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Screening of 11 Pathogens in Fur Animal Basesd on Oligonucleotide Microarray

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Abstract: Brucella, *E. coli* O157, *Staphylococcus aureus*, β-Streptococcus, *Erysipelothrix rhusiopathiae*, *Pseudomonas aeruginosa* and Bovine viral diarrhea virus, Sheep pox virus, Goat pox virus, Bluetongue virus, foot and mouth disease virus are common pathogens in fur animal. An oligonucleotide microarray able to detect simultaneously the 6 bacteria and 5 viruses is reported in the present study. The assay was highly specific for detecting the 6 bacteria and 5 viruses in single or multiple infections and as few as 100 copies of specific pathogens target fragments were detected successfully. The 320 archived samples were tested by this assay and the results were 100% consistent with previous results based on conventional PCR and sequencing. The assay is appropriate for the screening of 11 pathogens infections in fur animal due to its high throughput, low-cost, high specificity and sensitivity.

Key words: Fur animal, bacteria, viruses, oligonucleotide microarray, infection

INTRODUCTION

Brucella, *E. coli* O157, *Staphylococcus aureus*, β-streptococcus, *Erysipelothrix rhusiopathiae*, *Pseudomonas aeruginosa* and Bovine Viral Diarrhea Virus (BVDV), Sheep Pox Virus (SPV), Goat Pox Virus (GPV), Bluetongue Virus (BTV), Foot and Mouth Disease Virus (FMDV) are common pathogens in fur animal. To reduce bacterium and viruses contaminations in fur supplies and minimizing the prevalence of diseases, rapid and reliable methods are fundamental and needed. Current methods for detecting the 11 kind of pathogens include bacterial isolates, serological methods such as Enzyme-Linked Immunosorbent Assay (ELISA) (Storch, 2000), Immune Fluorescenc Assay (IFA) and Virus Neutralization Test (VNT) and various PCR-based assays (Belak *et al*., 2009). However, these methods have the limitations of routine diagnostic purposes. Pathogen isolation usually is time-consuming and laborious. Serology sometimes yields false positive results or false negative results due to cross-reaction and low sensitivity of the assay. Conventional individual PCR and real-time PCR assays fail to detect co-infection of multiple viruses. Although, multiplex PCR or real-time PCR assays have been developed to detect several pathogens simultaneously these methods also restrict the numbers of targeted fragments simultaneously in a single reaction. To solve these limitations, oligonucleotide microarray technology (Mothershed and Whitney, 2006) has recently been developed to detection of animal pathogens. This method has been further applied for bacteria identification (Suo *et al*., 2010) viral identification (Cannon *et al*., 2010) single nucleotide polymorphism detection (Nannya *et al*., 2005) and disease genotyping (Pasquini *et al*., 2008).

MATERIALS AND METHODS

Primers and probes design: The specific gene targets used for detection of 11 pathogen were searched, blast on National Center for Biotechnology Information (NCBI). *16S rDNA* and *gyrB* genes were chosen as targets of the 6 bacteria and the specific probes were designed to detect Brucella, *E. coli* O157, *Staphylococcus aureus*, β-streptococcus, *Erysipelothrix rhusiopathiae*, *Pseudomonas aeruginosa*, respectively. The universal primers and probes were designded by Capitalbio Corporation (Beijing, China). The multiple specific primers and oligonucleotide probes of BTV, FMDV, GPV, SPPV and BVDV were designed by Primer Premier 5.0 Software.

Each forward primer of the 11 kinds of pathogens and position control probe were labeled with TAMARA fluorophore and all of the oligonucleotide probes which the length can not reach to 35-mer were added T base to 35-mer at the 5' end. All the oligonucleotides were custom synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). Sequences of the primers and probes are shown in Table 1.

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Table 1: Primers and probes sequences used in this study

