JCM Accepts, published online ahead of print on 4 March 2009
J. Clin. Microbiol. doi:10.1128/JCM.01873-08

Multiplex PCR testing detected higher than expected rates of cervical *Mycoplasmas, Ureaplasmas, Trichomonas* and viral agents in sexually active Australian women.

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Keywords: Cervicitis; multiplex PCR; sexually transmitted diseases

Running heading: Detection of agents associated with cervicitis

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ABSTRACT

Knowing the prevalence of potential aetiologic agents of non-gonococcal and non-chlamydial cervicitis is important for improving 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 -1 (HSV-1) and Herpes simplex -2 (HSV-2) (VDL05); *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma genitalium*, *Mycoplasma hominis* (VDL06); *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, and group B streptococci (VDL07); and adenovirus species A-E (VDL09). The mPCRs were used to test 233 cervical swabs from 175 women attending a sexual health clinic in Sydney Australia, during 2006-2007. The agents detected alone or in combination in all cervical swabs (percentage total swabs) include: 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-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 micro-organisms 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. However the aetiology 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 prevalence of other cervical infectious agents (1, 41, 44, 45, 58). An under appreciation of the prevalence and role played by these non-gonococcal and non-chlamydial agents potentially jeopardize 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 HIV 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 detection of a range agents whose aetiologic role in genital infections needs to be further investigated, including the viruses: cytomegalovirus (CMV), herpes virus types 1 and 2 (HSV-1, -2) (4, 43), adenovirus (AV) (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 polymerase chain reaction (mPCR) assays, based on a conventional platform, for the detection of 19 microorganisms in cervical swabs including *Treponema pallidum* and *Chlamydia trachomatis*, *Trichomonas vaginalis*, group B streptococci and five adenovirus species in addition to the aforementioned. The assays were developed using cervical swabs from different women taken on one or more occasion during different visits to a sexual health clinic.

METHODS

Patients. Cervical swabs (n = 233) were taken from 175 women consecutively attending a sexual health clinic in Sydney (between one and three visits) during 2006-2007 who were all eligible for recruitment to an as yet unpublished case-control study investigating cervicitis. This included women with and without cervicitis. All women were aged ≥ 18 yrs, sexually active in the preceding three months, who required an internal examination regardless of symptoms, had not been treated with antibiotics or received gynaecologic intervention in the preceding month, did not have an intra uterine 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 women. Study protocol and data management were approved by the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee.

Sampling procedure. The cervix was accessed using a sterile metal speculum and prepared for swabbing by removing exudate with a large non-sterile swab (Multigate Medical Products, China). An initial swab of the endocervix was taken with a sterile cotton swab (Copan, CA, USA) and used to screen bacterial agents, before being placed in viral transport medium 199 (GIBCO Invitrogen, NY, USA) and stored at -70^{o}C. Two consecutive specimens of the same region were taken using viral transport swabs (Copan Diagnostics, Corona, USA), suspended in viral transport medium and stored at -70^{o}C. These latter two specimens were tested by the assays described below.

Nucleic acid extraction and PCR amplification. 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 testing within 48 hours of collection.

Detection of Mollicutes (VDL06). A single round-multiplex PCR using Qiagen OneStep RT-PCR (Qiagen, Gemany) master mix was designed to detect *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma genitalium* and *Mycoplasma hominis*. This commercial master mix was used to conform to other molecular procedures currently used in diagnostic laboratory allowing simplified quality assurance and workflow. The reactions were prepared in accordance with manufacturer's instructions for a 50µl reaction and consisted of 5.8µl of RNase-free water, 10.0µl of buffer, 2.0µl of deoxynucleoside triphosphate mix, 2.5µl of each primer (including primers for internal control at a final concentration = 0.5µM) (Table 1), 2.0µl Qiagen OneStep RT-PCR enzyme mix at a final activity = 0.5U, 0.2µl digoxigenin-11-dUTP (Roche, Germany), and 10µl of template. Cycling procedures include a reverse transcription step at 50°C for 30 min; denaturation at 95°C for 15 min; then 50 cycles of: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for one min; a final extension of 7 min at 72°C; and a 4°C hold. Products were visualized by gel electrophoresis and amplicons identified by probe-hybridization followed by PCR ELISA (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 and 0.5 µl of AmpErase (uracil N-glycosylase) (Applied Biosystems), 0.10 µM of each primer (38) was included in a 50 µl reaction mixture of 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 (digoxigenin) (Roche, Germany), 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). Products were visualised by gel electrophoresis and amplicons identified by probe-hybridisation (as above).

