Development of Multiplex PCRs for Detection of Common Viral Pathogens and Agents of Congenital Infections

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Potential causes of congenital infection include *Toxoplasma gondii* and viruses such as cytomegalovirus (CMV), enterovirus, hepatitis C virus, herpes simplex virus types 1 and 2 (HSV-1 and -2), human herpesvirus types 6, 7, and 8, lymphocytic choriomeningitis virus, parvovirus, rubella virus, and varicella-zoster virus. Testing for each of these agents using nucleic acid tests is time consuming and the availability of clinical samples such as amniotic fluid or neonatal blood is often limited. The aim of this study was to develop multiplex PCRs (mPCRs) for detection of DNA and RNA agents in the investigation of congenital infection and an mPCR for the viruses most commonly requested in a diagnostic virology laboratory (CMV, Epstein-Barr virus, enterovirus, HSV-1, HSV-2, and varicella-zoster virus). The assays were assessed using known pathogen-positive tissues (cultures, placenta, plasma, and amniotic fluid) and limits of detection were determined for all the agents studied using serial dilutions of plasmid targets. Nested PCR was performed as the most sensitive assay currently available, and detection of the amplicons using hybridization to labeled probes and enzyme-linked immunosorbent assay detection was incorporated into three of the four assays. This allowed detection of 10 to 10² copies of each agent in the samples processed. In several patients, an unexpected infection was diagnosed, including a case of encephalitis where HSV was the initial clinical suspicion but CMV was detected. In the majority of these cases the alternative agent could be confirmed using reference culture, serology, or fluorescence methods and was of relevance to clinical care of the patient. The methods described here provide useful techniques for diagnosing congenital infections and a paradigm for assessment of new multiplex PCRs for use in the diagnostic laboratory.

Nucleic acid testing has allowed more sensitive and specific detection of infectious agents and is being increasingly adopted by diagnostic laboratories. The technology is particularly useful in virology as it can replace conventional culture methods that are often expensive and labor intensive, detect fastidious organisms such as hepatitis C virus (HCV), detect low-copy-number agents such as herpes simplex virus (HSV) in cerebrospinal fluid, and improve turn-around times for detection of treatable agents such as herpesviruses (30, 42, 48, 50). In the clinical and diagnostic setting, accurate and rapid diagnosis of the causative agent of disease is paramount. Testing for various agents using multiple primer sets in multiplex PCR (mPCR) reactions is an innovation that offers significant benefits in costs, time and accurate diagnosis (20, 35). Furthermore, for any given clinical syndrome there a number of candidate agents that may be implicated, particularly with regard to congenital infection.

In the diagnostic setting, standardization of assays, use of quality controlled (usually commercially available) reagents, extensive validation of the assays used, and sensitive detection using standard techniques are all required. Standardization of

PCRs with improved ease of use has resulted from the availability of commercial master mixes that include hot-start *Taq* polymerase and novel formulations to enhance amplification (20). These properties were utilized in the development of applied mPCRs that are simple to prepare and can be validated and individualized for the clinical situation to maximize efficacy for diagnostic use (12, 29, 42, 43, 48).

There is wide range of putative agents implicated in congenital infections (4, 8, 24, 25, 33, 34) and clinical samples such as amniotic fluid and neonatal blood may be limited. The aim of this study was to develop and validate mPCRs that would facilitate this testing. Three nested mPCRs were designed to detect the majority of agents commonly associated with congenital infections (VDL01, VDL03, and VDL04). In addition, a generic nested mPCR was developed for the detection of six viruses commonly tested in a routine diagnostic laboratory (VDL05). As far as possible, identical preparation and conditions were employed to minimize complexity, facilitate use in a high volume, routine diagnostic laboratory, and allow rapid design and implementation of additional mPCR tests.

MATERIALS AND METHODS

Samples. Amniotic fluid collected during the first trimester of pregnancy, placenta, culture isolates, and clinical specimens were provided by the Virology Diagnostic and Research Laboratories of the Microbiology Department (South Eastern Area Laboratory Services), Prince of Wales Hospital. Ethics committee

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approval and patient consent was obtained for the examination of amniotic fluid and placenta. The presence of infectious agents was previously determined by one or more other methods including culture in MRC5 (human embryonic lung tissue) and LLCMK2 (monkey kidney tissue cells); serology: Biotrin parvovirus B19 immunoglobulin G (IgG) enzyme immunoassay (Biotrin International, Ireland), Biotrin parvovirus B19 IgM enzyme immunoassay (Biotrin International, Ireland); or monoplex nucleic acid testing: Cobas HCV Amplicor Monitor 2.0 (Roche) and CMV Oligodetect (Light Diagnostics) and developmental mPCRs (described below).