Bacteria virus	Target gene	Serial No.	Sequence (5'-3')
	Sample application QC		TAMARA-GTCACATGCGATGGATCGAGCTCCTTTATCATCGTTCCCACCTTAATGCA
	Positive hybrid QC		GTCCAGTATTTCAAGGGCAACCG
		16S rDNA-F	TAMARA-GGTTTCGGATGTTACAGCGTAGAGTTTGATCCTGGCTCAG
		16S rDNA-R	GACGGGCGGTGTGTRCA
		gyrB-F	TAMARA-CCAGGYATGTAYATYGG
		gyrB-R	CATYTCKCCYARMCCYTTRTAWCGYTG
Brucella	16S rDNA	Probe 1	ttttttttttttTGTCTCCACTAACCGCGACC
		Probe 2	ttttttttttttGGGCCGATCATTTGCCGATA
P. aeruginosa	gyrB	Probe 1	ttttttttttttCCTTCAGACCGCCTTCGTAC
		Probe 2	ttttttttttttACGTTGAAGTGGAATACCTC
E. coli O157	16S rDNA	Probe 1	ttttttttttttCTGCGGGTAACGTCAATGAG
	gyrB	Probe 2	ttttttttttttTACACCGTGTTCGTAGATCT
		Probe 3	ttttttttttttCGACAACTCACGCAGACGTT
E. rhusiopathiae	16S rDNA	Probe 1	ttttttttttttCTCGTTGTGATGTATCCCAT
		Probe 2	ttttttttttttTAAGCCCATCTCGTTGTGAT
		Probe 3	ttttttttttttTCCCTCTTCCTATCGTTCTT
		Probe 4	ttttttttttttTCTTAGATTAACGTATCCGG
S. aureus	16S rDNA	Probe 1	ttttttttttttAGAAGCAAGCTTCTCGTCCG
β-streptococcus	16S rDNA	Probe 1	ttttttttttttttACCGTCACTTGGTGGATT
BTV		BTV-F	TAMARA-ATCCGGGCTGATCCAA
		BTV-R	CCTTCTCCGCCTTCTGT
		BTV Probe 1	ttttttttttCGGTTGCCCTTGAAATACTGGAC
FMDV		FMDV-F	TAMARA-TGGGACCATACAGGAGAAG
		FMDV-R	CCAACGCAGGTAAAGTGAT
		FMDV Probe 1	ttttttttttTGGCAGGACTCGCCGTCCATTCT
GPV		GPV-F	TAMARA-TCACAGAAGAACAAGTTGGAGA
		GPV-R	GAGGTTGCTGGAAATG
		GPV Probe 3	ttttttttAGAAACGAGGTCTCGAAGCAATACC
SPPV		SPPV-F	TAMARA-ATGTAGTATTTGCCAC
		SPPV-R	AACTTTGATTTACGGT
		SPPV Probe 3	ttttTATACTTTCTTTGAACAGATCAGTATTTCCG
BVDV		BVDV-F	TAMARA-GTGGTGAGTTCGTTGGA
		BVDV-R	TCAGTAGCAATACAGTGGG
		BVDV Probe 3	ttttttttttAGGCTAGCCATGCCCTTAGTAGGAC

R-(A, G), Y-(C, T), K-(G, T), M-(A, C), W-(A, T), TAMRA = 6-carboxytetramethylrhodamine

Slide preparation: The 5'-amino-C6-modified oligonucleotide probes were diluted to 30 μmol L^{-1} by spotting solution (CapitalBio) and spotted in the aldehyde-modified glass microscope slides. Spotting was achieved with Smart Arrayer[48] microarray spotter (CapitalBio, Beijing, China) at 80% relative humidity. After the slides were spotted they were placed in a calorstat at 80°C to covalently link the oligonucleotides. The layout of microarray and probe locations is shown in Fig. 1.

Asymmetric PCR and multiple PCR: Genomic DNA of the 6 bacteria were extracted to use Bacterial Genomic DNA Extraction kit (Takara, Dalin, China). The asymmetric PCR of 16S rDNA and gyrB was established, respectively. The reaction mix was prepared as follows: 2×Taq PCR MasterMix, 12.5 μL; sense primer 5 μL (20 μM) reverse pimrer 0.5 μL (20 μM); template DNA 2 μL, add ddH_2O to 25 μL. Thermal cycling conditions were: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 55°C for 45 sec and 72°C for 50 sec and 72°C for 10 min.

Total RNA/DNA of 5 viruses were extracted by MiniBEST Viral RNA/DNA Extraction kit (TaKaRa, Dalian, China). Single PCR products were cloned to PMD 18 vector. Each multiplex PCR reaction was carried out similarly to the single PCR with some optimizations. Three RNA viruses (BTV, FMDV and BVDV) and two DNA viruses (GPV, SPPV) were amplified separately in two Multiplex PCR Systems. The reaction was optimized as follows: the reaction mixture was prepared in a final volume of 25 μL containing 50 ng of DNA/cDNA or plasmid template, 1×PCR buffer with 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer and 1.5 U Taq DNA polymerase. Annealing temperature was 55 and 43°C, respectively.

