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Development of multiplexed bead arrays for the simultaneous detection of nucleic acid from multiple viruses in bat samples

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ABSTRACT

Virus surveillance of wildlife populations is important for identifying, monitoring, and predicting the emergence of pathogens that pose a potential threat to animal and human health. Bats are identified as important wildlife hosts of many viruses capable of causing fatal human disease, including members of the henipaviruses, coronaviruses, rhabdoviruses and filoviruses. As global warming and habitat change are thought to impact upon pathogen transmission dynamics and increase the risk of spillover, virus surveillance in bat populations remains a significant component of efforts to improve the prediction and control of potential future disease outbreaks caused by bat-borne viruses.

In this study we have developed two fluid bead array assays containing customized panels that target multiple bat-borne viruses. These assays detect up to 11 viral RNA's simultaneously in urine samples collected from wild bat populations in Australia and Bangladesh. The assays developed show high specificity for the target viruses and the analytical sensitivity compares favorably to qRT-PCR. These assays enhance the ability to monitor multi-pathogen dynamics and identify patterns of virus shedding from bat populations, thus informing key approaches to outbreak response and control.

1. Introduction

Bats are hosts to and co-exist with a multitude of viruses, many of which are highly pathogenic in other mammalian species (Clayton et al., 2013; Field et al., 1999; Halpin et al., 2011). Over the past decade significant effort has been devoted to the study of these unique mammals, particularly the mechanisms by which they harbor zoonotic viruses in the absence of clinical signs of disease. Examples include Hendra (HeV) and Nipah (NIV) viruses (genus Henipavirus, family Paramyxoviridae). HeV sporadically spills-over to horses and subsequently humans, causing fatal disease in over 75% and 60% of cases respectively (Field et al., 2011). Surveillance of bat populations is crucial to understanding the dynamics of viral shedding and the biological and environmental drivers that lead to spillover.

Virus surveillance in bat populations is important for the identification of viruses however the process is time consuming, expensive and complex. Molecular methods predominantly reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR) are routinely employed for the detection of viral RNA. These assays are highly sensitive and specific for a single intended target but are not suitable to detect and differentiate multiple targets, in a multiplex format these methods are labor intensive and costly (Barnard et al., 2011; Mahony et al., 2010). MagPlex-TAG, a multi-analyte suspension array commercialized by Luminex Corporation®, provides a multiplexed bead array system with the potential to combine up to 500 different nucleic acid targets in a single reaction. Each bead set is differentiated and identified by unique internal dyes and the beads are conjugated to specific nucleotide tags that bind to complementary synthesized targets on specific primer sets. A DNA hybridization step incorporates target specific primer extension (TSPE) reactions to a given head set. A fluorescent conjugate is utilized to detect positive reactions in each bead set by passing through a flow cell where lasers in a Bio-plex 200 instrument identify and sort each bead (Bio-Rad, 2012).
Hercules, USA) with results delivered in real-time (Christopher-Hennings et al., 2013; Bossart et al., 2007; Foord et al., 2014; Yu et al., 2011).

In this paper we describe the development of two molecular bat virus panel assays (BVPA) for screening field samples collected from bat populations using MagPlex-TAG technology (X-TAG). Bat virus panel assay 1 (BVPA-1) was designed to identify a broad range of paramyxoviruses in Australian bats. The second panel, bat virus panel assay 2 (BVPA-2) was designed to identify paramyxoviruses and filoviruses in non-Australian bat populations. The assays were capable of detecting up to 11 different virus targets simultaneously. Simultaneous surveillance of multiple viral RNA’s, adds value to samples collected in the field and allows for the assessment of multi-pathogen transmission dynamics. Surveillance efficiency was further enhanced through the development of a method for pooling samples. This greatly increased the number of samples that could be tested per assay without sacrificing assay sensitivity.

2. Materials and methods

2.1. Virus stocks and field samples

Reference viruses were derived from tissue culture supernatant stocks maintained at the CSIRO Australian Animal Health Laboratory, Geelong, Australia. Viruses used included Hendra virus (HeV), Cedar virus (CedPV), Yeppoon virus (YepPV), Grove virus (GroPV), Menangle virus (MenPV), Hervey virus (HerPV), Tioman virus (TioPV), Tsviot virus (TevPV), Yaara Bend paramyxovirus (YBMPV; GenBank accession number KM359176) and Geelong paramyxovirus (GPMV; GenBank accession number KM359175). Nipah virus Bangladesh (NiBD), Nipah virus Malaysia (NiV-MY), Ebola Reston virus (EborV) and Ebola Zaire virus (EboZV). The inaugral isolation of the lesser-knownYepPV, GroPV, HerPV, and TevPV are detailed by (Barr et al., 2014). YBPMV and GPMV are novel paramyxovirus sequences detected but not isolated from Australian bats (unpublished). Synthesized positive controls of YBPMV and GPMV were used.

Bat urine and RNA extracted from urine samples were obtained from the Queensland Centre for Emerging Infectious Diseases (QCEID), Biosecurity Queensland, Department of Agriculture, Fisheries & Forestry (DAFF), and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). RNA from all the above viruses have been identified in excreted bat urine from a variety of bat species.

