informed consent was obtained from all of the women. The study protocol and data management were approved by the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee.

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TABLE 1. Oligonucleotides used in molecular detection methods

mPCR	Agent	Oligonucleotide[a]	Code	Oligonucleotide sequence (5′–3′)	Target (bp)[b]	LD[c]	Reference
For *Mollicutes*	Urea- and	A	My-ins	GTA ATA CAT AGG TCG CAA GCG TTA TC	16S tRNA gene (520)		59
(VDL06)	mycoplasma	B	MGSO-2-Bi	CAC CAT CTG TCA CTC TGT TAA CCT C			
	M. hominis	P	Mhom-P10-Am	GAC ACT AGC AAA CTA GAG TTA G		10^3	
	M. genitalium	P	Mgen-P3-Am	TCG GAG CGA TCC CTT CGG T		10^3	
	U. parvum	A	UMS-57	(T/C)AA ATC TTA GTG TTC ATA TTT TTT AC	Multiple banded antigen gene (326–327)[d]		28
		B	UMA222	GTA AGT GCA GCA TTA AAT TCA ATG			
		P	UP-PROBE1	CTG AGC TAT GAC ATT AGG AGT TAC C		10^3	
	U. urealyticum	A	UMS-170	GTA TTT GCA ATC TTT ATA TGT TTT CG	Multiple banded antigen gene (476)		54
		B	UMA263	TTT GTT GTT GCG TTT TCT G			
		P	UU-PROBE1	CTG AAT TCA ATG TTG CAA TTA CAT CAG CTG A		10^3	
For nonviral agents (VDL07)	*C. trachomatis*	A	CT-OF	TTG CAA GCT CTG CCT GTG GGG AAT[e]	Omp1 (931/378)		
		B	CT-OR	TCA CAT CGC CAG CTC CAG CAA TAG[e]			
		C	CT-IF	ACA TTA GGA GCC ACC AGT GGA TAT C[e]			
		D	CT-IR	ATC CTT AGT TCC TGT CGC AGC ATC T[e]			
		P	CT-PROBE	TGC GTG GAG CGT CGG CGC TCG CGC A[e]		10^2	
	T. vaginalis	A	TricV-OF	CTA TTG TCG AAC ATT GGT CTT ACC CTC[f]	G3 (264/206)		31
		B	TricV-OR	TCT GTG CCG TCT TCA AGT ATG CCC			
		C	TricV-IF	CTC AGT TCG CAA AGG CAG TCC TTG A[e]			
		D	TricV-IR	GCT TGG AGA GGA CAT GAA CTT CGG A[e]			
		P	TricV-PROBE	CTA CAA CAA ATT CTT CTC C[e]		10^1	
	T. pallidum	A	TP7-OF	CTC AGC ACT GCT GAG CGT AG	*bmp* gene (616/506)		39
		B	TP8-OR	AAC GCC TCC ATC GTC AGA CC			
	C	TP3-IF		CAG GTA ACG GAT GCT GAA GT			
	D	TP4-IR		CGT GGC AGT AAC CGC AGT CT			
	P	TP5-PROBE		GAC CTG AGG ACT CTC AAA TC		10^5	
	Group B streptococci	A	STB-OF	AAC CAG CCA ACC GGT TTA CCG TGA[e]	*scpB* (418/260)		12
		B	STB-OR	GGT CAA CCT TCT CGT ACT CTA GAG AAA[e]			
		C	STB-IF	ACA ACG GAA GGC GCT ACT GTT CC			
	D	STB-IR		GTT TTA CCT GGT GTT TGA CCT GAA CTA TC			
	P	STB-PROBE		ACA ACG GAA GGC GCT ACT GTT CC[e]		10^2	
Adenovirus species specific (VDL09)	Adenovirus sp. A	A	AdA1	GCT GAA GAA MCW GAA GAA AAT GA	Fiber (1,444–1,537)		57
		B	AdA2	CRT TTG GTC TAG GGT AAG CAC		10^3	
	Adenovirus sp. B	A	AdB1	TST ACC CYT ATG AAG ATG AAA GC	Fiber (670–772)		
		B	AdB2	GGA TAA GCT GTA GTR CTK GGC AT		10^3	
	Adenovirus sp. C	A	AdC1	TAT TCA GCA TCA CCT CCT TTC C	Fiber (1,988–2,000)		
		B	AdC2	AAG CTA TGT GGT GGT GGG GC		10^4	
	Adenovirus sp. D	A	AdD1	GAT GTC AAA TTC CTG GTC CAC	Fiber (1,205–1,221)		
		B	AdD2	TAC CCG TGC TGG TGT AAA AAT C		10^4	
	Adenovirus sp. E	A	AdE1	TCC CTA CGA TGC AGA CAA CG	Fiber (967)		
		B	AdE2	AGT GCC ATC TAT GCT ATC TCC		10^3	

