Appl Microbiol Biotechnol (2015) 99:919–928
DOI 10.1007/s00253-014-6337-8

METHODS AND PROTOCOLS

Bead-based suspension array for simultaneous differential detection of five major swine viruses

Ru Chen · Xiao-Lu Yu · Xiao-Bo Gao · Cun-Yi Xue · Chang-Xu Song · Yan Li · Yong-Chang Cao

Received: 8 September 2014 / Revised: 14 December 2014 / Accepted: 17 December 2014 / Published online: 6 January 2015

Abstract A novel multiplex detection array based on Luminex xMAP technology was developed and validated for simultaneous detection of five major viruses causing swine reproductive diseases. By combining one-step asymmetric multiplex reverse transcription polymerase chain reaction (RT-PCR) with xMAP bead-based hybridization and flow cytometry analysis, the resulting multiplex assay was capable of detecting single and mixed infections of PRRSV, PCV-2, PRV, CSFV, and PPV in a single reaction. The assay accurately detected and differentiated 23 viral strains used in this study. The low detection limit was determined as 2.2–22 copies/μL (corresponding to 0.5–6.8 fg/μL DNA template) on plasmid constructs containing viral fragments. The intra-assay and inter-assay variances (CV%) were low that ranged from 2.5 to 5.4 % and 4.1 to 7.6 %, respectively. The assay was applied to test field samples and detected single and mixed viral infections. The detection rate was higher than that of uniplex conventional PCR and RT-PCR methods. The detection of PRRSV by the bead-based multiplex assay was comparable with a commercially available real time RT-PCR kit. The test procedure on purified DNA or RNA samples could be completed within 2 h. In conclusion, the bead-based suspension array presented here proved to be a high-throughput practical tool that provided highly specific and sensitive identification of single and multiple infections of five major viruses in pigs and boar semen.

Keywords xMAP technology · Bead-based suspension array · Swine virus · Multiplex detection

R. Chen (✉)
Animal Inspection and Quarantine Laboratory, Technical Center, Guangdong Entry–Exit Inspection and Quarantine Bureau, No.66 Huacheng Dadao Ave., Zhujiangxincheng, Guangzhou 510623, China
e-mail: gd_chenr@sina.com

X.-L. Yu · C.-Y. Xue · Y.-C. Cao
School of Life Sciences, Sun Yat-Sen University, Guangzhou, China

X.-B. Gao
Department of Genetics, National Research Institute for Family Planning, Beijing 100081, China

C.-X. Song · Y. Li
Veterinary Medicine Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China

Introduction

Swine reproductive diseases involve a number of etiological agents, especially viruses. Porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), porcine pseudorabies virus (PRV), classical swine fever virus (CSFV), and porcine parvovirus (PPV) are five well-known pathogens causing swine reproductive failures, including infertility, abortion, mummified fetuses, and stillbirth. PRRSV and PCV-2 are also major contributors to the porcine respiratory disease complex. Mixed infections of two or more of these viruses occurred in intensive swine production (Huang et al. 2004; Yue et al. 2009). A definitive diagnosis of co-infections of these viruses is often difficult based only on clinical symptoms. Moreover, experimental and epidemiological data demonstrated transmission of these viruses through boar semen (Guerin and Pozzi 2005; Maes et al. 2008). Virus contamination of semen can be due to infections of the boar or occur during collection, processing, and storage (Maes et al. 2008). Prevention on transmission of pathogens through semen is important for swine industry where artificial insemination is extensively used. Development of multiplex detection methods enables rapid screening and differential

detection of pathogens in pigs and boar semen, which is important for epidemiological surveillance and disease prevention.

Molecular biology-based methods, which allow rapid, accurate, and sensitive identification of pathogens, have been widely developed and increasingly used in the fields of food safety, human, and animal health protection (Shisong et al. 2011; Barbau-Piednoir et al. 2013; Hu et al. 2014; Nagarajan and Loh 2014). Several nucleic acid-based molecular techniques were reported for simultaneous detection of swine viruses. Conventional and real-time multiplex polymerase chain reaction (PCR) or multiplex reverse transcription polymerase chain reaction (RT-PCR) assays were developed for simultaneous detection of different swine viruses (Giammarioli et al. 2008; Ogawa et al. 2009; Yue et al. 2009; Jiang et al. 2010; Wernike et al. 2012; Xu et al. 2012; Haines et al. 2013). Recently DNA microarrays were reported to successfully detect swine pathogens (Jack et al. 2009; Wang et al. 2013). However, all these methods have certain limitation. Conventional multiplex PCR and RT-PCR methods relied on gel-based analysis are laborious and less sensitive. Real-time PCR and RT-PCR methods are rapid and highly sensitive but limited in multiple capacities. Printed microarray that based on DNA probes array on a solid support (most commonly a glass microscope slide) has advantage in high-throughput analysis. But the technology requires several equipments to carry out complicate procedures which include probe array printing, probe hybridization, and scanning of signals. Therefore, it is expensive and inconvenient to use this technology on a routine basis.

The Luminex Multi-Analyte Profile (xMAP) technology basing on 5.6 μm microscopic polystyrene beads is capable of analyzing and reporting up to 100 different targets in a single reaction, which provides a new platform for cost-effective, high-throughput nucleic acid, and protein analysis (Dunbar 2006; Schmitt et al. 2006; Dunbar and Jacobson 2007). An array of 100 different microspheres (bead sets) currently available can be uniquely defined based on their specific spectral addresses using flow cytometry (Dunbar 2006). For nucleic acid analysis, potential DNA targets are amplified using a biotinylated primer and then hybridized to beads coupled with target-specific probes. The hybridization occurring on surface of beads is measured by using the streptavidin–phycoerythrin (SAPE) fluorochrome as reporter. Each bead set can be conjugated with a different probe. By using a combination of bead sets with different spectral address and coupled with different probes, the array is capable of differential detection and quantification of multiple targets. The suspension bead array analysis can be conducted in a reaction vessel without using special equipments. The only specific device required is a flow cytometer with two separate lasers. The red laser is used to determine bead spectrum, and the green laser is used to excite the SAPE reporter and quantify the probe-target hybridization signals.