Hybridization and washing: The 7 μL labeled PCR products were mixed with 8 μL hybridization solution (47% formamide, 3×SSC, 0.1% SDS and 5×Denhardts solution). The mixed solution was denaturated at 95°C for 10 min, quickly chilled on ice for 5 min and then placed on the microarray and hybridized for 4 h at 42°C in the hybrid instrument (CapitalBio, Beijing, China) with 5 rpm. Following the hybridized slide was washed thrice for 3 min in 2×SSC containing 0.2% SDS at 42°C, thrice for 3 min in 0.2×SSC with emundant instrument. Then, the slides were rinsed with deionized water and briefly dried at a low speed of 300 rpm before scanning.

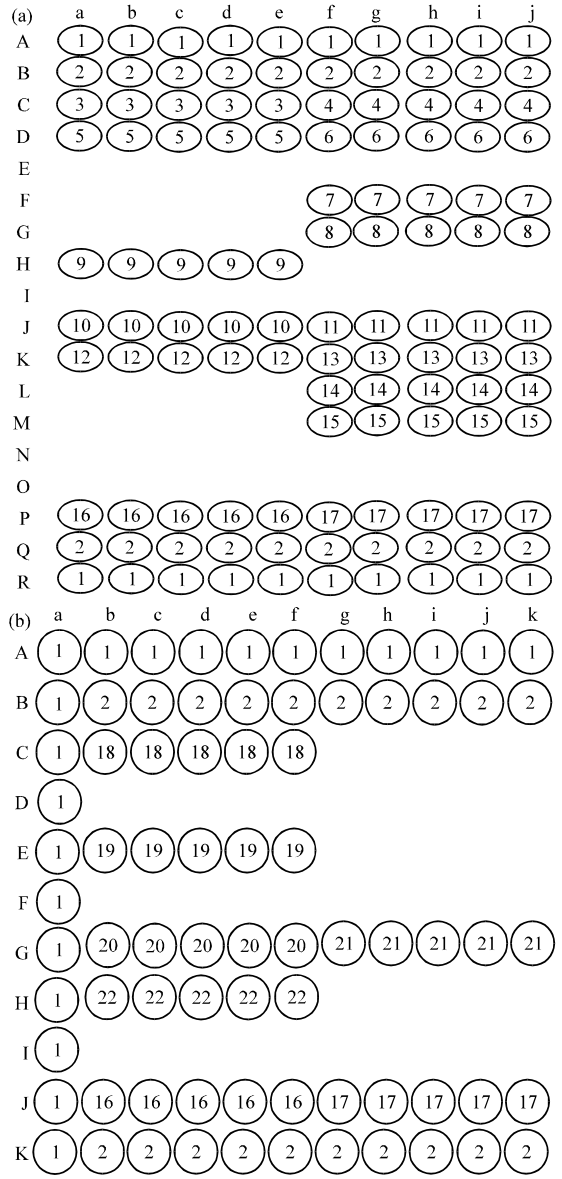

Fig. 1: a) Layout of oligonucleotide probes on the bacteria and b) viral detection microarray; 1: Spotting positive quality control; 2: Hybridization positive quality control; 3: *E. coli* O157 Probe 1; 4: *E. coli* O157 Probe 2; 5: *E. coli* O157 Probe 3; 6: *S. aureus* Probe 1; 7: β-streptococcus Probe 1; 8: *P. aeruginosa* Probe 1; 9: *P. aeruginosa* Probe 2; 10: *E. rhusiopathiae* Probe 1; 11: *E. rhusiopathiae* Probe 2; 12: *E. rhusiopathiae* Probe 3; 13: *E. rhusiopathiae* Probe 4; 14: Brucella Probe 1; 15: Brucella Probe 2; 16: spotting solution; 17: ddH_2O; 18: BTV probe; 19: FMDV probe; 20: GPV probe; 21: SPPV probe; 22: BVDV probe

Scanning and signal analysis: The microarrays were scanned with LuxScanIM10K Scanner (CapitalBio, Beijing, China) at an excitation wavelength of 532 nm. Fluorescent images about fluorescent signals and local background were analysed and recorded using LuxScan3.0 Software. Only results in which all 5 repeated spots had a ratio of the fluorescence intensity (minus background) to the noise in the background of >5 were considered positive.

