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Original research article

Development of a DNA-based microarray for the detection of zoonotic pathogens in rodent species

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ABSTRACT

The demand for diagnostic tools that allow simultaneous screening of samples for multiple pathogens is increasing because they overcome the limitations of other methods, which can only screen for a single or a few pathogens at a time. Microarrays offer the advantages of being capable to test a large number of samples simultaneously, screening for multiple pathogen types per sample and having comparable sensitivity to existing methods such as PCR. Array design is often considered the most important process in any microarray experiment and can be the deciding factor in the success of a study. There are currently no microarrays for simultaneous detection of rodent-borne pathogens. The aim of this report is to explicate the design, development and evaluation of a microarray platform for use as a screening tool that combines ease of use and rapid identification of a number of rodent-borne pathogens of zoonotic importance. Nucleic acid was amplified by multiplex biotinylation PCR prior to hybridisation onto microarrays. The array sensitivity was comparable to standard PCR, though less sensitive than real-time PCR. The array presented here is a prototype microarray identification system for zoonotic pathogens that can infect rodent species.

1. Introduction

Prompt detection of pathogens is a significant issue in diagnostic testing for both human and veterinary health. This is particularly relevant when slow-growing or fastidious organisms are involved and the limitations of some existing diagnostic tools are driving researchers to consider alternative methods, as demands on quantity and rapidity of testing methods are increasing [1]. Serological methods provide an indication of exposure to a pathogen and are best used for screening populations. However, they also require an adequate time post-infection/exposure for antibodies to develop and may be unable to distinguish between different strains or antigenic types of pathogen. Zoonotic pathogens make up the majority (75%) of emerging diseases and wildlife are a major source of these pathogens [2]. Early detection of pathogens in wild animals would be useful in identifying risk factors associated with disease transmission to humans or domestic animals, and this could help prevent a possible outbreak. It has also been suggested that prevention of disease, which could be aided by an effective surveillance system, is better than reacting to an outbreak, or to finding a cure [3]. Microarrays offer the advantage of testing large numbers of samples simultaneously, coupled with screening a single sample for multiple pathogens. Use of this technology would enable timely, accurate and inexpensive detection of pathogens, which could lead to more effective control of these infectious diseases, which has positive implications for public health [4]. There are a wide ranging number of potential applications for pathogen detection arrays; and have been used for the detection of novel pathogens, as in the case of severe acute respiratory syndrome (SARS) [5], simultaneous detection of Newcastle disease virus and avian influenza virus in birds [6] and detection of viruses that can cause vesicular or vesicular-like lesions in livestock [7].

Although microarrays are used widely, the fluorescence-based glass slide arrays are relatively expensive. Alternatives to the glass slide microarray are the ArrayTube™ (AT) and ArrayStrip™ (AS) platforms from Alere Technologies GmbH (Jena, Germany). These are much less expensive, and can be used without highly specialised equipment [8]. The AT (up to 225 spots) and AS (up to 600 spots per well) platforms make the use of a small array surface of size 4 × 4 mm placed on the bottom of a plastic vial or well.

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Hybridisation and analysis are simple and rapid, using standard laboratory methods, and hybridisation signals are detected following an enzyme-catalysed precipitation reaction [9]. The use of plastic tube-integrated arrays and fast non-fluorescent labelling and hybridisation protocols results in a system that is cost-effective, time saving, and allows high sample throughput, in a 96 well format [1].

There are currently no microarrays for the detection of multiple rodent-borne pathogens. The aim of this report is to explicate the design, development and evaluation of a microarray platform for use as a screening tool, which combines ease of use and rapid identification of a number of rodent-borne pathogens of zoonotic importance.

2. Materials and methods

2.1. Probe design

An initial literature search was performed to identify zoonotic pathogens which are transmissible by rodents. A microarray was then developed to screen for the presence of these pathogens. Table 1 shows the list of pathogens to be screened for, including the source of any reference material if available. Unfortunately, not all of the pathogens for which the array was designed to detect could be sourced. Therefore the probes for, Hepatitis E Virus, *Bartonella*, MRSA, *Rickettsia typhi* and *Streptobacillus monilliformis* were not evaluated. RNA from an infected *Rattus norvegicus* sample was supplied but several attempts at PCR proved unsuccessful and it was concluded that the RNA had degraded too much to be of use. A further literature search was conducted to identify particular genes or target regions which had been previously used for identification purposes in other diagnostic tests such as PCR. The DNA sequences were obtained from the NCBI database and aligned using ClustalX2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalW2_phylogeny/help/faq.html#5) software. Oligonucleotide sequences (probes) were designed for each pathogen from regions targeted by species-specific or generic primers. Two freely available software packages were used for probe design: Unique Probe Selector (UPS) [10] and OligoWiz [11,12]. Both types of software were used to compensate for any limitations in the other. OligoWiz, at present, can only be used to design probes for bacteria.

An optimal length of 60-nucleotide probes was assigned, and parameters for both OligoWiz and UPS included cross-hybridisation, delta-Tm, low-complexity, position and folding. An *in silico* analysis was performed on all the probes using the BLAST tool on the NCBI database to determine if cross-hybridisation would occur with any other known sequences. The selected probes were synthesised at Metabion International (Jena, Germany) with the following specifications: NH_2 modification at the 3′ end, no modification at the 5′ end, purification with HPLC, 0.04 μmol scale, and absolutely biotin-free.

The AT platform was used for initial evaluation for individual pathogens, and the best performing probes were transferred to the AS platform making a pool of probes from different pathogens. For both platforms, each probe was directly spotted onto the array surface at a 15 μM concentration with each probe printed in duplicate.

2.2. Primer design

Generic primers were designed from conserved flanking regions of the target sequence using Primer3 (http://primer3.ut.ee/). Species-specific primers were designed from more variable regions of a sequence that were specific to certain pathogens. The amplicon size was set between 250 and 750 bases, with an optimum of 500 bases. Primer sequences for both multiplex PCR and real-time PCR can be seen in Table 2.

2.3. Nucleic acid amplification

Several amplification methods were tested including sequence-independent amplification [13] using a random pentadecamer primer and a primer tag, and sequence-dependent amplification using pathogen-specific primers.

Table 1
A list of all the reference materials that were available for this study.

Pathogen	Sample type
Bartonella	Unavailable
Campylobacter spp	*C. jejuni* strain NCTC11168H
Cowpox virus	RNA of strain Compiegne 2009 isolate KP/LP from culture
E. coli spp	TUV93-0 stx −ve
	Sakai stx +ve
Hepatitis E Virus	Unavailable
Lymphocytic Choriomeningitis virus	Entry clone LCMV L1 domain (EVA catalogue number 231)
Leptospira spp	DNA from culture and clinically infected animals
Methicillin-Resistant *Staphylococcus aureus*	Unavailable
Puumala Hantavirus	Unavailable
Rickettsia typhi	Unavailable
Salmonella spp	DNA from culture
	S. typhimurium
	S. enteritidis
	S. gallinarum
	S. heidelberg
	S. infantis
	S. dublin
	S. hadar
	S. pullorum
Seoul Hantavirus	Unavailable
Streptobacillus monilliformis	Unavailable
Toxoplasma gondii	*T. gondii* parasites, RH-type, grown in tissue culture on a RK13 cell line (rabbit kidney), WHO type.
Yersinia enterocolitica	*Y. enterocolitica* 8081
Yersinia pestis	*Y. pestis* NCTC5923 Type strain
Yersinia pseudotuberculosis	*Y. pseudotuberculosis* YpIII

Table 2
Primers used during the evaluation of the arrays. The majority were designed during the study but others were obtained either from the literature or colleagues.

