Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Multiplex RT-PCR and Automated Microarray for Detection of Eight Bovine Viruses

O. Lung[1,†], T. Furukawa-Stoffer[1], K. Burton Hughes[1,‡], J. Pasick[2], D. P. King[3] and D. Hodko[4]

[1] Lethbridge Laboratory, National Centres for Animal Disease, Canadian Food Inspection Agency, Lethbridge, AB, Canada
[2] National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada
[3] The Pirbright Institute, Woking, Surrey, UK
[4] Nexogen, Inc., San Diego, CA, USA

Keywords:
multiplex PCR; microarray; automation; rinderpest; bovine viral diarrhoea; bovine herpesvirus; malignant catarrhal fever; bluetongue

Correspondence:
O. Lung. National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, 1015 Arlington Street, Winnipeg, MB R3E 3M4, Canada.
Tel.: +1 204 789 2014;
Fax: +1 204 789 2038;
E-mail: oliver.lung@inspection.gc.ca

Current address:
†CFIA National Centre for Foreign Animal Disease, 1015 Arlington Street, Winnipeg, MB R3E 3M4, Canada
‡Agriculture and Agri-Food Canada/ Agriculture et Agroalimentaire Canada, 5403 1st Ave., S Lethbridge, AB T1J 4B1, Canada

Received for publication April 14, 2016

doi:10.1111/tbed.12591

Summary

Microarrays can be a useful tool for pathogen detection as it allow for simultaneous interrogation of the presence of a large number of genetic sequences in a sample. However, conventional microarrays require extensive manual handling and multiple pieces of equipment for printing probes, hybridization, washing and signal detection. In this study, a reverse transcription (RT)–PCR with an accompanying novel automated microarray for simultaneous detection of eight viruses that affect cattle [vesicular stomatitis virus (VSV), bovine viral diarrhoea virus type 1 and type 2, bovine herpesvirus 1, bluetongue virus, malignant catarrhal fever virus, rinderpest virus (RPV) and parapox viruses] is described. The assay accurately identified a panel of 37 strains of the target viruses and identified a mixed infection. No non-specific reactions were observed with a panel of 23 non-target viruses associated with livestock. Vesicular stomatitis virus was detected as early as 2 days post-inoculation in oral swabs from experimentally infected animals. The limit of detection of the microarray assay was as low as 1 $TCID_{50}$/ml for RPV. The novel microarray platform automates the entire post-PCR steps of the assay and integrates electrophoretic-driven capture probe printing in a single user-friendly instrument that allows array layout and assay configuration to be user-customized on-site.

Introduction

The global cattle and buffalo population is estimated to be approximately 1.7 billion heads in 2013 (http://faostat3.fao.org/browse/Q/QL/E). Infectious diseases have a significant impact on the productivity and efficiency of the livestock industry. Current methods routinely used for detection of pathogens that affect livestock are primarily tests that detect a single agent. Assays that simultaneously detect multiple pathogens in a single reaction can potentially reduce the time, labour and cost of diagnostic testing and disease surveillance. Microarrays can be used to identify the presence of a large number of genetic sequences in parallel and have been used to detect and subtype viruses that affect livestock (Banér et al., 2007; Barrette et al., 2009; Jack et al., 2009; Jiang et al., 2011; Lung et al., 2011, 2015; Nicholson et al., 2011).

In this study, we describe the development and initial laboratory evaluation of a multiplex RT-PCR and

automated microarray assay for simultaneous detection of eight viruses that affect cattle: vesicular stomatitis virus (VSV, *Vesiculovirus* genus, family *Rhabdoviridae*), bluetongue virus (BTV, *Orbivirus* genus, family *Reoviridae*), bovine viral diarrhoea virus (BVDV, *Pestivirus* genus, family *Flaviviridae*) type 1 and type 2, malignant catarrhal fever virus (MCFV, *Rhadinovirus* genus, family *Herpesviridae*), bovine herpesvirus-1 (BoHV-1, *Varicellovirus genus,* family *Herpesviridae*), parapox virus complex (PPV, *Parapoxvirus genus,* family *Poxviridae*) and rinderpest virus (RPV, *Morbillivirus* genus, family *Paramyxoviridae*). The Global Rinderpest Eradication Programme (GREP) has successfully eradicated rinderpest circulating in the wild in 2011, but rinderpest remains a notifiable disease, and surveillance systems must be maintained for detection of clinical cases resulting from accidental or intentional release of the virus from the laboratory. The novel assay and microarray platform is a potential tool for simultaneous detection of multiple viruses that affect cattle.