Extraction. DNA and RNA were extracted from amniotic fluid using the MiniElute viral spin kit (QIAGEN) following manufacturer's instructions. Phenol-chloroform extraction methods based on those of Chomczynski and Sacchi (15) and Sambrook et al. (45) were used to extract RNA and DNA from placenta, respectively. Extractions from cultures and clinical samples were performed using High Pure viral nucleic acid kit (Roche, Germany) and COBAS HCV extraction Amplicor Monitor (Roche, Germany), and using semiautomated extraction on robots (MagnaPure, Roche, Germany, or BioRobot M8, QIAGEN, Germany) as indicated in Table 2. Extracts were stored at -20°C for less than 1 month before testing.

Developmental multiplexes for congenital diseases. Three mPCRs designated VRL01, VRL03, and VRL04 were developed by the Virology Research Laboratory of this department for systematic screening of amniotic fluid and placenta for congenital diseases. VRL01 was designed as a screen for DNA agents including *Toxoplasma gondii*, herpes simplex virus types 1 and 2 (HSV-1 and -2), cytomegalovirus (CMV), parvovirus, and varicella-zoster virus; VRL03 for CMV, human herpesvirus (HHV)-6, HHV-7, and HHV-8; and VRL04 for RNA viruses: lymphocytic choriomeningitis virus, rubella virus, hepatitis C virus (HCV), and enterovirus.

Unless indicated the primers used were derived from previous publications (Table 1). The potential cross-reactivity of the oligonucleotides and target specificity was elucidated using the basic local alignment search tool (BLAST) program on the National Centre for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>) (38).

For the VRL01 array (DNA agents) the final PCR mixture for a 50- μl reaction (first and second rounds) contained $1\times$ Taq buffer (Promega), 2 mM MgCl_2 (Promega), 0.2 mM deoxynucleoside triphosphates, 0.20 μM of each primer (*T. gondii*, HSV, CMV, parvovirus, varicella-zoster virus) (outer and inner sense), 1.5 U Taq polymerase (Promega), and 5 μl of extracted template or first-round product. First-round amplification conditions included denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 min; 7 min final extension at 72°C ; and a 4°C hold. The second-round conditions included denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds; 7 min final extension at 72°C ; and a 4°C hold.

The final PCR mixture for VRL03 array (herpes viruses) in a 50- μl reaction included $1\times$ Taq buffer (Promega); 3.5 mM MgCl_2 (Promega); 2 mM deoxynucleoside triphosphate mixture; 0.16 μM of CMV and HHV-8 primers, 0.30 μM HHV-6 and 0.20 μM HHV-7 primers (outer and inner sense); 2.5 U Taq polymerase (Promega); and 4 μl of template (both rounds). Amplification conditions for both rounds were the same and included denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 seconds, 58°C for 40 seconds, and 72°C for 50 seconds; 3 min final extension at 72°C ; and a 4°C hold.

The final reverse transcription (RT)-PCR mixture (first round) for the VRL04 array (RNA viruses) in a 50- μl reaction contained $1\times$ Taq buffer (Promega); 2 mM MgCl_2 (Promega), 0.4 mM deoxynucleoside triphosphates; 0.01 mM dithiothreitol; 0.40 μM of rubella virus, hepatitis C virus, and enterovirus primers, and 0.80 μM of lymphocytic choriomeningitis virus primers (outer and inner sense); 3 U avian myeloblastosis virus reverse transcriptase (Promega); 1.5 U Taq polymerase (Promega); and 10 μl of extracted template. Amplification conditions included a reverse transcription step at 42°C for 40 min; denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; 7 min final extension at 72°C ; and a 4°C hold. The composition of the second-round PCR for VRL04 array was the same as for the VRL01 array using the same primer concentrations (above) and 5 μl of first-round product was used (Promega). Amplification conditions included denaturation at 94°C for 2 min; followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; 7 min final extension at 72°C ; and a 4°C hold.

Glyceraldehyde-3-phosphate dehydrogenase PCR detection was used to validate extraction and was performed in parallel to mPCR. The glyceraldehyde-3-phosphate dehydrogenase primers were not included in the mPCR because of observed interference with reaction kinetics and cross-reaction.

Amplicons for the above methods were detected by electrophoresis.

Applied multiplexes. The above multiplexes (VRL01, VRL03, and VRL04) were modified for application as a screening tool in a diagnostic laboratory and are designated VDL01, VDL03, and VDL04, respectively. Modifications included using commercial master mixes, different primer concentrations and cycling conditions. The modified Herpes virus mPCR (VDL03) did not include detection of CMV. In addition, a multiplex designated VDL05 was developed for the detection of viral agents most commonly requested in our diagnostic laboratory, based upon review of 6 years testing (data not shown): HSV-1, HSV-2, CMV, varicella-zoster virus, Epstein-Barr virus, and enterovirus.

The same primers for each agent above were used in these modified mPCRs using the following concentrations of each outer and inner sense primer in a final PCR volume of 50 μl : VDL01: 0.10 μM of each *T. gondii*, HSV, CMV, parvovirus, and varicella-zoster virus; VDL03: 0.38 μM HHV-6, 0.25 μM HHV-7, and 0.2 μM HHV-8; VDL04: 0.13 μM of rubella virus, hepatitis C virus, and enterovirus, and 0.25 μM of lymphocytic choriomeningitis virus; and VDL05: 0.10 μM of each (HSV, CMV, varicella-zoster virus, Epstein-Barr virus, and enterovirus). The composition of the master mixes and the cycling conditions for the first and second-round reactions for each of the applied multiplexes are identical.