[a] A, outer sense primer; B, outer antisense primer; C, inner sense primer; D, inner antisense primer; P, probe.
[b] First-round product/second-round product.
[c] LD, limit of detection (number of copies per reaction).
[d] 326 bp for *U. parvum* serovars 1 and 3/14 and 327 bp for serovar 6 (28).
[e] Oligonucleotide designed by Nikolas Rismanto.
[f] Underlined sequences are modifications of the published primers cited.

Sampling procedure. The cervix was accessed using a sterile metal speculum and was prepared for swabbing by removing exudate with a large nonsterile swab (Multigate Medical Products, China). An initial swab of the endocervix was taken with a sterile cotton swab (Copan, California) and was used to screen bacterial agents before being placed in viral transport medium 199 (Gibco Invitrogen, New York) and stored at −70°C. Two consecutive specimens from the same region were taken using viral transport swabs (Copan Diagnostics), suspended in viral transport medium, and stored at −70°C. These two specimens were tested by the assays described below.

Nucleic acid extraction and PCR amplification. The swabs were suspended in 500 μl of universal viral transport medium before extraction of the total nucleic acid using a robotic extraction machine (MagNaPure LC; Roche, Germany) applying the Total NA protocol according to the manufacturer's instructions (Roche, Germany). Extracts were stored at 4°C before being tested within 48 h of collection.

Detection of *Mollicutes* species (VDL06). A single-round mPCR using Qiagen OneStep reverse transcription (RT)-PCR (Qiagen, Gemany) master mix was designed to detect *U. parvum*, *U. urealyticum*, *M. genitalium*, and *M. hominis*. This commercial master mix was used to conform to other molecular procedures currently used in diagnostic laboratories, allowing simplified quality assurance and workflow. The reaction mixtures were prepared in accordance with the manufacturer's instructions for a 50-μl reaction and consisted of 5.8 μl of RNase-free water, 10.0 μl of buffer, 2.0 μl of deoxynucleoside triphosphate mix, 2.5 μl of each primer (including primers for internal control at a final concentration of 0.5 μM) (Table 1), 2.0 μl Qiagen OneStep RT-PCR enzyme mix at a final activity of 0.5 U, 0.2 μl digoxigenin-11-dUTP (Roche, Germany), and 10 μl of template. The cycling procedures included an RT step at 50°C for 30 min, denaturation at 95°C for 15 min, and then 50 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, a final extension of 7 min at 72°C, and a 4°C hold. The products were visualized by gel electrophoresis, and the amplicons were identified by probe hybridization, followed by PCR enzyme-linked immunosorbent assay (digoxigenin detection) (Roche, Germany). Known-positive clinical samples were used as reaction controls for the PCR.

Virus detection (VDL05). A nested mPCR of the same designation previously described (38) was used without modification for the detection of CMV, HSV-1, HSV-2, EBV, EV, and VZV. Briefly, a first-round reaction comprising 20 μl of template, 0.5 μl of AmpErase (uracil *N*-glycosylase) (Applied Biosystems), and 0.10 μM of each primer (38) was included in a 50-μl reaction mixture of the Qiagen OneStep RT-PCR kit (Qiagen, Germany). A second-round reaction was undertaken using 2 μl of first-round product, 0.2 μl digoxigenin-11-dUTP (Roche, Germany), and 0.10 μM of each primer in a 50-μl reaction mixture of Ampli*Taq* Gold PCR Master Mix (Applied Biosystems). The thermocycling conditions used for each round were as previously described (38). The products were visualized by gel electrophoresis, and the amplicons were identified by probe hybridization (as described above).