Materials and Methods

Samples

Thirty-seven strains of the eight targeted viruses used in this study are listed in the figure legend of Fig. 1. Research on rinderpest virus (RPV) was completed before May 2011 as required by the 79th OIE General Session (May 2011) resolution no. 18. Archived DNA and RNA from seven FMDV isolates representing each of the seven serotypes (A/Iran/99, O1 Manisa, C1 Noville, Asia1 Shamir, SAT1 Ken 4/98, SAT2 Swa 1/69 and SAT3 Zim 4/81) and border disease virus (BDV), classical swine fever virus (CSFV), elk herpesvirus, cervid herpesvirus, rangifer herpesvirus, caprine herpesvirus, epizootic haemorrhagic disease virus (EHDV), vesicular exanthema of swine virus (VESV), swine vesicular disease virus (SVDV), African swine fever virus (ASFV), porcine circovirus 1 (PCV 1), porcine circovirus 2 (PCV 2), swine influenza virus (SIV), transmissible gastroenteritis coronavirus (TGEV), porcine coronavirus (PCoV) and porcine reproductive and respiratory syndrome virus (PRRSV) were used as non-target controls to evaluate the specificity of the assay.

The details of the inoculation studies in animals and clinical samples were as previously described (Lung et al., 2011) or will be described elsewhere for other viruses. All animal studies complied with the guidelines of the Canadian Council on Animal Care. Oral ($n = 8$) and nasal ($n = 8$) swabs from eight healthy animals were used as clinical sample material during the spiking of BVDV type 1, BVDV type 2, BoHV-1, bovine papular stomatitis virus