The first-round amplification reaction for all mPCRs utilized the QIAGEN OneStep RT-PCR kit (QIAGEN) as the master mix for reverse transcription and PCR amplification. The use of a common master mix for RNA and DNA agents simplifies the procedures for a diagnostic laboratory. The reaction components were prepared in accordance with the manufacturer's instructions for a 50 μl reaction and consisted of 10.5 μl of RNase-free water, 10.0 μl of buffer, 2.0 μl of deoxynucleoside triphosphate mix, 5 μl of primer mix, 2.0 μl QIAGEN OneStep RT-PCR enzyme mix, 0.5 μl AmpErase (uracil N-glycosylase) (Applied Biosystems), and 20 μl of template. Cycling conditions included a reverse transcription step at 50°C for 30 min; denaturation at 95°C for 15 min; then 35 cycles of 94°C for 45 seconds and 57°C for 45 seconds; and 72°C for one min; 7-min final extension at 72°C ; and a 4°C hold. A culture of enterovirus virus was included in each VDL04 run to control the reverse transcription step.

The second-round master mix (50 μl) comprised of 17.8 μl RNase-free water, 25 μl of AmpliTaq Gold PCR Master Mix (Applied Biosystems), 5 μl of primer mix, 0.2 μl Digoxigenin-11-dUTP (digoxigenin) (Roche, Germany), and 2 μl of first-round product. PCR was performed with denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 20 seconds, 57°C for 20 seconds and 72°C for 20 seconds; 7 min final extension at 72°C ; and a 4°C hold.

Plasmid controls for applied multiplexes. Plasmid constructs of the target genes were used as reaction controls and to measure the limit of detection for each agent. First-round amplification products (Table 1) were separately cloned using the pGEM-T Easy Vector System II (Promega) and constructs extracted using the Wizard PCR Preps DNA purification system (Promega). Genomic concentration was calculated using absorbance measurements at 260 nM (γ) (Beckman Du, National Technologies Laboratories). Sequences were verified on an ABI 3730 DNA capillary sequencer using the ABI PRISM Big Dye kit (Perkin-Elmer) and elucidated using NCBI BLAST (as above). The limit of detection for each target was defined as the lowest dilution detected of a series of serially diluted (1:10) plasmid constructs of the target sequence.

Plasma samples spiked with plasmid constructs were used as qualitative positive controls (high range) for the agents found rarely in clinical samples (HHV-6, HHV-7, HHV-8, lymphocytic choriomeningitis virus, and rubella virus) and two common agents (hepatitis C virus and enterovirus). Cultures of JM109 bacterial cells containing plasmid constructs (above) were grown overnight on horse blood agar plates (Oxoid, United Kingdom) (37°C) and suspended in 0.85% saline to give an absorbance of 0.75 at 640 nM (γ) in a Spectronic 20 spectrophotometer (Milton Roy). This suspension is equivalent to 10^8 to 10^9 CFU/ml (36) of which 125 μl of two different clone suspensions (including either enterovirus or hepatitis C virus) were added to 400 μl of plasma (Red Cross blood donation) before extraction. Doubling dilutions of plasmid constructs in different plasma were also prepared to test a low range of 10^3 to 10^5 copies/reaction of either HHV-6, HHV-7, HHV-8, lymphocytic choriomeningitis virus, rubella virus, hepatitis C virus, or enterovirus.

Amplicon detection for applied multiplexes. Products were visualized by gel electrophoresis. Additionally, the remainder of the reaction (45 μl) from the VDL01, VDL04 and VDL05 mPCRs was used to confirm the identity of amplicons by hybridization with biotin-labeled oligonucleotide probes (Proligo, Australia) (Table 1), followed by enzyme-linked immunosorbent assay (ELISA) detection of the digoxigenin-labeled products using PCR ELISA (digoxigenin detection) (Roche, Germany). The manufacturer's instructions for the detection reaction were modified by using Amplicor wash buffer (Roche, Germany) and 3,3',5,5'-tetramethylbenzidine (Sigma) to substitute the wash buffer and horseradish peroxidase substrate provided, respectively. These changes were found to