Pathogen	Primer	Sequence	Reference
C. jejuni	Camp/flgS/776650/F	CTTGCAAGCATGGGTAGTGT	[35,36]
	Camp/flgS/777053/R	GGCTTTTCACATTCGCTTTC	
Cowpox	Cow/HA/178301/F	ATGACACGATTGCCAATACTTC	[37]
	Cow/HA/178929/R	CTTACTGTAGTGTATGAGACAGC	
E. coli	E.coliStxF	CCTGTGAGCTATACGGAAAGTAC	(David Gally and Sean McAteer
	E.coliStxR	TCAGATGCCATTCTGGCAACTCG	Personal Communication 2013)
Francsiella	FT RD-F	TTTATATAGGTAAATGTTTTACCTGTACCA	[38]
	FT RD-R	GCCGAGTTTGATGCTGAAAA	
HEV	HEV/ORF1/4109/F	GTTGAGGCCATGGTGGAG	This study
	HEV/ORF1/4724/R	AGAGGACCACCGAATCATCA	
LCMV	LCMV/NP/779/F	CCTGGGAAAACCACTGCACA	This study
	LCMV/NP/1448/R	TTGACAAGGTGCAGGAAGATGC	
Leptospira	Lept/sphH/3501394/F	ACGCGGATCCTTCTACTCCT	This study
	Lept/sphH/3501818/R	GGCTGATCGAATCTTTCCAA	
Leptospira	Lept/hemo/3184769/F	ACGCGGATCCTTCTACTCCT	This study
	Lept/hemo/3185246/R	GGCTGATCGAATCTTTCCAA	
MRSA	MRSA/mecA/2343/F	GGCCAATACAGGAACAGCAT	This study
	MRSA/mecA/2925/R	TTCACCTGTTTGAGGGTTGA	
Mycobacterium	TB 16S-F	TGCACTTCGGGATAAGCCTG	(Abu-Bakr Abu-Median,
	TB 16S-R	TAGCATGTGTGAAGCCCTGG	personal communication 2013)
PUUV	PUUV/S/29/F	CGAGAAAGACTGGAATGAGTGA	This study
	PUUV/S/475/R	CACGCGTTGAAAGCATGTA	
Rickettsia typhi	Rick/17kD/1047487/F	GCTCTTGCAGCTTCTATGTT	This study
	Rick/17kD/1047921/R	CCGCCAACCTGACGGGCAATGG	
Salmonella	Salm/flag/1366055/F	GGTAGAAATTCCCAGCGGGTACTGG	[39]
	Salm/flag/1366482/R	GCCAACCATTGCTAAATTGGCGCA	
SEOV	SEOV/S/472/F	AGAGGCAGGCAGACTTCAAA	This study
	SEOV/S/971/R	CCAGCAAACACCCATATTGA	
S. monilliformis	S.monil/asp/F	ACAGGAACTCTTACACATGTTGC	This study
	S.monil/asp/R	GACTTTGCCCTATTTTCAGCA	
S. typhimurium	Salm/CDP/2167279/F	CCAGCACCAGTTCCAACTTGATAC	[39]
	Salm/CDP/2005357/R	GGCTTCCGGCTTTATTGGTAAGCA	
T. gondii	T.gon/B1/12/F	CTCCGTCGTCCGTCGTAATA	This study
	T.gon/B1/510/R	TCGACAATACGCTGCTTGAA	
Yersinia spp.	Y.gen/YOPH/62151/F	AAAGCCATTTCCGTATGCTG	This study
	Y.gen/YOPH/62664/R	TGTACTCGCATTTGGCTGAC	
Y. enterocolitica	Y.ent/YE4072/4447336/F	TTTCTGGCCTTTCTGCTGTT	This study
	Y.ent/YE4072/4447834/R	AGATGCTGGGAATGTCGTGT	
Y. pestis	Y.pes/pPCP/8374/F	CCCGAAAGGAGTGCGGGTAA	This study
	Y.pes/pPCP/8902/R	CGCCCCGTCATTATGGTGAA	
Y. pseudotuberculosis	Y.pse/YPTB0154/178134/F	GGTGTTTGGGCCAGAGATAA	This study
	Y.pse/YPTB0154/178436/R	AGATTGCGTGATGCATCCTT	

2.3.1. Sequence-independent amplification

Any RNA present in the sample was reverse transcribed into cDNA with 1.0 μl of primer A (GTT TCC CAG TCA CGA TCN NNN NNN NNN NNN NN) (40 μM), 1.0 μl of 10 mM dNTP mix (Invitrogen), and variable amounts of water and template (minimum 50 ng/μl) were mixed in a PCR tube to a total volume of 13 μl. The volume of water was variable to allow for different concentrations of template. This was then heated to 65 °C for 5 min using a thermal cycler. The mixture was placed on ice for at least 1 min. A separate mixture containing 4.0 μl of 5x Reverse Transcriptase Buffer (Invitrogen), 1.0 μl of 0.1 M Dithiothreitol (DTT) (Invitrogen), 1.0 μl of RNase inhibitor, RNaseOUT (Invitrogen), and 1.0 μl of SuperScript III Reverse Transcriptase (Invitrogen) was added to the PCR tube contents, and mixed by pipetting. The 20 μl reaction was incubated using a thermal cycler at 25 °C for 5 min, then at 50 °C for 1 h and finally at 70 °C for 15 min to inactivate the reaction. The reaction was left at room temperature for 5 min, followed by 1 min on ice. The mix was then heated to 94 °C for 2 min, and rapidly cooled to 10 °C in the thermal cycler for 5 min 10 μl of Klenow mix (1.0 μl 10x Klenow buffer (Promega UK), 8.7 μl water, 0.3 μl Klenow polymerase (Promega)) was then added. For any DNA already present in the sample primer extension was effected with 1.0 μl Primer A (40 μM), 1.0 μl 10x Klenow buffer and variable amounts of water and template (minimum 50 ng/μl) to make a total volume of 10 μl. This sample mixture was then heated to 94 °C for 2 min and then allowed to cool to 10 °C in a thermal cycler for 5 min. The following 5.05 μl reaction mix was added to the sample mixture during its incubation at 10 °C: 0.5 μl 10x Klenow buffer, 1.5 μl 3 mM dNTPs, 0.75 μl 0.1 M DTT, 1.5 μl 500 μg/ml BSA, 0.3 μl Klenow polymerase (Promega UK), 0.5 μl water. The reaction was left at room temperature for 5 min, followed by 1 min on ice. The mix was then heated to 94 °C for 2 min, and rapidly cooled to 10 °C in the thermal cycler for 5 min 10 μl of Klenow mix (1.0 μl 10x Klenow buffer, 8.7 μl water, 0.3 μl Klenow polymerase) was then added.

