Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

Multiplex PCR and Microarray for Detection of Swine Respiratory Pathogens

O. Lung[1], S. Ohene-Adjei[1,*], C. Buchanan[1], T. Joseph[2], R. King[3], A. Erickson[1], S. Detmer[4] and A. Ambagala[1]

[1] Lethbridge Laboratory, National Centres for Animal Disease, Canadian Food Inspection Agency, Lethbridge, AB, Canada
[2] Animal Health Centre, BC Ministry of Agriculture, Abbotsford, BC, Canada
[3] Animal Health and Assurance Division, Alberta Agriculture and Rural Development, Edmonton, AB, Canada
[4] Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

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Correspondence:
O. Lung. Lethbridge Laboratory, National Centres for Animal Disease, Canadian Food Inspection Agency, Township Road 9-1, Lethbridge, AB T1J 3Z4, Canada.
Tel.: +1 403 382 5589;
Fax: +1 403 381 1202;
E-mail: oliver.lung@inspection.gc.ca

*Current address: Department of Animal Sciences, College of Basic and Applied Sciences, University of Ghana, Legon, Accra, Ghana

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Summary

Porcine respiratory disease complex (PRDC) is one of the most important health concerns for pig producers and can involve multiple viral and bacterial pathogens. No simple, single-reaction diagnostic test currently exists for the simultaneous detection of major pathogens commonly associated with PRDC. Furthermore, the detection of most of the bacterial pathogens implicated in PRDC currently requires time-consuming culture-based methods that can take several days to obtain results. In this study, a novel prototype automated microarray that integrates and automates all steps of post-PCR microarray processing for the simultaneous detection and typing of eight bacteria and viruses commonly associated with PRDC is described along with associated multiplex reverse transcriptase PCR. The user-friendly assay detected and differentiated between four viruses [porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus, porcine circovirus type 2, porcine respiratory corona virus], four bacteria (*Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Salmonella enterica* serovar Choleraesuis, *Streptococcus suis*), and further differentiated between type 1 and type 2 PRRSV as well as toxigenic and non-toxigenic *P. multocida*. The assay accurately identified and typed a panel of 34 strains representing the eight targeted pathogens and was negative when tested with 34 relevant and/or closely related non-target bacterial and viral species. All targets were also identified singly or in combination in a panel of clinical lung samples and/or experimentally inoculated biological material.

Introduction

The global pig industry produced approximately 963 million pigs and 109 million metric tons of pork in 2011 (http://faostat.fao.org). Respiratory diseases are considered to be one of the main contributors to economic losses in the swine industry (Opriessnig et al., 2011). The 2006 United States Department of Agriculture (U.S.D.A.) National Animal Health Monitoring System (NAHMS) study of 435 swine production sites with 100 or more pigs from 17 major pork-producing States showed that respiratory problems are the main cause of nursery deaths (53.7%) and grower/finisher pig mortality (60.1%) (United States Department of Agriculture, 2008). In Canada, 37–78% of pigs going for slaughter have cranioventral bronchopneumonia (Hansen et al., 2010a). Porcine respiratory disease complex (PRDC) is multifactorial, with both infectious and non-infectious factors contributing to respiratory disease and predominantly seen in pigs between the ages of 3 and 6 months (Opriessnig et al., 2011). The interaction of viral and bacterial pathogens, environmental factors, pig-specific factors and management conditions all contribute to the development and impact the severity of PRDC (Opriessnig et al., 2011).

The type of pathogens involved in PRDC is specific to the regions and countries where production occurs (Opriessnig et al., 2011). However, viruses most commonly associated with PRDC include porcine reproductive and respiratory syndrome virus (PRRSV) (Rammohan et al., 2012), porcine circovirus type 2 (PCV2) (Ellis et al., 2004; Genzow et al., 2009), influenza A virus (IAV) and porcine respiratory corona virus (PRCV) (Pensaert et al., 1986; Jung et al., 2009; Renukaradhya et al., 2010). Bacteria such as *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) (Hansen et al., 2010b), *Pasteurella multocida* (*P. multocida*) (Davies, 2004), *Salmonella enterica* serovar Choleraesuis (*S.e* Choleraesuis) (Reed et al., 1986; Asai et al., 2010) and *Streptococcus suis* (*S. suis*) (Done and Paton, 1995; Silva et al., 2006; Baums et al., 2007) are also commonly associated with PRDC.

Porcine reproductive and respiratory syndrome virus is a major cause of swine production losses worldwide, and in the United States, reproductive and growing pig losses are an estimated $560 million per year (Neumann et al., 2005). PRRSV (genus *Arterivirus*, family *Arteriviridae*) is an enveloped virus with a single-stranded, positive-sense RNA genome of approximately 15 kb. PRRSV is classified into two types with type 1 predominating in Europe and type 2 predominating in North America and Asia. PCV2 (genus *Circovirus*, family *Ciroviridae*) is a non-enveloped virus with a circular, covalently closed single-stranded DNA genome of 1767–1768 nucleotides (Meehan et al., 1997; Hamel et al., 1998). IAV (genus *Influenzavirus A*, family *Orthomyxoviridae*) are enveloped viruses with a genome composed of eight single-stranded negative-sense RNA segments. PRCV (genus *Alphacoronavirus*, family *Coronaviridae*) are enveloped viruses with a single-stranded positive-sense RNA genome of approximately 28.5 kb. It was first isolated in Belgium in 1984, and it is a natural variant of transmissible gastroenteritis virus (TGEV) (Pensaert et al., 1986) that contains a 5′ deletion (621–681 nt in size) in the S gene which is used to differentiate PRCV from TGEV in PCR-based assays (Kim et al., 2000; Costantini et al., 2004). PRCV often causes subclinical infections. *M. hyopneumoniae* (Hansen et al., 2010b) causes mycoplasmal pneumonia of swine and is known to be one of the most common and economically important diseases found in pig farms worldwide, having low mortality, but high morbidity (Otagiri et al., 2005). *P. multocida* is a commensal found in the upper respiratory tract of pigs, but can also act as a primary or secondary pathogen responsible for pneumonia (Davies, 2004) and atrophic rhinitis in pigs (Tang et al., 2009). *S. e.* Choleraesuis is a host adapted *Salmonella* serovar responsible for almost all types of salmonellosis in pigs in North America and Europe (Kingsley and Baumler, 2000). *S. suis* is a Gram-positive, zoonotic bacterial pathogen important in polyserositis, septicaemia, arthritis, pneumonia and endocarditis in pigs (Done and Paton, 1995; Silva et al., 2006; Baums et al., 2007). Some of these bacteria are of low pathogenicity and exist as commensals in the upper respiratory tract of healthy pigs. They can cause severe respiratory disease by invading tissues already damaged due to a primary pathogen(s) (virus or bacteria), general immunosuppression and other factors such as poor environmental conditions and poor management practices.