TABLE 1. Oligonucleotides used in mPCRs

Agent (reference)	Function ^a	Code	Oligonucleotide sequence (5'-3')	Target products (bp) ^b
Cytomegalovirus (26)	A	CMVOF	AAG GTT CGA GTG GAC ATG GT ^c	Major intermediate early region (396/229)
	B	CMVOR	CAG CCA TTG GTG GTC TTA GG ^c	
	C	CMVIF	GAG CCT TTC GAG GAG ATG AA	
	D	CMVIR	GGC TGA GTT CTT GGT AAA GA	
	P		AGG CCC GTG CTA AAA AGG ATG	
Enterovirus (55)	A	EV1	CAA GCA CTT CTG TTT CCC CGG	5' untranslated region (479/156)
	B	EV4	CAC YGG ATG GCC AAT CCA A ^c	
	C	EV2	TCC TCC GGC CCC TGA ATG CG	
	D	EV3	ATT GTC ACC ATA AGC AGC CA	
	P		AAA CAC GGA CAC CCA AAG TA	
Epstein-Barr virus (37)	A	p23-1	ATC AGA AAT TTG CAC TTT CTT TGC	<i>BLRF2</i> gene (482/363)
	B	p23-2	CAG CTC CAC GCA AAG TCA GAT TG	
	C	p23-3	TTG ACA TGA GCA TGG AAG AC	
	D	p23-4	CTC GTG GTC GTG TTC CCT CAC	
	P		AAC TAC CCG CAA TGA AAT GG ^c	
Hepatitis C virus (51)	A	Hep 14 (KY80)	GCA GAA AGC GTC TAG CCA TGG CGT	5' untranslated region (260/217)
	B	Hep 15 (KY78)	CTC GCA AGC ACC CTA TCA GGC AGT	
	C	Hep 21b	GAG TGT CGT RCA GCC TCC AGG	
	D	Hep 15	As above	
	P		CAG ACC ACT ATG GCT CTC CC ^c	
Herpes simplex viruses 1 and 2 (42)	A	HSVOF	ATC CGA ACG CAG CCC CGC TG	Glycoprotein D (382/280)
	B	HSVOR	TCC GG(G/C) GGC AGC AGG GTG CT	
	C	HSVIF	GCG CCG TCA GCG AGG ATA AC	
	D	HSVIR	AGC TGT ATA (G/C)GG CGA CGG TG	
	P	(HSV-1)	AAG GGC TCC TGT AAG TAC ACC C	
	P	(HSV-2)	CGC GCC TCC TGC AAG TAC GCT C	
Human herpesvirus type 6	A	H6-1216	GCA CTG CTG TTC TCC AAA GT ^c	Immediate early protein (808/562 (variant A) and 808/911 (variant B))
	B	H6-2353	AGG AGT GAC CTC TGG TGG TG ^c	
	C	HHV6is	ATG ATG ATA CTG GTT TGA TTA ^c	
	D	HHV6ias	ATC AGT TTC ATC ATT GTT ATC ^c	
Human herpesvirus type 7 (53)	A	H73132	CCT GGT TTC GCT GCG ACT TT ^c	Major capsid protein (U57 region) (700/408)
	B	H73918	ATC AGT GTC GCT GTC CAG GC ^c	
	C	H73315	AGT TCC AGC ACT GCA ATC G	
	D	H73703	CAC AAA AGC GTC GCT ATC AA	
Human herpesvirus type 8 (13, 31)	A	HHV8EF	CCA GCT AGC AGT GCT ACC CCC ATT	Glycoprotein M (300/233)
	B	HHV8ER	ATG GAC AGA TCG TCA AGC ACT CGC	
	C	KS1	AGC CGA AAG GAT TCC ACC AT	
	D	KS2	TCC GTG TTG TCT AGT CCA G	
Lymphocytic chorio-meningitis virus	A	LCMVOF	TGC AGT CCA TGA GTG CAC AGT ^c	Nucleoprotein (644/457)
	B	LCMVOR	AGG TGA AGG RTG GCC ATA CAT ^c	
	C	LCMVIF	CCA GCC ATA TTT GTC CCA CAC ^c	
	D	LCMVIR	GCC AGY GCC TGG AGC AGC AGG ^c	
	P		ACT CCA AGT ACT CAC ACG GCA TG ^c	
Parvovirus B19 (54)	A	PVOF	AAG GAA GTT TGC CGG AAG TT ^c	Viral particle-1 (556/104)
	B	PVOR	TCC TGA CCT TGC CCT AAC AC ^c	
	C	PVIF	CAA AAG CAT GTG GAG TGA GG	
	D	PVOR	CCT TAT AAT GGT GCT CTG GG	
	P		GGA AAA TGT ACA AGT TAC AGA GTT AGC ^c	
Rubella virus (19)	A	DL.RUBa	TGC TTT GCC CCA TGG GAC CTC GAG	E1 and E2 groups (321/276)
	B	DL.RUBb	GGC GAA CAC GCT CAT CAC GGT	
	C	DL.RUBc	CGA GAT CCC CAC TGA TGT CT ^c	
	D	DL.RUBd	GGC GAA CAC GCT CAT CAC GGT ^c	
	P		TGG CAC ACA GCG CGC GTG CAC CTT CT ^c	
<i>Toxoplasma gondii</i> (22)	A	TGOF	CAG ATG TGC TAA AGG CGT CA ^c	B1 gene (445/131)
	B	TGOR	ATT GCC GCA CGA TAC TAG GT ^c	
	C	TGIF	TGA AGA GAG GAA ACA GGT GGT CG	