Nucleic acid assays based on Luminex xMAP technology have been described for the detection and genotyping of bacterial, viral, and parasite pathogens. (Schmitt et al. 2006; Dunbar and Jacobson 2007; Landlinger et al. 2009; Righter et al. 2011; Ros-García et al. 2013). However, there are few applications of this technology on veterinary study. Recently, xMAP protein assays for animal viruses were reported (Lin et al. 2011; van der Wal et al. 2012). To date, bead-based xMAP assays for porcine disease diagnosis have mainly focused on the detection of antibodies to specific pathogens (Christopher-Hennings et al. 2013).

In the present study, we developed a novel multiplex nucleic acid array for sensitive and clinically applicable detection of swine viruses based on this new technology. By combining one-step asymmetric multiplex RT-PCR with bead-based hybridization and flow cytometry analysis, the resulting bead-based multiplex assay could simultaneously identify PRRSV, PCV-2, PRV, CSFV, and PPV in a single reaction. The bead-based suspension array assay was evaluated on specificity, sensitivity, reproducibility, and clinical performance.

Materials and methods

Virus and vaccine

A variety of virus or vaccine strains of PRRSV (n=11), PCV-2 (n=5), PRV (n=3), CSFV (n=2), and PPV (n=2) were used in this study. The strains listed below were all maintained in the authors' laboratories: PRRSV ATCC-VR2332 strain (NA type, genotype 2) was maintained in the laboratory of Guangdong entry–exit inspection and quarantine bureau (GDCIQ); PRRSV CH-1a strain (NA type, isolated in China in 1996) was maintained in laboratory of Sun Yat-Sen University; PRRSV BLC isolated strain, XF isolated strain, ZLT-H isolated strain, QY B8-20 isolated strain, JX isolated strain, and YF isolated strain were all highly pathogenic NA type stains isolated in China and maintained in laboratory of Guangdong Academic of Agricultural Sciences (GAAS); PCV-2 GDHY isolated strain, HY-song isolated strain, BH-song isolated strain, and PY-song isolated strain were all isolated in China and maintained in the laboratory of GAAS; PRV HS isolated strain of China was maintained in the laboratory of GAAS; PPV NADL-2 strain was maintained in the laboratory of Sun Yat-Sen University. Purified RNA of wild-type CSFV Shimen strain was kindly provided by

Professor Chen JD of South China Agriculture University. For virus propagation and titration, we used Marc-145 cell line for PRRSV and PRV, PCV 1-free PK-15 cell line for PCV-2, and ST cell line for PPV and CSFV.

PRRSV genotype 1 (EU type) strain vaccine (AMERVAC-PRRS, LABORATORIOS HIPRA, Spain), PRRSV CH-1R strain vaccine (Shanghai Hile Bio-Technology Co. Ltd., China), PRRSV JXA1-R strain vaccine (Guangdong Dahuanong Animal Health Products Co. Ltd., China), PCV-2 LG strain vaccine (Harbin Weike Biotechnology Development Company, China), PRV Batha K-61 strain vaccine (LABORATORIOS HIPRA, Spain), PRV HB-98 strain vaccine (Wuhankeqian Animal Biological Products Co., Ltd, China); CSFV C strain vaccine (cell line origin, Guangdong Winsun Biological Pharmaceutical Ltd., China), and PPV vaccine (Shanghai Hile Bio-Technology Co. Ltd., China) were all purchased in China.

Other pathogens and materials

Nucleic acids from other swine pathogens and pathogen-free cell lines and pig tissues were also used in the specificity evaluation, which included genomic DNA or RNA of Japanese encephalitis virus, food and mouth diseases virus, H1 and H3 subtype of swine influenza virus, porcine transmissible gastroenteritis virus, and virus-free PK-15, Marc-145, ST cell lines, and pig tissues.

Swine specimen

Swine tissue (n=42), blood (n=106), and semen (n=70) specimens were collected from 36 pig farms in Guangdong province in south China during December 2012 to January 2014. These included 182 specimens from 33 pig farms with suspected infection cases and 36 semen samples from three breeding farms without clinical symptoms in the time of sample collection. All specimens were freshly processed or stored at −30 °C until analysis.

Nucleic acid extraction

Virus infected cell harvests, tissue homogenates, and pig semen were frozen and thawed repeatedly followed by centrifugation in low speed. The supernatants were used immediately or stored at −30 °C. Lyophilized vaccine samples were resuspended in phosphate buffered saline. Blood samples were directly used for nucleic acid extraction. The viral genomic DNA and RNA were extracted from the supernatants or liquid samples by using the TIANamp Virus DNA/RNA kit (TIANGEN Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. Each extracted sample was finally eluted in 50 μL RNase-free ddH_2O and stored at −20 °C until subsequent analysis.

Primer pairs and probes

Primer pairs and probes for the five viruses were designed based on GenBank sequences listed in Table 1. Inosine-containing primer and probe for PRRSV were designed to yield superior amplification and efficient hybridization. All the primers and probes were designed by using the Array Designer 4.0 software (Premier Biosoft International, CA, USA) and assessed using an online NCBI Blast analysis. The 5′-end of the forward primer for PRRSV and the reverse primers for the other four viruses were labeled with biotin. The probes were 5′ amine-C12-modified (Table 1).

Multiplex nucleic acid amplification

The five-plex nucleic acid amplifications were performed using the optimized asymmetry one-step RT-PCR assay. RT-PCRs were carried out in a total volume of 50 μL containing 1 μL of GoldScript RT/Platinum® Taq enzyme Mix (Invitrogen, CA, USA), 25 μL 2 x Reaction Mix buffer (containing 0.4 mM of each dNTP, 2.4 mM of $MgSO_4$), 2–5 μL of nucleic acid template. A number of unlabeled to biotinylated primer ratios were tested to select the ratios that give the best signal. And the optimized final concentrations of primers in the five-plex reaction mixture were as follows—0.8 /0.1 μM (biotinylated forward primer/reverse primer) for PRRSV, 0.04/0.4 μM (forward primer/biotinylated reverse primer) for PCV-2, 0.1/0.8 μM (forward primer/biotinylated reverse primer) for PRV, 0.04/0.4 μM (forward primer/biotinylated reverse primer) for CSFV, and 0.04 /0.2 μM (forward primer/biotinylated reverse primer) for PPV. Amplifications were conducted in a thermocycler (ABI 2700, USA) with the optimized condition. Firstly, cDNA synthesis for RNA virus was conducted at 50 °C for 15 min, followed by denaturation at 94 °C for 2 min. Then, DNA amplification was carried out by 40 cycles of 94 °C for 10 s, 55 °C for 15 s and 72 °C for 10 s; followed by final extension at 72 °C for 1 min.