RESULTS

Asymmetric PCR and multiple PCR results: *S. aureus* ATCC29213 and 26003-5a7 strains, β-streptococcus 32210-4a strain, *P. aeruginosa* strain, *E. rhusiopathiae* CVCC123, *B. cereus* CVCC2002 strain, *B. thuringiensis* S1 and S6 strains, *E. coli* O157 NCTC12900 and the identified *E. coli* O18, Brucella, Listeria, Salmonella and *E. sakazakii* genomes were extracted and amplicated with the 16S rDNA and gyrB primers. The results showed in Fig. 2. Single PCR of each five virus was first performed and then the target genes were cloned to PMD-18 Vector (Takara, Dalin, China). Then, combinations of the three RNA viruses and the duplex DNA viruses were used in multiplex PCR, the respective virus amplicons were produced and no nonspecific products appeared (Fig. 3).

Specificity of the microarray: To test the specificity of the bacteria probes presented on the microarray. DNA of the 6 kinds of bacteria were extracted and amplificated according to the procedure 2.3. The products were hybrizated in the prepared microarray. Each bacterium was tested at least twice on the microarray. The probes only detected the presence of the 6 bacteria neither non-specific nor cross-reactive signal was observed (Fig. 4).

RNA/DNA of 5 viruses and other virus, PRV, AIV, NDV were extract by Viral Extraction kit (TaKaRa, Beijing, China), respectively. After amplication, hybridization, washing and scanning, the fluorescence signals on the array were unambiguously distinguished for each of the species and obtained the key probe, respectively and no cross hybridizations were observed (Fig. 5). Briefly, the preparation of bacterial and viral microarray had a strong specificity and no cross reaction.

Sensitivity of the microarray: The 10 fold serial dilution of the bacteria genome from 10^1-10^6 copies μL^{-1} was prepared. The resulting PCR products were then labeled and applied to the array. The minimum copy number of Brucella, *E. coli*, *Staphylococcus aureus*, β-streptococcus, *Erysipelothrix rhusiopathiae* and *Pseudomonas aeruginosa* was ~100 copies.

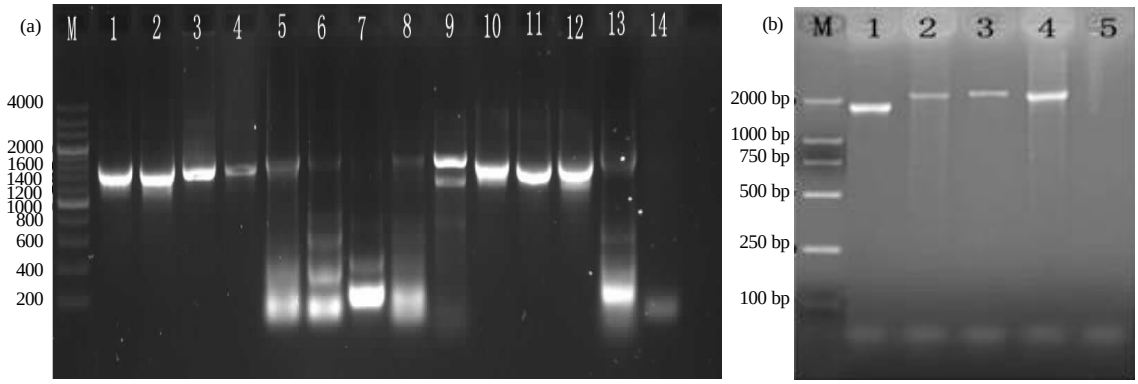

Fig. 2: a) The PCR results used *16S RNA* gene primers and M: 200 bp Marker; 1: *E. coli* O157 NCTC12900; 2: *E. coli* O18; 3: Salmonella f1-10; 4: *P. aeruginosa* ATCC15442; 5: *S. aureus* ATCC29213; 6: *S. aureus* 26003-5a7; 7: Brucella; 8: *E. sakazakii*; c9: *E. rhusiopathiae*; 10: *B. thuringiensis* S1; 11: *B. thuringiensis* S2; 12: β-streptococcus 32210-4a; 13: Listeria; 14: negtive control; b) the PCR results used *gyrB* gene primers and M: Marker I DNA Ladder; 1: *B. thuringiensis*; 2: *P. aeruginosa*; 3: *P. aeruginosa*; 4: *E. coli* O157