2.2. Viral RNA extraction

Urine samples were extracted individually or as a pool of 4 samples using the MagMAX 96 Express instrument (Life Technologies, Carlsbad, USA) using an in-house modification of loaded software (KingFisher v 2.6, ThermoFisher). Briefly, extraction of individual samples used 60 μL of sample with 150 μL MagMAX lysis buffer (Ambion®) following manufacturer’s guidelines. RNA was eluted in 70 μL elution buffer. For extraction of pooled urine samples, 125 μL x4 samples were first combined into a pool of 500 μL and added to 1200 μL MagMAX lysis buffer. Following extraction the RNA was eluted in 100 μL elution buffer.

2.3. Design of bat virus panel assays (BVPA)

Primer sequences of virus targets in each panel were based on either existing PCR assays or designed using available virus sequences (Supplementary data). Robust assay conditions were developed to amplify a diverse range of virus targets. Designed primers were between 18 and 25 nucleotides in length and were evaluated for their suitability using Primer Express 3.0.1 software (Applied Biosystems).

Panel BVPA-1 was constructed as an 11 Plex. In the first round of amplification, paramyxovirus family-based degenerate primers (PAR-FI & PAR-R) amplified a portion of the polymerase L-gene (Tong et al., 2006). To allow for direct sequencing, an M13-tag was incorporated on the 5’ end of the reverse primer (Supplementary data 1.1). BVPA-1 targets the following viral RNA’s: HeV, CedPV, YepPV, GroV, MenPV, HerPV, TevPV, NiV-BD/MY, TioV, YBPMV and GPMV.

BVPA-2 was constructed as an 8 Plex assay, virus species or virus genus specific first round primers allowed for the detection of paramyxoviruses and filoviruses (Supplementary data 1.2). BVPA-2 targets the following viruses: HeV, NiV-BD, NiV-MY, CedPV, MenPV, TioPV, EboRV and EboZV.

Target specific primer extension (TSPE) primers for both assays were designed as a chimera with a 24 base anti-TAG sequence on the 5’ end and a virus sequence specific oligonucleotide on the 3’ end. The anti-TAG sequence is complementary to and binds to the X-TAG sequence that is specific to each bead set. Both assays were designed to be run in a 96 well plate format.

2.4. Multiplex array assay procedure

2.4.1. Bat virus panel assay 1

One step RT-PCR was performed using the Superscript III One-Step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, USA) under the following conditions: 25 pmol forward and reverse primers, and 2.0 mM MgSO4 in a 25 μL reaction volume with reaction buffer. Thermal cycling conditions were: 60°C for 1 min, 30 min at 48°C (RT reaction), 2 min at 94°C (Taq activation), 40 cycles of 15 s at 94°C, 30 s at 49°C and 1 min at 68°C, followed by 68°C for 5 min, then hold at 4°C. Reactions were performed in a 96 well PCR plate, sealed with Microseal A film (Bio-Rad, Hercules, USA). The unincorporated dNTPs and primers from the initial RT-PCR were removed by treating each reaction with ExoSAP-IT® (Affymetrix, Santa Clara, USA) as per manufacturer’s instructions.

2.4.2. Bat virus panel assay 2

One step reverse transcription (RT-PCR) was performed using Superscript III One-Step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, USA) under the following conditions: 25 pmol of each forward and reverse primers from four sets of primer pairs to specific virus species or virus families made up to a 25 μL reaction volume with reaction buffer. Thermal cycling conditions: 30 min at 50°C (RT reaction), 2 min at 94°C (Taq activation), 45 cycles of 30 s at 94°C, 40 s at 50°C and 40 s at 68°C, followed by 68°C for 5 min then hold at 4°C in a 96 well PCR plate, and sealed with Microseal A film (Bio-Rad). The unincorporated dNTPs and primers from the initial RT-PCR were removed by treating with ExoSAP-IT (Affymetrix). Following ExoSAP-IT treatment, both panels could immediately progress to the TSPE reaction stage or stored at −20°C until further processing.

2.4.3. Target specific primer extension

This reaction initiated PCR extension of targeted regions with biotinylated dCTP nucleotides. The incorporation of biotin allows for binding of streptavidin R-phycocerythrin (Life Technologies Carlsbad, USA) for the detection on the Bio-plex 200 instrument (Foord et al., 2014; Mahony et al., 2010). The TSPE reaction additionally incorporates anti-TAG sequence corresponding to specific bead sets that will be utilized during the hybridization step. Each TSPE reaction contained 2 μL of 10× PCR buffer (Qiagen,
2.4.4. Hybridization to microspheres and assay analysis