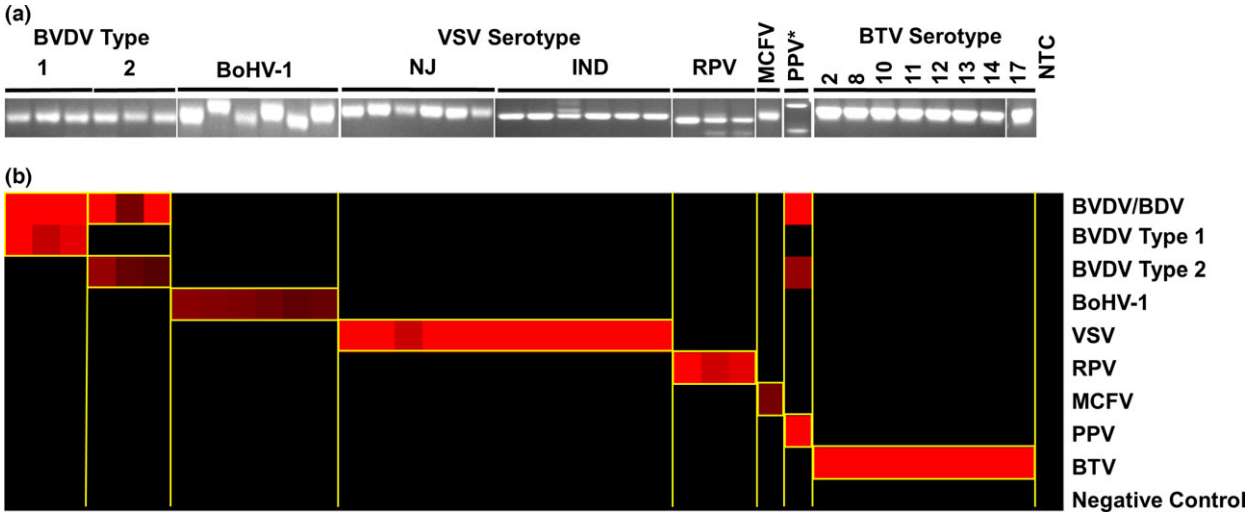

Fig. 1. Amplification and detection of 37 strains of eight viruses that affect cattle. (a) Agarose gel image of RT-PCR amplicons and (b) heat map of the reactivity of the minimal set of 10 probes. Probes and viral strains are grouped according to species and serotype and are listed on the right or above the heat map, respectively. The order of viral strains is BVDV type 1 strains New York 1 (Type 1b), Singer (Type 1a), Hastings (Type 1b); BVDV type 2 strains 24515, 890, Ames 125c; BoHV-1 strains Colorado #34, V654, V135, Edmonton #5, Edmonton #39, V76; VSV New Jersey serotype strains 0185 PNB, 0804 COE1, 1184 HDB, 89 GAS, 92 CLB, New Mexico 95; VSV Indiana serotype strains 85 CLB, 94 GUB, 97 CRB, 98 COE, Indiana (ATCC), IND1 02V1008; RPV strains Kabete O, Eldoret, Buffalo Pakchong; MCFV strain WC11 9101; PPV strain Orf NZ2; BTV serotype 2, 8, 10, 11, 12, 13, 14 17; non-template control (NTC). Positive reactions (positive-to-negative, P : N ratio greater or equal to 2) are indicated in shades of red that corresponds with the strength of the signal, and negative reactions (P : N ratio <2) are indicated in black. Note that the PPV (Orf virus) sample generated two amplicons which reacted as expected with the PPV-specific capture probe, and also with the probe for type 2 BVDV and a BVDV/BDV probe designed to detect both BVDV 1, 2 and BDV. NTC represents the non-template control, and negative control probe represents a non-specific-binding control probe. The approximate amplicon size for each virus is different and is listed in Table 1.

(BPSV) and pseudocowpox virus (PCPV) at 1×10^5 $TCID_{50}$/ml and 1×10^6 $TCID_{50}$/ml for MCFV. A total of 10–20 μl of neat cell culture-derived virus was used for spiking of BoHV-1 and contagious ecthyma virus (CEV).

Primer and probes

Virus-specific primer and probe sequences were either identified from the literature and used with or without modification, or designed in-house (Table 1). Primers were evaluated with Primer-BLAST (Ye et al., 2012), and ones which did not return hits with less than four mismatches with non-target viruses in GenBank's Viridae database were selected. An *in silico* analysis using ePCR (Schuler, 1997; Rotmistrovsky et al., 2004) was also conducted to ensure none of the 420 unique primer pairs in the multiplex PCR returned hits with less than four mismatches against non-target viruses in the GenBank Viridae database. Although hits were observed against two non-related viruses at a mismatch threshold of four mismatches per primer, the ThermoBLAST program (DNA Software™, Ann Arbor, MI, USA; SantaLucia, 2007) was used as an *in silico* simulation and modelling tool to demonstrate amplicons will not be generated due to unfavourable thermodynamic conditions used for the PCR. Capture probes were similarly evaluated *in silico*. All capture probes had 5′-biotinylation, and the reverse primers of the multiplex RT-PCR were synthesized with the reverse complementary sequence of the NGEN™ Red Universal Reporter Probe at the 5′ end (Lung et al., 2011). All primers and probes were synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA).

Nucleic acid extraction

Total DNA or RNA was extracted from viruses and clinical samples as described previously (Lung et al., 2011, 2012) or using QIAamp Viral RNA Kit (Qiagen Inc., Toronto, ON, Canada) and the Ambion MagMax Kit (Thermo Fisher Scientific, Mississauga, ON, Canada) according to the manufacturer's recommendations. RNA was eluted in 50 μl or 80 μl elution buffer.

Multiplex reverse transcriptase–PCR

A multiplex RT-PCR was developed in this study to amplify selected genomic regions of eight bovine viruses (MCFV, RPV, VSV, BVDV type 1 and type 2, BoHV-1, BTV and PPV) in a single reaction. Primers used in the RT-PCR for specific amplification of the targeted viruses were used with or without a primer pair for enterovirus armored RNA to

