Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 223 (2015) 5–12

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

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

Victoria Boyd[a,*], Ina Smith[a], Gary Crameri[a], Amy L. Burroughs[a], Peter A. Durr[a], John White[a], Christopher Cowled[a], Glenn A. Marsh[a], Lin-Fa Wang[a,b]

[a] CSIRO Biosecurity Flagship & Australian Animal Health Laboratory, Geelong, Victoria, Australia
[b] Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School Singapore, Singapore

Article history:
Received 19 March 2015
Received in revised form 8 July 2015
Accepted 14 July 2015
Available online 17 July 2015

Keywords:
Bats
Virus surveillance
Multiplex
Luminex
MagPlex-TAG

A B S T R A C T

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 tags on specific primer sets. A DNA hybridization step incorporates target specific primer extension (TSPE) reactions to a given bead 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,

Abbreviations: BVPA, bat virus panel assay; BVPA-1, bat virus panel one; BVPA-2, bat virus panel two; X-TAG, MagPlex-TAG technology; TSPE, target specific primer extension.

* Corresponding author at: CSIRO Biosecurity Flagship & Australian Animal Health Laboratory, Victoria Private Bag 24, Geelong, Victoria 3220, Australia.
E-mail addresses: Vicky.Boyd@csiro.au (V. Boyd), Ina.Smith@csiro.au (I. Smith), Gary.Crameri@csiro.au (G. Crameri), amy_in_paris@hotmail.com (A.L. Burroughs), Peter.Durr@csiro.au (P.A. Durr), John.White@csiro.au (J. White), Chris.Cowled@csiro.au (C. Cowled), Glenn.Marsh@csiro.au (G.A. Marsh), Linfa.Wang@csiro.au (L.-F. Wang).

http://dx.doi.org/10.1016/j.jviromet.2015.07.004
0166-0934/

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), Teviot virus (TevPV), Yarra Bend paramyxovirus (YBPMV; 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 inaugural isolation of the lesser-known YepPV, 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 ×4 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., 2008). 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 $MgSO_4$ 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-phycoerythrin (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,

Hilden, Germany), 0.5 μL of 50 mM $MgCl_2$, 0.15 μL of 5 U/μL Tsp polymerase (Invitrogen), 0.5 μL of 0.2 mM dATP, dGTP, and dTTP (Invitrogen), 0.25 μL of 400 μM Biotin-dCTP (Invitrogen), 0.5 μL of each TSPE primer at 1 μM (Geneworks), 5 μL of ExoSAP-IT treated PCR product, and water to produce a final volume of 20 μL. The reaction mixture was then amplified at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, hold at 4 °C. This reaction was performed in a PCR plate sealed using a Microseal A film plate cover (Bio-Rad).

2.4.4. Hybridization to microspheres and assay analysis

Each X-TAG bead set is unique, containing a different fluorescent 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/L streptavidin-R-phycoerythrin (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 the 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 multiplex 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 henipavirus (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 (Budczies 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

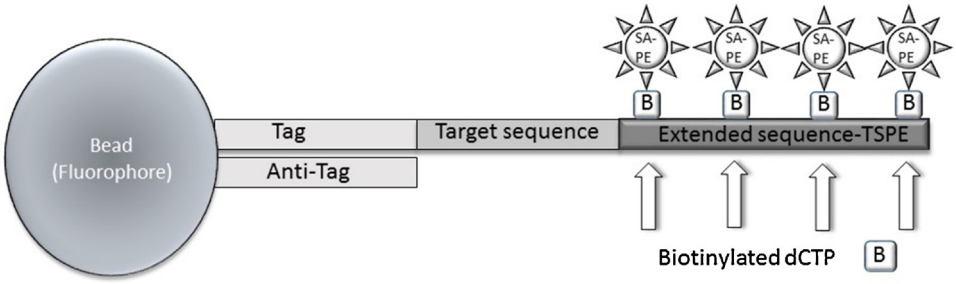

Fig. 1. Target specific primer extension products (TSPE) are detected and identified by hybridization onto microsphere beads. Anti-tag oligonucleotides on the beads, hybridize to the TSPE products containing a complementary tag oligonucleotide. The microbeads are sorted by the Bio-plex 200 flow cell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