Detection of adenovirus species A-E (VDL09). This method is based on that previously described by Xu et al. (56) and was modified to conform to the above 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 Biosystems). The thermocycling conditions included a preliminary denaturation step at 94^{o}C for 5 min; 30 cycles of amplification (denaturation at 94^{o}C for one min, annealing at 54^{o}C for 45 sec, extension at 72^{o}C for 2 min); and a final extension at 72^{o}C for 5 min. Products were visualized using gel electrophoresis.

Detection of other agents (VDL07). Using the primers listed in Table 1, a multiplex PCR was developed using the same protocol as for VDL05 for the detection of *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema 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 sensitivity of the assays was assessed by measuring the limit of detection of plasmid constructs of the target sites for each agent. Briefly, amplification products from first (or single) round reactions were cloned using pGEM-T Easy Vector System II (Promega) and constructs extracted using the Wizard PCR Preps DNA purification system (Promega). Genomic concentration was measured using the NanoDrop® Spectrophotmeter (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. Specificity was determined by testing proven-negative samples.

Controls. Positive controls for the above assays were derived from either culture- or molecular-proven sources. Amplification from all samples of the gene Glyceraldehyde-3-phosphate dehydrogenase using primers previous described (38) was applied to validate extraction and PCR reactions.

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 testing between 17 and 96 proven-negative control samples of each agent.

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

VDL07 (for other agents). *Chlamydia trachomatis* and Group B streptococci were detected in < 1% of these samples whilst *T. pallidum* was not detected.

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

DISCUSSION

Multiplex PCR assays allow simultaneous detection of multiple agents in a single reaction and have been applied here to detect a broader range of micro-organisms. 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 workflow allowing performance of these assays in a routine diagnostic laboratory with basic molecular facilities. The choice of a commercial mastermix inclusive of 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 whilst 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 reaction was used to detect *M. hominis*, *M. genitalium*, *U. parvum*, and *U. urealyticum* (VDL06). The

method developed by Yoshida et al. (2003) was first considered for the detection of these agents. However, our evaluation of this method showed cross-reactions with the hybridisation 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 the *U. parvum* (28) and *U. urealyticum* (54). The latter primer sets allow for differentiation of the ureaplasmas by characteristic electrophoretic band sizes which are confirmed by probe hybridization.

Cytomegalovirus was the most frequent virus detected using the VDL05 mPCR. Cytomegalovirus is not a common cause of cervicitis in immunocompetent women (37). However, studies in China shown detection rates of 5.1% in a prospective study of women with cervical human papilloma virus (HPV) (58) and 14.0% in erosive cervicitis (44) possibly as a result of cervical carriage and reactivation by localised 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 and is a prevalence not previously reported in Australian clinics, suggesting the need for considering routine testing in pregnant high risk patients.

Previous studies have suggested most genital HSV infections are caused by HSV type 2 (13, 42, 43, 52). Consistent with more recent findings increasingly implicating HSV 1(6, 13, 17, 29, 56) 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 cause 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 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 is 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 clinical significance was not determined (25).