For both RNA and DNA sequence-independent steps the mixture was then heated to 37 °C for 8 min, and then held at 37 °C for a further 8 min. This was followed by a rapid increase to 94 °C for 2 min after which the mix was cooled to 10 °C for 5 min, during which 1.2 μl of diluted Klenow (1:4) was added. The temperature was again increased to 37 °C for 8 min followed by a hold of 8 min at 37 °C, and then the reaction was terminated by placing the mixture on ice for 5 min. Standard PCR was then conducted using Primer B (GTT TCC CAG TCA CGA TC) (100 μM) to amplify the round A product with the following cycle parameters one step at 95 °C for 10 s; 35 cycles of 30 s at 94 °C, 30 s at 40 °C, 30 s at 50 °C, 2 min at 72 °C and one final extension step of 72 °C for 2 min. A 50 μl reaction mix was prepared from the following: 39.0 μl water, 1.5 μl 50 mM Magnesium chloride ($MgCl_2$) (Invitrogen UK), 5.0 μl 10x

Mg-free buffer (Invitrogen UK), 0.5 μl 25 mM dNTP mix, 0.5 μl Primer B, 0.5 μl *Taq* polymerase (5 U/μl) (Invitrogen UK) and 3.0 μl Round A product.

2.3.2. Sequence-specific amplification

Sequence-specific PCR was performed using a 50-μl reaction containing 37.5 μl of nuclease-free water, 2.0 μl of 50 mM $MgCl_2$ (Invitrogen UK), 5.0 μl of 10x Mg-free Buffer (Invitrogen UK), 1.0 μl of 25 mM dNTP mix, 1.0 μl of 10 μM forward primer, 1.0 μl of 10 μM reverse primer, 0.5 μl of *Taq* DNA polymerase (5 U/μl) and 2.0 μl of cDNA or DNA (optimal concentration 50 ng/μl). Cycling parameters were one step of 94 °C for 2 min; 30 cycles of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C and one final extension step of 10 min at 72 °C. In addition the Qiagen Multiplex PCR *Plus* kit was tested with the sequence-dependent primer sets. This was carried out with both non-biotinylated and biotinylated primers. Multiplex PCR was performed using a 50-μl reaction containing 25 μl Multiplex Master Mix, 5 μl 10x primer mix (2 μM each primer) and variable volumes of water and template (50 ng/μl). The recommended protocol in the Qiagen Multiplex PCR *Plus* handbook was followed with cycling parameters of one step at 95 °C for 5 min; 40 cycles of 30 s at 95 °C, 90 s at 60 °C and 90 s at 72 °C and one final extension step of 10 min at 68 °C.

Real-time PCR was carried out using the Applied Biosystems 7500 Fast Real-Time PCR System. Real-time PCR was performed using a 10-μl reaction containing 5 μl of TaqMan® Universal PCR Master Mix 2x (Life Technologies), 1 μl of 300 nM forward primer, 1 μl of 300 nM reverse primer, 1 μl TaqMan® probe (2.5 μM), 1 μl of nuclease-free water and 1 μl of sample (or water as a negative control). The recommended protocol was followed with cycling parameters of one step at 50 °C for 2 min, another step at 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Each sample was run in triplicate.

2.4. Microarray hybridisation

Prior to hybridisation of the labelled sample onto the array, the AS was conditioned by washing with 150 μl of water for 20 min at 30 °C. After the water was removed using a pipette, a pre-hybridisation buffer (5x saline-sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS), 4x Denhardt's solution) was pipetted into each well for 30 min at 50 °C. Both washes were performed using a thermomixer (BioShake iQ, QUANTIFOIL Instruments GmbH, Jena Germany) at 550 rpm, which was used in all subsequent incubation steps unless otherwise stated. A 10-μl aliquot of the biotin-labelled sample was added to 90 μl of hybridisation buffer (5x SSC, 1% SDS, 4x Denhardt's solution). The mixture was denatured at 95 °C for 3 min and then kept on ice. The denatured sample (100 μl) was then pipetted into the AS well and allowed to hybridise for 30 min at 55 °C at 550 rpm. The sample solution was then removed and the AS was washed successively for 20 min at 60 C at 550 rpm with 150 μl wash buffer 1 (1x SSC, 0.2% SDS), wash buffer 2 (0.1x SSC, 0.2% SDS), and wash buffer 3 (0.1x SSC). This buffer was then removed and vacant binding sites on the microarray were blocked by incubation with a blocking solution (100 μl) of 2% biotin-free milk in PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween™ 20 for 60 min at 30 °C at 300 rpm. The blocking solution was replaced with 100 μl conjugation solution (Streptavidin Poly-Horseradish peroxidase (HRP) diluted 1:100 in the blocking solution), and the array incubated for 15 min at 30 °C at 300 rpm. Post-conjugation washes were performed using wash buffers 1–3 as described for the post-hybridisation washes. After removal of wash buffer 3, 100 μl of a tetramethylbenzidine (TMB)-hydrogen peroxide (H_2O_2) solution, in this instance TrueBlue™ (Insight BioTechnology LTD, UK), was added and incubated for 10 min at 25 °C without shaking. After removing the solution, the AS was then inserted into the ArrayMate and the array image was recorded with raw data generated. The recorded image was analysed using Alere's integrated IconoClust software and analysis script. Iconoclust processes the signals and automatically normalises the signal value after an algorithm processes the average intensity of the spot and the local background noise. The output range of the signals was between 0 and 1, with 0 being negative and 1 being the maximal possible signal value. The normalised intensity of the spots was automatically calculated by subtracting the local background noise from the average intensity of the automatically recognised spot.

3. Results

3.1. PCR amplification and hybridisation

A 327 probe ArrayStrip was produced, and the number of probes per pathogen are given in Table 3. During the evaluation stage, it was determined that sequence-independent amplification resulted in lower hybridisation signals on the array than sequence-dependent amplification. Some of the pathogens (e.g. Cowpox, *Toxoplasma gondii* and *Campylobacter jejuni*), when amplified by their specific primers produced good quality, detectable, hybridisation signals, but when random amplification was used, they showed no or weak hybridisation. A DNA sample of *C. jejuni* for example was amplified using sequence-independent PCR and the product was then hybridised onto the array. A measurable signal was seen with 26.6% of the *C. jejuni* probes on the array. With specific amplification there was 100% probe hybridisation at significantly higher signal strength (data not shown).

Fig. 1A–H shows the images recorded after hybridisation with a variety of *Salmonella* species following sequence-dependent and sequence-independent amplification. The spots indicated by arrows are the biotin markers, which act as assay controls. Numerous probes showed cross-hybridisation in these images, albeit at low signal intensities, so they were removed from the final version of the array. It is also apparent that the cross-hybridising probes were only visible in the images which show samples that had been amplified using sequence independent-amplification (Fig. 1A–F).

Table 3
The number of probes for each pathogen that were spotted on the WT_Rodent_Chip_03 ArrayStrip.