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TABLE 1—*Continued*

Agent (reference)	Function ^a	Code	Oligonucleotide sequence (5'-3')	Target products (bp) ^b
	D	TGIR	CCG CCT CCT TCG TCC GTC GTA	
	P		GCA AGA GAA GTA TTT GAG GTC A ^c	
Varicella-zoster virus (33)	A	VZVOF	ACG GGT CTT GCC GGA GCT GGT	Open reading frame 29 (272/208)
	B	VZVOR	AAT GCC GTG ACC ACC AAG TAT AAT	
	C	VZVIF	ACC TTA AAA CTC ACT ACC AGT	
	D	VZVIR	CTA ATC CAA GGC GGG TGC AT	
	P		GAG AAC GGT TTG GGT TTT CA ^c	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	A	HKOF	GAC CCC TTC ATT GAC CTC AAC ^c	GAPDH (804/323)
	B	HKOR	CAA AGT TGT CAT GGA TGA CC ^c	
	C	HKIF	CCA TGG AGA AGG CTG GGG ^c	
	D	HKIR	CTA AGC AGT TGG TGG TGC AG ^c	

^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 Sequence was designed in-house.

consistently offer clear distinction between blank and positive control absorbance values (data not shown).

Confirmatory tests for applied multiplexes. Results of other tests that may have been performed on mPCR-positive clinical samples were collated. Such tests include: culture (as above), enterovirus direct fluorescent antibody panels (Light Diagnostics), HSV 1/HSV 2 direct specimen direct fluorescent antibody (Trinity Biotech, Ireland), Merifluor VZV direct fluorescent antibody (Meridian), Cobas CMV Amplicor Monitor (Roche, Germany), CMV IgG AXSYM System (Abbott, Germany), CMV IgM ELISA (DiaSorin, Italy), HerpeSelect 1 ELISA IgG (Focus Technologies), HerpeSelect 2 ELISA IgG (Focus Technologies), herpes simplex 1 and 2 IgM ELISA (Diagnostic Systems Laboratories), parvovirus (as above), Enzygnost anti-varicella-zoster virus/IgG (Dade Behring), and Enzygnost anti-varicella-zoster virus/IgM (Dade Behring).

Statistics. Sensitivity is defined as the ability of the mPCR to give a positive finding for samples previously established as positive by an alternate method. Similarly, specificity is the reproducibility of an established negative finding. Both measures are expressed as percentages and for values less than 100%, estimation of the population parameter (95% confidence interval [CI]) were calculated using a method for proportions (GraphPad InStat).

RESULTS

Screening of congenital agents using developmental multiplexes. Infectious agents were not detected in 191 amniotic fluid samples screened by the developmental mPCRs with VRL01 (DNA) and VRL04 (RNA) arrays. Furthermore, herpes viruses (CMV and HHV-6, -7, and -8) were not detected in 15 of placentae screened using the VRL03 mPCR. Samples with sufficient volume remaining were used for the assessment of the applied mPCRs.

Assessment of applied multiplexes. A summary of the assessment of the four mPCRs: VDL01, VDL03, VDL04, and VDL05 is shown in Table 2. For all agents tested, the multiplexes showed a sensitivity of $\geq 95\%$ or a 95% CI that includes values in this range, and a specificity of 100%. Plasmid constructs for the four mPCRs diluted from 0.2 to 200 000 copies per reaction (10^0 to 10^6 copies/ml) showed a limit of detection range from 2 to 200 copies per reaction (10^1 to 10^3 copies/ml). All cultures and spiked samples were positively identified using multiplexes. A notable discrepancy was observed for CMV detection in VDL01 where only 78% of PCR-proven CMV-positive samples were detected. In contrast, a higher sensitivity was observed for CMV detection using VDL05 when different extracts were used than those for VDL01 evaluation.

During the post-PCR probe detection stage, several isolates

of enterovirus cross-reacted with the HSV-1 probe (data not shown). Furthermore, weak reactions sometimes occurred between HSV-2 probes and HSV-1 amplicons. Given these observations, probe hybridization was used only to confirm the identity of electrophoretic bands and this was particularly useful when nonspecific amplification products such as primer dimers were present.

Since this assessment, VDL01 and VDL05 have been used in our routine diagnostic laboratory for six months during 2004 to 2005 (Table 3). Using VDL01 an agent was detected in 13.5% of samples tested and 15 of 17 (88.2%) mPCR-positive specimens were confirmed by additional tests (including repeat tests on consecutive samples, culture, antigen detection by direct fluorescent antibody, CMV quantification and IgM serology). Similarly, the overall detection rate of the VDL05 was 22.2% of all samples and 35/37 (94.6%) were confirmed by additional testing (as above). During this period of diagnostic use alternative agents to that clinically suspected were detected for some cases. These episodes included six requests for HSV that were positive for CMV (a corneal ulcer swab and two cerebrospinal fluid specimens), varicella-zoster virus (a swab of facial cellulitis), and Epstein-Barr virus (two conjunctival swabs); Epstein-Barr virus detected in two specimens of bronchial washings where CMV testing was requested and one stool sample where enterovirus was requested; and four specimens where varicella-zoster virus was requested that were found to be positive for HSV-1 (swab of skin lesion) and HSV-2 (swab of skin vesicle and two blisters).