Detection of adenovirus species A to E (VDL09). The adenovirus detection method is based on that previously described by Xu et al. (56) and was modified to conform to the above-mentioned protocols. A single-round reaction was performed comprising 5 μl of template and 0.20 μM of each primer (Table 1) in a 50-μl reaction mixture of Ampli*Taq* Gold PCR Master Mix (Applied Biosys-

TABLE 2. Use of mPCRs for screening nongonococcal agents in cervical swabs

Microorganism(s)	mPCR	No. (%) detected	
		Total cervical swabs (n = 233)	Total women (n = 175)
Mollicutes	VDL06		
U. parvum		112 (48.0)	93 (53.1)
U. urealyticum		6 (2.6)	6 (3.4)
M. hominis		15 (6.4)	13 (7.4)
M. genitalium		3 (1.3)	3 (1.7)
U. parvum + *M. hominis*		15 (6.4)	13 (7.4)
U. urealyticum + *M. hominis*		2 (0.9)	2 (1.1)
U. parvum + *U. urealyticum*		6 (2.6)	5 (2.9)
Viruses	VDL05		
CMV		14 (6.0)	11 (6.3)
EV		5 (2.1)	5 (2.8)
EBV		6 (2.6)	6 (3.4)
HSV-1		6 (2.6)	6 (3.4)
HSV-2		2 (0.8)	2 (1.1)
VZV		10 (4.3)	9 (5.1)
VZV + HSV-2		1 (0.4%)	1 (0.6)
Adenovirus species	VDL09		
A, B, C, D, E			0
Other agents	VDL07		
C. trachomatis		1 (0.4)	1 (0.6)
T. vaginalis		8 (3.4)	7 (4)
T. pallidum		0	0
Group B streptococci		1 (0.4)	1 (0.6)

tems). The thermocycling conditions included a preliminary denaturation step at 94°C for 5 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 54°C for 45 s, and extension at 72°C for 2 min), and a final extension at 72°C for 5 min. The products were visualized using gel electrophoresis.

Detection of other agents (VDL07). Using the primers listed in Table 1, an mPCR was developed using the same protocol as for VDL05 for the detection of *C. trachomatis*, *T. vaginalis*, *T. pallidum*, and group B streptococci.

Measurement of sensitivity and specificity. As an adequate number of positive controls (cultures or proven positives) were not available for most agents, the sensitivities of the assays were assessed by measuring the limit of detection of plasmid constructs of the target sites for each agent. Briefly, amplification products from first-round (or single-round) reactions were cloned using the pGEM-T Easy Vector System II (Promega), and constructs were extracted using the Wizard PCR Preps DNA purification system (Promega). The genomic concentration was measured using the NanoDrop Spectrophotometer (NanoDrop Technologies). The limit of detection for each target site was defined as the lowest dilution of a series of serially diluted (1:10) plasmid constructs that was amplified by each assay. The specificity was determined by testing proven-negative samples.

Controls. Positive controls for the above-mentioned assays were derived from either culture-proven or molecularly proven sources. Amplification of the glyceraldehyde-3-phosphate dehydrogenase gene from all samples using previously described primers (38) was performed to validate extraction and PCRs.

RESULTS

The limit of detection (Table 1) for each agent ranged from 10 (for *T. vaginalis*) to 10^5 (for *T. pallidum*) copies per reaction. False positives were not detected when between 17 and 96 proven-negative control samples of each agent were tested.