Luminex xMAP suspension array procedure

Oligonucleotide coupling, hybridization, and measurement Five capture probes were included in the bead-based suspension array (Table 1). The capture probes modified with an amino-C12 linker at the 5′ end were bound to different carboxylated bead sets (Luminex, USA) as previous described (Chen et al. 2010). Biotinylated amplified DNA products were hybridized

Table 1 Primer pairs and probes in the bead-based multiplex assay

Targeted virus	Target gene	Sequence (5′-3′)	Size (bp)	Bead set number
PRRSV	Envelope protein gene (accession no. U87392.3)	Forward primer: biotin-GCCATTCTGTTTGGGTTCAC Reverse primer: TAGAGCGCG***I***ACGGAG[a]	88	28
		Probe: AmMC12-CGGAGCAAACCA***I***TCTGA[a]		
PCV-2	Capsid protein gene (accession no. JN639856.1)	Forward primer: CGGGAGTGGTAGGAGAAG Reverse primer: biotin-TACTACAGAATAAGAAAGGTTAAGG	194	37
		Probe: AmMC12-GTGGCGGGAGGAGTAGTTTA		
PRV	gD gene (accession no. JF797217.1)	Forward primer: CACCGCACGTACAAGTTC Reverse primer: biotin-TACCAGTAGTTCACCACCTC	125	33
		Probe: AmMC12-GATTCCTGACGCCGTTCTAC		
CSFV	Polyprotein gene (accession no. JX028204.1)	Forward primer: GGATTAGGAACTTCACCAACTG Reverse primer: biotin-TCTTCTTGACCTGGTATTTTACTC	201	56
		Probe: AmMC12-CCCAAAAGAGCATGAGAAGGA		
PPV	NS1 gene (accession no. AY502114.1)	Forward primer: ATGGAGTAGATGGTTAATAATGC Reverse primer: biotin-TTGTGAGTGTAGGTTAGTAGC	117	52
		Probe: AmMC12-GAATTAGCAGAGGATGGTGAGTG		

[a] The inosine(I) bases are bold and italic

to coupled bead sets according to the following procedure. Each coupled bead set was pellet and resuspended in 1.5× TMAC buffer (Sigma, USA). Hybridizations were carried out in a total volume of 50 μL containing 33-μL mixtures of the five coupled bead sets (each of 1,000 in number), 5 μL multiplex amplification products, and 12 μL TE buffer, pH8.0. The mixture was subjected to denaturing at 95 °C for 5 min in a thermocycler (ABI 2700, USA) before hybridization incubation. The optimal temperature and incubation time for hybridization were proved to be 57 °C and 10 min with shaking at 300 rpm in a Thermomix Comfort (Eppendorf, Germany). After hybridization, pellet the beads at 10,000 rpm for 5 min and then wash three times with 1× TMAC buffer at 5,000 rpm for 3 min. After the final wash, removed the supernatant and incubated the bead pellet with 80 μL streptavidin–phycoeritryn (Invitrogen, CA, USA) in 1×TMAC buffer at 57 °C for 10 min. Finally, the signals produced for each bead sets were analyzed using the Luminex™ 200 system (Luminex, TX, USA).

Data analysis and cutoff definition Data were calculated by the software of the instrument. The result is expressed as the median fluorescence intensity (MFI) of at least 100 beads per set. The sample net MFI values were subtracted from the mean background fluorescence determined by the parallel analysis of control samples containing all components except nucleic acid template. The cut-off value for a positive result was defined as net MFI greater than two times the mean background.

Conventional PCR and RT-PCR methods for comparison study

Uniplex conventional PCR for PRV, PCV-2, PPV and RT-PCR for PRRSV, CSFV were all developed and validated in our laboratory. Each of these methods targeted at the same viral gene as the bead-based assay but amplified different regions within the gene. The amplified product size for PRRSV, PCV-2, PRV, CSFV and PPV was 272, 334, 187, 407, and 481 bp, respectively. PCR was conducted in a total volume of 50 μL containing 1 μL of Taq DNA polymerase (TIANGEN, Beijing, China), 5 μL 10× Reaction Mix buffer (containing 200 mM Tris–HCl, 200 mM KCl, 100 mM $(NH_4)_2SO_4$, and 15 mM $MgCl_2$), 5 μL dNTP Mixture (2.5 mM), 1 μL of each primer (10 μM), and 5 μL of nucleic acid template. RT-PCR used a total volume of 25 μL reaction mixture containing the same reagent panel as in the bead-based assay, containing 0.2 μM of each primer and using 5 μL nucleic acid template. DNA amplification was carried out in 94 °C for 2 min; followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s; and final extension at 72 °C for 2 min. One-step viral RNA amplification was carried out as follows: firstly, cDNA synthesis at 50 °C for 30 min, then denaturation at 94 °C for 2 min; followed by DNA amplification by 40 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s; and final extension at 72 °C for 1 min.

Real-time RT-PCR for comparison study

We used validated commercial real-time RT-PCR reagent for PRRSV (SAVICHAS Biotechnology, Beijing, China) in the

comparison study for testing field samples. The testing was carried out in total volume of 25 μL by 40 cycles according to the manufacture's protocol, using 5 μL of nucleic acid template in each reaction.