DISCUSSION

Although monoplex PCR and real-time assays have considerable benefits in targeting detection of specific organisms, they do not necessarily allow detection of the causative agent, due to the specificity of the primer sets used. Increasingly, detection of the causative agent using multiplexes in respiratory specimens (16, 50), gastrointestinal specimens (7), the eye (14), conditions causing lymphadenopathy (39), cerebrospinal fluid (11, 49) are replacing pathogen specific (versus clinical conditions specific) detection (32). Both forms of multiplex testing are useful—the VDL03 (herpesviruses) and VDL04

TABLE 2. Assessment of applied multiplex PCRs

Test	Agent	Sample type ^a	Positive samples		Negative samples		Performance		
			No. tested	No. positive	No. tested	No. negative	Sensitivity, % (95% CI)	Specificity, %	Limit of detection (copies per reaction)
DNA multiplex (VDL01)	HSV-1	Amn. fluid	0		34	34			
		Cultures	2	2	0				
		Plasma (M)	(6)	6	16 (1)	17			
		Total	8	8	51	51	100	100	20
	HSV-2	Amn. fluid	0		35	35			
		Cultures (M)	4 (1)	5	0				
		Plasma (M)	(9)	9	16 (1)	17			
		Total	14	14	52	52	100	100	20
	CMV	Amn. fluid	0		34	34			
		Cultures (M)	11 (2)	13	0				
		Plasma (M)	30 (1)	25	62 (1)	63	86.4 (73–95)		
		Total	44	38	97	97		100	2
	<i>T. gondii</i>	Amn. fluid	0		31	31			
		Cultures (M)	1 (1)	2					
		Total	2	2	31	31	100	100	2
	Parvovirus	Amn. fluid	0		35	35			
		Cultures (M)	0 (1)	1					
		Plasma (M)	(10)	10	3 (1)	4			
		IgM ⁺	28	27					
		IgG ⁺ , IgM ⁺			30	30			
		IgG ⁺ , IgM ⁺			11	11	97.4 (87–100)		
Herpesvirus multiplex (VDL03)	VZV	Amniotic fluid	0		35	35			
		Culture (M)	8 (16)	24	0	8 (1)	9		
		Total	24	24	44	44	100	100	20
	HHV-6	Amniotic fluid	0		27	27			
		Spiked plasma (high) (B)	(35)	35					
		Spiked plasma	8	8					
		Plasma	4	4					
	HHV-7	Amniotic fluid	0		27	27			
		Spiked plasma (high) (B)	(38)	38					
		Spiked plasma (low)	8	8					
		Total	46	46	27	27	100	100	200
	HHV-8	Amniotic fluid	0		27	27			
		Spiked plasma (high) (B)	(38)	38					
		Spiked plasma (low)	8	8					
		Plasma	2	2					
RNA multiplex (VDL04)	Lymphocytic choriomeningitis virus	Total	48	48	27	27	100	100	200
		Spiked plasma (high) (B)	0 (40)	40					
		Spiked plasma (low)	8	8					
		Plasma	0		20	20			
	Rubella virus	Total	48	48	20	20	100	100	20
		Spiked plasma (high) (B)	0 (40)	40					
		Spiked plasma (low)	8	8					
		Plasma	0		20	20			
	TOTAL	Total	48	48	20	20	100	100	200

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TABLE 2—*Continued*

Test	Agent	Sample type ^a	Positive samples		Negative samples		Performance		
			No. tested	No. positive	No. tested	No. negative	Sensitivity, % (95% CI)	Specificity, %	Limit of detection (copies per reaction)
Common pathogens multiplex (VDL05)	HCV	Spiked plasma (high) (B)	0 (40)	40					
		Spiked plasma (low)	8	8					
		Plasma (B)	0 (8)	8	0 (32)	32	98.4 (90–100)	100	20
		Plasma (C)	0 (8)	7	0 (15)	15			
		Total	64	63	47	47			
	Enterovirus	Spiked plasma (high) (B)	0 (40)	40					
		Spiked plasma (low)	8	8					
		Cultures	15	15					
		Plasma	0	0	32	32	100	100	20
		Total	63	63	32	32			
	CMV	Plasma (M)	34 (6)	38	12 (6)	18	95.0 (83–99)	100	20
		Total	40	38	18	18			
	Epstein-Barr virus	Plasma	6	5	13	13	83.3 (36–100)	100	2
		Total	6	5	13	13			
	Enterovirus	Plasma	31	31	29	29	100	100	20
		Total	31	31	29	29			
	HSV-1	Plasma (M)	19 (4)	23	17 (3)	20	100	100	NT
		Total	23	23	20	20			
	HSV-2	Plasma (M)	32 (4)	36	17 (2)	18	100	100	20
		Total	36	36	18	18			
	VZV	Plasma	25	25	31	31	100	100	200
		Total	25	25	31	31			

^a Samples designated plasma are clinical samples that have been previously tested by another method. Unless indicated, samples were extracted by HPA (Roche). The number extracted by an alternative method is given in parentheses. The number of samples extracted using a MagNaPure LC (total nucleic acid isolation kit) (Roche, Germany) (M), BioRobot M8 (viral isolation kit) (QIAGEN, Germany) (B), or COBAS Amplicor (Roche) (C) is shown in parentheses. Amn., amniotic.