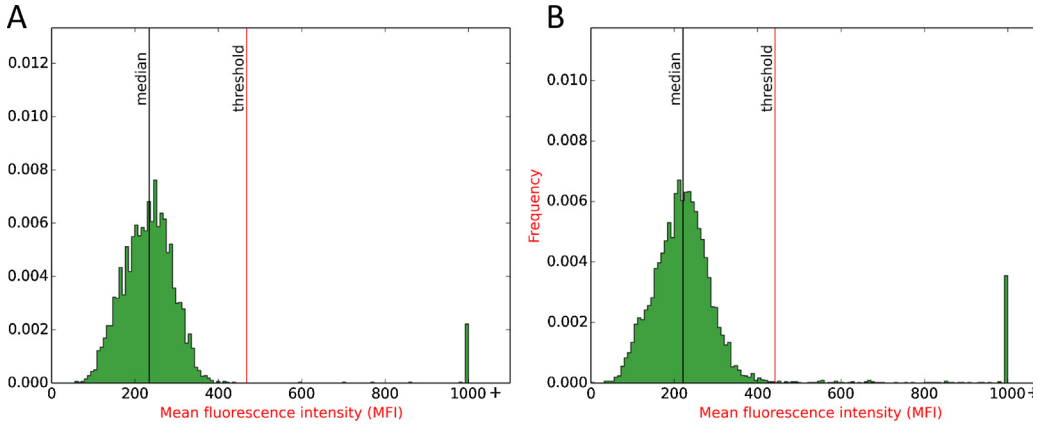

Fig. 2. Histogram of frequency median/threshold results based on Australian urine samples analyzed on the 11 plex assay (A) corresponding to 5852 data points, and bat urine samples from Bangladesh analyzed on the 8 plex assay (B) corresponding to 4320 data points. From all combined results as well as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

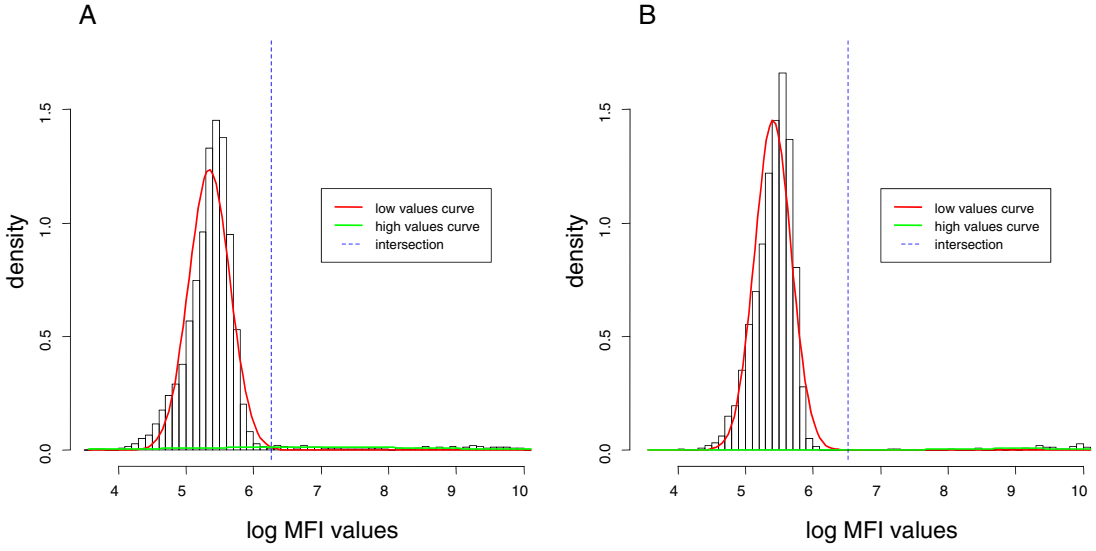

Fig. 3. Histogram of the natural log of the MFI values for thresholds superimposed on the histogram are the two Guassian distributions fitted by the *normalmixEM* function. The chosen threshold corresponded to the value where the two probability density functions intersected, which when back-transformed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

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).

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

Table 1
Specificity of BVPA-1.