Results

Specificity test

The five-plex bead-based assay was tested against various strains of the five viruses and other swine pathogens listed above. Nucleic acid from negative pig tissue was also tested to check cross-reaction with pig genomic DNA. Three control samples using RNase-free ddH_2O to replace nucleic acid template were run in each experiment to determine the mean background signal. The MFI values obtained in the five-plex assay for each probe are showed in Fig. 1. All 23 strains of PRRSV, PCV-2, PRV, CSFV, and PPV were correctly identified by specific probes, with net MFI values significantly greater than the cut-off value. No cross-reaction was observed among the five virus specific probes. MFI values of negative samples including other pathogens, pig tissue, and virus-free cell lines were all clearly below the cut-off value and did not significantly differ from the background.

A test on mixture of viral nucleic acid was conducted to evaluate the performance of the assay on mixed infections. Genomic DNA or RNA of the five viruses were mixed (100 ng each) and used as the template for five-plex amplification and hybridization analysis. As showed in Table 2, the bead-based multiplex assay identified all the five viral target regions on the viral mixture, demonstrating the assay's capability of simultaneous differential detection on mixed infections.

Sensitivity test

The analytical sensitivity of the five-plex bead-based assay was determined by preparing tenfold serial dilutions of recombinant plasmids containing PRRSV-, PCV-2-, PRV-, CSFV-, or PPV-targeted fragments. Five recombinant plasmids were constructed by cloning virus target sequence into pMD-19 T Vector (Takara Biotechnology Dalian, China). Recombinant plasmid DNAs were extracted and purified; the concentrations at A_{260} were measured by using a spectrophotometer (NanoDrop, USA), and the corresponding genome copies were calculated based on the concentration and molecular weight of the plasmid. Serial tenfold diluted recombinant plasmid templates were tested by the bead-based multiplex assay. The multiplex amplification hybridization detection steps were performed in triplicate for each dilution series. The limit of detection was defined as the highest dilution at which the three replicates were above the cut-off value. The mean MFI values obtained for each virus specific probe and their corresponding plasmid dilutions (10^4–10^{10}) were showed in Fig. 2. The MFI deviation and the corresponding concentration

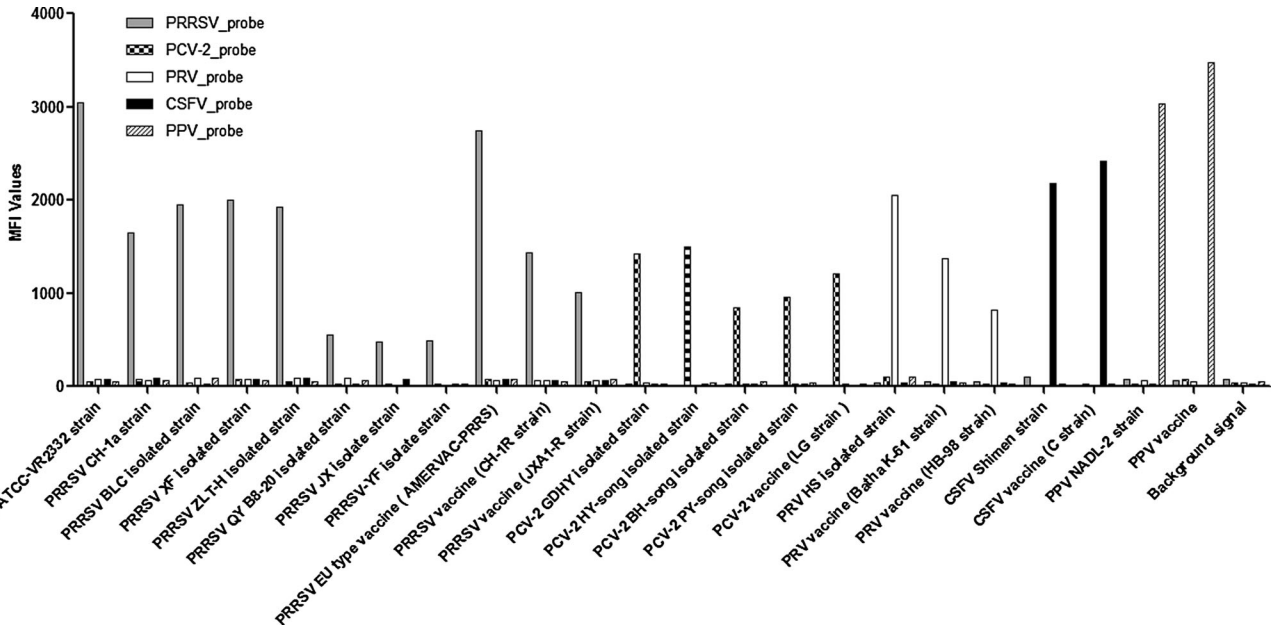

Fig. 1 Specific detection by the xMAP bead-based multiplex assay on the five swine viruses. *MFI*, median fluorescent intensity; the *X*-axis represents viral or vaccine strains of the five viruses

Table 2 Detection on viral mixture by the bead-based multiplex assay

Template	MFI values for each probe				
	PRRSV_ probe	PCV-2_ probe	PRV_ probe	CSFV_ probe	PPV_ probe
Five-plex mixed sample[a]	1024	1374	1943	2173	965
Five-plex mixed sample 2[a]	946	1530	1856	2067	952
Background	42	29	32	39	45

[a] Five-plex mixed sample contained viral genomic nucleic acid from PRRSV VR2332 strain, PCV-2 GDHY isolated strain, PRV Batha K-61 strain, CSFV C strain, and PPV NADL-2 strain

were detailed in Table 3. As a result, the analytical low detection limit of the bead-based five-plex assay on PRRSV, PCV-2, PRV, CSFV, and PPV was 2.2×10^{1}, 2.2, 3.3, 1.6×10^{1} and 3.4 copies/μL (corresponding to 6.8, 7.4×10^{-1}, 1.1, 5.3×10^{-1} and 1.1 fg/μL of DNA template), respectively.

The detection sensitivity on viral fragment mixture was also evaluated by using mixture of recombinant plasmid templates of PRRSV, PCV-2, and PRV on equal amounts. Serial tenfold dilutions of the template mixture were tested by the multiplex assay; each dilution was tested in three replicates, and the limit of detection was defined as mentioned above. The low detection limit of the assay on PRRSV, PCV-2, and PRV viral fragment in the triple-mixture was 2.2×10^{1}, 2.2, and 3.3 copies/μL, respectively, equivalent to the detection on single template.