The results of the screening of 233 cervical swabs from 175 women by the four mPCRs are shown in Table 2. The total agents detected among the 175 participating women at the initial and subsequent visits are also shown. The *Mollicutes* were the most common group of organisms detected, and were recovered from 159 (68.2%) of the 233 cervical swabs tested. Either alone or in combination with another member of the *Mollicutes*, *U. parvum* was the species most commonly detected (57.0%), followed by *M. hominis* (13.7%), *U. urealyticum* (6.1%), and *M. genitalium* (1.3%). CMV was the predominant virus detected (6.0%), followed by VZV (4.3%). The remaining viruses (EV, EBV, HSV-1, and HSV-2) were each detected in <3% of the samples, and adenoviruses (A to E) were not detected. *T. vaginalis* (4.0%) was the commonest agent detected by VDL07 (for other agents). *C. trachomatis* and group B streptococci were detected in <1% of these samples, while *T. pallidum* was not detected.

Multiple infections were detected in 42 (24.0%) of the 175 women tested. Two of these patients had multiple infections on two separate occasions. All 44 coinfections included a *Mollicutes* sp., most commonly *U. parvum* (88.6%). Of the eight patients diagnosed with trichomoniasis, six (75.0%) had coinfections with *U. parvum*. Two of these patients were also coinfected with either VZV or CMV.

DISCUSSION

mPCR assays allow simultaneous detection of multiple agents in a single reaction and were applied here to detect a broad range of microorganisms. The mPCRs developed in this study are based on those we previously described for the detection of viruses in a routine diagnostic laboratory (38) utilizing identical reagent and cycling conditions. This simplifies the workflow, allowing performance of these assays in a routine diagnostic laboratory with basic molecular facilities. The choice of a commercial master mix including a reverse transcriptase reaction benefits a busy laboratory environment where both RNA and DNA agents are being detected.

The mPCRs VDL05 and VDL07 are nested PCRs to increase sensitivity, while specificity is enhanced with a post-PCR probe hybridization assay. The adenovirus mPCR (VDL09) was limited to a single-round reaction without post-PCR probe hybridization because of variation in regions targeted by the species-specific primers (57). A single-round PCR was used to detect *M. hominis*, *M. genitalium*, *U. parvum*, and *U. urealyticum* (VDL06). The method developed by Yoshida et al. (59) was first considered for the detection of these agents. However, our evaluation of this method showed cross-reactions with the hybridization reactions for *U. parvum* (serotypes 6 and 149) and *U. urealyticum* and weak reactions for *U. parvum* with wild strains of *M. hominis* (data not shown). The mPCR employed in this study utilizes the method of Yoshida et al. for the detection of *Mycoplasma* spp. and includes specific primers for *U. parvum* (28) and *U. urealyticum* (54). These primer sets allow differentiation of the ureaplasmas by characteristic electrophoretic-band sizes, which are confirmed by probe hybridization.

CMV was the most frequent virus detected using the VDL05 mPCR. CMV is not a common cause of cervicitis in immunocompetent women (37). However, studies in China have shown detection rates of 5.1% in a prospective study of women with cervical human papillomavirus (58) and 14.0% in erosive cervicitis (44), possibly as a result of cervical carriage and reactivation by localized inflammation (36). Infection in pregnancy

may cause spontaneous abortion, and there is a significant risk of fetal infection with congenital abnormalities (3, 8, 37). In this study, CMV was detected in 6.3% of female patients tested, which is a prevalence not previously reported in Australian clinics, suggesting the need to consider routine testing in pregnant high-risk patients.

Previous studies have suggested that most genital HSV infections are caused by HSV-2 (13, 42, 43, 52). Consistent with more recent findings (6, 13, 17, 29, 56) increasingly implicating HSV-1 in genital infection, we detected HSV-1 (3.4%) more commonly than HSV-2 (1.7%) in the female patients tested. An early study in China showed detection rates in erosive cervicitis to be as high as 26.5% (44), with asymptomatic shedding potentially an important means of transmission (13). None of the 175 women in our study had genital erosions or clinical signs of acute HSV infection at the time of testing.