Pathogen	Number of probes
Mycobacterium	27
Francisella	44
C. jejuni	10
Salmonella	7
S. typhimurium	5
E. coli	12
T. gondii	15
Leptospira	14
LCMV	14
Bartonella	15
MRSA	15
Rickettsia	15
S. monilliformis	15
Cowpox	14
Y. enterocolitica	10
Y. pestis	14
Y. pseudotuberculosis	13
Generic *Yersinia*	11
SEOV	15
PUUV	13
Hantavirus	14
HEV	15

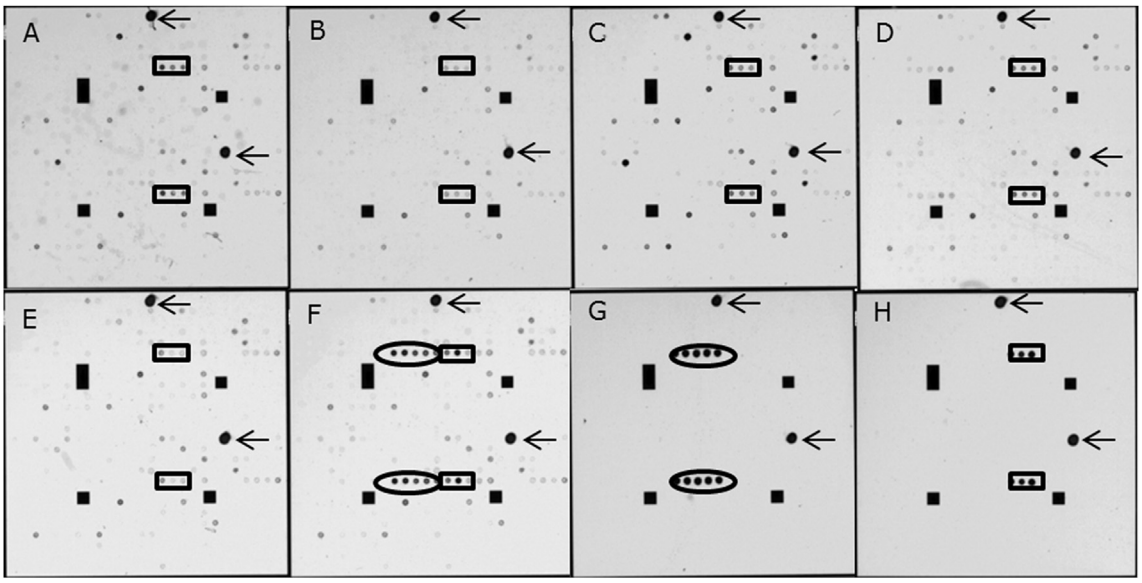

Fig. 1. Images produced after hybridisation of various *Salmonella* species on WT_Rodents_2_1.0 array. The spots indicated by arrows are the biotin markers. The solid square and rectangular areas are the orientation markers. *A. S.* Gallinarum hybridisation following random amplification. *B. S.* Dublin hybridisation following random amplification. *C. S.* Pullorum hybridisation following random amplification. *D. S.* Enteritidis hybridisation following random amplification. *E. S.* Hadar hybridisation following random amplification. *F. S. typhimurium* amplification following random amplification. *G. S. typhimurium* amplification with primers Salm/flag/1366055/F and Salm/flag/1366482/R (*S. typhimurium*-specific). *H. S. typhimurium* amplification with primers Salm/CDP/2167279/F and Salm/CDP/2005357/R (Generic *Salmonella* species).

The images which show hybridisation following sequence-specific amplification were much cleaner (Fig. 1G–H), and had the expected hybridisation profile. The three spots indicated by the rectangular box in all images except Fig. 1G are probes that were designed to hybridise with a wide range of *Salmonella* species. Fig. 1H shows the amplification of *S. typhimurium* with a set of primers designed to amplify this region which is common to multiple *Salmonella* species. *S.* Gallinarum (Fig. 1A), *S.* Dublin (Fig. 1B), *S.* Pullorum (Fig. 1C), *S.* Enteritidis (Fig. 1D), *S.* Hadar (Fig. 1E), and *S. typhimurium* (Fig. 1F–H) were tested on the array. Although it was visible on the array, the signal strength is low in comparison to Fig. 1H. This is particularly noticeable for *S.* Dublin and *S.* Hadar from Fig. 1B and E.

Both sequence-independent and sequence-specific amplification were used for *S. typhimurium*. The images produced after hybridisation can be seen in Fig. 1F and G. Fig. 1F shows sequence-independent amplification, and the circled probes were designed to be specific for *S. typhimurium*. This set of probes did not show any detectable hybridisation in the other images so it appeared these were good probes for distinguishing *SS. typhimurium* from other *Salmonella* species. Fig. 1G shows the sequence-specific amplification with a set of primers designed to amplify the *S. typhimurium*-specific region. The probes for all of the pathogens tested which produced a hybridisation signal can be seen in Table A.1 in the Appendix.

3.2. Multiplex PCR amplification and hybridisation

The primer sets which performed well in singleplex PCR reactions were then tested in a multiplex reaction. As it is unlikely that a sample would contain all of the pathogens tested, the effectiveness of the primer mix in detecting a pathogen was tested using a sample of rodent liver DNA which was spiked with individual pathogen DNA (DNA concentration ranged from 1.66 to 112.5 ng/μL, and copy number from 2.33×10^9–2.09×10^{11}). Fig. 2A shows a gel image of the result of amplification of individual pathogens from the spiked material when the multi-pathogen primer mix was used. Table 4 shows the pathogen detected in each lane from Fig. 2A. As can be seen from the figure, the majority of lanes had a strong band. The two bands in Lane 9 represent the specific *S. typhimurium* amplicons (663 bp) and the generic *Salmonella* amplicons (428 bp). This was expected, as the multi-pathogen primer mix had primers specific for *S. typhimurium*, and also had primers to amplify a region common to many *Salmonella* species. Fig. 2B shows the spots that hybridised after using the multiplex primer mix (with biotinylated primers) on a pooled nucleic acid sample from all pathogens for which reference samples were available. Although the band seen in Lane 12 for *T. gondii* was quite faint, careful analysis of the recorded image indicated that all of the pathogens in the sample, including *T. gondii*, hybridised with the expected specific probes on the array.

3.3. Array sensitivity testing

The sensitivity of the array was tested by performing real-time PCR using serially diluted *Yersinia pestis* DNA. The pathogenic DNA in the sample was no longer detectable using real-time PCR (Fig. 3) at copy numbers less than 4.39×10^2. As expected, there was no amplification for the negative control sample. Samples in Fig. 3B–E (copy number 3.47×10^9, 1.76×10^7, 8.57×10^4, 4.39×10^2) were detectable by real-time PCR. The DNA in sample B was detectable after 18 cycles. For samples C, D, and E the cycle number at which detection occurred was 24, 32 and 36, respectively. The DNA in sample F appeared to have been too low for real-time PCR to detect and no amplification was observed.

The array images shown in Fig. 3 were produced after hybridisation of the products of standard PCR amplification. These were the same samples that were tested by real-time PCR for *Y. pestis* on the Yersinia_01 ArrayTube. The biotin markers on each array are indicated with an arrow. On the Yersinia_01 array only two probes were expected to hybridise with the primer set used (Y.pes/pPCP/8374/F Y.pes/pPCP/8902/R). As can be seen from the images produced after hybridisation, the two expected probes hybridised with samples B, C and D. For samples E and F there was no apparent hybridisation.