The presence of enterovirus 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 in the vaginal secretions of 16.3% young girls with protracted forms of vulvovaginitis (33). More recently, a study in Central Africa detected enterovirus RNA in nearly 10% of childbearing-aged women and may be the basis of possible antenatal or perinatal transmission from mother-to-child (24). Detection of enterovirus in 2.8% women in our study indicates the proportion of patients at risk, but again clinical relevance is 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 high prevalence of this 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 a larger genome and was reduced to four detectable agents to minimize template competition. In this assay, *T. vaginalis* was the most commonly detected agent in women 4.0%. and was included as there has been 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 enables detection of trichomonas which is sexually transmissible and often asymptomatic. *Trichomonas vaginalis* is associated with pelvic inflammatory diseases, adverse birth outcomes (51) and is also linked to the increased risk of HIV 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 where significant morbidity may arise if Group B Strep is not detected and eradicated (23). *Chlamydia trachomatis* is commonly associated with cervicitis and 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 then expected for this population and could in part be explained by the exclusion of women with PID and recent antibiotic treatment in this study population. Again evident 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 in men with urethritis show 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 cinera* (16). Further, the diagnosis of this pathogen is a simple and expedient process using conventional microcopy and culture techniques.

As shown, improved screening has demonstrated higher than expected rates of organisms, particularly the Mollicutes, in the cervices of women attending sexual health clinics. These mPCR assays will facilitate further clarificaion of the significance of these organisms in genital infections, distinguishing pathogens from commensals. Ultimately, the improvement of 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, risk to fetal development, control of sexually transmitted diseases and improved reproductive health at the public health level.

ACKNOWLEDGEMENTS

We thank all staff at the Short Street Sexual Health Clinic, St George Hospital for collection of samples; and assistance by scientists at the Virology Diagnostic Laboratory (SEALS), Prince of Wales Hospital.

Financial support. Partial support provided by HARP Unit, SESIAHS, National Health and Medical Research and Novartis.

Potential conflicts of interest. JL was partially supported by Novartis research scholarship.

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TABLE 1. Oligonucleotides used in molecular detection methods.

Agent (reference)	Oligo[a]	Code	Oligonucleotide sequence (5' - 3')	Target[b]	LD[c]
Multiplex for *Mollicutes* (VDL06)					
Urea- and Mycoplasma	A	My-ins	GTA ATA CAT AGG TCG CAA GCG TTA TC	16S tRNA gene	
(Yoshida et al. [59])	B	MGSO-2-Bi	CAC CAT CTG TCA CTC TGT TAA CCT C		
Mycoplasma hominis	P	Mhom-P10-Am	GAC ACT AGC AAA CTA GAG TTA G		10^3
Mycoplasma genitalium	P	Mgen-P3-Am	TCG GAG CGA TCC CTT CGG T		10^3
Ureaplasma parvum	A	UMS-57	(T/C)AA ATC TTA GTG TTC ATA TTT TTT AC	Multiple	
(Kong et al. [28])	B	UMA222	GTA AGT GCA GCA TTA AAT TCA ATG	banded antigen	
	P	UP-PROBE1	CTG AGC TAT GAC ATT AGG AGT TAC C	gene (326-327)[d]	10^3
Ureaplasma urealyticum	A	UMS-170	GTA TTT GCA ATC TTT ATA TGT TTT CG	Multiple	
(Teng et al. [54])	B	UMA263	TTT GTT GTT GCG TTT TCT G	banded antigen	
	P	UU-PROBE1	CTG AAT TCA ATG TTG CAA TTA CAT CAG CTG A	Gene (476)	10^3
Multiplex PCR for non-viral agents (VDL07)					
Chlamydia trachomatis	A	CT-OF	TTG CAA GCT CTG CCT GTG GGG AAT *	Omp1	
	B	CT-OR	TCA CAT CGC CAG CTC CAG CAA TAG *	(931/378)	
	C	CT-IF	ACA TTA GGA GCC ACC AGT GGA TAT C *		
	D	CT-IR	ATC CTT AGT TCC TGT CGC AGC ATC T *		
	P	CT-PROBE	TGCGTGGAGCGTCGGCGCTCGCGCA *		10^2