There have been reports of EBV-associated genital ulcers in women (2, 7, 14, 21, 25, 32, 53, 55). This condition is underrecognized and may be incorrectly attributed to HSV infection (7, 32, 53). However, the clinical relevance of our detection of EBV in 3.4% of female patients in this study has yet to be established. A recent study showed strong evidence for sexual transmission of the virus from a partner infected with infectious mononucleosis (55). In a study in Thailand of women with HSV-associated genital herpes, 17/30 (56.7%) cases were found to have EBV DNA present, although the clinical significance was not determined (25).

The presence of EV in the female genital tract may also be a predisposition to antenatal and perinatal infection (3). An early study in Russia detected antigens of coxsackie A and B virus in the vaginal secretions of 16.3% of young girls with protracted forms of vulvovaginitis (33). More recently, a study in Central Africa detected EV RNA in nearly 10% of women of childbearing age, which may be the basis for possible antenatal or perinatal transmission from mother-to-child (24). Detection of EV in 2.8% of the women in our study indicates the proportion of patients at risk, but again, the clinical relevance has yet to be determined.

The *Mollicutes* detected in this study are associated with infections of the genitourinary tract, reproductive failure, and neonatal morbidity and mortality. Our detection rates of the four species of the *Mollicutes* putatively associated with genital infection are consistent with previous studies, with *U. parvum* being the commonest (26, 45, 49). Detection of *M. genitalium* is becoming increasingly important because of recent reports of a high prevalence of the organism in women with cervicitis (15, 41, 45). Furthermore, the high prevalence of infected sexual partners supports its role as a sexually transmitted infection (15).

The VDLO7 mPCR screens organisms with larger genomes and was reduced to four detectable agents to minimize template competition. In this assay, *T. vaginalis* was the most commonly detected agent in women at 4.0% and was included, as there has been a proven advantage of molecular techniques over the insensitive traditional methods of direct visualization and wet-mount microscopy (47, 51) and Pap smear. Inclusion in this assay enabled detection of trichomoniasis, which is sexually transmissible and often asymptomatic. *T. vaginalis* is associated with pelvic inflammatory diseases and adverse birth outcomes (51) and is also linked to an increased risk of human immunodeficiency virus transmission (48). Vaginal colonization with group B streptococci is not normally symptomatic or associated with sexual transmission. However, cervical colonization is relevant to pathology of the fetus and newborn, and significant morbidity may arise if group B streptococcus is not detected and eradicated (23). *C. trachomatis* is commonly associated with cervicitis and is the most frequent cause of bacterial sexually transmitted infection worldwide (9, 15, 18, 22). The detection rate of chlamydial infections (<1%) in this study is lower than expected for this population and could be explained in part by the exclusion of women with pelvic inflammatory disease and recent antibiotic treatment from the study population. As was evident again here, syphilitic cervicitis is uncommon but is important to diagnose because infection may clinically and colposcopically simulate a primary advanced cervical cancer (19, 20). Ideally, the assay for this agent should be more sensitive and should be performed as a monoplex to increase sensitivity for high-risk patients.

Although uncommon, adenovirus has been associated with genital infections (5, 6, 50). We did not detect adenovirus in the women examined here. Recent Australian studies of men with urethritis showed that the infection is uncommon and seasonal (6).

A test for *N. gonorrhoeae* was not considered in this development because of reports of cross-reactivity in commercial and published methods with closely related strains, such as *Neisseria subflava* and *Neisseria cinerea* (16). Furthermore, the diagnosis of this pathogen is a simple and expedient process using conventional microcopy and culture techniques.

As shown here, improved screening has demonstrated higher-than-expected rates of occurrence of organisms, particularly the *Mollicutes*, in the cervices of women attending sexual-health clinics. These mPCR assays will facilitate further clarification of the significance of these organisms in genital infections, distinguishing pathogens from commensals. Ultimately, the improvement of the diagnosis of cervicitis and other genital infections will guide the use of appropriate interventions targeted against specific pathogens. Efficacious treatment of cervicitis has important implications for the reduction of gynecologic infections and risk to fetal development, for the control of sexually transmitted diseases, and for improved reproductive health at the public health level.

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