Routine diagnostic methods for detection of viruses implicated in PRDC include virus isolation in cell culture, antigen detection by direct fluorescent antibody staining, and enzyme immunoassay and culture-based methods for bacteria. Such methods (Grau-Roma and Segales, 2007) are time-consuming and require independent tests for each pathogen. Furthermore, the detection of bacterial pathogens typically depends on time-consuming culture-based methods that can take several days to obtain results. Due to their high sensitivity and ease of use, PCR and real-time PCR tests have been developed for several agents implicated in the PRDC; however, these tests typically target single pathogens (Lierz et al., 2008; Lomonaco et al., 2009; Tang et al., 2009). A diagnostic test capable of simultaneous detection of multiple pathogens involved in PRDC (Atashpaz et al., 2009; Wernike et al., 2012; Xu et al., 2012) would save time, labour and cost by providing more information with each test performed. A multiplex PCR assay capable of detecting five porcine viruses including two porcine respiratory viruses was developed (Giammarioli et al., 2008). Duplex and triplex real-time PCR for porcine respiratory viruses have also been recently described (Chang et al., 2014; Wu et al., 2014). However, to date, there are no diagnostic tests capable of simultaneous detection of multiple major viral and bacterial porcine respiratory pathogens in a single reaction. Microarray technology, with its capacity to incorporate a large number of capture probes, is a potentially useful tool for multiplexed detection and typing of pathogens. Here, a microarray assay with associated multiplex RT-PCRs for detection and differentiation of four viruses and four bacteria involved in PRDC using a novel user-friendly electronic microarray in which capture probe printing, hybridization, washing and reporting are fully integrated and automated is described. The electronic microarray contains 400 test sites which can be activated independently via electrodes and utilizes electrophoretically driven hybridization that can be completed instantaneously.

Materials and Methods

Sequence databases

Databases containing all available full and partial sequences of the genomic regions encoding the matrix proteins of

IAV (n = 4373) and PRRSV (n = 1829), PRCV spike protein (n = 24), and PCV2 capsid protein (n = 2048) were compiled from the National Centre for Biotechnology Information (NCBI). Similarly, databases were compiled for the *kmt1* (n = 16) and *toxA* (n = 50) genes of *P. multocida, sly* (n = 106) and *orfB* (n = 48) genes of *S. suis,* a 640 bp portion of intergenic region ig-070/071 (Gardner and Minion, 2010) of *M. hyopneumoniae* (n = 6) and a 708 bp region between open reading frames SC4343 and SC4353 previously identified as a metabolic island for *S. e.* Choleraesuis (n = 1). Sequences were retrieved by searching NCBI's 'Nucleotide' database using the gene names as keywords, as well as performing BLAST homology searches (Altschul et al., 1990) with a representative sequence for each target, and downloading the aligned portion of all BLAST hits. Redundant sequences were removed based on accession numbers. Multiple sequence alignments for each genetic target were generated with ClustalX v. 2.0 (Thompson et al., 1997; Larkin et al., 2007) or MAFFT v. 7.0 (Katoh and Standley, 2013) using default settings. Databases were maintained with either Mega4 (Tamura et al., 2007) or BioEdit Sequence Alignment Editor v. 7.1.9 (Hall, 1999) to ensure that all sequences were correctly oriented and aligned. Similarly, representative whole-genome sequences, as well as full and partial sequences of homologous genes from related and unrelated non-targets such as TGEV, porcine circovirus type 1 (PCV1), as well as other *Salmonella enterica* serovars, and *Mycoplasma* species were downloaded for *in silico* analysis of probe specificity.

Primer and probe design

Several published PCR primers suitable for the assay were adopted from the literature (Table 1). Additional PCR primers (Table 1) and all target-specific capture probes (Table 2) were designed using either AlleleID (Premier Biosoft International, Palo Alto, CA, USA) or BioEdit Sequence Alignment Editor v. 7.1.9 (Hall, 1999) based on the databases described above. Primers and probes were designed to be 18–25 bp in length, with the melting temperatures between 54 and 65°C and minimal secondary structures ($\Delta G \geq -8.0$ kcal/mol). Primers and probes identified by each software were compiled and examined *in silico* for specificity by BLAST (Altschul et al., 1990) analysis using custom in-house databases containing representative whole-genome sequences, as well as full or partial sequences of homologous genes from related and relevant non-targets. Primers or probes that showed significant homology to closely related or unrelated non-target species were excluded from further investigation.

Table 1. Primers used in this study

Organism	Genomic region	Primer name	Sequence (5′–3′)	Amplicon size (bp)	Reference
Virus					
PCV2	Capsid	CircoV-1222F	GTAATCAATAGTGGAATCTAGGAC	534	This study
		CircoV-1760R	TTCGTTTTCAGATATGACGTATC		
PRCV	Spike	PCoV-2 -24597F	GTAGTACAGGTTGCTGTGGATG	522	Nicholson et al. (2011)
		PCoV-2-25437R	AGTTGTTGTAACAATGCCATCA		
PRRSV	Matrix	PRRS-Mtrx-F2	AAGGTAAGTCGCGGCCGAC	379	This study
		PRRS-Mtrx-R2	TGCCRCCCAACACGAGGC		
IAV	Matrix	AIV-M-407F	GCATGGGYCTCATATACAACMGRATGG	280	Lung et al. (2012)
		AIV-M-696R	GATGAGTCCCAATKGTYCKCA		
Bacteria					
M. hyopneumoniae	Intergenic space	M. hyopn 80251F	CGGTTTTATAAGAATTAGTTGCTCC	421	This study
		M. hyopn 80628R	TTGGCAAGCCGCCGTCATT		
P. multocida	*kmt1*	PM-KMT1 1084F	GAGTTTTATGCCACTTGAAATGGG	205	This study
		PM-KMT1 1281R	CACAAGGAAATATAAACCGGCAAAT		
	toxA	PmToxA 2094 F	ATCTTAGATGAGCGACAAGG	247	Lichtensteiger et al. (1996)
		PmToxA 2340 R	TTGCCTCTGGAATCGCACC		
S. e. Choleraesuis	Metabolic Island	CsPcSC4352 262F	TCGAGGGTTAAAGATGGGG	708	Woods et al. (2008)
		CsPcSC4352 95R	TACCACACGCTAAGCAACC		
S. suis	*sly*	Suilysin 29F	GCTCAATAGTCAGTTTGGCACTC	443	Silva et al. (2006)
		Suilysin 472R	GAAGGTTATTCACCCCTGTTC		
	orfB	p-SlyB 443-462 F	GAATAGCTAAGGCTGTTGCA	240	This study
		p-SlyB 683-659 R	GATGTAGATGATACGCTTTATGATC		
Internal Control	N/A	Dengue F	AAACCGTGCTGCCTGTAG	229	Sudiro et al. (1997)
		Dengue R	TCTCTCCCAGCGTCAA		