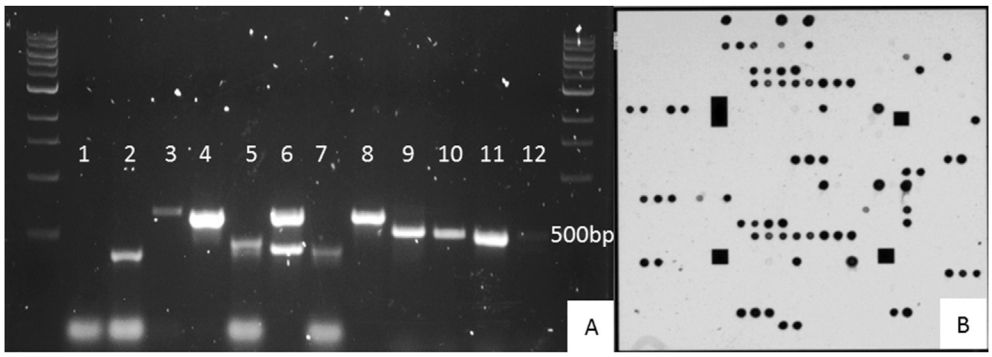

Fig. 2. A. Agarose gel electrophoresis image produced after amplification of nucleic acid of individual pathogens using the multiplex primer mix. B. Profile produced after hybridisation of a mixture of all the pathogens following amplification with the multiplex primer mix for which reference samples were available.

Table 4
The negative control sample and reference pathogen samples in lanes 1–12 from Fig. 2A.

Lane	Pathogen	Concentration ng/μL	Amplicon size (bp)
1	Negative control (water)	NA	NA
2	*Campylobacter jejuni*	84.55	404
3	Cowpox virus	35.17	629
4	Lymphocytic choriomeningitis virus	1.66	670
5	*Leptospira*	62.34	477
6	*Salmonella typhimurium*	1.89	663 and 428
7	*Salmonella* Enteritidis	8.76	428
8	*Escherichia coli*	38.12	620
9	*Yersinia pestis*	61.97	529
10	*Yersinia pseudotuberculosis*	78.94	303
11	*Yersinia enterocolitica*	112.5	499
12	*Toxoplasma gondii*	10.78	499

4. Discussion

Collecting good quality samples for disease surveillance can often be a time and cost intensive process. Therefore, it is important that any technology used is as efficient as possible. We report the development of a DNA microarray for simultaneous detection of multiple pathogens of rodents, comprised of 327 probes derived from a variety of genes in each of the target pathogens. The

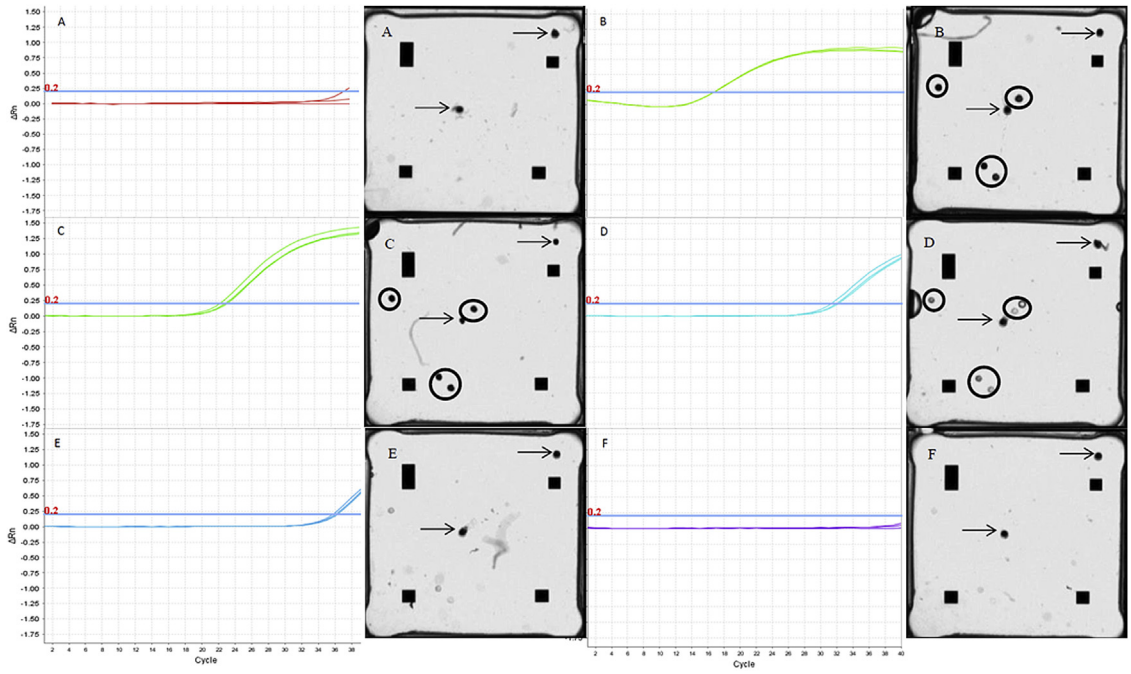

Fig. 3. Real-time PCR sensitivity testing was performed with serial dilutions of *Y. pestis*. Sample A was a negative control sample (water). The copy number in samples B to F was 3.47×10^9, 1.76×10^7, 8.57×10^4, 4.39×10^2, and 1. Array images after hybridisation of the standard PCR products from the same *Y. pestis* amplification on WT_Yersinia_01 are also shown. Biotin markers are indicated by arrows, and *Y. pestis* probes that showed hybridisation are circled.

technology presented here represents a simple but effective system which is affordable and compatible with standard laboratory equipment, and has been used for a variety of purposes over recent years [1,14–18].

The design of oligonucleotide probes is a complex process for a variety of reasons, including identifying the best target sequences to be screened and understanding the thermodynamics of probe-target interactions during hybridisation [19]. An oligonucleotide length of 60 bp was selected, as several studies have indicated that this offers the best combination between specificity and sensitivity [20–22]. Shorter oligonucleotide probes (15–25 mers) have a very high specificity, but they have been criticised for having a lack of sensitivity, whereas longer oligonucleotides (40–90 mers) are thought to have a good sensitivity whilst maintaining a high specificity [19]. It has been reported that 60-mer oligonucleotides have an eight-fold higher sensitivity than 25-mers [23].

Variation within microarray experiments can still occur regardless of careful probe design. The typical sources of variation can be broadly divided into three main categories: variation within the biological sample, the performance of the technology itself, and finally, variation in the spot signal measurements. The majority of variance in microarray experiments is generally biological rather than technical [24]. Arrays are generally made in batches and variation can occur between batches. These can include different probe concentrations, which can lead to incorrect conclusions being drawn from data [25]. Variation at the array production stage can occur for a number of reasons including, for example the particular printing pin used, the humidity, and temperature during printing. These variables can lead to slight differences in the amount of probe that is deposited on the slide surface, the amount that remains on the array surface after processing, and the level of deviation from the expected spot location. All of these factors can have an impact on the amount of labelled target that can bind to the probe, and on the efficiency of subsequent spot finding and data extraction steps [19]. The level of deviation from the expected location can result in the array reader making inaccurate readings, the signals of neighbouring probes becoming merged, or the spot can become invalid and cannot be accurately detected by the analysis software. In order to reduce the variability that is inherent in all biological experiments, experimental replication is essential. One obvious form of technical replication is through array probe replication. It is advantageous to at least have duplicates, or preferably multiples, of all probes spotted on the same array, however this may not be possible due to spotting density constraints. The precision of particular probe measurements will be more reliable if the spot intensities of the replicate spots are averaged for each sample [26].