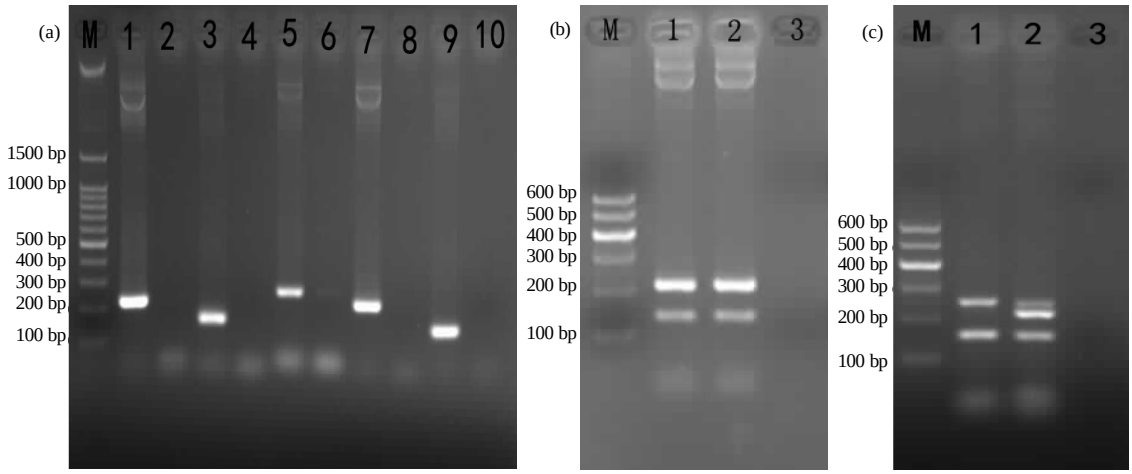

Fig. 3: PCR amplification results of five viruses. a) Sigle PCR of five viruses and M: 100 bp DNA Marker; 1: BTV; 3: SPPV; 5: GPV; 7: BVDV; 9: FMDV; 2, 4, 6, 8 and 10: ddH_2O; b) triple PCR amplification results of BTV, FMDV and BVDV and M: Marker; 1, 2: Triple PCR amplification results of BTV, FMDV and BVDV; 3: Negative control; c) duplex PCR amplification results of GPV and SPPV and M: Marker; 1: Double PCR amplification of GPV and SPPV; 2: Triple PCR amplifications of BTV, GPV and SPPV; 3: Negative control

To determine the detection limits of the viral microarray assays, each target gene of five virus was cloned to PMD-18 vector. Each of the five recombinant vectors was prepared 10 fold serial dilutions, ranging from 10^1-10^7 copies μL^{-1}. Following each viral PCR products were hybrided with the preparation microarray respectively and scanned using LuxScanIM10K Scanner (CapitalBio, Beijing, China). Analysis of the fluorescent signal indicated that the microarray-based assay could detect virus at least approximately 10 copies of the recombinant plasmid.

Validation of the microarray and detection of single and mixed infections: In order to validate the bacteria microarray and evaluate its performance, 210 archived animal fur samples were tested by the array. Of the positive samples, three were Brucella two were *S. aureus* positive and 215 were negative. The results were identical with that of real-time PCR and then were further confirmed by conventional PCR.

To determine the applicability of virus microarray, the recombinant virus plasmids were added to 110 animal fur samples randomly. Then, artificial positive fur were