BVPA-1	Median fluorescence intensity (MFI) values for each of the 11 probes/targets[a]										
Control samples	TevPV	YepPV	GroPV	CedPV	HeV	MenPV	HerPV	YBPMV	GPMV	TioPV	NiV
HeV	131	201	149	151	**12,289**	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	**21,219**	185	122
YBPMV	217	144	221	131	206	227	213	**14,941**	264	183	148
NiV	191	160	183	204	204	204	187	201	203	185	**4940**
MIX[b]	**8512**	**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[c]	**6071**	**731**	**11,879**	359	326	**19,573**	**11,870**	314	413	330	345
B	**1073**	166	160	217	**16,476**	256	**7713**	230	273	181	215
C	**9539**	122	**2661**	223	**6577**	291	275	202	218	152	165
D	221	161	166	204	**731**	**833**	179	212	188	170	184
E	**17,242**	**1911**	**5055**	297	222	330	237	**2258**	251	257	220
F	299	245	**2724**	324	299	299	232	295	310	298	253
G	**624**	101	106	199	139	**10,484**	78	156	143	148	98
No template control	123	107	102	182	157	143	101	199	156	122	146

[a] Virus targets; TevPV = Teviot virus, YepPV = Yeppoon virus, GroPV = Grove virus, CedPV = Cedar virus, HeV = Hendra virus, MenPV = Menangle virus, HerPV = Hervey virus, YBPMV = Yarra Bend paramyxovirus, GPMV = Geelong paramyxovirus, TioPV = Tioman virus, NIV = Nipah virus; samples considered positive are highlighted.
[b] A mixture of TevPV, YepPV, GroPV, MenPV, HerPV
[c] Sample confirmed by PCR or sequencing to be positive for TevPV, YepPV, GroPV, MenPV, and HerPV.

Table 2
Specificity of BVPA-2.

BVPA-2	Median fluorescence intensity (MFI) values for each of the 8 probes[a]							
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	177	148	**13,421**	**21,472**	117	176
EboRV	161	237	**5876**	119	197	139	162	149
EboZV	136	167	153	**14,170**	168	134	169	185
MenV	197	195	174	114	174	151	144	**16,252**
Field urine samples								
A[b]	189	225	180	195	**768**	**1065**	153	195
B[b]	151	235	209	127	**858**	**1130**	147	203
C[b]	288	238	324	275	**1289**	**1770**	299	365
D[b]	279	238	240	185	**599**	**703**	221	248
E	152	168	154	120	200	155	135	175
F	153	167	138	132	168	153	154	189
G	169	149	210	177	162	128	127	184
No template control	121	112	169	129	109	117	125	124

[a] 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, MenvPV = Menangle virus; samples considered positive are highlighted.
[b] Samples confirmed positive to NiV by sequencing.

on all remaining bead sets indicated the absence of cross reactivity in biological samples.

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 3
Comparison of X-Tag analytical sensitivity to qRT-PCR.

(3.1) qRT-PCR and 8 Plex assay[a]

Assay	qPCR Cedar P-gene	X-Tag Cedar P-gene	qPCR Nipah N-gene	X-Tag Nipah N-gene
	Ct	MFI	Ct	MFI
10-1	29	21,994	23	21,367
10-2	33	21,417	27	21,498
10-3	37	17,941	30	19,103
10-4	40	6415	33	8847
10-5	U[b]	208	36	953
10-6			U[b]	121

(3.2) qRT-PCR and 11 Plex assay[a]

Assay	qPCR Cedar P-gene	X-Tag Cedar L-gene	qPCR HeV N-gene	X-Tag HeV L-gene
	Ct	MFI	Ct	MFI
10-1	27	19,078	24	19,332
10-2	31	2483	28	18,569
10-3	35	257	31	13,774
10-4	39.5 (1/2)	235	35	4593
10-5	U[b]		U[b]	1264
10-6				153

[a] *Abbreviations*: U = undetected, MFI = median fluorescence intensity; results are in duplicate. All positive results are shaded in gray (MFI values 676 or above are considered positive).
[b] qRT-PCR cut-offs were Ct ≤ 40 positive, ≥45 negative, 40–45 indeterminate.