Table 1. PCR primers and probes used in this study

Virus	Primers and probes (5′-3′)	Binding Region	Amplicon Length (bp)	Reference
VSV	Primer: GGATGGAGTATCSTBAATCT	L-gene	270	This study
	Primer: CCATARTTYAARTARTCTGC			This study
	Probe: RTAAATGAYGATGARACYATGCAATC			Lung et al., 2011
RPV	Primer: GCTCTGAACGCTATTACTAAG	P-gene	235	Forsyth and Barrett, 1995
	Primer: CTGCTTGTCGTATTTCCTCAA			Forsyth and Barrett, 1995
	Probe: GAGTTCYACYACCAGTAGGAG			This study
MCFV	Primer: TACGGGTGCCCTGACATTTCATCTCTTTTG	ORF 50	405	Traul et al., 2005
	Primer: ATAACTGGTTGATGTGGCAGATGCATCTAT			Traul et al., 2005; Li et al., 2000
	Probe: CGATGATGTTCCTGAAGCAC			This study
BVDV	Primer: CATGCCCRYAGTAGGACTAGC	5′-UTR	277	Deregt et al., 2006
	Primer: ATGTGCCATGTACAGCAGAG			Deregt et al., 2006
	BVDV/BDV Probe: GGGTAGCAACAGTGGTGA GTTCGTTGGATGGCT			Deregt et al., 2006
	Type 1 Probe: CAGGTAAAAGCAGTTTTAACCGA CTGTTACGAATACAGCCTGATA			Deregt et al., 2006
	Type 2 Probe: GACACTCCATTAGTCGAGGAGTCTCGAGATGCC			Deregt et al., 2006
BoHV-1	Primer: TGAGGCCTATGTATGGGCAGTT	Adjacent gB	432	Hindson et al., 2008
	Primer: GGACACAACAAACAATGCGG			This study
	Probe: CCACAAAGCACATTTGACCC			This study
BTV	Primer: GTTAAAATGCAATGGTCGCAAT	Gene segment 1	342	Toussaint et al., 2007 (modified)
	Primer: TCCGGATCAAGTTCACTCC			Toussaint et al., 2007
	Probe A: TGAGGTGTACGTGAACTCAATTTTGCCGT			This study
	Probe B: CGAAGTTTACATCAATTCAATTTTACCGT			This study
PPV	Primer: TCGATGCGGTGCAGCAC	BL2	84	Nitsche et al., 2006
	Primer: GCGGCGTATTCTTCTCGGAC			Nitsche et al., 2006
	Probe: TCGAGCGTGCGGTAGAAGCC			Nitsche et al., 2006 (modified)

evaluate the potential for incorporation of an internal control. The reactions were carried out using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Burlington, ON, Canada) on an Applied Biosystems Gene Amp 9700 thermocycler (Life Technologies, Burlington, ON, Canada). Reverse transcription was carried out for 30 min at 50°C and followed by denaturation at 94°C for 2 min. The PCR consisted of 40 cycles of 94°C for 1 min; 50°C for 1 min; and 68°C for 1.5 min, followed by a final extension step of 68°C for 5 min. The 50 μl reaction mixture consisted of either 1 μl of RNA from laboratory samples or 5 μl of RNA from clinical samples, 1 μl of the enzyme mix in 1× reaction buffer and final primer concentrations of 0.1 μM (MCFV), 0.2 μM (BVDV, BoHV-1, RPV), 0.3 μM (PPV), 0.8 μM (VSV) and 1.4 μM (BTV). Following PCR of all samples during assay development, 5 μl of unpurified material was loaded on a 1% agarose gel for visualization of amplicons to allow comparison with downstream microarray results.

Electronic microarray

Electronic microarray reactions were performed in duplicates for all samples with 8 μl of neat unpurified amplicons on the NanoChip 400 electronic microarray (Nanogen Inc, San Diego, CA, USA) as described previously (Lung et al., 2011, 2015). The exception is that amplicons were electronically addressed at 995 nA for 120 s to selected electrodes containing bound capture probes. The results were analysed and displayed as described previously (Lung et al., 2011, 2015). Samples that produced P : N ratios greater than an empirically determined cut-off of 2 for both replicates for at least one virus-specific probe were considered positive, except for MCFV where 1.5 was used as the cut-off.

Results

RT-PCR amplification

The multiplex RT-PCR successfully amplified DNA of the expected size for a panel of 37 strains of the target viruses, which includes strains representing both VSV serotypes and eight BTV serotypes (Fig. 1a). Non-target viruses that cause similar clinical signs in infected animals (e.g. FMDV, SVDV) and non-target viruses related to targets (e.g. CSFV, non-target caprine, rangifer, cervid herpesviruses and EHDV), as well as nucleic acid extracted from oral swab material from healthy animals (n = 5), did not generate any significant detectable RT-PCR products except for BDV. Border disease virus generated a 277-bp product due to the use of pestivirus RT-PCR primers designed against the conserved 5′ UTR region (data not shown). After RT-PCR, one tissue culture-amplified sample (PPV, Orf virus) had the expected 84-bp amplicon product that corresponded to that of PPV, but also had an extra 277-bp product that corresponded to the expected size of the BVDV amplicon (Fig. 1a, lane 29).