(RNA viruses) multiplexes used here were designed to allow more efficient testing of infrequently requested agents, thereby allowing more rapid turn-around times, and more efficient testing of commonly requested agents that were determined on the basis of requesting, rather than on the basis of any common pathologies (VDL05 mPCR).

The four applied mPCRs described here were developed for use in a large routine diagnostic laboratory. Appropriate to this setting, we aimed to develop methods of simplicity, robustness and minimal complexity without compromise to sensitivity and specificity. To achieve this, we used common protocols for master mixes (for both the RNA and DNA agents), thermocycling conditions for first and second-round reactions and amplicon detection. The RT-PCR was also used for the DNA agents for conformity of methodology to reduce complexity between methods and may have enhanced sensitivity by transforming RNA transcripts, though this is speculative and was

not investigated. In VDL01, VDL04 and VDL05 mPCRS, uracil *N*-glycosylase was incorporated in the first-round reaction to lower the risk of cross contamination linked to carryover of digoxigenin labeled amplicons.

We used hot-start PCR to minimize nonspecific reactions such as primer dimers when annealing occurs during precycling temperatures (20). Furthermore, nested PCRs were used to enhance sensitivity and specificity. To improve sensitivity in specimens of low genomic content, we used 20 µl of template in a 50-µl reaction. These applied mPCRs generally showed high sensitivity when testing a variety of specimens of differing genomic content that were extracted by different methods and were either recently extracted or had been stored at –20°C for up to one month. The only exception was the sensitivity (86.4%) for CMV by VDL01 for which the limit of detection was 2 copies per reaction. It is likely that measurements of sensitivity for this virus may have been compromised by testing

TABLE 3. Detection of agents using VDL01 and VDL05 (applied multiplexes) in the routine diagnostic laboratory

mPCR and specimen		No. of each agent detected (%)					
VDL01	Total	CMV	HSV-1	HSV-2	Parvovirus	<i>T. gondii</i>	VZV
Amniotic fluid	15	0	0	0	0	0	0
CSF	34	0	1 (2.9)	0	1 (2.9)	0	1 (2.9)
Plasma	119	10 (8.4)	0	0	4 (3.4)	0	5 (4.2)
Swabs	23	0	3 (13.0)	0	0	0	0
Other	17	1 (5.9)	0	0	0	0	1 (5.9)
VDL05	Total	CMV	Epstein-Barr virus	Enterovirus	HSV-1	HSV-2	VZV
CSF	144	3 (2.1)	5 (3.5)	10 (6.9)	0	3 (2.1)	1 (0.7)
Plasma	136	10 (7.4)	8 (5.9)	0	0	0	0
Swabs	128	0	2 (1.6)	0	16 (12.5)	9 (7.0)	17 (13.2)
Other	51	0	5 (9.8)	2 (3.9)	2 (3.9)	3 (5.9)	6 (11.8)

predominantly stored extracts. The small number of samples tested for Epstein-Barr virus would account for the low sensitivity measured for detection of this virus. However, the sensitivity of the mPCRs for other agents was $\geq 95.0\%$ and no false-positives were recorded.

The specificity of the mPCRs is enhanced by using both electrophoresis and probe-amplicon hybridization methods. The multiplexes were optimized to reduce spurious amplification products such as due to the formation of primer dimers and nonspecific interaction with nucleic acid rich extracts. Post-PCR probe-hybridization assisted in identifying amplicons when nonspecific electrophoresis products were present. However, probes were not developed for the VDL03 multiplex as it tests for only three agents (HHV6, -7, and -8) and the products were easily discernible by electrophoresis. Given our observations of cross-hybridization between enterovirus and the HSV-1 probe and between the HSV-2 probe and HSV-1 amplicons, post-PCR detection by this method was used to confirm the electrophoresis band and not used independently, i.e., as a means of increasing sensitivity of detection.

The use of VDL01 and VDL05 mPCRs in our diagnostic laboratory over a six month period not only enhanced our validation, but showed the resourcefulness of the array of detectable agents by each method-demonstrated by detection rates of 13.5% and 22.2%, respectively (Table 3). The VDL01 mPCR was developed for detection of common intrauterine infections caused by DNA containing agents, and demonstrated its utility as a diagnostic screen for other infections such as viral meningitis and skin lesions. The array for VDL05 was selected on the basis of commonly requested pathogens and evident by the high detection rate, implementation has resulted in cost efficiencies and timely reporting of results. Further diagnostic benefits were demonstrated when alternate agents to that clinically suspected were detected. This had occurred in 11 requests, including requests for varicella-zoster virus in swabs of skin lesions when HSV-1 and HSV-2 were detected, and requests for HSV in cerebrospinal fluid when CMV was detected.