Assessment of diagnostic reproducibility

To test reproducibility of the five-plex bead-based assay, multiplex one-step RT-PCR and hybridization steps for all the five viruses were performed in six parallel reactions using virus genomic nucleic acid at concentration of 1 ng/μL. The inter-assay variation (CV%) obtained from this test of the five viruses ranged from 4.1 %–7.6 % (Table 4). The intra-assay probe detection variations were also assessed by carrying out six parallel multiplex hybridizations and measurements on product from the same panel of virus amplification, which also revealed assay variances bellowed 10 % (Table 4).

Application detection on field samples

The five-plex bead-based assay was applied to test 218 swine samples, including 182 specimens (blood, tissue, and semen) from pig farms with suspected infection and 36 semen samples collected randomly from clinical healthy boars. The assay detected positive of PRRSV (n=58), PCV-2 (n=18), PRV (n=33), CSFV (n=11) or PPV (n=25) in the 182 suspected specimens (Table 5). Co-infections were detected in three sample types. Totally, 98 specimens were tested as positive, with 43 specimens identified as co-positive of two or three viruses. Furthermore, the assay detected seven single positive in the 36 semen samples from clinically healthy boars, including five of PRRSV and two of PCV-2 positive; no co-positive was detected.

Conventional uniplex PCR or RT-PCR methods were also used to test the field samples, and the result was detailed in

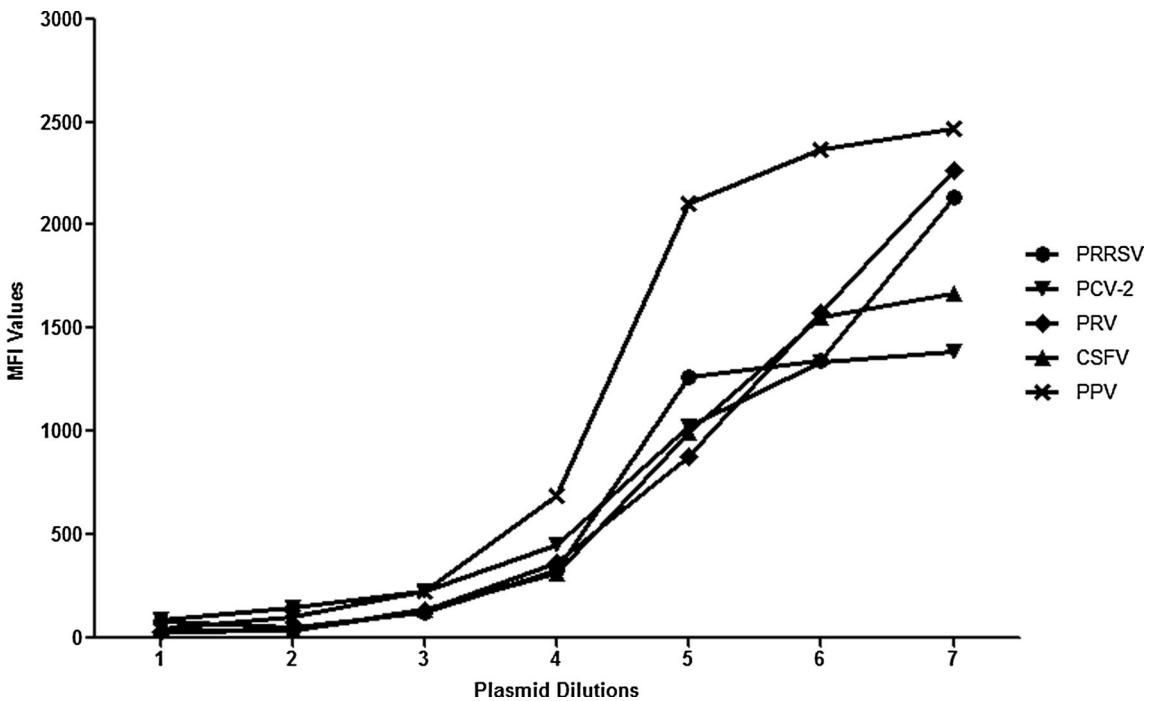

Fig. 2 Detection of tenfold serial dilution of recombinant plasmid templates by the xMAP bead-based multiplex assay. *MFI*, median fluorescent intensity; the *X*-axis represents plasmid dilutions 1–7, corresponding to 10^{10} to 10^{4} dilution of each constructed plasmid template

Table 3 Detection variation on constructed plasmid templates

Plasmid template		Probes				
		PRRSV	PCV-2	PRV	CSFV	PPV
10^{4a}	Concentration (copies/uL)	2.2×10^{4}	2.2×10^{4}	3.3×10^{4}	1.6×10^{5}	3.4×10^{4}
	(fg/uL)	6.8×10^{3}	7.4×10^{3}	1.1×10^{4}	5.3×10^{3}	1.1×10^{4}
	MFI deviation (CV%)[b]	0.4	3.4	3.0	3.8	3.6
10^{5}	Concentration (copies/uL)	2.2×10^{3}	2.2×10^{3}	3.3×10^{3}	1.6×10^{4}	3.4×10^{3}
	(fg/uL)	6.8×10^{2}	7.4×10^{2}	1.1×10^{3}	5.3×10^{2}	1.1×10^{3}
	MFI deviation (CV%)	6.4	4.1	2.8	1.6	2.7
10^{6}	Concentration (copies/uL)	2.2×10^{2}	2.2×10^{2}	3.3×10^{2}	1.6×10^{3}	3.4×10^{2}
	(fg/uL)	6.8×10^{1}	7.4×10^{1}	1.1×10^{2}	5.3×10^{1}	1.1×10^{2}
	MFI deviation (CV%)	15.1	10.6	4.1	2.3	9.2
10^{7c}	Concentration (copies/uL)	2.2×10^{1}	2.2×10^{1}	3.3×10^{1}	1.6×10^{2}	3.4×10^{1}
	(fg/uL)	6.8	7.4	1.1×10^{1}	5.3×10^{1}	1.1×10^{1}
	MFI deviation (CV%)	19.1	12.1	17.6	17.9	3.2
10^{8c}	Concentration (copies/uL)	ND	2.2	3.3	1.6×10^{1}	3.4
	(fg/uL)	ND	7.4×10^{-1}	1.1	5.3×10^{-1}	1.1
	MFI deviation (CV%)	ND	13.5	8.5	8.2	17.1