Each X-TAG bead set is unique, containing a different fluorochrome dye and specific X-TAG sequence (Gastaldelli et al., 2011). Hybridization of biotinylated TSPE products to X-TAG beads occurred via the anti-TAG sequence that was incorporated as part of the virus specific TSPE primer (Fig. 1). 10 μL of TSPE reaction product was added to 40 μL of microsphere mix containing 1500 beads of each X-TAG set in 1× hybridization buffer (0.2 M NaCl/0.1 M Tris/0.08% Triton X-100, pH 8.0) in a low profile 96-well PCR plate. The hybridization mixture was incubated at 96 °C for 90 s followed by 37 °C for 30 min. The plate was placed on a magnetic separator and the supernatant removed, and washed with two washes of 1× hybridization buffer. Hybridization buffer (75 μL) containing 2 mg/mL streptavidin-R-phycocerythin (Invitrogen) was added to each well and the mixture incubated in the dark at 37 °C for 15 min with moderate shaking. Hybridized microspheres were analyzed on a Bio-Plex Array System integrated with Bio-Plex Manager Software (v 6.0) (Bio-Rad Laboratories, Inc., CA, USA). The reporter target channel (RP1) was set on high for the fluorescent identification of beads. Reporter conjugate emission wavelengths were maintained using a Bio-Plex Calibration Kit (Bio-Rad). Consistent optical alignment, fluidics performance, doublet discrimination and identification of individual bead signatures were assured using a Bio-Plex Validation Kit (v 4.0, Bio-Rad). X-TAG assays were analyzed at 37 °C on the 96 well plate heated platform. One hundred beads of each set were analyzed per well. Fluorescence signals were expressed as the Median Fluorescence Intensity (MFI) (Hwang et al., 2014).

2.5. Comparative assessment of assays

2.5.1. Sensitivity of BVPA to qRT-PCR assays

The analytical sensitivity of the BVPAs was assessed by direct comparison to qRT-PCR using a subset of viruses from both multiple assays. The gene targets for this analysis were based on pre-existing validated qRT-PCR assays targeting NiV and HeV N genes, and the CedPV P gene (Feldman et al., 2009; Marsh et al., 2012). Serial dilutions (10-fold) of control viral RNA were performed and run in parallel in the BVPA and the qRT-PCR to determine the limit of detection (LOD) for the assays. Conditions for qRT-PCR were as follows; for CedPV, Superscript III Platinum Taq One-Step qRT-PCR system (Invitrogen), was used with 5 pmol/μL probe and 10 pmol/μL forward and reverse primers. Thermal cycling was 50 °C for 5 min, 90 °C for 2 min followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. For the HeV and NiV qRT-PCR assays, the conditions were performed using AgPath-ID One-Step RT-PCR Reagents (Life Technologies, Carlsbad, USA) with 250 nM probe and 900 nM forward and reverse primers. Thermal cycling was 45 °C for 10 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. For both assay conditions, the cycle threshold (Ct) values equal to or below 40 were considered positive, values equal to or above 45 were considered negative. Results between 40 and 45 were deemed indeterminate (Foord et al., 2013).

2.5.2. Extraction sensitivity of pooled samples

The effect of the process of pooling samples on detection sensitivity was investigated using a known HeV RNA positive urine sample as the control for both individual and pooled samples. The HeV control sample was extracted individually and in combination with three HeV RNA negative samples. qRT-PCR assays for the HeV N gene, NiV N gene and the hemipavirus (HeV and NiV) P gene were performed to assess any change in detection sensitivity between individual and pooled samples. The extracted RNA was tested neat and at a 1/10 dilution for both individual and pooled samples.

3. Results

3.1. Analysis of field sample data and determination of the threshold value

Over 3000 bat urine samples were extracted and tested in the BVPAs providing more than 25,000 unique MFI readings (data points). The results from a total of 532 Australian field samples were used for further analysis. These Australian field samples were analyzed using the 11 Plex BVPA-1 which gave 5852 data points. The results from a total of 540 Bangladesh field samples were used for further analysis. The Bangladesh field samples were analyzed using the 8 Plex BVPA-2 which gave 4320 data points. Two methods were used to determine the most appropriate threshold for categorizing MFI values and thus samples as either positive or negative for each specific virus RNA. The first was an initial exploratory method based on a histogram plot of the values. The second involved a more rigorous statistical method applying “mixture modelling”.

For the exploratory method, a program was written in Python (the Luminex Analyzer Program or “LAP”) which reads data directly from the Luminex platform. The LAP sorted the individual data points below 1000 MFI into 124 “bins” of equal size (8 MFI units/bin), while values ≥1000 MFI were grouped into the 125th bin (Fig. 2). To ascertain the consistency of median values across all virus targets, the individual assay targets were analyzed and median values determined for each. As a first approach, we used simple exploratory methods (i.e. visualization of MFI values and taking 2 × the median value as our threshold). A value twice the highest median MFI (YBPMV = 245) of the individual targets was used to arrive at an MFI value of 490 as an initial threshold.

Finite mixture modeling is a generic framework which identifies different populations by their differing probability distributions. In this case, our two populations were animals with samples positive and negative for the individual targets. To fit the two mixture distributions, we adapted the approach used by (Budzies et al., 2012), whereby two Gaussian distributions were aligned to the histogram of the natural log of the each of the two MFI datasets, viz, for the samples from Australia and Bangladesh. The actual distribution fitting used the function normalmixEM from the R package mixtools (Benaglia et al., 2009). The optimum cutoff was determined as the value where the probability density functions of the mixing distribution coincide (Fig. 3). This natural log cut-off value was back-transformed to give the optimal cutoff value of 527.7 MFI for Australian bat samples and 676.3 for Bangladesh bat samples.