Table 2. Probes designed and used in this study

Organism	Gene	Probes	Sequence (5′–3′)	Assay[a]	
				Slide array	Electronic array
Virus					
PCV2	Capsid	1576	ATATCCGAAGGTGCGGGAT	✓	✓
		1657	GACGAGCCAGGGGCGGCGGC	✓	✓
		1332	TGAGGGCTGTGGCCTTTGTT	✓	✓
		1384	GCCCACTCCCCTGTCACCCTG	✓	
		1460	TCAAAGGGCACAGAGC	✓	
		1508	TCATTAATATTGAATCTCA	✓	
		1521	ATCTCATCATGTCCACCGCCCAG	✓	
		1546	AGGGCGTTCTGACTGTGGTTCGC	✓	
PRCV	Spike	2–3	CTATTGAAGAAGTAAACACACAAAATC	✓	✓
		2–2	GGATAAAGCATAAGCTTGGTCT	✓	
		2–1	ATCTGCTATACTGGTAAATTACTTGTG	✓	
		2–4	TACACAACAAGTTAAGACGTGTGTC	✓	
PRCV/TGEV	Spike	1–1	GTTATAGTACAACAGCATCAGG	✓	✓
		2	GTTAGTGCTAGAACACAAAACT	✓	
		3	AGAGTTCAGCATCGCTGTACTCT	✓	
PRRSV	Matrix	322–346 COM	TACATTCTGGCCCCTGCCCATCACG	✓	✓
Type 2		361	GGCTTTCATCCGATTGCGGCAAATG	✓	✓
Type 2		378	GGCAAATGATAACCACGCATTTG	✓	✓
Type 2		282	CATCACCTCCAGATGCCGTTTGTG	✓	
Type 2		303	GTGCTTGCTAGGCCGCAAGTACA	✓	
Type 2		324	CATTCTGGCCCCTGCCCACCACGT	✓	
Type 2		210	CGCGCTCACTATGGGAGCAGT	✓	
Type 2		262	GCCATAGAAACCTGGAAATTCATCA	✓	
Type 2		405	GCGTCCCGGCTCCACTACGGT	✓	
Type 2		430	AACGGCACATTGGTGCCCGGGTT	✓	
Type 1		233–252 EU	TTGTCACCCTTCTGTGGGGC	✓	✓
Type 1		380–402 EU	CGTCTGGTAACCGAGCATACGCT	✓	✓
Type 1		287–310 EU	CATCACCTCCAGATGCCGTTTGTG	✓	
IAV	Matrix	M 594	ATGGARCARATGGCTGGRTC	✓	✓
		M 624	GCAGCRGARGCYATGGA	✓	✓
Bacteria					
M. hyopneumoniae	Intergenic space	80353	AACGGTAAACTCCTACCTGG	✓	✓
		80516	AGAGTGAGATTTTTTAAAATGGA	✓	✓
		80321	AACATATAAAAGGGTGTT	✓	
		80573	ATGACGGCGGCTTGCCAA	✓	
P. multocida	*kmt1*	1135–1156	GTGAGTGGGCTTGTCGGTAGTC	✓	✓
		1252–1273	GGACGTTATTTATTACTCAGCT	✓	✓
		1229–1249	CCTTGACAACGGCGCAACTGA	✓	
		1195–1213	TGGGCGGAGTTTGGTGTGT	✓	
	toxA	2190	CGTGAACTGCGTACTCAATTAGA	✓	✓
		2276	AGGTTCTGGTGCCGCTCGAT	✓	✓
		2308	CAGCCATGAATGAAATGGC	✓	✓
S. e. Choleraesuis	Metabolic Island	760	TCAACGCTTGAAACGCAGCAACA	✓	✓
		897	CCCGACACAAGACTCTGCTAT	✓	✓
		598	GATCGCGCACAGAAAGCTGATA	✓	
		683	ATCAGGAAGTCGAGGGAG	✓	
		810	ATTGGCGCGAGCAACAATCTCC	✓	
S. suis	*sly*	298–319	GGTGCTTTATTGCGTGCTGACC	✓	✓
		351–372	CAGTATTGCGCGGGGAGATCTG	✓	✓
		44–65	TGGCACTCGTAGGGGTCACACC	✓	
		166–189	GATAATCCGCCAGCAACAACTGGT	✓	
		391–413	CCTGGTTTGGCCAATGGGGATAG	✓	
		138–158	GATTCTTACAAATGAGGGAG	✓	
	orfB	576	GCGAAAGGAAAGGTAGAGTGGT	✓	✓
		632	CCAAAGCACGTTCAAACGGTTG	✓	✓
		468	CACCTGATACCCAAAATCTGCCA	✓	✓

(continued)

Table 2. (continued)

Organism	Gene	Probes	Sequence (5′–3′)	Assay[a]	
				Slide array	Electronic array
Control probes	N/A	137–155	GGAAGCTGTATCCTGGTGG		✓
		57–75	CCATGGAAGCTGTACGCAT		✓
		84–100	CCTCCCAAAACATAACGC	✓	✓
		301–316	GAGGTTAGAGGAGACC	✓	✓
		NSBP[b]	CAAAGTGGGAGACGTCGTTG	✓	✓

[a]'✓' indicates probes that were tested on each platform.
[b]NSBP probe was adapted from Hindson et al. (2008).

Samples and sample preparation

A list of the viruses, bacteria and clinical samples used in this study is presented in Table 3. The yield of the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada), QIAamp Viral RNA Mini Kit (Qiagen) and MagMAX Total Nucleic Acid Kit (Ambion, Austin, TX, USA) was evaluated with PCV2 (a non-enveloped DNA virus) and PRRSV (an enveloped RNA virus) as per the manufacturers' recommendation. For these viruses, 100 μl of a quantified stock was serially diluted (10^{-2} to 10^{-6}) in swab material from 2-day-old piglets (Prairie Swine Centre, Saskatoon, SK, Canada), previously tested to be negative for the targets. Similarly, the UltraClean Tissue and Cells DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), DNeasy Blood and Tissue Kit (Qiagen) and MagMAX Total Nucleic Acid Kit were tested to evaluate the nucleic acid extraction efficiency of a Gram-positive bacteria (*S. suis*) and a Gram-negative bacteria (*P. multocida*). For these bacteria, 100 μl of culture of known CFU/ml was serially diluted in the same swab material as above and extracted using each kit in parallel according to the manufacturers' instructions. The efficiency of the extraction kits was evaluated based on the limits of detection observed after RT-PCR amplification of the extracted material. The DNeasy Blood and Tissue Kit and the Viral RNA Mini extraction kit were the most efficient for the tested bacteria and virus targets, respectively (data not shown). Therefore, all subsequent nucleic acid extractions of laboratory amplified strains were performed using these kits. Following preparation of nucleic acid extractions, the samples were subjected to PCR and microarray analysis.