Continued on next page

TABLE 1-*Continued*

Agent (reference)	Oligo.[a]	Code	Oligonucleotide sequence (5' - 3')	Target[b]	LD[c]
Trichomonas vaginalis	A	TricV-OF	CTA TTG TCG AAC ATT GGT CTT ACC CTC	G3	
(Lawing, L.F et al. [31])	B	TricV-OR	TCT GTG CCG TCT TCA AGT ATG CCC	(264/206)	
	C	TricV-IF	CTC AGT TCG CAA AGG CAG TCC TTG A *		
	D	TricV-IR	GCT TGG AGA GGA CAT GAA CTT CGG A *		
	P	TricV-PROBE	CTACAACAAATTCTTCTCC*		10^1
Treponema pallidum	A	TP7-OF	CTC AGC ACT GCT GAG CGT AG	*bmp* gene	
(Noordhoek et al. [39])	B	TP8-OR	AAC GCC TCC ATC GTC AGA CC	(616/506)	
	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 *	*scpB*	
(Dimitriev A. et al. [12])	B	STB-OR	GGT CAA CCT TCT CGT ACT CTA GAG AAA*	(418/260)	
	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*		10^2
Adenovirus species-specific multiplex (VDL09)					
(Xu et al. [57])					
Adenovirus sp. A	A	AdA1	GCTGAAGAAMCWGAAGAAAATGA	Fiber	
	B	AdA2	CRTTTGGTCTAGGGTAAGCAC	(1444-1537)	10^3
Adenovirus sp. B	A	AdB1	TSTACCCYTATGAAGATGAAAGC	Fiber	
	B	AdB2	GGATAAGCTGTAGTRCTKGGCAT	(670-772)	10^3
Adenovirus sp. C	A	AdC1	TATTCAGCATCACCTCCTTTCC	Fiber	
	B	AdC2	AAGCTATGTGGTGGTGGGGC	(1988-2000)	10^4

Continued on next page

TABLE 1-*Continued*

Agent (reference)	Function[a]	Code	Oligonucleotide sequence (5' - 3')	Target[b]	LD[c]
Adenovirus sp. D	A	AdD1	GATGTCAAATTCCTGGTCCAC	Fiber	
	B	AdD2	TACCCGTGCTGGTGTAAAAATC	(1205-1221)	10^4
Adenovirus sp. E	A	AdE1	TCCCTACGATGCAGACAACG	Fiber	
	B	AdE2	AGTGCCATCTATGCTATCTCC	(967)	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] Limit of detection (copies per reaction).

[d] 326 bp for *U. parvum* serovars 1 and 3/14 and 327 for serovar 6 (Kong et al. [28])

* Oligonucleotides designed by Nikolas Rismanto

Note: Underlined sequences are modifications of published primers cited.

TABLE 2 Use of mPCRs for screening non-gonococcal agents in cervical swabs.

Micro-organism(s)	No. (%) detected	
	Total cervical swabs (*n* = 233)	Total women (*n* =175)
***Mollicutes* (VDL06)**		
Ureaplasma parvum	112 (48.0)	93 (53.1)
Ureaplasma urealyticum	6 (2.6)	6 (3.4)
Mycoplasma hominis	15 (6.4)	13 (7.4)
Mycoplasma 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)		
Cytomegalovirus	14 (6.0)	11 (6.3)
Enterovirus	5 (2.1)	5 (2.8)
Epstein Barr virus	6 (2.6)	6 (3.4)
Herpes simplex virus – 1	6 (2.6)	6 (3.4)
Herpes simplex virus – 2	2 (0.8)	2 (1.1)
Varicella-zoster virus	10 (4.3)	9 (5.1)
Varicella-zoster virus + Herpes simplex virus – 2	1 (0.4%)	1 (0.6)
Adenovirus species (VDL09)		
Species: A, B, C, D, E	0	0
Other agents (VDL07)		
Chlamydia trachomatis	1 (0.4)	1 (0.6)
Trichomonas vaginalis	8 (3.4)	7 (4)
Treponema pallidum	0	0
Group B Streptococci	1 (0.4)	1 (0.6)