3.2. Specificity of microsphere suspension array assays

The analytical specificities of the BVPAs were assessed using RNA extracted from previously characterized tissue culture virus preparations that were used as positive controls for each target virus. Specificity was consistent for all targets when tested against homologous individual control RNA in the multiplexed format.
Target specific primer extension products (TSPE) are detected and identified by hybridization onto microsphere beads. Anti-tag oligonucleotides on the beads, hybridize to the TSPE products containing a complementary tag oligonucleotide. The microbeads are sorted by the Bio-plex 200 flow cell instrument, which identifies spectrophotometrically different color beads imbedded with fluorophores with one laser, and a phycoerythrin signal on the beads with a second laser.

Fig. 1. Target specific primer extension products (TSPE) are detected and identified by hybridization onto microsphere beads. Anti-tag oligonucleotides on the beads, hybridize to the TSPE products containing a complementary tag oligonucleotide. The microbeads are sorted by the Bio-plex 200 flow cell instrument, which identifies spectrophotometrically different color beads imbedded with fluorophores with one laser, and a phycoerythrin signal on the beads with a second laser.

Fig. 2. Histogram of frequency median/threshold results based on Australian urine samples analyzed on the 11 plex assay (A) corresponding to 5852 data points, and bat urine samples from Bangladesh analyzed on the 8 plex assay (B) corresponding to 4320 data points. From all combined results as well as the median results for each individual virus targets, which ranged from 206 to 245, the overall threshold we chose was $2 \times$ the median of the individual assay with the highest background fluorescence resulting in a MFI of “490” for Australian and Bangladesh bat urine samples.

Fig. 3. Histogram of the natural log of the MFI values for thresholds superimposed on the histogram are the two Guassian distributions fitted by the normalmixEM function. The chosen threshold corresponded to the value where the two probability density functions intersected, which when back-transformed equates to an MFI value of “527.7” for (A) Australian bat urine samples and “676.3” for (B) Bangladesh bat urine samples.

Bat urine samples collected in the field and previously identified to contain multiple viruses using virus isolation, sequencing or real-time PCR were also included in the BVPAs to assess the specificity of these assays (Tables 1 and 2). Results correlated to previous results with virus RNA targets correctly identified. Low background levels generating similar MFI values as those generated in single-plex format. No template control (NTC) wells confirmed no cross reactivity between the primers during the reactions, with all MFI values below 300 (below cutoff values generated from histogram and modeling fit).
3.3. Sensitivity of bat virus panel assays

The sensitivity of BVPA’s were assessed by serial dilution of a control RNA template and compared to qRT-PCR. The most sensitive qRT-PCR assay available was used for comparative purposes; this was not always directed to the same gene target as that used in the BVPA (Table 3). BVPA-1, was 10-fold less sensitive for CedPV but 10-fold more sensitive for HeV (Table 3.2). BVPA-2, both targets (CedPV and NiV) demonstrated equivalent sensitivities in both molecular assays (Table 3.1).

3.4. Impact of sample pooling on assay sensitivity

The volumes of sample extracted for the individual and pooled methods were 60 µL and 125 µL respectively. Bat urine containing HeV genome was used for the determination of extraction sensitivity. The extracted RNA was tested by qRT-PCR neat and diluted 1:10. Two qRT-PCR assays were run to establish comparative sensitivity, the HeV N-gene and Henipavirus P-gene. Both these qRT-PCR assays generated comparable results for both individual and pooled extraction samples when tested neat, while the pooled method appeared to show slightly better detection in both qRT-PCR assays when diluted 1:10. The results indicate that pooling samples for extraction in this way did not diminish sensitivity.

3.5. Field sample analysis

Based on the calculated threshold of 527.7 MFI, we classified 5633 results as “negative” and 218 as “positive” for the Australian field samples. Based on the calculated threshold of 676.3 MFI for Bangladesh bat samples, we classified 4172 results as “negative” and 75 as “positive”.

### Table 1

Specificity of BVPA-1.

| BVPA-1 | Median fluorescence intensity (MFI) values for each of the 11 probes/targets<sup>a</sup> |  |
|--------|-------------------------------------------------------------------------|----|
| Control samples | TePV | YepPV | GroPV | CedPV | HeV | MenPV | HerPV | YBMV | GPMV | TioPV | NiV |
| HeV     | 131 | 201 | 149 | 151 | 12,280 | 196 | 154 | 375 | 225 | 124 | 120 |
| MenPV   | 229 | 216 | 196 | 265 | 208 | 18,981 | 207 | 252 | 252 | 173 | 153 |
| CedPV   | 199 | 200 | 183 | 20,422 | 273 | 120 | 158 | 252 | 191 | 252 | 193 |
| GPMV    | 180 | 180 | 234 | 222 | 292 | 276 | 225 | 271 | 21,419 | 185 | 122 |
| YBMV    | 217 | 144 | 221 | 131 | 206 | 227 | 213 | 14,541 | 264 | 183 | 148 |
| NiV     | 191 | 160 | 183 | 204 | 204 | 204 | 187 | 201 | 203 | 185 | 490 |
| Mix<sup>b</sup> | 3,452 | 1296 | 7640 | 205 | 201 | 19,112 | 3983 | 269 | 307 | 235 | 175 |
| TioPV   | 142 | 155 | 109 | 169 | 245 | 192 | 198 | 189 | 201 | 19,520 | 183 |