ND not determined

[a] Dilutions of constructed plasmid template

[b] Variation of MFI values among the triplicate reactions of each dilution

[c] The low detection limit of PRRSV probe was at 10^{7} dilution of plasmid template, and at 10^{8} dilution of plasmid template for PCV-2, PRV, CSFV, or PPV probe, respectively

Table 5. These methods in total detected 90 samples with positive of PRRSV (n=48), PCV-2 (n=17), PRV (n=31), CSFV (n=10) or PPV (n=19) from the 182 suspected specimens. Thirty-two specimens were identified as co-positive of two or three viruses based on summary of the individual test results. It is noteworthy that samples tested as positive by these methods were all detected by the bead-based multiplex assay.

The clinical detection of the bead-based assay was also compared with a commercial real-time RT-PCR kit specific for PRRSV. Comparison of the detection result on the 182 suspected specimens was detailed in Table 6. The real-time assay detected four specimens which were tested as negative by the bead-based assay, but failed to detect seven specimens detected as positive by the bead-based method. It is noted that two of the seven specimens missed by the real-time assay were also tested as PRRSV-positive by the conventional RT-PCR method mentioned above. The overall detection rate for PRRSV was close between the two methods, which was 32 % (58/182) by the bead-based multiplex assay, compared with 30 % (55/182) by the real-time RT-PCR assay.

For the test on the 36 semen samples from clinically healthy boar, the detection result of conventional PCR or RT-PCR was consistent with the bead-based multiplex assay. The real-time RT-PCR detected five positive of PRRSV in the semen samples, with the same result as the other two methods.

Table 4 Result of assay reproducibility tests

Target		PRRSV	PCV-2	PRV	CSFV	PPV
Intra-assay	Mean MFI	2,405	1,327	1,653	2,442	1,571
	Variation (CV%)	2.6	5.3	5.4	3.6	2.5
Inter-assay	Mean MFI	2,480	1,714	1,605	2,568	1,543
	Variation (CV%)	4.1	4.1	4.8	7.6	6.8

Discussion

In nature, swine diseases are associated with a variety of pathogens. Multiple infections occur commonly in swine industry worldwide. High-throughput multiplex methods to detect many targets rapidly offer increased test capacity and reduce overall cost and time, which can effectively support fast diagnosis of swine diseases. To overcome limitations of previously reported methods, improvement of current multiplex detection techniques for swine pathogens by applying new technology platform is necessary and challenging.

The Luminex xMAP technology is an attractive approach suitable for high-throughput clinical detection. As compared with planar microarrays, the xMAP arrays are of lower feature

Table 5 Clinical detection by the bead-based multiplex assay and comparison with conventional PCR/RT-PCR methods

Specimen	Number	Method	Number of positive[a]															
			C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	PRRSV	PCV-2	PRV	CSFV	PPV	Total
Semen	34	Bead-based assay	0	0	0	1	2	2	3	0	2	2	4	1	1	1	3	22
		PCR/RT-PCR	0	0	0	1	2	2	3	0	2	2	4	1	1	1	2	21
Tissue	42	Bead-based assay	1	0	2	3	2	0	1	1	0	1	13	0	6	2	0	32
		PCR/RT-PCR	0	0	2	3	1	0	1	0	0	0	10	2	8	2	0	29
Blood	106	Bead-based assay	0	2	0	6	5	1	3	2	0	1	7	1	6	3	7	44
		PCR/RT-PCR	0	1	0	4	4	1	1	2	0	0	8	3	7	2	7	40

[a] C1: co-positive of PRRSV, PCV-2 and PRV; C2: co-positive of PRRSV, PCV-2 and PPV; C3: co-positive of PRRSV, PRV and PPV; C4: co-positive of PRRSV and PCV-2; C5: co-positive of PRRSV and PRV; C6: co-positive of PRRSV and CSFV; C7: co-positive of PRRSV and PPV; C8: co-positive of PCV-2 and PRV; C9: co-positive of CSFV and PRV; C10: co-positive of PRV and PPV; PRRSV, PCV-2, PRV, CSFV, PPV: single positive of the virus

density, but with advantages making this platform more practical for clinical application. Bead-based suspension arrays are three-dimensional arrays that provide faster hybridization kinetics, excellent sensitivity, specificity, and multiplexing capability of up to 100 different targets (Nolan and Mandy 2001; Dunbar 2006). The bead-based technology provides more flexibility in array preparation, making it feasible and relatively low cost for development of user-defined applications (Dunbar 2006; Miller and Tang 2009). Other benefits of the Luminex xMAP technology include rapid data acquisition and statistical superiority.

This study reports the development of a bead-based suspension array for quantitative, simultaneous detection of five important viruses associated with swine reproductive diseases. According to the test on field samples and manual viral mixture, the array was demonstrated to be enough accurate and efficient in detecting multiple infections in pigs and boar semen. It was rapid which took less than 2 h for multiplex detection on purified nucleic acid. By combining one-step RT-PCR procedure, the assay is capable of detecting DNA and RNA viruses in a single reaction, yielding quantitative results classified and measured by high-speed digital signal processing system.

We aimed at developing a screening assay that is capable of detecting all genotypes and varieties of target viruses. This purpose was followed in designing primers and probes. For example, the target sequence we chose for PRRSV is conserved in both genotype 1 (EU-type) and genotype 2 (NA-type) of the virus. Target regions of PCV-2, PRV, CSFV, and PPV were selected and assessed under similar considerations. Furthermore, inosine base was incorporated into PRRSV-specific primer and capture probe to enhance the capacity on detecting large varieties of virus strains. Specificity of the primers and probes used in the multiplex assay were assessed carefully by bioinformatics analysis apart from laboratory testing. The specificity of the comprehensive bead-based assay with all these primers and probes was validated through accurate identification of viruses without cross-reaction with other pathogens. Although this multiplex assay could not differentiate between wild-type viruses and vaccine strains, it was valuable in screening diagnosis where suspected clinical symptoms occurred.