For the determination of the analytical sensitivity of the assay for viral targets, selected genes were amplified using the SuperScript™ III One-Step RT-PCR System with Platinum®Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA) and cloned into the pJET1.2 cloning vector using the CloneJET PCR Cloning Kit (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturers' specifications. Plasmids were extracted from successfully transformed bacteria using the QIAPrep MiniPrep kit (Qiagen) according to the manufacturer's specifications and were confirmed by sequencing (Eurofins Genomics, Huntsville, AL, USA). Vectors containing the target genes for each virus, except for PCV2, were linearized with the Hind III restriction enzyme (Fisher Scientific) and subjected to *in vitro* transcription using the MEGAScript T7 Transcription Kit (Life Technologies) according to the manufacturer's specifications. Template DNA was eliminated using successive treatments with TURBO DNase (Life Technologies,) before quantifying the RNA using the RNA BR Assay Kit and the Qubit 2.0 fluorometer (Life Technologies) according to the manufacturers' specifications. The RNA was then serially diluted 1 : 10 in UltraPure Distilled water (Life Technologies). The copy number was inferred using an online tool (http://endmemo.com/bio/dnacopynum.php) taking into account the nucleic acid concentration and nucleotide composition of the amplified region of each target. The copy number for the plasmid containing PCV2 capsid protein coding region was inferred based on the nucleic acid concentration and nucleotide composition over the entire plasmid.

For the determination of the analytical sensitivity of the assay for bacterial targets (excluding *M. hyopneumoniae*), frozen cultures were streaked for single colonies onto 5% sheep blood agar plates (BBL™ Blood Agar Base Infusion Agar; BD Diagnostics, Sparks, MD, USA) and incubated at 37°C overnight. A single colony was inoculated into 5 ml of Miller's LB broth (Life Technologies) and grown overnight at 37°C on a shaking incubator (150 rpm). The overnight LB broths were serially diluted 1 : 10 in PBS, and 100 μl of material was spread onto blood agar plates in triplicate and grown overnight at 37°C for enumeration using the viable plate count method. The cultures were standardized to 3.33×10^8 CFU/ml (*S. e* Choleraesuis and *P. multocida*) and 3.33×10^6 CFU/ml (*S. suis*), so a 30 μl aliquot from each serial dilution in the series yielded CFUs to the nearest power of base 10 (i.e. 1×10^6, 1×10^5, etc.). For *M. hyopneumoniae*, an aliquot of genomic DNA was quantified on the Qubit 2.0 fluorometer, and a genomic copy number was inferred based on the nucleic acid

Table 3. Viral and bacterial isolates used in this study

Organism	Lab sample	Role	Heatmap	Assay		
				RT-PCR	Slide array	Electronic array
Virus						
PCV2	EF394779[7]	T	12	✓	✓	✓
PRCV	AR310[10]	T	13	✓		✓
	ISU-1[9]	T	14	✓	✓	✓
PRRSV	Vaccine 2,5[3]	T	1	✓		✓
	Vaccine 1,4[3]	T	2	✓		✓
	MLV[3]	T	3	✓		✓
	YNL[1]	T	4	✓	✓	✓
	93 44927[1]	T	5	✓		✓
	LV[1]	T	6	✓	✓	✓
IAV	H1+[3]	T	7	✓	✓	✓
	MN/07 (H3N2)[8]	T	8	✓		✓
	TX/98 (H3N2)[8]	T	9	✓		✓
	IA/04 (H1N1)[8]	T	10	✓		✓
	IL/08 (H1N1/H3N2)[8]	T	11	✓		✓
PCV1	AY184287[7]	RNT	16	✓	✓	✓
TGEV	TC1998[3]	RNT	15	✓	✓	✓
BPSV	Texas A&M/1976[1]	NT	17	✓		✓
BTV	BH254/07[1]	NT	18	✓		✓
BVDV	Hastings[1]	NT	19	✓		✓
CEV	152205 (Vaccine)[1]	NT	20	✓		✓
CSFV	Alfort[6]	NT	21	✓	✓	✓
BoHV-1	Edmonton[1]	NT	22	✓		✓
MCFV	WC11 Virus 9101[1]	NT	23	✓		✓
PCPV	Kansas (1973)[1]	NT	24	✓		✓
RPV	Kabette[1]	NT	25	✓		✓
SVDV	ITL 19/92[1]	NT	26	✓		✓
VESV	Cal[1]	NT	27	✓		✓
VSV	02V1008 (Indiana)[1]	NT	28	✓		✓
Bacteria						
M. hyopneumoniae	25934[10]	T	12	✓	✓	✓
P. multocida	4533 (Toxigenic)[5]	T	1	✓	✓	✓
	4837 (Toxigenic)[5]	T	2	✓	✓	✓
	1059[10]	T	3	✓	✓	✓
	Ser A[3]	T	4	✓	✓	✓
	Ser B[3]	T	5	✓	✓	✓
	Ser D[3]	T	6	✓	✓	✓
	Pm1[3]	T	7	✓	✓	✓
	Pm2[3]	T	8	✓		✓
	Pm3[3]	T	9	✓		✓
	Pm4[3]	T	10	✓		✓
	Pm5[3]	T	11	✓		✓
	MAFRI #21[3]	T		✓		
	MAFRI #42[3]	T		✓		
S. e. Choleraesuis	SGSC 4770[7]	T	13	✓	✓	✓
	(6,7:c:1,5)[2]	T	14	✓		✓
S. suis	MAFRI[3]	T		✓	✓	
	P1/7[10]	T	15	✓		✓
	Prairie Diagnostic[11]	T	16	✓		✓
	SS1[3]	T	17	✓		✓
	SS2[3]	T	18	✓	✓	✓
	SS3[3]	T	20	✓		✓
	SS5[3]	T	19	✓		✓

(continued)

Table 3. (continued)