To achieve an efficient hybridisation step, it is important to have probes with a narrow melting temperature distribution, because the hybridisation step takes place at the same temperature for all probes on the array [27]. The algorithms used in both OligoWiz and UPS are able to make minor adjustments to the length of each probe so that a narrow melting temperature range is achieved. Determination of melting temperature thresholds is a difficult task as this determines the conditions under which probes will bind to the target sequence. Melting temperatures can cause loss of signal if too high, and non-specific signal if too low [28]. As a single temperature is used during the hybridisation step, it is advisable that the narrowest melting temperature range be used to maximise signal detection [29].

The chip presented here represents a prototype microarray identification system for zoonotic pathogens that can infect rodents. The probes used on the microarray were based on genes that are unique to the pathogens selected. These genes were selected following a literature search to identify gene sequences which have been previously used to identify these pathogens, and a BLAST analysis to see if the sequences selected had similarity to any other pathogen sequences on the database. This is also the first report of biotinylated primers used in a multiplex format with up to 24 primer pairs. There was no apparent difference in the hybridisation signal produced when only a single pathogen was present in single and multiplex PCR reactions. More work needs to be done to determine the limits of detection and the sensitivity of the array, but as a proof of concept the array has demonstrated potential. Further improvements to this array could be made by obtaining reference material for pathogens which were unavailable and evaluating the probes for these pathogens. Whilst reference material was available for SEOV during the evaluation stage, several attempts at PCR proved unsuccessful. However, a number of rodent samples (nucleic acid was extracted from liver, kidney and lung of *R. rattus* and *R. norvegicus*, an aliquot of which was then pooled and amplified by multiplex PCR followed by hybridisation with the microarray) were screened on the array and two of these were identified as SEOV positive. This was later verified by both PCR and sequencing.

While *in silico* analysis of the gene and resulting probe sequences are important in eliminating the possibility of cross-hybridisation with other sequences already on the NCBI database, it does not rule out the possibility of cross-hybridisation with newly emerging organisms for which the gene sequence is unknown. As a result, microarrays can be used to identify novel as well as known pathogens. This can be achieved by designing probes at a genus level with additional probes designed for differentiating between species [30].

The presence of host nucleic acid in a sample presents another challenge in microarray experiments, as it can lower the sensitivity of the array. This occurs because in most situations the host DNA is present in much higher amounts than the pathogen nucleic acid, which makes the pathogen more difficult to detect. The sensitivity of an array may be improved by the removal of host nucleic acid by DNAses, i.e. enriching pathogen-derived nucleic acid, using dedicated methods and kits for this purpose prior to PCR amplification [31]. The sensitivity on this array was less than that of real-time PCR, as has been previously demonstrated with other pathogen detecting microarrays [14,32], this leads to a trade-off between sensitivity and cost-effectiveness. It would have been useful to have tested the array sensitivity for RNA pathogens as well. However, as mentioned earlier, there were no working RNA pathogens available during the evaluation stage. A critical step in the development of a microarray is sourcing reference samples with which the array can be evaluated.

The design stage can be the deciding factor in the success of any microarray experiment and the choice of array platforms or probe types can be challenging. However, it is now becoming increasingly clear that when a careful design is followed, the results obtained with different platforms are likely to be comparable [19,33,34]. The user can decide whether to invest time and resources in developing their own arrays, utilise one of the commercial providers who can assist with array design and fabrication, or use off-the-shelf commercial arrays. The relatively low cost of screening for many pathogens simultaneously in a single sample is an economical and efficient approach for rapid and sensitive diagnostics. This may be of particular use for wildlife samples which may be small in volume and are often irreplaceable.