Table 6 Clinical detection of PRRSV by the bead-based multiplex assay and comparison with real-time RT-PCR method

xMAP assay		Real-time RT-PCR	
		No. pos.	No. neg.
No. pos.	58	51	7
No. neg.	124	4	120

No. pos. number of positive by the method, *No. neg*. number of negative by the method

In developing the assay, optimizations of reagents and reaction conditions were carried out carefully in order to achieve optimal performance. The assay optimization included primer ratios in asymmetric multiplex amplification, annealing temperature and time during amplification, hybridization conditions, SAPE concentration, and incubation time. It was noted that optimization of biotinylated to unlabeled primer ratios in asymmetric PCR significantly enhanced bead-hybridization efficiency, which consequently enhanced assay's performance and sensitivity. Moreover, different with our previous study (Chen et al. 2010), the hybridization was conducted with shaking on a thermo device. This modification enhanced positive signal by 30 % without increasing background noises.

The analytical sensitivity of the assay was evaluated with serial dilutions of single and mixed recombinant plasmid DNA. The low detection limit on mixture of three constructed templates was equivalent to that on single template, which was tested as low as 2.2–22 copies/μL(equivalent to 0.53–6.8 fg/μL). The result indicated that the assay was highly sensitive on testing multiple infections. Considering that multiple infections of more than three of these viruses are not common in nature, we did not determine the low limit of

detection on mixture of more templates. However, it is easy to obtain such data if necessary.

Inter-assay and intra-assay variabilities were assessed with viral genome nucleic acid on medium concentration(1 ng/μL). However, the samples may contain cell genomic nucleic acid since they were extracted from virus whole cell culture; therefore, the true concentration of pure viral genomic DNA or RNA may be lower than what was measured and calculated. On the other hand, the inter-assay variation at different template concentration can be evaluated by data obtained from the test for determination of analytical sensitivity in this study, where the detection for each plasmid dilution series was performed in triplicate. The inter-assay variability could be evaluated by calculating the variation among triplicate reactions. The inter-assay MFI variation (CV%) was tested as 0.4-9.2 % at template concentration of 10^2–10^3 fg/μL (Table 3). And not surprisingly, at lower template, concentrations yielded higher variance; the highest variance was approximately 20 % at template concentration of 10^{-1}–10^1 fg/μL. Taken together, the present study displayed excellent reproducibility of the bead-based assay.

The utility and reliability of the assay was further demonstrated by testing on natural samples. The multiplex bead-based assay detected 54 % (98/182) total positive in suspected swine specimens, with 24 % (43/182) detected as co-positive of two or three viruses. Co-positives involved two viruses in 38 (21 %) samples and three viruses in five (2.7 %) samples. High detection rate was obtained in tissue (76 %, 32/42) and semen (65 %, 22/34) specimens. The assay also detected 14 % (5/36) positive of PRRSV and 5.6 % (2/36) positive of PCV-2 in semen samples from clinically healthy boars. This finding may support the importance of strict surveillance of etiological agents on boar semen destined for artificial insemination. The detection of viruses in semen by conventional techniques for virus isolation is time-consuming and difficult due to the cytotoxicity of semen materials. The bead-based array assay for viral nucleic acid detection with high-throughput feature is a suitable tool for monitoring pathogens on boar semen.

Previous report demonstrated a multiplex Luminex assay with clinically sensitivity and specificity comparable to real-time RT-PCR (Munro et al. 2013). In our study, we compared the bead-based assay with conventional PCR or RT-PCR methods and a PRRSV-specific real-time RT-PCR kit on clinical testing. As a result, the bead-based multiplex assay detected more positive of all the five viruses than uniplex conventional PCR and RT-PCR methods in field samples. The overall detection rate of PRRSV by the bead-based assay was closed to that of the real-time RT-PCR kit but with inconsistent results on 11 samples (Table 6). The inconsistence may due to the difference between the two methods on detection sensitivity and capacity for covering different varieties of virus strains. A large number of PRRSV strains with genomic variance have been isolated in nature, which increase difficulties for virus detection based on nucleic acid techniques. In designing PRRSV-specific primers and capture probe for the bead-based assay, inosine base was used to replace normal base in positions where high gene variance occurred, which was aimed at improving the capacity for detecting a large variety of virus strains.

In conclusion, the present study established a novel and successful multiplex method for swine diseases diagnosis. To our knowledge, this is the first report of an xMAP nucleic acid assay with five-plex capacity for swine viruses. The bead-based suspension array described here allows quantitative, highly sensitive, and accurate detection of five important swine viruses simultaneously in a single reaction. The assay was demonstrated to identify multiple infections effectively in field samples. This method should contribute to improve management of virus infection in pig industry and pigs for international trade. Furthermore, the flexibility of the bead-based system allows inclusion of more targets by simply adding appropriate bead sets to the multiplex mixture without redesign of an entire array. Our further study will aim at development of assays with increased multiplexing and capacity for differentiation of virus pathogenic strains.

Acknowledgments We thank Ms. Yan-Yu Duan and Ms. Zhi-Ling Liu for technical assistance. This work was supported by the research program (No. 2013IK029) of General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ), and the Science and Technology Research Program (No. 2012B020305003) of Guangdong province, China.