Organism	Lab sample	Role	Heatmap	Assay		
				RT-PCR	Slide array	Electronic array
Mycoplasma alkalescens	L'EAQ[4]	RNT	22	✓		✓
Mycoplasma bovigenitalium	L'EAQ[4]	RNT	23	✓		✓
Mycoplasma bovis	L'EAQ[4]	RNT	24	✓		✓
Mycoplasma hyorhinis	17981[10]	RNT	25	✓	✓	✓
Mycoplasma pneumoniae	29342[10]	RNT	26	✓	✓	✓
Pasteurella haemolytica	Z13[1]	RNT	21	✓	✓	✓
S. e. Arizonae	SGSC 4693[7]	RNT	27	✓		✓
S. e. Diarizonae	SGSC 4692[7]	RNT	28	✓		✓
S. e. Enteritidis	SGSC 4901[7]	RNT	29	✓		✓
S. e. Houtenar	SGSC 3074[7]	RNT	30	✓		✓
S. e. Indica	SGSC 3116[7]	RNT	31	✓		✓
S. e. Salamar	SGSC 3039[7]	RNT	32	✓	✓	✓
S. e. Typhimurium	71-471[1]	RNT	33	✓	✓	✓
	SGSC 452[7]	RNT		✓		
Streptococcus pyogenes	19615[10]	RNT	34	✓	✓	✓
Aeromonas hydrophila	Z22[1]	NT	35	✓	✓	✓
Bacillus cereus	14579[10]	NT	36	✓		✓
Enterococcus faecalis	29212[10]	NT	37	✓	✓	✓
Escherichia coli	25922[10]	NT	38	✓	✓	✓
Klebsiella pneumoniae	13883[10]	NT	39	✓	✓	✓
Pseudomonas aeruginosa	27853[10]	NT	40	✓	✓	✓

T, target; RNT, related non-target; NT, non-target; ✓, isolates that were tested on each platform.
Source information: 1 = Canadian Food Inspection Agency, 2 = Public Health Agency of Canada (OIE Reference Lab), 3 = Manitoba Agriculture, Food and Rural Initiatives, 4 = Enchantillons Provenance L'EAQ, 5 = United States Department of Agriculture's National Veterinary Services Laboratories, 6 = European Union Reference Laboratory for Classical Swine Fever, 7 = University of Calgary, 8 = University of Saskatchewan, 9 = Ohio State University, 10 = American-Type Culture Collection, 11 = Prairie Diagnostics Inc.

concentration and nucleotide composition over the entire genome.

The analytical specificity of the viral and bacterial multiplex PCR assays was assessed by amplifying panels of 14 non-target viruses and 21 bacteria, respectively (Table 3).

Multiplex PCR/RT-PCR

The forward primers were modified with 5′-phosphorylation (IDT, Coralville, IA, USA). All reverse primers were modified with either 5′-TYE665® fluorophore using SpC3® attachment chemistry for the slide microarray or were synthesized with the reverse complementary sequence of the reporter probe at the 5′ end for the electronic microarray as described in Lung et al. (2012). RT-PCRs were performed using the SuperScript™ III One-Step RT-PCR System with Platinum®*Taq* DNA polymerase. A multiplex RT-PCR with 10 primers targeting the genomic regions encoding the IAV and PRRSV matrix proteins, PRCV spike protein, PCV2 capsid protein, as well as an internal control, was developed (Table 1). A multiplex PCR with 14 primers targeting the *kmt1* and *toxA* genes of *P. multocida*, *sly* and *orfB* genes of *S. suis*, intergenic space of *M. hyopneumoniae,* metabolic island of *S. e.* Choleraesuis and an internal control was developed (Table 1). A plasmid containing a fragment of the dengue virus genome was used as an internal PCR control for both the bacterial and viral RT-PCRs. Both assays were optimized for buffer and magnesium concentration, annealing temperature, cycle number and internal control concentration. The finalized RT-PCR mixtures consisted of 1 μl of nucleic acid, 0.01 pg internal control, 1 μl of enzyme mix, 1 μM of each primer in 1× reaction buffer in a final volume of 25 μl. Reverse transcription was carried out for 15 min at 60°C, followed by 94°C for 2 min. PCR was carried out for 35 cycles of 94°C for 30 s, 50°C for laboratory samples and 58°C for clinical samples for 15 s, 68°C for 45 s, with a final extension step of 68°C for 7 min. Following PCR, unpurified material was assayed on the QIAxcel Capillary Gel Electrophoresis System (Qiagen) for visualization of amplicons. Analytical sensitivity of each multiplex assay was determined using serial dilutions of quantified DNA or reverse-transcribed RNA as appropriate for each pathogen. The serial dilutions were amplified using the multiplex PCR assays as well as the singleplex PCR for each target. All PCR amplifications were carried out on the Veriti thermocycler (Life Technologies) and visualized on the QIAxcel using the

BioCalculator v. 3.0 software (Qiagen). The limit of detection was considered to be the last dilution where amplification was greater than the default threshold on the electropherogram and described in terms of the approximate total number RNA or DNA copies in the sample.

Microarray

A total of 30 probes for the detection of four target viruses, 25 probes for the detection of four bacterial targets and three control probes were initially screened by passive hybridization on low-cost conventional Epoxy glass slide microarrays (Corning, Corning, NY, USA) that were printed and processed in-house according to protocols described previously (Lung et al., 2011). The probes were screened against a panel of five isolates representing the four target viruses, and three non-target viruses, and 11 strains representing the four target bacteria, and 11 non-target bacteria (Table 3). Microarray data were represented using the mean pixel intensity for each probe reaction. Probe reactivity was calculated using the mean pixel fluorescent intensity (MFI) of all probes as a ratio of the non-template control. Probe reactions above 2× the ratio of the non-template control were considered positive. Probes that showed good reactivity and specificity were selected for testing and validation on a novel automated electronic microarray in which capture probes are printed on streptavidin-containing acrylamide hydrogels and hybridization, washing and reporting are automated and computer controlled. Capture probes used on the electronic microarray were modified with 5′-biotin group to allow attachment to streptavidin-containing test sites (IDT, Coralville, IA, USA). A selected set of 12 probes targeting the viruses, 14 probes targeting the bacteria and three control probes (a negative probe and two probes targeting the internal control), which exhibited high reactivity and specificity on glass slide microarrays, were selected for validation on the electronic microarray. The viral probes were tested against an expanded panel of 14 strains or isolates of the four target viruses and 14 non-target viruses (Table 3). Similarly, the bacterial probes were tested against a panel of 20 strains or isolates representing the four target bacteria and 20 related or unrelated non-target bacteria (Table 3). The electronic microarray assays were run using a protocol previously described (Lung et al., 2012) with modifications. The modifications included the replacement of the 'touch down' washing protocol with a 'touch up' protocol in which washing steps were carried out using Low Salt Buffer (Nexogen, Inc., San Diego, CA, USA) with incremental increases rather than decreases in temperature. Images were captured at each temperature increment. The Red Universal Reporter Probe was replaced with a 5′-Alexa Fluor 647 modified locked nucleic acid (LNA) variant (5′-/5Alex647N/TGT+CA+AGC-G+AT+AT+ACT+GC-3′) (IDT, Coralville, IA, USA) to increase its thermal stability over a more robust range of wash temperatures. All electronic microarray hybridizations were performed in duplicate, and a non-template PCR control (NTC) was included in all experiments. Raw fluorescent intensity (FI) data from all utilized electrodes at each temperature increment were obtained and analysed using Microsoft Excel. For each probe, positive-to-non-template control (P : N) ratios were calculated by dividing the FI value from each analyte by the FI value produced by the NTC. For each assay, samples that produced P : N ratios of 2.0 or greater were considered positive. Average P : N data derived from the microarrays were visualized with a heat map generated using TreeView v. 1.60 (Eisen et al., 1998).

