**ORIGINAL ARTICLE**

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

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**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 TCID₅₀/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 (Baner 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, Asial 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.
(BPSV) and pseudocowpox virus (PCPV) at $1 \times 10^5$ TCID$_{50}$/ml and $1 \times 10^6$ 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 TM, 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

| Virus | Primers and probes (5’-3’) | Binding Region | Amplicon Length (bp) | Reference |
|-------|---------------------------|----------------|----------------------|-----------|
| VSV   | Primer: GGATGGAGTATCSTBAATCT  | L-gene       | 270                  | This study |
|       | Primer: CCATARTTAARTCAGTCG  |               |                      | This study |
|       | Probe: RTAATGAYGATGARACYATGCAATC |              |                      | Lung et al., 2011 |
| RPV   | Primer: GCTCTGAACGCTATTACTAAG | P-gene       | 235                  | Forsyth and Barrett, 1995 |
|       | Primer: CTGCTCTGCTATTCTCTCAA |               |                      | Forsyth and Barrett, 1995 |
|       | Probe: GAGTTCYACYACGATTGAGG |              |                      | This study |
| MCFV  | Primer: TACGCTGCCCCTGACATTCTCTTCTTTG | ORF 50       | 405                  | Traul et al., 2005 |
|       | Primer: AATACTGCTGAGTGGCAGATGCATTCTAT |               |                      | Traul et al., 2005; Li et al., 2000 |
|       | Probe: CGATGAGTCTGAGTGAACAC |              |                      | This study |
| BVDV  | Primer: CATGCCCRYAGTAGGACTAGC | 5’-UTR        | 277                  | Dereggt et al., 2006 |
|       | Primer: ATGGCCCATGTCACGAG |               |                      | Dereggt et al., 2006 |
|       | BVDV/BVDV Probe: GGATGCAAACATGGTGGA |             |                      | Dereggt et al., 2006 |
|       | GTCGGTGGATGCGGT |              |                      | Dereggt et al., 2006 |
|       | Type 1 Probe: CAGGTAAGAGCAGTICAACCGGA |           |                      | Dereggt et al., 2006 |
|       | CTGGTACGAATACGCTTCTGA |              |                      | Dereggt et al., 2006 |
|       | Type 2 Probe: GACACTCCATTATGCGAGAGTGTCGAGATGCC | | | Dereggt et al., 2006 |
| BoHV-1 | Primer: TGAGGCCCTATGATGGCAGTT | Adjacent gB   | 432                  | Hindson et al., 2008 |
|       | Primer: GGAACAACAAACAAATGCGG |               |                      | This study |
|       | Probe: CCACAACAGGACACATTGACCC |              |                      | This study |
| BTV   | Primer: GTAAAATGCAATGTCGCAGAT | Gene segment 1 | 342                  | Toussaint et al., 2007 (modified) |
|       | Primer: TCCGGATCAAGTTCCTCACCC |               |                      | This study |
|       | Probe A: TGAGGGTAGTACGTAACCTAACTTCGGCT |           |                      | This study |
|       | Probe B: CGAAGTCTCAATCAACATTCTTTACCCT |          |                      | This study |
| PPV   | Primer: TCGATGCGGTGACGCAC | BL2           | 84                   | Nitsche et al., 2006 |
|       | Primer: GCGGGCTATTTTCTCGGAC |               |                      | 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, rinderpest, 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 ampiclon product that corresponded to that of PPV, but also had an extra 277-bp product that corresponded to the expected size of the BVDV ampiclon (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 TCID50/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, n = 5) were accurately detected by the multiplex RT-PCR.

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

| Virus    | RT-PCR (TCID50/ml) | Microarray (TCID50/ml) |
|----------|--------------------|------------------------|
| VSV      | 2 × 10^2           | 2 × 10^2               |
| 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               |

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n = 8; BVDV type 1 and type 2, n = 6) produced ampli-
cons 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 infec-
tion 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% spec-
ificity 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 cap-
ture 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 cap-
ture 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 labor-
atories without the electronic microarray instrumen-
tation 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 elec-
tronic 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 unpub-
lished 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 typ-
ing. The ability to computer-control the printing of all cap-
ture 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 prede-
termine 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 detec-
tion can be modulated by computer-controlled washing
and reporting temperature, and the use of temperature gra-
dients (stepwise increase or decrease in temperature). By
increasing the stringency for detection sequences found in
different strains, viruses with a minimum of three mis-
matches can be successfully differentiated (O. Lung, un-
published results). New instrumentation that further
integrates PCR amplification with the automated microar-
ray in a single computer-controlled instrument has been
developed that will further streamline the workflow of
microarray-based assays. Despite a thorough in silico evalu-
ation 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 pro-
ject, 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 valida-
tion, electronic microarray assays have potential as a tool
for surveillance of multiple targets or may be used in con-
junction 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.

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