Microarray detection and differentiation

A minimal set of 10 virus-specific capture probes (Table 1), of 49 tested, accurately detected the entire panel of target viruses (Fig. 1b). No cross-reactivities with heterologous viruses, non-target viruses and clinical material from healthy animals were observed. The cell culture-amplified Orf virus, which produced two bands after RT-PCR, reacted with the capture probe for the parapox virus as expected. This sample also reacted with a probe that detects both BVDV type 1 and type 2 and BDV, and the probe specific for type 2 BVDV. This result suggested the sample was contaminated with a type 2 BVDV and that the assay could simultaneously detect two different target viruses in the same sample.

Limits of detection

Nucleic acid extracted from 10-fold serial dilutions of viruses of known titre was used to determine the limit of detection of the assay for each virus (Table 2). The multiplex microarray assay was most efficient for detection of RPV and had a low limit of detection of 1 $TCID_{50}$/ml.

Testing of clinical and spiked samples

A total of 47 clinical samples from cattle experimentally infected with VSV, RPV or BTV viruses were tested with the multiplex assay. Both VSV serotypes were detected in oral samples as early as 2 days post-inoculation (dpi). RPV was detected in 6 dpi oral and nasal samples, while the earliest detection of BTV was at 8 dpi in whole-blood samples from experimentally infected cattle.

Clinical oral and nasal material experimentally inoculated with MCFV, PPV, BoHV-1, BVDV type 1 and type 2 was also examined and shown that there was no interference with detection. All 22 samples spiked with target viruses examined (MCFV, n = 2; PPV, n = 6; BoHV-1,

Table 2. Limit of detection of multiplex PCR and microarray assay

Virus	RT-PCR ($TCID_{50}$/ml)	Microarray ($TCID_{50}$/ml)
VSV	2×10^3	2×10^3
MCFV	312.5	625
RPV	1	1
BVDV	30	30
BoHV-1	68	6.8×10^3
PPV	2.1×10^{-1}	2.1×10^3
BTV	1×10^3	1×10^3

n = 8; BVDV type 1 and type 2, n = 6) produced amplicons of the expected size after the multiplex PCR, and the viruses were accurately detected by the microarray (data not shown).

In addition to samples taken prior to experimental infection at 0 dpi, nucleic acid extracted from an additional 42 oral and nasal swabs from 34 healthy cattle did not produce detectable bands on agarose gels after the multiplex RT-PCR and no reactivity above background was observed subsequently on the microarray, demonstrating 100% specificity for these samples (data not shown).

Discussion

Automation of complex diagnostic tests for the detection of multiple pathogens in a single reaction is desirable for a diagnostic laboratory as it can potentially reduce the cost and improve the efficiency and accuracy of diagnostic testing and surveillance. This study describes the development and initial evaluation of a novel microarray platform that integrates and automates capture probe printing with all post-amplification steps of a microarray assay (e.g. hybridization, washing, reporting) for detection and differentiation of eight bovine viruses. The post-PCR automated electronic microarray process described here takes approximately 4 h to complete and requires a NanoChip 400 instrument and microfluidic electronic microarray cartridges with 400 test sites. The multiplex PCR and microarray assay accurately detected a panel of 37 strains of the target viruses, and the capture probes showed no cross-reactivity with heterologous viruses, 23 non-target viruses including ones that are related genetically, or cause similar clinical signs in ruminants, and samples from healthy animals. For laboratories without the electronic microarray instrumentation and specialized consumables, the multiplex PCR may potentially be used as a stand-alone assay as the size of the amplicons for each target with the exception of BVDV and VSV is different. As seen in previous electronic microarray assays developed for pathogens that affect poultry and porcine, the assay was able to detect viruses in clinical samples and also mixed infections in samples with two different viruses (Lung et al., 2012, 2015). Based on available data for VSV, the electronic microarray results were comparable to an in-house real-time RT-PCR. Both tests detected the VSV-IND strain tested at 2 dpi, and the electronic microarray detected the tested VSV-NJ strain at 2 dpi, 1 day earlier than the real-time RT-PCR. Although not thoroughly investigated in this study, previous published and ongoing unpublished work on the electronic microarray platform have shown that depending on the assay and the target, the sensitivity of multiplex PCRs can be comparable, or sometimes less than the component singleplex PCRs. In this study, we further observed that the downstream electronic microarray had a lower sensitivity for BoHV-1 and PPV when compared with the multiplex PCR. This reduced sensitivity for some targets on the microarray when compared with the preceding multiplex PCR has been observed in other electronic microarray assays (O. Lung, unpublished results).