Field samples and experimental inoculation of oral and nasal swab material

Oral and nasal swabs were obtained from specific pathogen free pigs at the CFIA Ottawa Laboratory Fallowfield, Ontario, Canada. Oral and nasal swabs were screened for the target viral and bacterial pathogens, and pools of oral and nasal material that were negative for the target bacteria and viruses were used for spiking with target pathogens. Bacterial strains were grown and quantified as described above, and supernatants containing virus from cell culture were used. For *M. hyopneumoniae*, culture was not performed and a freeze-dried cell pellet purchased from ATCC was used after re-suspension in PBS and 60% glycerol. For inoculations with single agents, 120 μl aliquots of oral and nasal samples were experimentally inoculated with 20 μl of each live virus or bacteria. Samples inoculated with multiple agents were prepared by pooling 20 μl of each pathogen together, and adding 20 μl of the pooled pathogens into 120 μl of oral and nasal material (Table 4). Nucleic acid from the full 140 μl volume of the samples was extracted, PCR amplified and assayed on the electronic microarray as described in previous sections.

Approximately 30–40 mg of a panel of lung tissue submitted in 2015 to Manitoba Agriculture, Food and Rural Initiatives (MAFRI) for diagnosis of porcine respiratory pathogens was ground in 1.5 ml of RLT buffer (Qiagen), transferred to 400 μl of MagMax lysis buffer and processed for nucleic acid extraction in the KingFisher 96 instrument (Ambion). Five to 8 μl of extracted RNA from 90 μl of elution buffer was tested by real-time RT-PCR at MAFRI, and 5 μl was tested at CFIA by electronic microarray.

Results

Multiplex PCR/RT-PCR

Two separate multiplex PCRs were developed for amplification of selected genes of four viruses and four bacteria involved in PRDC, respectively. The multiplex PCR for

Table 4. Detection of targets in lung tissue from clinical cases and experimentally inoclulated porcine and nasal material on the electronic microarray

Sample	PCV2	PRCV	PRRSV	IAV	*M. hyopneumoniae*	*P. multocida*	*S. e* Choleraesuis	*S. suis*
LT-01[b]				+				
LT-02[c]			+		+			
LT-03[c]				+	+	+		
LT-04[c]	+					+		
LT-05[c]	+		+		+			
LT-06[c]			+					
LT-07[c]	+					+		+
NM-01[a]	+							
NM-02[a]		+						
NM-03[a]			+					
NM-04[a]					+			
NM-05[a]						+		
NM-06[a]							+	
NM-07[a]								+
NM-08[a]			+			+		
NM-09[a]	+		+			+		
NM-10[a]						+	+	+
OM-01[a]	+							
OM-02[a]		+						
OM-03[a]			+					
OM-04[a]					+			
OM-05[a]						+		
OM-06[a]							+	
OM-07[a]								+
OM-08[a]			+			+		
OM-09[a]	+		+			+		
OM-10[a]						+	+	+

LT = porcine lung tissue from clinical cases, NM = experimentally inoculated porcine nasal material; OM = experimentally inoculated porcine oral material.

[a]The isolates used for experimental inoculations were PCV2 EF394779, PRCV AR310, PRRSV YNL, *M. hyopneumoniae* A TCC 25934, *P. multocida* MAFRI#42, *S. e*. Choleraesuis SGSC 4770 and *S. suis* P1/7.

[b]Sample provided by University of Saskatchewan.

[c]Samples provided by Manitoba Agriculture, Food and Rural Initiatives.

'+' indicates target was detected by the assay.

bacteria consisted of a total of 12 primers for the detection of the four target bacteria, typing of *P. multocida* and a pair of primers for an internal control (Table 1). Two genes from *S. suis* and *P. multocida* were each targeted for amplification by the multiplex PCR. Amplification and detection of the *toxA* gene of *P. multocida* allowed for differentiation of virulent or pathogenic strains from non-pathogenic strains. Primers for the *orfB* gene were added to a previously designed multiplex PCR with primers for the *sly* gene to allow detection of *S. suis* strains that lack the *sly* gene. The multiplex PCR generated products of the expected sizes ranging from approximately 205–708 bp (Table 1) when a panel of 23 isolates representing the target species, including different serotypes of *S. suis* were tested (Fig. 1).

Similarly, a multiplex RT-PCR with 10 primers was developed and used to detect the four viruses and an internal control. The multiplex RT-PCR successfully amplified a panel of 14 targeted viruses and generated amplicons of the expected size of approximately 229–534 bp (Table 1). The samples represented both genotypes of PRRSV, as well as different subtypes of IAV (Fig. 1).

The internal control variably amplified in both the bacterial and viral multiplex PCRs as a result of competitive PCR. In instances where targets were strongly amplified, amplification of the internal control was either weak or absent.

Microarray

Conventional glass slide microarrays were processed manually as an initial low-cost screening tool to assess the specificity of the probe set (n = 30) designed to detect the four target viruses, distinguish between genotypes 1 and 2 of PRRSV, as well as differentiate PRCV and TGEV, and the probe set (n = 25) designed to detect the four target bacteria and differentiate toxigenic and non-toxigenic strains of

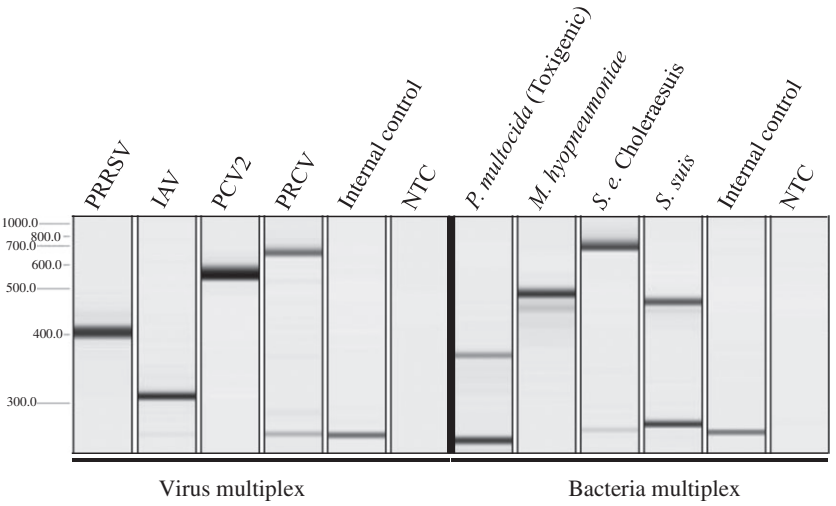

Fig. 1. QIAxcel image of viral and bacterial targets amplified with the multiplex PCR assays. NTC = no template control. Amplification of the internal control is not always observed when a target is present in high amounts.