| Field urine samples |  |
|---------------------|----|
| A<sup>c</sup> | 6071 | 731 | 11,879 | 359 | 326 | 19,573 | 11,870 | 314 | 413 | 330 | 345 |
| B<sup>c</sup> | 1079 | 166 | 160 | 217 | 6752 | 160 | 256 | 2713 | 230 | 273 | 181 | 215 |
| C<sup>c</sup> | 9529 | 122 | 2661 | 223 | 6577 | 291 | 275 | 202 | 218 | 152 | 165 |
| D<sup>c</sup> | 221 | 161 | 166 | 204 | 731 | 833 | 179 | 212 | 188 | 170 | 184 |
| E<sup>c</sup> | 17,242 | 1911 | 5055 | 297 | 222 | 330 | 237 | 2258 | 251 | 257 | 220 |
| F<sup>c</sup> | 299 | 245 | 2724 | 324 | 299 | 299 | 232 | 295 | 310 | 298 | 253 |
| G<sup>c</sup> | 624 | 101 | 105 | 199 | 139 | 10,484 | 78 | 156 | 143 | 148 | 98 |
| No template control | 123 | 107 | 102 | 182 | 157 | 143 | 101 | 199 | 156 | 122 | 146 |

<sup>a</sup> Virus targets; TePV = Teiotavirus, YepPV = Yegpoon virus, GroPV = Groove virus, CedPV = Cedar virus, HeV = Hendra virus, MenPV = Menangle virus, HerPV = Hervey virus, YBMV = Yarra Bend paramyxovirus, GPMV = Geelong paramyxovirus, TioPV = Tioman virus, NiV = Nipah virus; samples considered positive are highlighted.

<sup>b</sup> A mixture of TePV, YepPV, GroPV, MenPV, HerPV

<sup>c</sup> Sample confirmed by PCR or sequencing to be positive for TePV, YepPV, GroPV, MenPV, and HerPV.

### Table 2

Specificity of BVPA-2.

| BVPA-2 | Median fluorescence intensity (MFI) values for each of the 8 probes<sup>a</sup> |  |
|--------|------------------------------------------------------------------|----|
| Control samples | HeV | CedPV | EboRV | EboZV | Henipa | NiV | TioPV | MenPV |
| HeV     | 19,949 | 169 | 161 | 118 | 12,874 | 202 | 154 | 180 |
| Cedar   | 190 | 23,419 | 168 | 103 | 259 | 141 | 115 | 124 |
| TioPV   | 203 | 233 | 175 | 107 | 174 | 148 | 23,768 | 224 |
| Nipah-BD | 210 | 237 | 223 | 132 | 13,531 | 20,196 | 169 | 219 |
| Nipah-MY | 211 | 174 | 127 | 148 | 11,421 | 21,472 | 117 | 176 |
| EboRV   | 161 | 237 | 5876 | 119 | 197 | 139 | 162 | 149 |
| EboZV   | 136 | 167 | 153 | 147,10 | 168 | 134 | 169 | 185 |
| MenPV   | 197 | 195 | 174 | 114 | 174 | 151 | 144 | 16,252 |

| Field urine samples |  |
|---------------------|----|
| A<sup>c</sup> | 189 | 225 | 180 | 195 | 768 | 1065 | 153 | 195 |
| B<sup>c</sup> | 151 | 235 | 208 | 127 | 858 | 1130 | 147 | 203 |
| C<sup>c</sup> | 288 | 238 | 224 | 275 | 1289 | 1779 | 299 | 365 |
| D<sup>c</sup> | 279 | 238 | 240 | 185 | 599 | 703 | 221 | 248 |
| E<sup>c</sup> | 152 | 168 | 154 | 120 | 200 | 155 | 135 | 175 |
| F<sup>c</sup> | 153 | 167 | 138 | 132 | 168 | 153 | 154 | 189 |
| G<sup>c</sup> | 169 | 149 | 210 | 177 | 162 | 128 | 127 | 184 |
| No template control | 121 | 112 | 169 | 129 | 109 | 117 | 125 | 124 |

<sup>a</sup> Virus targets; HeV = Hendra virus, CedPV = Cedar virus, EboRV = Ebola-Reston, EboZV = Ebola-Zaire virus, Henipa = Hendra or Nipah virus, NiV = Nipah virus, TioPV = Tioman virus, MenPV = Menangle virus; samples considered positive are highlighted.

<sup>b</sup> Samples confirmed positive to NiV by sequencing.
A selection of seven representative BVPA results for field samples are shown in Tables 1 and 2 respectively. For some samples, multiple virus targets were detected. For example, for sample A, Table 1, five virus targets were detected. These results are unlikely to be a result of cross-reactions as the results were confirmed by other molecular methods including PCR and/or sequencing.

4. Discussion and conclusion

Virus surveillance in wildlife populations is essential to efficiently monitor the risk of emerging infectious disease outbreaks. Internationally, virus surveillance in bats remains a major component of this important effort. Disease and virus surveillance is an arduous process with constraints on sample collection, transport and analysis particularly in remote locations. Sample collection needs to be well coordinated, comprehensive, and longitudinal which demands significant resources both in the field and during subsequent analyses (Hoye et al., 2010). Although these constraints are significant, surveillance of wildlife species is crucial to the identification and tracking of trends in viral shedding and its association with environmental and population conditions. This was highlighted in Australia where an extensive surveillance study of more than 40 different animal species in Queensland was required to identify bats as the natural reservoir of Hendra virus (Young et al., 1996). The development of the two novel bat virus panel assays described here represents an important step toward a more effective screening platform for the simultaneous detection of multiple viral pathogens and allows for high sample throughput fulfilling the objectives of a reliable, accurate and cost-effective process of generating surveillance information. The BVPAs developed show high specificity to the targeted viruses and the analytical sensitivity compares favorably to qRT-PCR. The flexible nature of the platform allows for a high degree of customization through the expansion and/or modification of viral targets.