The electronic microarray platform has several unique features as a potential tool for pathogen detection and typing. The ability to computer-control the printing of all capture probes, all post-PCR steps of a microarray analysis on a single instrument, and activate each of the 400 test sites of the microarray independently gives the user the ability to customize assay configurations on-site for any subset or the entire set of target viruses. Thus, there is no need to predetermine targets and stockpile pre-printed array. The ability of the platform to electrophoretically address amplicons to the array also allows instantaneous hybridization when compared to passive hybridization. The stringency of detection can be modulated by computer-controlled washing and reporting temperature, and the use of temperature gradients (stepwise increase or decrease in temperature). By increasing the stringency for detection sequences found in different strains, viruses with a minimum of three mismatches can be successfully differentiated (O. Lung, unpublished results). New instrumentation that further integrates PCR amplification with the automated microarray in a single computer-controlled instrument has been developed that will further streamline the workflow of microarray-based assays. Despite a thorough *in silico* evaluation of the primers and probes against publically available sequences of the genetically variable BTV serotypes 1–24 at the time of assay design, further evaluation of primers and probe sequences against new sequences as they become available is necessary. Due to limited resources in this project, additional validation with true field samples, and incorporation of an internal control, is desirable, especially with upcoming new instrumentation that further integrates PCR with the electronic microarray. With further validation, electronic microarray assays have potential as a tool for surveillance of multiple targets or may be used in conjunction with a real-time RT-PCR for FMDV (Reid et al., 2002, 2003; King et al., 2006; Moniwa et al., 2007) for rapid and simultaneous differential diagnosis of FMD-like vesicular diseases in cattle.

Acknowledgements

This work was funded by Defence Research and Development Canada, Centre for Security Science's Chemical, Biological, Radiological-Nuclear, and Explosives Research and Technology Initiative (CRTI) project 07-132TA. The

authors acknowledge Dr. Dirk Deregt for providing advice on the research and manuscript, Mr. Cody Buchanan, Mr. Rodrigo Ortega-Polo and Ms. Kristen Hahn for technical support.

References

Banér, J., P. Gyarmati, A. Yacoub, M. Hakhverdyan, J. Stenberg, O. Ericsson, M. Nilsson, U. Landegren, and S. Belák, 2007: Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes. *J. Virol. Methods* 143, 200–206.

Barrette, R. W., S. A. Metwally, J. M. Rowland, L. Xu, S. R. Zaki, S. T. Nichol, P. E. Rollin, J. S. Towner, W. J. Shieh, B. Batten, T. K. Sealy, C. Carrillo, K. E. Moran, A. J. Bracht, G. A. Mayr, M. Sirios-Cruz, D. P. Catbagan, E. A. Lautner, T. G. Ksiazek, W. R. White, and M. T. McIntosh, 2009: Discovery of swine as a host for the Reston ebola virus. *Science* 325, 204–206.

Deregt, D., S. Gilbert, S. Dudas, J. Pasick, S. Baxi, K. Burton, and M. Baxi, 2006: A multiplex DNA suspension microarray for simultaneous detection and differentiation of classical swine fever virus and other pestiviruses. *J. Virol. Methods* 136, 17–23.

Forsyth, M., and T. Barrett, 1995: Evaluation of polymerase chain reaction for the detection and characterisation of rinderpest and peste des petits ruminants viruses for epidemiological studies. *Virus Res.* 39, 151–163.

Hindson, B. J., S. M. Reid, B. R. Baker, K. Ebert, N. P. Ferris, L. F. Tammero, R. J. Lenhoff, P. Naraghi-Arani, E. A. Vitalis, T. R. Slezak, P. J. Hullinger, and D. P. King, 2008: Diagnostic evaluation of multiplexed reverse transcription-PCR microsphere array assay for detection of foot-and-mouth and look-alike disease viruses. *J. Clin. Microbiol.* 46, 1081–1089.