Table A.1

Probe ID.	Probe sequence (5′-3′)	Amplification method	Pathogen tested	Gene target	Probe mean signal	Standard deviation
Campylo_Owiz_472	AATATTCCAATACCAACATTAGTGTGCGATGGAAGGACGCTTAGGGCTAAGGGGGCTA	Sequence-independent	C.jej	23srRNA	0.470 (8)	0.053
Campylo_Owiz_473	CTAAGTTTAGCTAATGTTGCCCGTACCGTAAACCGACACAGGTGGGTGGGATGAGTATTC	Sequence-independent	C.jej	23srRNA	0.493 (11)	0.063
Campylo_Owiz_474	GTAAACCGACACAGGTGGGTGGGATGAGTATTCTAAGGCGCGTGGAAGAACTCTCTTTA	Sequence-independent	C.jej	23srRNA	0.510 (12)	0.093
Campylo_Owiz_475	TTTGAAAAATTTGATAGTGATTATGTTTTTGGATGGCACAATATGCCTTTTGGTAGCGAT	Camp/hipO/1662/F and Camp/hipO/1984/R	C.jej	hipO	0.879 (4)	0.019
Campylo_Owiz_476	TTTTGGATGGCACAATATGCCTTTTGGTAGCGATAAGAAATTTTATCTTAAAAAAGGTGC	Camp/hipO/1662/F and Camp/hipO/1984/R	C.jej	hipO	0.883 (4)	0.009
Campylo_Owiz_480	ATATTTTAATCAGCGGTAGCAATCTTTCTTCTGCAGGTTTTGGTGCAACTCAATTTAT	Sequence-independent	C.jej	hipO	0.563 (8)	0.128
Campylo_Owiz_481	AGAAGAGTGTGAAAGTGCCATTAACTCTTATAATTTCACTTCTCAAATAGACTTTGAAA	Camp/flgS/776650/F and Camp/flgS/777053/R	C.jej	flgS	0.881 (6)	0.022
Campylo_Owiz_482	TAAGCATATAGTTCTTGAAATCCAAAAGGCTATTGCTAGAGTGGAGCGTATCGTAAAT	Camp/flgS/776650/F and Camp/flgS/777053/R	C.jej	flgS	0.860 (6)	0.026
Campylo_Owiz_483	GAAATTTTACCACTCGCCAAAGTGCCCCAAAATGCTATGATATGAACGCTAGTATTTA	Camp/neuA3/1316412/F and Camp/neuA3/1316858/R	C.jej	neuA3	0.826 (6)	0.048
Campylo_Owiz_484	TAATTCCAATCTCATCACCGCAGTTCCTGCTAGACGTAATCCTTATTTTAACCTCGTAGA	Camp/neuA3/1316412/F and Camp/neuA3/1316858/R	C.jej	neuA3	0.670 (6)	0.193
CowpoxUPS_821	ATGAGTGCTTGGTATAAGGAGCCCAATTCCATTATTCTCTTGGCTGCTAAAAGTGATGTC	Cow/HA/178301/F and Cow/HA/178929/R	CPXV	HA gene	0.872 (10)	0.017
CowpoxUPS_822	ACCTGATTATATAGATAATTCTAATTGCTCGTCGGTATTCGAAATCGCGACTCCGGGACC	Cow/HA/178301/F and Cow/HA/178929/R	CPXV	HA gene	0.816 (10)	0.018
CowpoxUPS_823	TCCACAACAGACGAGACTCCGGAATCAATTACTGATAATGAAGAAGATCACGCTGTCTCA	Cow/HA/178301/F and Cow/HA/178929/R	CPXV	HA gene	0.867 (10)	0.867
E.coliUPS_519	ATGTCGCATAGTGGAACCTCACTGACGCAGTCTGTGGCAAGAGCGATGTTACGGTTTGTT	E.coliStxF and E.coliStxR	E.col	Stx1	0.885 (10)	0.025
E.coliUPS_520	GGGGAAGGTTGAGTAGTGTCCTGCCTGATTATCATGGACAAGACTCTGTTCGTGTAGGAA	E.coliStxF and E.coliStxR	E.col	Stx1	0.860 (10)	0.038
LCMVUPS_595	ACTGGGTGCTTGTCTTTCAGCCTTTCAAGATCATTAAGATTTGGATACTTGACTGTGTA	Sequence-independent	LCMV	Glycoprotein C	0.865 (4)	0.034
LCMVUPS_597	TTCTATCCAGTAAAAGGATGGGTCAGATTGTGACAATGTTTGAGGCTTTGCCTCACATCA	Sequence-independent	LCMV	Glycoprotein C	0.885 (4)	0.016
LCMVUPS_601	ACCCCAGTGTGCATTTTGCATAGCCAGCCATATTTGTCCCACACTTTATCTTCATATTCT	Sequence-independent	LCMV	S segment	0.857 (4)	0.031
LCMVUPS_603	CCGTGTGAATACTTGGAGTCCTGCTTGAATTGCTTCTGGTCCGTAGGTTCCCTGTAAAAA	Sequence-independent	LCMV	S segment	0.487 (4)	0.058
LCMVUPS_606	AGATCTGGGAGCCTTGCTTTGGAGGCGCTTTCAAAAATGATGCAGTCCATGAGTGCAC	Sequence-independent	LCMV	Glycoprotein C	0.876 (4)	0.021
LCMVUPS_606	AGATCTGGGAGCCTTGCTTTGGAGGCGCTTTCAAAAATGATGCAGTCCATGAGTGCAC	LCMV/S/1742/F and LCMV/S/2391/R	LCMV	S segment	0.640 (4)	0.195
LCMVUPS_611	TTATCGTGATCACGGGTATCAAGGCTGTCTACAATTTTGCCACCTGTGGGATATTCGCAT	Sequence-independent	LCMV	Nucleoprotein	0.852 (4)	0.014
LCMVUPS_612	GACCGGCGAAACTAGTGTCCCCAAGTGCTGGCTTGTCACCAATGGTTCTTACTTAAATGA	Sequence-independent	LCMV	Nucleoprotein	0.831 (4)	0.030
LCMVUPS_612	GACCGGCGAAACTAGTGTCCCCAAGTGCTGGCTTGTCACCAATGGTTCTTACTTAAATGA	LCMV/NP/779/F and LCMV/NP/1448/R	LCMV	Nucleoprotein	0.895 (6)	0.004
LCMVUPS_613	TCTTGCTGCAGAGCTTAAGTGTTTCGGGAACACAGCAGTTGCGAAATGCAATGTAAATCA	Sequence-independent	LCMV	Nucleoprotein	0.784 (4)	0.039
LCMVUPS_613	TCTTGCTGCAGAGCTTAAGTGTTTCGGGAACACAGCAGTTGCGAAATGCAATGTAAATCA	LCMV/NP/779/F and LCMV/NP/1448/R	LCMV	Nucleoprotein	0.867 (6)	0.033
LCMVUPS_614	GTCCAGGATTCTCCTTTCCCAAGAGAAGACTAAGTTCCTCACTAGGAGACTAGCGGGCAC	Sequence-independent	LCMV	Nucleoprotein	0.819 (4)	0.038
LCMVUPS_614	GTCCAGGATTCTCCTTTCCCAAGAGAAGACTAAGTTCCTCACTAGGAGACTAGCGGGCAC	LCMV/NP/779/F and LCMV/NP/1448/R	LCMV	Nucleoprotein	0.892 (6)	0.009
LCMVUPS_615	TTCCTACTTCTGGCTGGCAGGTCCTGTGGCATGTACGGTCTTAAGGGACCCGACATTTAC	Sequence-independent	LCMV	Nucleoprotein	0.823 (4)	0.017
LCMVUPS_616	CAGGAAGCCGATAACATGATTACAGAGATGTTGAGGAAGGATTACATAAAGAGGCAGGGG	Sequence-independent	LCMV	Nucleoprotein	0.794 (4)	0.018
LCMVUPS_616	CAGGAAGCCGATAACATGATTACAGAGATGTTGAGGAAGGATTACATAAAGAGGCAGGGG	LCMV/NP/779/F and LCMV/NP/1448/R	LCMV	Nucleoprotein	0.892 (6)	0.002
LCMVUPS_617	ACTTTGTCAGACTCTTCAGGGGTGGAGAATCCAGGTGGTTATTGCCTGACCAAATGGATG	Sequence-independent	LCMV	Nucleoprotein	0.822 (4)	0.027
LCMVUPS_617	ACTTTGTCAGACTCTTCAGGGGTGGAGAATCCAGGTGGTTATTGCCTGACCAAATGGATG	LCMV/NP/779/F and LCMV/NP/1448/R	LCMV	Nucleoprotein	0.895 (6)	0.005
LeptospiraUPS_623	TTCTCCAGAATTAAAGCAGTCAGGTTCACGAGCTTGTAGCTGAGTTCCGATAACATGAAA	Sequence-independent	Lep	LA1027	0.465 (6)	0.036
LeptospiraUPS_625	TCTGGAGAGCGTGGATTTGAAGAGTCGGAATCCGAAGGGGCGTTGTCGTTGGCATTTGCG	Sequence-independent	Lep	LA1027	0.563 (8)	0.076
LeptospiraUPS_629	CATGTCATCGGAACCCACGCTCAGTCTCAGGATCAGAACTGTTCAAATCTAGGAATACCA	Sequence-independent	Lep	LA1029	0.488 (7)	0.067
LeptospiraUPS_630	CGGATCACTATCCGATATACGGTTTTGTATACGCGGATCCTTCTACTCCTACAAAGTCCG	Lept/hemo/3184769/F and Lept/hemo/3185246/R	Lep	LA1029	0.672 (4)	0.033
LeptospiraUPS_636	AGCTACTCTGGATTATTATTATCTTACGATTTGGGATGGTGGTAATTGGGATGGATATT	Sequence-independent	Lep	SphH	0.514 (14)	0.059
LeptospiraUPS_636	AGCTACTCTGGATTATTATTATCTTACGATTTGGGATGGTGGTAATTGGGATGGATATT	Lept/sphH/3501394/F and Lept/sphH/3501818/R	Lep	SphH	0.873 (4)	0.028
SalmonellaUPS_709	TGGCGCAGAAGTTAGGTTGTCGATGAGAAGCGCTATACGGCGCGTAGAAAGATAACGGAG	Sequence-independent	Sal	Endonuclease	0.508 (12)	0.121
SalmonellaUPS_709	TGGCGCAGAAGTTAGGTTGTCGATGAGAAGCGCTATACGGCGCGTAGAAAGATAACGGAG	Salm/CDP/2167279/F and Salm/CDP/2005357/R	Sal	Endonuclease	0.880 (10)	0.023
SalmonellaUPS_710	TTACCATCATCATGCCGGACGAAGATAGCGATTTTCGTCTGTGTCGAAGGTTGTGCGCCA	Sequence-independent	Sal	Endonuclease	0.527 (10)	0.170
SalmonellaUPS_710	TTACCATCATCATGCCGGACGAAGATAGCGATTTTCGTCTGTGTCGAAGGTTGTGCGCCA	Salm/CDP/2167279/F and Salm/CDP/2005357/R	Sal	Endonuclease	0.863 (10)	0.034
SalmonellaUPS_711	TTGCGACTATCAGGTTACCGTGGAGGCTATCGAACATAAAGCGAAGCCAGTGCTGACGCT	Sequence-independent	Sal	Endonuclease	0.404 (8)	0.071
SalmonellaUPS_711	TTGCGACTATCAGGTTACCGTGGAGGCTATCGAACATAAAGCGAAGCCAGTGCTGACGCT	Salm/CDP/2167279/F and Salm/CDP/2005357/R	Sal	Endonuclease	0.875 (10)	0.035
SalmonellaUPS_733	TCACCATCCCCAGGCCCATATACATGCTCTAATCGCATGTTTACAAATGAAATGTCATGC	Sequence-independent	Sal	CDP-abeguose synthase	0.802 (4)	0.015