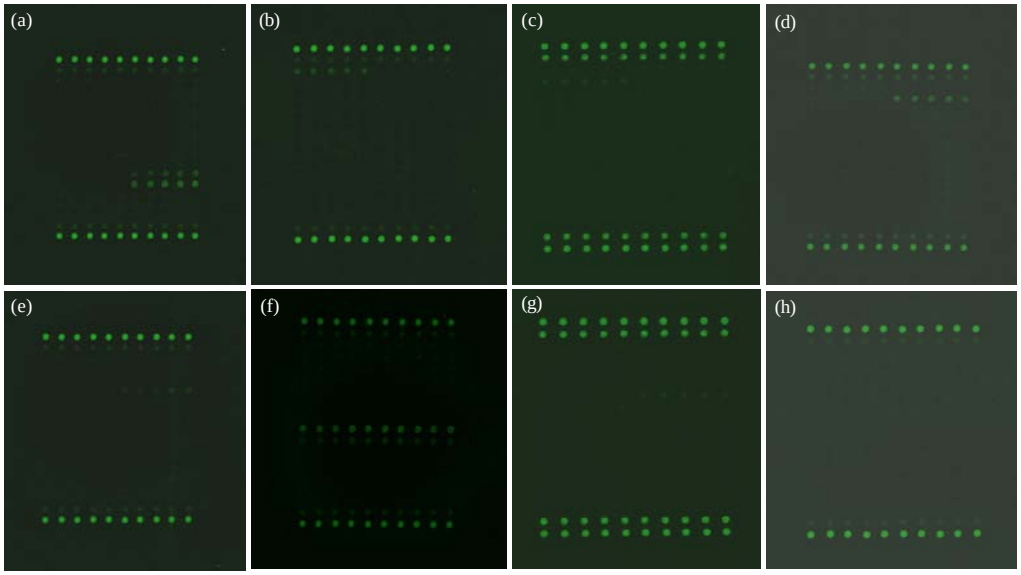

Fig. 4: The specific results of bacteria microarray. a, b, d, e, f was 16S rDNA of Brucella, *E. coli* O157, *S. aureus*, β-streptococcus detection of the microarray, respectively. C, G was gyrB products of *E. coli* O157, *P. aeruginosa*

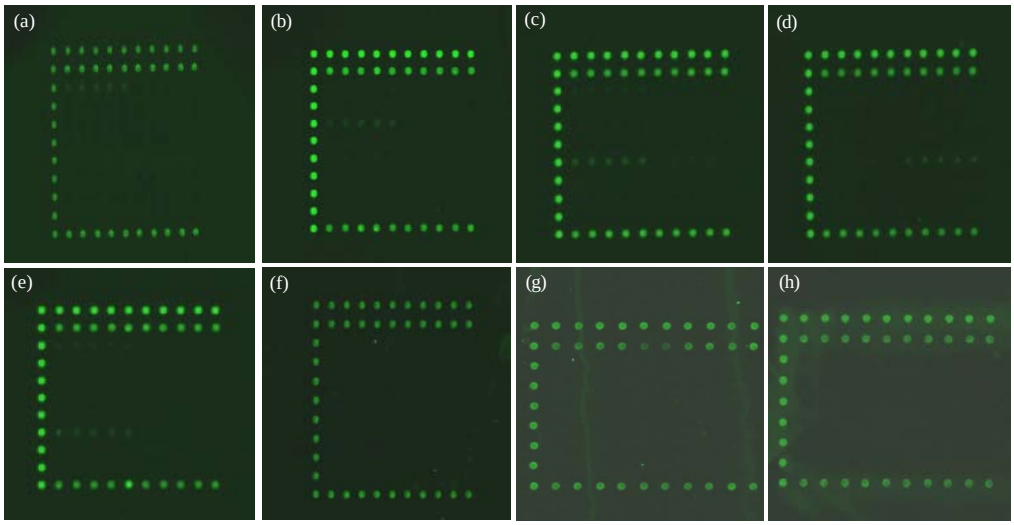

Fig. 5: Specificity of microarray in detecting viral pathogens. Genomic DNA from each of the following strains was amplified in single specific PCR and then analyzed by microarray; a-h) was the special hybrid results of BTV, FMDV, GPV, SPPV, BVDV, AIV, NDV, PRV, respectively

parallel detected by the array and each of virus commercial PCR Test kit (Vipotion, Guangzhou, China). The results obtained by microarray and PCR/RT-PCR techniques were in concordance with 100% of agreement.

DISCUSSION

Because of the different epidemic diseases of different countries, fur animal and animal products carried different pathogens. The 6 most prominent bacteria and 5 viral pathogens were common in exported and imported fur animal. At present, traditional methods, mainly relied on serological methods and Polymerase Chain Reaction (PCR) to detect these pathogens however these methods are time-consuming and fail to detect co-infection of multiple pathogens. In this study, researchers used fluorophore to mark the upstream primer and then established a Microarray Detection Method for the 6 bacteria and 5 viruses.

One of the key factors affecting the microarray chip testing veracity was the design of probe. The position of oligonucleotide probe in sequence of nucleic acid, probe length, Tm value of the oligonucleotide probe fragments and binding affinities will affect the specificity, sensitivity and fluorescence signal strength of the microarray chips (Ratushna *et al*., 2005; Southern *et al*., 1999). In this study, in order to ensure the high throughput detection at a certain temperature in the same microarray chip, researchers fully consider the Tm value when designing the oligonucleotide probes.