Following nucleic acid extraction the BVPAs take significantly longer than qRT-PCR (approximately 7 h total), however in this time the BVPAs generated results equivalent to 8–11 individual qRT-PCR assays. Like any amplification method, precautions need to be taken to prevent contamination. Despite the fact that these multiplex assays have been designed to a 96-well plate format, basic aseptic precautions ensured that no detectable contamination occurred in any of the 3000 bat urine samples tested.

In this study, the use of first round PCR primers together with specific TSPE extension primers and unique bead sets allowed for the detection of up to 11 viral targets. The background signals for all bead sets in no-template-controls were low, in the range of 50–300 MFI. The two assay approaches utilized different first round amplification strategies; strategy 1 (BVPA-1) employed a single degenerate primer pair for Paramyxoviruses while strategy 2 (BVPA-2) utilized multiple primers to different virus families. BVPA-2 allowed for detection of multiple viral families however the presence of multiple primer sets in the first round amplification increased the potential for cross reactivity, so careful assay design and validation was critical. BVPA-1 also provides for the addition of M13-tags to the primers allowing direct sequencing of products from first round amplification. The pooling strategy described for the extraction of RNA from field samples makes significant savings in reagent cost and processing time however it does increase the likelihood of multiple targets.

Establishing appropriate thresholds or “cutoff” values is challenging for wildlife population research, due to the fact there will potentially be animal samples collected at differing stages of the infection cycle and no naïve control samples were available (Gardner et al., 2010; Peel et al., 2013). It needs to be recognized that any threshold will exclude positive individuals that are at the beginning or end of the infection cycle. Although the assay platform is not empirically quantitative, the MFI values are indicative of the level of viral RNA present in the sample. Another important consideration in multiplex assays is the issue of competition when samples have multiple viral RNA’s present. In this case it is possible that MFI values will be affected by the competition for assay reagents, thus generating lower values for all targets than samples with single viral RNA’s. This competition for reagents and subsequent impact on results make multiplex assays less applicable in diagnostic applications. However, for population surveillance applications the need for an absolute determination for each sample is less important than the spatial and temporal trend of virus activity.

The commonest method to evaluate the diagnostic sensitivity (DSe) and specificity (DSP) of the Luminex xTAG assay is to
compare their performance against validated qRT-PCR tests for each of the viruses (Pabaraju et al., 2008; Foord et al., 2013). However, this process of evaluation and validation for emerging viral diseases in wildlife is problematic as it can be very difficult to assemble a “gold standard” dataset, with samples from both truly positive and negative animals. This is made even more challenging for the viral diseases we are testing for in the flying foxes, as most do not cause any illness or pathology in these animals. Furthermore, as we tested pooled under roost urine specimens, it is impossible to know exactly which animal(s) contributed to the specimen. A third challenge is that many of these emerging viral diseases have only been detected infrequently, and thus the number of potentially positive samples is quite low, and insufficient to undertake validation statistical procedures such as ROC curve analysis to define an optimum threshold value (Greiner et al., 2000).

To overcome these challenges, we have adopted a strategy to determine an appropriate assay threshold value that is both pragmatic and incremental. As a first approach, we used simple exploratory methods (i.e. plotting the MFI values and taking 2× the median value as our threshold). This was expanded on through the use of the more objective mixture model method. Further work could be undertaken to derive a more biologically justifiable threshold. In particular, the distribution of log values in Fig. 3 indicates that there may be in fact three populations: “negative”, “intermediate” and “positive”. In order to determine the “true” biological status of samples in this indicated intermediate group qRT-PCR could be used for validation, however these samples are often reflected by indeterminate results in this assay as well. The real benefit of our incremental approach is that it enabled us to focus our follow-on validation work to a restricted sub-sample, resulting in the determination of a robust threshold in a cost-effective and timely manner.

The flexibility of multiplex bead array technologies has led to many applications from animal and human health to the detection of bio-threat agents (Janse et al., 2012; Jokela et al., 2012; LeBlanc et al., 2010; Washington et al., 2010). Although the two bead panels were designed for application to Australian and Bangladesh bat urine samples, the technology is highly flexible allowing the incorporation of different virus targets and its application to broader hosts and situations. Such as Coronavirus excretion in Eurasian bat colonies or the spatial and temporal analysis of different NB Bluetongue virus strains in Europe. The technology provides a reliable and cost effective approach for a wide range of diagnostic, surveillance and epidemiological applications (Babady et al., 2012; Baner et al., 2007; Pabaraju et al., 2008; Wu et al., 2014).