P. multocida. A selected set of 12 probes targeting the viruses, 14 probes targeting the bacteria and three control probes (a negative probe and two probes targeting the internal control), which exhibited the highest reactivity and specificity on slide microarrays, were selected for validation on the user-friendly automated electronic microarray.

All target viruses and bacteria were accurately detected, and PRRSV and *P. multocida* were accurately typed using the viral and bacterial probe set on the electronic microarray platform (Fig. 2). The assay also successfully detected targeted pathogens in clinical lung specimens, as well as porcine oral and nasal swab material experimentally inoculated with single or multiple targets (Table 4). The results obtained were consistent with those obtained by singleplex assays with the exception of LT-7 which was positive for *P. multocida* based on the electronic microarray assay, but negative by bacterial culture.

Analytical sensitivity and specificity

For both the RT-PCR and microarray assays, the analytical sensitivity varied with the different targets. For some targets, the multiplex assay had comparable sensitivity with the respective singleplex assay, while for other targets, the singleplex assays were more sensitive (Table 5). The multiplex assay was most sensitive for detection of IAV and *S. suis* for viral and bacterial targets, respectively (Table 5).

Non-target bacteria samples did not react with the probes on either the conventional slide microarray or electronic microarray. Other than TGEV, a natural variant and highly related virus to PRCV, no other non-target viruses showed amplification in the virus multiplex RT-PCR (data not shown). Due to the strong amplification of TGEV by the multiplex RT-PCR, the internal control failed to amplify (Fig. 2b, sample 21). However, PRCV and TGEV were differentiated based on amplicon size, and in subsequent microarray characterization (Fig. 2).

Discussion

A user-friendly microarray for the simultaneous detection and differentiation of four viruses and four bacteria associated with PRDC was developed as a test case for a novel automated 'amplicon-to-answer' electronic microarray technology. The electronic microarray integrates and automates all post-PCR steps required for microarray analysis (capture probe printing, hybridization, washing, reporting) and allows for simultaneous identification of eight pathogens, differentiation of the two PRRSV genotypes and pathogenic versus non-pathogenic *P. multocida* strains.

Although the amplification of bacterial DNA did not require a reverse transcriptase phase, an RT step was included in the PCR protocol as the amplicon yield was better than without the RT step. Using the same protocol would also allow potential combination of the bacterial and viral multiplex PCR into a single multiplex PCR targeting all eight pathogens. A likely explanation for the increased amplification yield observed with RT-PCRs for bacterial targets is RT-PCR could utilize not just genomic DNA, but also RNA transcripts of target genes as template. In addition, the proprietary quantity of Taq polymerase in the RT-PCR kit that was used in the RT-PCRs may be higher than that used in the PCR.

Initial screening of capture probes was performed using a conventional slide microarray platform due to the lower cost of screening large number of probes that were printed in conjunction with other projects. Subsequently, the assay was adapted to a novel, rapid and user-friendly microarray platform that automates and integrates capture probe printing with all post-PCR steps of the assay, including

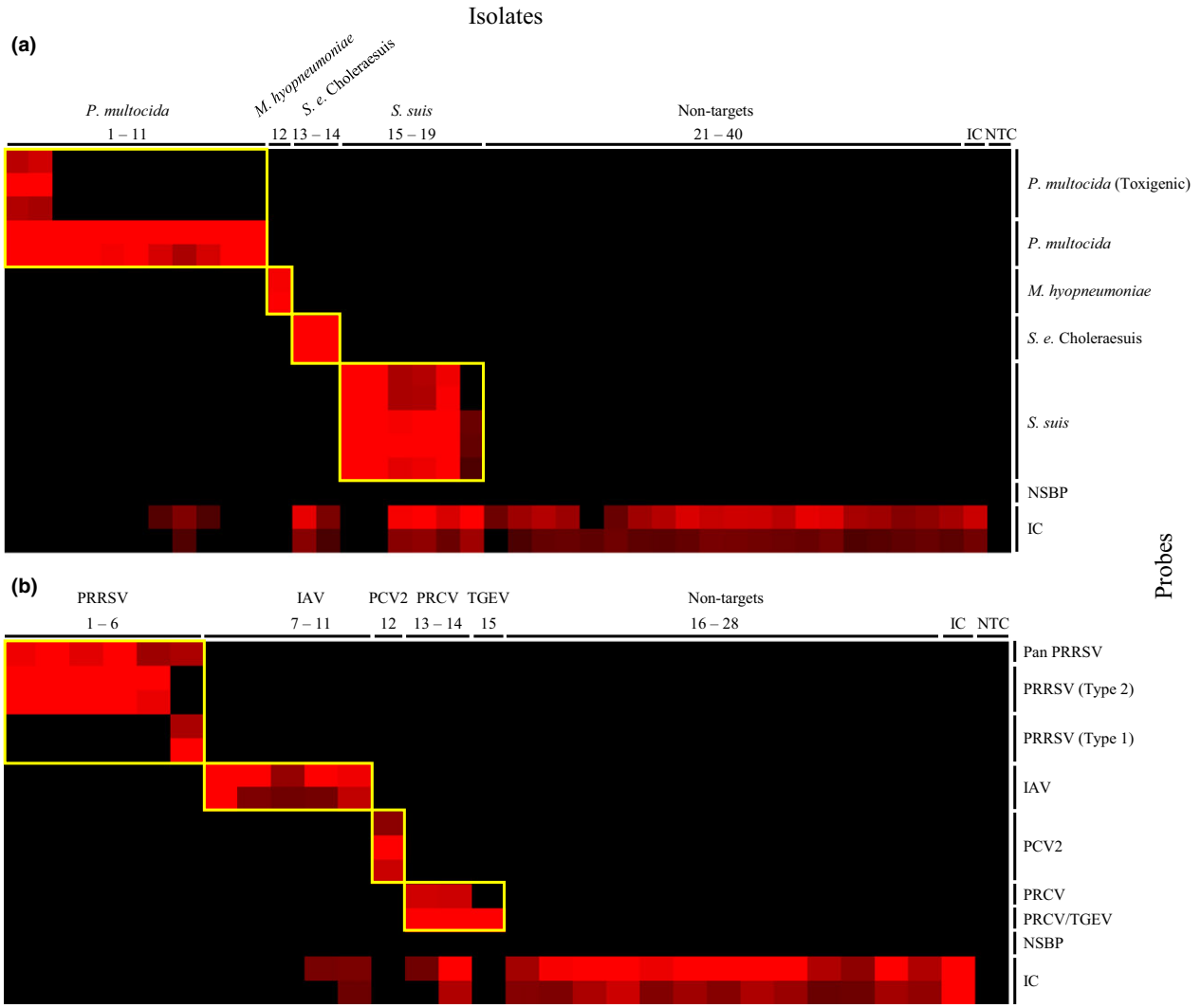

Fig. 2. Summary of microarray results from the electronic microarray representing the four bacterial targets (a), the four viral targets (b) on the electronic microarray. The reactivity of specific reactions between targets and each pathogen-specific probes is outlined in yellow. NSBP = non-specific binding probe negative control. P : N ratios ≥ 2.0 are shown in red, and P : N ratios < 2.0 are in black. NTC = no template control. IC = internal control. Amplification and detection of the internal control are not always observed when a target is present in high amounts.