Jack, P. J., R. N. Amos-Ritchie, A. Reverter, G. Palacios, P. L. Quan, O. Jabado, T. Briese, W. I. Lipkin, and D. B. Boyle, 2009: Microarray-based detection of viruses causing vesicular or vesicular-like lesions in livestock animals. *Vet. Microbiol.* 133, 145–153.

Jiang, Y., Y. Guo, P. Wang, Q. Dong, T. Opriessnig, J. Cheng, H. Xu, X. Ding, and J. Guo, 2011: A novel diagnostic platform based on multiplex ligase detection-PCR and microarray for simultaneous detection of swine viruses. *J. Virol. Methods* 178, 171–178.

King, D. P., N. P. Ferris, A. E. Shaw, S. M. Reid, G. H. Hutchings, A. C. Giuffre, J. M. Robida, J. D. Callahan, W. M. Nelson, and T. R. Beckham, 2006: Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription-polymerase chain reaction assays. *J. Vet. Diagn. Invest.* 18, 93–97.

Li, H., N. Dyer, J. Keller, and T. Crawford, 2000: Newly recognized herpesvirus causing malignant catarrhal fever in white-tailed deer (*Odocoileus virginianus*). *J. Clin. Microbiol.* 38, 1313–1318.

Lung, O., M. Fisher, A. Beeston, K. Burton Hughes, A. Clavijo, M. Goolia, J. Pasick, W. Mauro, and D. Deregt, 2011: Multiplex RT-PCR detection and microarray typing of vesicular disease viruses. *J. Virol. Methods* 175, 236–245.

Lung, O., A. Beeston, S. Ohene-Adjei, J. Pasick, D. Hodko, K. Burton Hughes, T. Furukawa-Stoffer, M. Fisher, and D. Deregt, 2012: Electronic microarray assays for avian influenza and Newcastle disease virus. *J. Virol. Methods* 185, 244–253.

Lung, O., S. Ohene-Adjei, C. Buchanan, T. Joseph, R. King, A. Erickson, S. Detmer, and A. Ambagala, 2015: Multiplex PCR and microarray for detection of swine respiratory pathogens. *Transbound. Emerg. Dis.* doi:10.1111/tbed. 12449 [Epub ahead of print].

Moniwa, M., A. Clavijo, M. Li, B. Collignon, and P. R. Kitching, 2007: Performance of a foot-and-mouth disease virus reverse transcription-polymerase chain reaction with amplification controls between three real-time instruments. *J. Vet. Diagn. Invest.* 19, 9–20.

Nicholson, T. L., D. Kukielka, A. L. Vincent, S. L. Brockmeier, L. C. Miller, and K. S. Faaberg, 2011: Utility of a panviral microarray for detection of swine respiratory viruses in clinical samples. *J. Clin. Microbiol.* 49, 1542–1548.

Nitsche, A., M. Büttner, S. Wilhelm, G. Pauli, and H. Meyer, 2006: Real-time PCR detection of parapoxvirus DNA. *Clin. Chem.* 52, 316–319.

Reid, S. M., N. P. Ferris, G. H. Hutchings, Z. Zhang, G. J. Belsham, and S. Alexandersen, 2002: Diagnosis of all seven serotypes of foot-and-mouth disease by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. Virol. Methods* 105, 67–80.

Reid, S. M., S. S. Grierson, N. P. Ferris, G. H. Hutchings, and S. Alexandersen, 2003: Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Methods* 107, 129–139.

Rotmistrovsky, K., W. Jang, and G. Schuler, 2004: A web server for performing electronic PCR. *Nucleic Acids Res.* 32, W108–W112.

SantaLucia, J., 2007: Physical principles and visual-OMP software for optimal PCR design. *Methods Mol. Biol.* 402, 3–34.

Schuler, G., 1997: Sequence mapping by electronic PCR. *Genome Res.* 7, 541–550.

Toussaint, J. F., C. Sailleau, E. Breard, S. Zientara, and K. De Clercq, 2007: Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J. Virol. Methods* 140, 115–123.

Traul, D. L., S. Elias, N. S. Taus, L. M. Herrmann, J. L. Oaks, and H. Li, 2005: A real-time PCR assay for measuring alcelaphine herpesvirus-1 DNA. *J. Virol. Methods* 129, 186–190.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. Madden, 2012: Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13, 134.