Our novel BVPA's are a significant surveillance tool for viral RNA detection in bats. The platform allows for the expansion of the panels to include further viral agents or host gene targets such as those that identify gender and species of bat. These assays enhance our ability to identify patterns of virus shedding informing key approaches to outbreak response and control. The methodology provides vastly expanded scope and efficiency in the critical and complex analysis of virus and disease surveillance for wildlife populations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.viromet.2015.07.004

References

Babady, N.E., Mead, P., Stiles, J., Brennan, C., Li, H., Shugart, S., Stratton, C.W., Tang, Y.-W., Kamboj, M., 2012. Comparison of the LumineX xTAG RVP Fast assay and the Idaho Technology FilmArray RP assay for detection of respiratory viruses in pediatric patients at a children’s hospital. J. Clin. Microbiol. 50, 2282–2288, http://dx.doi.org/10.1128/JCM.00618-11
Baner, J., Gyarmati, P., Yacoub, A., Halkhverdy, M., Stenberg, J., Ericsson, O., Nilsson, M., Landegren, U., Belak, S., 2007. Microarray-based molecular debility of foot-and-mouth disease, vesicular stomatitis, and swine respiratory diseases, using padlock probes. J. Virol. Methods 143, 200–206, http://dx.doi.org/10.1016/j.viromet.2007.03.004
Barnard, R.T., Hall, R.A., Gould, E.A., 2011. Expecting the unexpected: nucleic acid-based diagnosis and discovery of emerging viruses. Expert Rev. Mol. Diagn. 11, 409–423, http://dx.doi.org/10.1586/erm.11.24
Barr, J., Smith, C., Smith, I., de Jong, C., Todd, S., Melville, D., Broos, A., Cramer, S., Haining, J., Marsh, C., Cramer, G., Field, H., Wang, L.-F., 2014. Isolation of multiple novel paramyxoviruses from pteropod bat urine. J. Gen. Virol., http://dx.doi.org/10.1099/vir.0.068160-0
Benaglia, T., Chauveau, D., Hunter, D.R., Young, D.S., 2009. mixtools: an R package for analyzing finite mixture models. J. Stat. Softw. 32, 1–29.
Boskant, N.K., McCaugh, J.A., Hickey, A.C., Choudhry, V., Dimitrov, S., Eaton, B.T., Wang, L.-F., 2007. Neutralization assays for different henipavirus serology using Bio-Plex protein array systems. J. Virol. Methods 142, 29–40, http://dx.doi.org/10.1016/j.viromet.2007.01.003
Budczies, J., Klauchen, F., Sinn, B.V., Györföly, B., Schmitt, W.D., Darb-Esfahani, S., Denkert, C., 2012. Cutoff finder: a comprehensive and straightforward Web application enabling calculation of an optimal cutoff from raw data. PloS ONE 7, e51862, http://dx.doi.org/10.1371/journal.pone.0051862
Christopher-Hennings, J., Araujo, K.P.C., Souza, C.J.H., Fang, Y., Lawson, S., Nelson, E.A., Clement, T., Dunn, M., Lunney, J.K., 2013. Opportunities for bead-based multiplex assays in veterinary diagnostic laboratories. J. Vet. Diagn. Investig. 15, 671–691, http://dx.doi.org/10.1177/1040637813507256
Clayton, B.A., Wang, L.F., Marsh, G.A., 2013. Henipaviruses: an updated review focusing on the pteropod reservoir and features of transmission. Zoonoses Public Health 60, 69–83, http://dx.doi.org/10.1111/zph.12150.x
Field, H., McCall, B., Barrett, J., 1999. Australian Bat Lyssavirus infection in a captive juvenile black flying fox. Emerg. Infect. Dis. 5, 438–440.
Field, H., de Jong, C., Melville, D., Smith, C., Smith, I., Bull, S.J., Kung, Y.H.N., McLaughlin, A., Zedelmann, A., 2011. Hendra virus infection dynamics in Australian fruit bats. PLoS ONE 6, e26878, http://dx.doi.org/10.1371/journal.pone.0026878
Foord, A.J., White, J.R., Colling, A., Heine, H.G., 2013. Microsphere suspension array assays for detection and differentiation of Hendra and Nipah viruses. BioMed Res. Int. 2013, 289295, http://dx.doi.org/10.1155/2013/289295
Foord, A.J., Boyd, V., White, J.R., Williams, D.T., Colling, A., Heine, H.G., 2014. Flavirus detection and differentiation by a microsphere array assay. J. Virol. Methods 203, 65–72.
Gardner, L.A., Greiner, M., Dubey, J.P., 2010. Statistical evaluation of test accuracy studies for Toxoplasma gondii in animal intermediate hosts. Zoonoses Public Health 57, 82–94, http://dx.doi.org/10.1111/j.1863-2378.2009.01281.x
Gastaldelli, M., Stefani, E., Lettini, A.A., Pozzano, N., 2011. Multiplexed typing of Mycobacterium avium subsp. para tuberculosis types I, II, and III by luminex xMAP suspension array. J. Clin. Microbiol. 49, 385–391, http://dx.doi.org/10.1128/JCM.01761-10