electrophoretically driven hybridization, washing and reporting. The automated electronic microarray assay reduces the labour, time and number of instruments needed to acquire microarray results when compared with conventional microarrays which typically require substantial manual processing, slower passive hybridization of amplicons with capture probes and multiple pieces of equipment. The electronic microarray has an open platform format with 400 test sites that can be individually activated and utilizes a single integrated instrument that automates capture probe printing and microarray processing using parameters set by the user. Thus, the novel technology also reduces the skill level required to perform microarray assays and allows immediate, on-site modification of assays depending on the needs of the end-user. These unique features eliminate the need for anticipation of future needs, and the procurement and storage of manufactured arrays that are designed for a specific set of predetermined pathogens. For example, the system will allow immediate switching between assays that detects/types all eight pathogens simultaneously to one that detects/types a subset of the pathogens, or to assays for detection and typing of other pathogens. As the user is able to control hybridization, wash and reporting temperature the assay can have excellent specificity and can be used for differentiating variants that are genetically very similar. However, the automated electronic microarray assay requires specialized arrays and investment in instru-

Table 5. Comparison of the limit of detection (copies/μl[a]) for each target by RT-PCR and the electronic microarray

	RT-PCR		Electronic microarray	
Target[b]	Singleplex	Multiplex	Singleplex	Multiplex
PCV2	1.4×10^3	1.4×10^3	1.4×10^3	1.4×10^4
PRCV	62	620	620	620
PRRSV	3.8×10^3	3.8×10^3	3.8×10^4	3.8×10^4
IAV	160	160	160	160
M. hyopneumoniae	480	480	480	4.8×10^3
P. multocida	1.0×10^3	1.0×10^3	1.0×10^3	1.0×10^3
S. e. Choleraesuis	1.0×10^3	1.0×10^4	1.0×10^3	1.0×10^4
S. suis	10	100	100	100

[a]For the viral pathogens (excluding PCV2), target genes were cloned, reverse-transcribed, and the copy number calculated based on the RNA concentration and nucleotide composition of the amplicon. For each bacterial pathogen (excluding *M. hyopneumoniae*), the copy number was determined by plate count enumeration of a 10-fold serially diluted overnight culture. The copy number for PCV2 and *M. hyopneumoniae* was calculated based on the DNA concentration and nucleotide composition of the entire plasmid and genome, respectively.
[b]The isolates used were PCV2 (EF394779), PRCV ISU-1, PRRSV YNL, A/swine/St. Hyacinthe (H1N1), *M. hyopneumoniae* ATCC 25934, *P. multocida* MAFRI#42, *S. e.* Choleraesuis SGSC 4770 and *S. suis* P1/7.

mentation. The cost of consumables for testing a sample with an electronic microarray is expected to be between one and a few real-time PCR assays and will depend on the assay design (i.e. reducing the number of probes will decrease the cost by allowing more samples to be tested on each array with 400 test sites). In addition to glass and hydrogel (acrylamide)-based microarray matrices used in this study, the final probe sets have been tested successfully on reverse dot blots performed on nylon membranes (unpublished results). Thus, the probe set described should have broad applications in hybridization-based assays that use a variety of different matrices.

Assays for simultaneous detection of multiple bacteria and virus implicated in PRDC have not been described previously. The limit of detection of a LAMP assay for PCV2 has been shown to be approximately 1 copy of DNA plasmid, much more sensitive than conventional PCR whose detection limit was 1×10^4 copies (Zhou et al., 2011). However, LAMP assays typically detect a single pathogen and are difficult to multiplex. In multiplex real-time PCR assays, a duplex PCR assay had a detection limit of 1 $TCID_{50}$/ml for PCV2 and 6.3 $TCID_{50}$/ml for PRRSV (Chang et al., 2014), while a triplex real-time PCR assay had a detection sensitivity of 10 copies/μl for PCV2 and PRSV and 100 copies/μl for PPV (Wu et al., 2014). The multiplex PCR described here did not reduce the limits of detection for five targets although the detection limit of the PCR for three pathogens was an order of magnitude lower in the multiplex format. Thus, further improvements in sensitivity are desirable and may be partly achieved by increasing the number of PCR cycles, reducing amplicon lengths or reducing the amount of internal control used in the assay. The limit of detection for PCV2, PRRSV, and *M. hyoneumoniae* was lower with the electronic microarray in comparison with the multiplex RT-PCR indicating the use of the electronic microarray platform reduces sensitivity, and unpublished results show that only amplicons that were visible after agarose gel electrophoresis can be detected by the electronic microarray. Despite the reduced analytical sensitivity of the electronic microarray assay for some of the targets, the bacterial and viral targets were detected singly or in combination in clinical samples submitted for laboratory diagnosis. Furthermore, the assay detected *P. multocida* in a clinical lung sample that was not detected by traditional culture methods. This discrepancy may be due to the higher sensitivity of the microarray assay, or the lack of active infection (e.g. the use of antibiotics).

While primers and probes were evaluated using all sequences available on NCBI at the time of assay design and samples representative of both target and non-target bacteria and viruses were tested in this study, regular re-evaluation of the coverage of the primers and probes and additional validation with clinical samples is desired. The four swine respiratory viruses targeted in this study have also been successfully detected with the Virochip panviral array which consists of probes for detection of all known viruses at the time of design (Nicholson et al., 2011). The high-density Virochip is a useful tool for virus discovery, but needs approximately 24 h to obtain results and requires high viral titre for positive detection. In contrast, the electronic microarray assay described here can be completed in less than 4 hours with little user handling plus approximately 1.5 h for the RT-PCR described. New instrumentation that further simplifies the workflow by integrating the PCR and array processes is now commercially available. To our knowledge, the automated microarray assay described here is the first one that simultaneously

detects multiple bacterial and viral pathogens implicated in PRDC and in livestock.

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Conflict of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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