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, 139). 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* (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.
OneStep reverse transcription (RT)-PCR (Qiagen, Gemany) master mix was applied to the extracts, which were stored at 4°C before being tested within 48 h. Nucleic acid extraction and PCR amplification was performed comprising 5 min denaturation at 94°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, followed by a final extension of 7 min at 72°C, and a 4°C hold. The products were identified by probe hybridization (as described above).

### Sampling procedure.

The cervix was accessed using a sterile metal speculum of 0.5 cm, 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 0.5 ml of universal viral transport medium before extraction of the total nucleic acid using a robotic extraction machine (MagNaPure LC; Roche, Germany). Known-positive clinical samples were used as positive controls for the PCR. The adenovirus detection assay was performed comprising 20 ml of template, 0.5 ml of AmpErase (uracil N-glycosylase) (Applied Biosystems), and 0.2 ml of deoxynucleoside triphosphate mix, 102 M of each primer in a 50-ml reaction mixture of 0.5 U, 0.2 ml digoxigenin-11-dUTP (Roche, Germany), and 0.1 ml of AmpErase (uracil N-glycosylase) (Applied Biosystems), and 0.10 ml of RNase-free water, 10.0 ml of buffer, 2.0 ml of deoxycholate triphosphate mix, 2.5 ml of each primer (including primers for internal control at a final concentration of 0.5 μM) (Table 1), 2.0 ml Qiagen OneStep RT-PCR enzyme mix at a final activity of 0.5 U, 0.5 ml of digoxigenin-11-dUTP (Roche, Germany), and 10 ml 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 ml of template, 0.5 ml of AmpErase (uracil N-glycosylase) (Applied Biosystems), and 0.10 ml of deoxynucleoside triphosphate mix, 102 M of each primer was included in a 50-ml reaction mixture of 0.5 U, 0.2 ml digoxigenin-11-dUTP (Roche, Germany), and 10 ml 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.

### Detection of adenovirus species (VDL09).

A single-round mPCR using Qiagen OneStep reverse transcription (RT)-PCR (Qiagen, Germany) master mix was designed to detect U. parvum, U. urealyticum, M. genitalis, and M. hominis. This commercial master mix was used to conform to other molecular procedures currently being 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-ml reaction and consisted of 5.8 ml of RNase-free water, 10.0 ml of buffer, 2.0 ml of deoxycholate triphosphate mix, 2.5 ml of each primer (including primers for internal control at a final concentration of 0.5 μM) (Table 1), 2.0 ml Qiagen OneStep RT-PCR enzyme mix at a final activity of 0.5 U, 0.2 ml digoxigenin-11-dUTP (Roche, Germany), and 10 ml 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 (as described above).
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 (57.8%), 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 adenosviruses (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 trichomoniassis, six (75.0%) had coinfections with *U. parvum*. Two of these patients were also coinfected with either VZV or CMV.

### RESULTS

The 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 adenosivirus 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 *Mollicutes* by localized inflammation (36). Infection in pregnancy

### DISCUSSION

mPCR being detected by 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

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

<table>
<thead>
<tr>
<th>Microorganism(s)</th>
<th>mPCR</th>
<th>Total cervical swabs (n = 233)</th>
<th>Total women (n = 175)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mollicutes</em></td>
<td>VDL06</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>U. parvum</em></td>
<td></td>
<td>112 (48.0)</td>
<td>93 (53.1)</td>
</tr>
<tr>
<td><em>U. urealyticum</em></td>
<td></td>
<td>6 (2.6)</td>
<td>6 (3.4)</td>
</tr>
<tr>
<td><em>M. hominis</em></td>
<td></td>
<td>15 (6.4)</td>
<td>13 (7.4)</td>
</tr>
<tr>
<td><em>M. genitalium</em></td>
<td></td>
<td>3 (1.3)</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td><em>U. parvum + M. hominis</em></td>
<td></td>
<td>15 (6.4)</td>
<td>13 (7.4)</td>
</tr>
<tr>
<td><em>U. urealyticum + M. hominis</em></td>
<td></td>
<td>2 (0.9)</td>
<td>2 (1.1)</td>
</tr>
<tr>
<td><em>U. parvum + U. urealyticum</em></td>
<td></td>
<td>6 (2.6)</td>
<td>5 (2.9)</td>
</tr>
<tr>
<td>Viruses</td>
<td>VDL05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td></td>
<td>14 (6.0)</td>
<td>11 (6.3)</td>
</tr>
<tr>
<td>EV</td>
<td></td>
<td>5 (2.1)</td>
<td>5 (2.8)</td>
</tr>
<tr>
<td>EBV</td>
<td></td>
<td>6 (2.6)</td>
<td>6 (3.4)</td>
</tr>
<tr>
<td>HSV-1</td>
<td></td>
<td>6 (2.6)</td>
<td>6 (3.4)</td>
</tr>
<tr>
<td>HSV-2</td>
<td></td>
<td>2 (0.8)</td>
<td>2 (1.1)</td>
</tr>
<tr>
<td>VZV</td>
<td></td>
<td>10 (4.3)</td>
<td>9 (5.1)</td>
</tr>
<tr>
<td>VZV + HSV-2</td>
<td></td>
<td>1 (0.4%)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Adenovirus species</td>
<td>VDL09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, B, C, D, E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other agents</td>
<td>VDL07</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. trachomatis</em></td>
<td></td>
<td>1 (0.4)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td><em>T. vaginalis</em></td>
<td></td>
<td>8 (3.4)</td>
<td>7 (4)</td>
</tr>
<tr>
<td><em>T. pallidum</em></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group B streptococci</td>
<td></td>
<td>1 (0.4)</td>
<td>1 (0.6)</td>
</tr>
</tbody>
</table>
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). This condition is under-recognized 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 microscopy 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.

**ACKNOWLEDGMENTS**

We thank all staff at the Short Street Sexual Health Clinic, St George Hospital, for collection of samples, and we acknowledge the assistance of scientists at the Virology Diagnostic Laboratory (SEALS), Prince of Wales Hospital. Partial financial support was provided by the HARP Unit, SESIAHS, National Health and Medical Research, and Novartis. Potential conflicts of interest: J.L. was partially supported by a Novartis research scholarship.

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