In addition, the length of the oligonucleotide probe effect the binding property which accommodates highly stringent microarray hybridization (Koltai and Weingarten-Baror, 2008). Therefore, in order to enhance the space occupied effect of probes and strengthen the fluorescent signal and specificity, researchers extended the probe length which is <35-mer by adding T bases to 35-mer at the 5' end. The experimental results showed that in full consideration of the earlier three conditions this method can detect 11 pathogens simultaneously with stronger fluorescent signal, specificity and higher sensibility.

The different hybridization condition of the formamide concentration of the hybrid liquid medium, temperature and hybrid time were others key factors affecting specificity and sensitivity of the microarray chip which even caused the false positive hybrid signal (Rodaree *et al*., 2011). In theory, researchers know that the optimization is generally carried out experimentally by establishing probe denaturation profiles with pure cultures or clones of target and non-target organisms an option that is clearly not feasible for high density microarrays given the large number of probes (Yilmaz *et al*., 2012), researchers did show the proof of principle for formamide denaturation in microarrays of perfect and mismatched oligonucleotide probes. In this study, researchers optimized four kinds of hybrid liquid mediums with different concentration of armor amide and optimized the hybrid time simultaneously. Finally, researchers obtained the stronger signals with heavy specificity and high sensitivity up to approximately 10 gene-copies at the efficient hybridization conditions which were rotated 2 h with 5 rpm in 47% formamide at 42°C in the hybrid instrument.

The array was a high throughput rapid diagnosis with strong specificity and sensibility and suitable for the batch animal fur samples testing in modern entry-exit inspection and quarantine departments.

CONCLUSION

In this study, a DNA microarray platform was developed to simultaneously detect the 6 most prominent bacteria and the 5 viral pathogens in animal fur with species-specific gene probes.

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REFERENCES

Belak, S., P. Thoren, N. LeBlanc and G. Viljoen, 2009. Advances in viral disease diagnostic and molecular epidemiological technologies. Expert Rev. Mol. Diagn., 9: 367-381.

Cannon, G.A., M.J. Carr, Z. Yandle, K. Schaffer and R. Kidney *et al*., 2010. A low density oligonucleotide microarray for the detection of viral and atypical bacterial respiratory pathogens. J. Virol. Methods, 163: 17-24.

Koltai, H. and C. Weingarten-Baror, 2008. Specificity of DNA microarray hybridization: Characterization, effectors and approaches for data correction. Nucleic Acids Res., 36: 2395-2405.

Mothershed, E.A. and A.M. Whitney, 2006. Nucleic acid-based methods for the detection of bacterial pathogens: Present and future considerations for the clinical laboratory. Clinica Chimica Acta, 363: 206-220.

Nannya, Y., M. Sanada, K. Nakazaki, N. Hosoya and L. Wang *et al*., 2005. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res., 65: 6071-6079.

Pasquini, G., M. Barba, A. Hadidi, F. Faggioli and R. Negri *et al*., 2008. Oligonucleotide microarray-based detection and genotyping of *Plum pox virus*. J. Virol. Methods, 147: 118-126.

Ratushna, V.G., J.W. Weller and C.J. Gibas, 2005. Secondary structure in the target as a confounding factor in synthetic oligomer microarray design. BMC Genomics, Vol. 6. 10.1186/1471-2164-6-31.

Rodaree, K., T. Maturos, S. Chaotheing, T. Pogfay and N. Suwanakitti *et al*., 2011. DNA hybridization enhancement using piezoelectric microagitation through a liquid coupling medium. Lab Chip, 11: 1059-1064.

Southern, E., K. Mir and M. Shchepinov, 1999. Molecular interactions on microarrays. Nat. Genet., 21: 5-9.

Storch, G.A., 2000. Essentials of Diagnostic Virology. Churchill Livingstone Inc., New York, USA., ISBN-13: 9780443076329, Pages: 340.

Suo, B., Y. He, G. Paoli, A. Gehring, S.-I. Tu and X. Shi, 2010. Development of an oligonucleotide-based microarray to detect multiple foodborne pathogens. Mol. Cell. Probes, 24: 77-86.

Yilmaz, L.S., A. Loy, E.S. Wright, M. Wagner and D.R. Noguera, 2012. Modeling formamide denaturation of probe-target hybrids for improved microarray probe design in microbial diagnostics. PloS ONE, Vol. 7. 10.1371/journal.pone. 0043862.