References

Barbau-Piednoir E, Bertrand S, Mahillon J, Roosens NH, Botteldoorn N (2013) SYBR®Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels. Appl Microbiol Biotechnol 97:9811–9824

Chen R, Bi Y, Yang G, Liu Z, Liu Z, Zeng B, Tong T (2010) Development of a fluorescent microsphere-based multiplex assay for simultaneous rapid detection of *Mycobacterium tuberculosis* complex and differentiation of *M. tuberculosi*s and *M. bovis* in clinical samples. Diagn Mol Pathol 19:172–179

Christopher-Hennings J, Araujo KPC, Souza CJH, Fang Y, Lawson S, Nelson EA, Clement T, Dunn M, Lunney JK (2013) Opportunities for bead-based multiplex assays in veterinary diagnostic laboratories. J Vet Diagn Invest 25:671–691

Dunbar SA (2006) Applications of Luminex xMAP technology for rapid, high-throughput multiplexd nucleic acid detection. Clin Chim Acta 363:71–82

Dunbar SA, Jacobson JW (2007) Quantitative, multiplexed detection of *Salmonella* and other pathogens by Luminex xMAP suspension array. Methods Mol Biol 394:1–19

Giammarioli M, Pellegrini C, Casciari C, De Mia GM (2008) Development of a novel hot-start multiplex PCR for simultaneous detection of classical swine fever virus, African swine fever virus, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus and porcine parvovirus. Vet Res Comm 32:255–262

Guerin B, Pozzi N (2005) Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. Theriogenology 63:556–572

Haines FJ, Hofman MA, King DP, Drew TW, Crooke HR (2013) Development and validation of a multiplex, real-time RT-PCR assay for the simultaneous detection of classical and African swine fever viruses. PLoS One 8:e71019

Hu Z, Zhu C, Chang H, Guo W, Liu D, Xiang W, Wang X (2014) Development of a single-tube duplex EvaGreen real-time PCR for the detection and identification of EHV-1 and EHV-4. Appl Microbiol Biotechnol 98:5719–5735

Huang CJ, Hung JI, Wu CY, Chien MS (2004) Multiplex PCR for rapid detection of pseudorabies virus, porcine parvovirus and porcine circoviruses. Vet Microbiol 101:209–214

Jack PJ, Amos-Ritchie RN, Reverter A, Palacios G, Quan PL, Jabado O, Briese T, Lipkin WI, Boyle DB (2009) Microarray-based detection of viruses causing vesicular or vesicular-like lesions in livestock animals. Vet Microbiol 133:145–153

Jiang YH, Shang H, Xu H, Zhu LJ, Chen WJ, Zhao LY, Fang L (2010) Simultaneous detections of porcine circovirus type 2, classical swine fever virus, porcine parvovirus and porcine reproductive and respiratory syndrome virus in pigs by multiplex polymerase chain reaction. Vet J 183:172–175

Landlinger C, Preuner S, Willinger B, Haberpursch B, Racil Z, Mayer J, Lion T (2009) Species-specific identification of a wide range of clinically relevant fungal pathogens by use of Luminex xMAP technology. J Clin Microbiol 47:1063–1073

Lin K, Wang C, Murtaugh MP, Ramamoorthy S (2011) Multiplex method for simultaneous serological detection of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2. J Clin Microbiol 49:3184–3190

Maes D, Nauwynck H, Rijsselaere T, Mateusen B, Vyt P, de Kruif A, Van Soom A (2008) Diseases in swine transmitted by artificial insemination: An overview. Theriogenology 70:1337–1345

Miller MB, Tang YW (2009) Basic concepts of microarrays and potential application in clinical microbiology. Clin Microbiol Rev 22:611–633

Munro SB, Kuypers J, Jerome KR (2013) Comparison of a multiplex real-time PCR assay with a multiplex Luminex assay for influenza virus detection. J Clin Microbiol 51:1124–1129

Nagarajan K, Loh KC (2014) Molecular biology-based methods for quantification of bacteria in mixed culture: perspective and limitations. Appl Microbiol Biotechnol 98:6907–6919

Nolan JP, Mandy FF (2001) Suspension array technology: new tools for gene and protein analysis. Cell Mol Biol 47:1241–1256

Ogawa H, Taira O, Hirai T, Takeuchi H, Nagao A, Ishikawa Y, Tuchiya K, Nunoya T, Ueda S (2009) Multiplex PCR and multiplex RT-PCR for inclusive detection of major swine DNA and RNA viruses in pigs with multiple infections. J Virol Methods 160:210–214

Righter DJ, Rurangirwa FR, Call DR, McElwain TF (2011) Development of a bead-based multiplex PCR assay for the simultaneous detection of multiple *Mycoplasma* species. Vet Microbiol 153:246–256

Ros-García A, Barandika JF, García-Pérez AL, Juste RA, Hurtado A (2013) Assessment of exposure to piroplasms in sheep grazing in communal mountain pastures by using a multiplex DNA bead-based suspension array. Parasit Vectors 6:277

Schmitt M, Bravo IG, Snijders PJF, Gissmann L, Pawlita M, Waterboer T (2006) Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol 44:504–512

Shisong F, Jianxiong L, Xiaowen C, Cunyou Z, Ting W, Xing L, Xin W, Chunli W, Renli Z, Jinquan C, Hong X, Muhua Y (2011) Simultaneous detection of influenza virus type B and influenza A virus subtypes H1N1, H3N2 and H5N1 using multiplex real-time RT-PCR. Appl Microbiol Biotechnol 90:1463–1470

Van der Wal FJ, Achterberg RP, de Boer SM, Boshra H, Brun A, Maassen CB, Kortekaas J (2012) Bead-based suspension array for simultaneous detection of antibodies against the rift valley fever virus nucleocapsid and Gn glycoprotein. J Virol Methods 183:99–105

Wang XQ, Dang E, Gao JZ, Guo S, Li Z (2013) Development of a gold nanoparticle-based oligonucleotide microarray for simultaneous detection of seven swine viruses. J Virol Methods 191:9–15

Wernike K, Hoffmann B, Dauber M, Lange E, Schirrmeier H, Beer M (2012) Detection and typing of Highly pathogenic porcine reproductive and respiratory syndrome virus by multiplex real-time RT-PCR. PLoS One 7:e38251

Xu XG, Chen GD, Huang Y, Ding L, Li ZC, Chang CD, Wang CY, Tong DW, Liu HJ (2012) Development of multiplex PCR for simultaneous detection of six swine DNA and RNA viruses. J Virol Methods 183:69–74

Yue F, Cui S, Zhang C, Yoon K (2009) A multiplex PCR for rapid and simultaneous detection of porcine circovirus type 2, porcine parvovirus, porcine pseudorabies virus, and porcine reproductive and respiratory syndrome virus in clinical specimens. Virus Genes 38: 392–397