Greiner, M., Pfeiffer, D., Smith, R.D., 2000. Principles and practical application of the receiver operating Characteristic analysis for diagnostic tests. Prev. Vet. Med. 45, 23–41, 54 ref.
Halpin, K., Hyatt, A.D., Fogarty, R., Middleton, D., Bingham, J., Epstein, J.H., Rahman, S.A., Hughes, T., Smith, C., Field, H.E., Dazak, P., 2011. Pteropod bats are confirmed as the reservoir hosts of henipaviruses: a comprehensive experimental study of virus transmission. Am. J. Trop. Med. Hyg. 85, 946–951, http://dx.doi.org/10.4269/ajtmh.2011.10-0567
Hoye, B.J., Munster, V.J., Nishiyama, H., Klaassen, M., Fouche, R.A.M., 2010. Surveillance of wild birds for avian influenza virus. Emerg. Infect. Dis. 16, 1827–1834, http://dx.doi.org/10.3201/ed16100589
Hwang, S.M., Lim, M.S., Han, M., Hong, Y.J., Kim, T.S., Lee, H.R., Song, E.Y., Park, K.U., Song, J., Kim, E.C., 2014. Comparison of xTAG respiratory virus panel and veripogene respiratory virus plus for detecting influenza virus and respiratory syncytial virus. J. Clin. Lab. Anal. 6, 1–6, http://dx.doi.org/10.1002/jclal.21738
Janse, I., Bok, J.M., Hamidjaja, R.A., Hodemaekers, H.M., van Rotterdam, B.J., 2012. Development and comparison of two assay formats for parallel detection of four bioterror pathogens by using suspension microarrays. PLoS ONE 7, e31958, http://dx.doi.org/10.1371/journal.pone.0031958
Performance of the Luminex xTAG respiratory viral panel fast in a clinical laboratory setting. J. Virol. Methods 182, 82–86, http://dx.doi.org/10.1016/j.jviromet.2012.03.015
LeBlanc, N., Leijon, M., Jobs, M., Blomberg, J., Belák, S., 2010. A novel combination of TaqMan RT-PCR and a suspension microarray assay for the detection and species identification of pestiviruses. Vet. Microbiol. 142, 81–86, http://dx.doi.org/10.1016/j.vetmic.2009.09.046
Mahony, J.B., Chong, S., Lunstra, K., Petrich, A., Smieja, M., 2010. Development of a novel bead-based multiplex PCR assay for combined subtyping and oseltamivir resistance genotyping (H275Y) of seasonal and pandemic H1N1 influenza A viruses. J. Clin. Virol. 49, 277–282, http://dx.doi.org/10.1016/j.jcv.2010.08.006
Marsh, G.A., Jong, C.D., Barr, J.A., Tachedjian, M., Smith, C., Middleton, D., Yu, M., Todd, S., Foord, A.J., Haring, V., Payne, J., Robinson, R., Broz, L., Crameri, G., Field, H.E., Wang, L., 2012. Cedar virus: a novel henipavirus isolated from australian bats. PLoS Pathog. 8, e1002836, http://dx.doi.org/10.1371/journal.ppat.1002836
Pabbaraju, K., Tokaryk, K.L., Wong, S., Fox, J.D., 2008. Comparison of the Luminex xTAG respiratory viral panel with in-house nucleic acid amplification tests for diagnosis of respiratory virus infections. J. Clin. Microbiol. 46, 3056–3062, http://dx.doi.org/10.1128/JCM.00878-08
Peel, A.J., McKinley, T.J., Baker, K.S., Barr, J.A., Crameri, G., Hayman, D.T.S., Feng, Y.-R., Broder, C.C., Wang, L.-F., Cunningham, A.A., Wood, J.L.N., 2013. Use of cross-reactive serological assays for detecting novel pathogens in wildlife: assessing an appropriate cutoff for henipavirus assays in African bats. J. Virol. Methods 193, 295–303, http://dx.doi.org/10.1016/j.jviromet.2013.06.030
Tong, S., Chern, S.W., Li, Y., Mark, A., Anderson, L.J., Pallansch, M.A., 2008. Sensitive and broadly reactive reverse transcription-PCR assays to detect novel paramyxoviruses. J. Clin. Microbiol. 46, 2652–2658, http://dx.doi.org/10.1128/JCM.00192-08
Washington, C., Metzgar, D., Hazbón, M.H., Binn, L., Lyons, A., Coward, C., Kuschner, R., 2010. Multiplexed Luminex xMAP assay for detection and identification of five adenovirus serotypes associated with epidemics of respiratory disease in adults. J. Clin. Microbiol. 48, 2217–2222, http://dx.doi.org/10.1128/JCM.00029-10
Wu, W., Zhang, S., Qu, J., Zhang, Q., Li, C., Li, J., Jin, C., Liang, M., Li, D., 2014. Simultaneous detection of IgG antibodies associated with viral hemorrhagic fever by a multiplexed Luminex-based immunoassay. Virus Res. 187, 84–90, http://dx.doi.org/10.1016/j.virusres.2013.12.037
Young, P., Halpin, K., Selleck, P., 1996. Serologic evidence for the presence in Pteropus bats of a paramyxovirus related to equine morbillivirus. Emerg. Infect. Dis. 2, 239–240.
Yu, D., Wu, S., Wang, B., Chen, Y., Li, L., 2011. Rapid detection of common viruses using multi-analyte suspension arrays. J. Virol. Methods 177, 64–70, http://dx.doi.org/10.1016/j.jviromet.2011.06.017.