Both HSV-1 and HSV-2 infection cause dermatomal vesicular lesions not unlike those caused by varicella-zoster virus (52). However, CMV infection of the central nervous system is rare and can occur in infants following intrauterine infection (6). Epstein-Barr virus was detected in a variety of specimens, including: conjunctival swabs ($n = 2$), bronchial washings ($n =$

2), plasma ($n = 6$), and feces ($n = 1$). This virus can be isolated from oropharyngeal washings or from circulating lymphocytes of 80 to 90% of patients with infectious mononucleosis (46). However, serendipitous detection should receive careful consideration given the ubiquity of virus shedding in both healthy persons and in those with unrelated illnesses (46).

The motivation for the development of these novel mPCRs was for the screening of amniotic fluid for pathogens known to cause fetal loss (miscarriages and stillbirths). In Australia, common fetal pathogens include CMV, HSV, rubella virus, *T. gondii* and VZV (1, 9, 10, 28, 40). To our knowledge, there is no universal screening for any of these infections, and diagnosis is often difficult, particularly as detailed viral testing in this country is rarely performed in intrauterine deaths (40), in neonates and even in postnatal death from sudden infant death syndrome (23, 41).

The availability of these mPCRs in the routine diagnostic laboratory will enable more frequent testing of a broad spectrum of agents implicated in congenital disease including viruses whose association is suspected but has not been established in Australia. lymphocytic choriomeningitis virus is well-established as a cause of congenital disease in the United States and Europe (5) but not yet evident in this country (47). HHV-6, HHV-7 and HHV-8 are infectious agents with possible associations with congenital anomalies and stillbirth, on the basis of case reports, plausible animal models, or detection in placental or uterine tissue of affected and unaffected babies (2, 25, 34). Antenatal infections with enterovirus have been associated with neurodevelopmental delay (21), and infant diabetes (17, 27). Transplacental transmission of hepatitis C virus is uncommon although the risk of transmission may increase when the mother is coinfectd with human immunodeficiency virus (6).

Failure to detect infectious agents in the amniotic fluids tested by the developmental mPCRs reflects the rarity of congenital infections in a population of healthy pregnant women. Furthermore, amniotic fluid collected during the first trimester may be too early for detection of agents such as CMV (18) and *T. gondii* (44). The applied mPCRs that have been adapted for routine use will enable testing to be undertaken more frequently and on a larger scale, and where recent infection is suspected by illness or seroconversion.

CMV is the most common cause of intrauterine infection and most studies of the clinical significance of viral detection in

amniotic fluid has been done with this virus. A recent study (24) showed with 100% probability that the presence of $\geq 10^3$ genome equivalents/ml predicted mother-child infection, and $\geq 10^5$ genome equivalents predicted the development of a symptomatic infection. The limit of detection of this agent in VDL01 and VDL05 appears to be appropriately sensitive for the prediction of these clinical outcomes and should be augmented by quantitative PCR assessment.

The techniques used here have allowed mPCR detection of congenital agents from genomic material extracted from amniotic fluid (3) from plasma and has been compared with detection of known virus in clinical samples, cultures, or plasma spiked with plasmids (Table 2). This work also represents a standard approach to the assays, using commercial agents, and use of consistent and thorough assessment of these assays to give accurate figures for sensitivity, specificity and limit of detection (Table 2). The use of nested PCR and RT-PCR has meant these assays typically detect down to 10^1 to 10^2 copies of the etiological agent, an important element of detection in congenital infections, and infections of the central nervous system and cerebrospinal fluid particularly (14, 49). Although not examined in this study, a potential use of the mPCRs would be the detection of congenital agents in dried blood spots retrospectively collected from children postnatally diagnosed with conditions such as deafness after birth which is detectable months to years after birth (4).

The increase in diagnostic capacity of these mPCRs offers the cost benefits of less reagents and consumables, and improved turn-around time. Furthermore, the development enables testing for a wide range of agents using a small volume of clinical sample. The automation of the extraction process as used in this study (Table 2) further enhances efficiency. These mPCRs have suitable performance characteristics for the detection of a broad range of agents associated with congenital and other infections. The use of a common methodology is conducive to routine screening of small sample volumes, inclusive of the rarer agents such as lymphocytic choriomeningitis virus, and HHV-6, HHV-7, and HHV-8. The increase in testing will enhance our understanding of the role played by these agents in congenital disease within our epidemiologic setting. Furthermore, the benefits observed from the use of mPCRs in a routine diagnostic laboratory such as VDL05 for the detection of commonly tested viruses is the motivation for continuing development of other organ-specific mPCRs.

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