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 subsequence 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 (Pabbaraju 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 bat 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; Banér et al., 2007; Pabbaraju et al., 2008; Wu et al., 2014).

Our novel BVPAs 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.

Acknowledgements

The authors would like to thank the Queensland Centre for Emerging Infectious Diseases, Biosecurity Queensland, Department of Agriculture, Fisheries & Forestry, and International Centre for Diarrhoeal Disease Research, Bangladesh and EcoHealth Alliance, New York for providing bat samples used in this study. We also thank colleagues Hans Heine for advice on primer design and Michelle Baker and James Wynne for critical review of the manuscript.

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.jviromet.2015.07.004

References

Babady, N.E., Mead, P., Stiles, J., Brennan, C., Li, H., Shuptar, 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 cancer hospital. J. Clin. Microbiol. 50, 2282–2288, http://dx.doi.org/10.1128/JCM.06186-11

Banér, J., Gyarmati, P., Yacoub, A., Hakhverdyan, M., Stenberg, J., Ericsson, O., Nilsson, M., Landegren, U., Belák, S., 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, http://dx.doi.org/10.1016/j.jviromet.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., Crameri, S., Haining, J., Marsh, G., Crameri, G., Field, H., Wang, L.-F., 2014. Isolation of multiple novel paramyxoviruses from pteropid bat urine. J. Gen. Virol., http://dx.doi.org/10.1099/vir.0.068106-0

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.

Bossart, K.N., McEachern, J.A., Hickey, A.C., Choudhry, V., Dimitrov, D.S., Eaton, B.T., Wang, L.-F., 2007. Neutralization assays for differential henipavirus serology using Bio-Plex protein array systems. J. Virol. Methods 142, 29–40, http://dx.doi.org/10.1016/j.jviromet.2007.01.003

Budczies, J., Klauschen, F., Sinn, B.V., Győrffy, B., Schmitt, W.D., Darb-Esfahani, S., Denkert, C., 2012. Cutoff finder: a comprehensive and straightforward Web application enabling rapid biomarker cutoff optimization. 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/10.1177/1040638713507256

Clayton, B.A., Wang, L.F., Marsh, G.A., 2013. Henipaviruses: an updated review focusing on the pteropid reservoir and features of transmission. Zoonoses Public Health 60, 69–83, http://dx.doi.org/10.1111/j.1863-2378.2012.01501.x

Feldman, K.S., Foord, A., Heine, H.G., Smith, I.L., Boyd, V., Marsh, G.A., Wood, J.L.N., Cunningham, A.A., Wang, L.-F., 2009. Design and evaluation of consensus PCR assays for henipaviruses. J. Virol. Methods 161, 52–57, http://dx.doi.org/10.1016/j.jviromet.2009.05.014

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., Broos, A., Kung, Y.H.N., McLaughlin, A., Zeddeman, A., 2011. Hendra virus infection dynamics in Australian fruit bats. PLoS ONE 6, e28678, http://dx.doi.org/10.1371/journal.pone.0028678

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. Flavivirus detection and differentiation by a microsphere array assay. J. Virol. Methods 203, 65–72.

Gardner, I.A., Greiner, M., Dubey, J.P., 2010. Statistical evaluation of test accuracy studies for *Toxoplasma gondii* in food 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., Pozzato, N., 2011. Multiplexed typing of *Mycobacterium avium* subsp. paratuberculosis types I, II, and III by Luminex xMAP suspension array. J. Clin. Microbiol. 49, 389–391, 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., Daszak, P., 2011. Pteropid 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., Nishiura, H., Klaassen, M., Fouchier, R.A.M., 2010. Surveillance of wild birds for avian influenza virus. Emerg. Infect. Dis. 16, 1827–1834, http://dx.doi.org/10.3201/eid1612.100589

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 verigene respiratory virus plus for detecting influenza virus and respiratory syncytial virus. J. Clin. Lab. Anal. 6, 1–6, http://dx.doi.org/10.1002/jcla.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 biothreat pathogens by using suspension microarrays. PLoS ONE 7, e31958, http://dx.doi.org/10.1371/journal.pone.0031958

Jokela, P., Piiparinen, H., Mannonen, L., Auvinen, E., Lappalainen, M., 2012. 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., Luinstra, 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, I., 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.