SalmonellaUPS_733	TCACCATCCCCAGGCCCATATACATGCTCTAATCGCATGTTTACAAATGAAATGTCATGC	Salm/flag/1366055/F and Salm/flag/1366482/R	Sal	CDP-abeguose synthase	0.887 (4)	0.010
SalmonellaUPS_734	CCACATCATCTACAAAAATAAAGTCTCTTATCTGTTCGCCTGTTGTACATTTCACGCAAC	Sequence-independent	Sal	CDP-abeguose synthase	0.813 (4)	0.007
SalmonellaUPS_734	CCACATCATCTACAAAAATAAAGTCTCTTATCTGTTCGCCTGTTGTACATTTCACGCAAC	Salm/flag/1366055/F and Salm/flag/1366482/R	Sal	CDP-abeguose synthase	0.878 (4)	0.021
SalmonellaUPS_735	CCAGCACCAGTTCCAACTTGATACTCAGTATATGAAGGTACTTCTTTTCTATTTTCTAAT	Sequence-independent	Sal	CDP-abeguose synthase	0.654 (4)	0.018
SalmonellaUPS_735	CCAGCACCAGTTCCAACTTGATACTCAGTATATGAAGGTACTTCTTTTCTATTTTCTAAT	Salm/flag/1366055/F and Salm/flag/1366482/R	Sal	CDP-abeguose synthase	0.878 (4)	0.024
SalmonellaUPS_736	ATGCAGAATCAATTGATAACTCCTCGACTAATAATTCGATATTATCCCAACTGCACCATC	Sequence-independent	Sal	CDP-abeguose synthase	0.650 (4)	0.014
SalmonellaUPS_736	ATGCAGAATCAATTGATAACTCCTCGACTAATAATTCGATATTATCCCAACTGCACCATC	Salm/flag/1366055/F and Salm/flag/1366482/R	Sal	CDP-abeguose synthase	0.898 (4)	0.002
SalmonellaUPS_737	ATGCCCAATTTCATCAAAGTGTCTTTTAGTAATTATATAAGGCCGCATATGTTGATAATT	Sequence-independent	Sal	CDP-abeguose synthase	0.616 (4)	0.013
SalmonellaUPS_737	ATGCCCAATTTCATCAAAGTGTCTTTTAGTAATTATATAAGGCCGCATATGTTGATAATT	Salm/flag/1366055/F and Salm/flag/1366482/R	Sal	CDP-abeguose synthase	0.887 (4)	0.015
T.gondii_422	CCGGGAACCCCAGAAAGTGGGGGAGAGGCGGAGGCGGCCATCCAGGAAGCGGAGGATCGC	Sequence-independent	T.gon	SAG3	0.525 (4)	0.032
T.gondii_435	TTGCATCATAACAAGAGCTGTATTACCCGCTGGCAAATACAGGTGAAATGTACCTCCAGA	T.gon/B1/12/F and T.gon/B1/510/R	T.gon	B1	0.837 (4)	0.049
T.gondiiUPS_361	TCCGTCGTAATATCAGGCCTTCTGTTCTGTTCGCTGTCTGTCTAGGGCACCCTTACTGCA	T.gon/B1/12/F and T.gon/B1/510/R	T.gon	B1	0.687 (4)	0.155
T.gondiiUPS_364	TTCATTTTCGCAGTACACCAGGAGTTGGATTTTGTAGAGCGTCTCTATTCAAGCAGCGTA	T.gon/B1/12/F and T.gon/B1/510/R	T.gon	B1	0.830 (6)	0.038
T.gondiiUPS_755	TCGTCCCATGAAGTCGACCACCTGTTTCCTCTCTTCACTGTCACGTACGACATCGCATTC	T.gon/B1/12/F and T.gon/B1/510/R	T.gon	B1	0.500 (5)	0.120
T.gondiiUPS_756	GGTCCGCCCCCACAAGACGGCTGAAGAATGCAACATTCTTGTGCTGCCTCCTCTCATGGC	T.gon/B1/12/F and T.gon/B1/510/R	T.gon	B1	0.471 (4)	0.031
T.gondiiUPS_768	GGTGGGAATGAAGGCAGAGGTTACAGAGGCAGAGGTGAAGGAGGAGGCGAGGATGACAGG	Sequence-independent	T.gon	GRA6	0.734 (4)	0.029
Y.enteroco_Owiz_156	TCCGGCTCTATTACCCGAGGTGCTGGCATTACGTCAGGATGATGCACTCAAGTTGGCT	Sequence-independent	Y.ent	YE3228	0.517 (4)	0.062
Y.enteroco_Owiz_157	AAAAACACAAGATTCAACGCTGGCAACGGCGGGTTACGCCCGGTTATTTCTCCAGC	Sequence-independent	Y.ent	YE3228	0.555 (4)	0.013
Y.pestis_Owiz_120	CACACTCCACATATCACTGACGGAGCACAACGGAATAGTGAACAAACAACAACAAACTGC	Sequence-independent	Y.pes	pPCP	0.865 (8)	0.020
Y.pestis_Owiz_121	TGTCCGGGAGTGCTAATGCAGCATCATCTCAGTTAATACCAAATATATCCCCTGACAGC	Sequence-independent	Y.pes	pPCP	0.839 (8)	0.026
Y.pestis_Owiz_129	GAATCGCGCCCGGATATGTTTTAACGCGATTTTCAGACTCAGACAAATTCAGCAGAAT	Sequence-independent	Y.pes	pPCP	0.875 (8)	0.012
Y.pestisUPS_785	AATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAGGCCAGTATCGCAT	Sequence-independent	Y.pes	pPCP	0.819 (8)	0.040
Y.pestisUPS_785	AATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAGGCCAGTATCGCAT	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	Y.pes	pPCP	0.814 (8)	0.015
Y.pestisUPS_786	AATGATGAGCACTATATGAGAGATCTTACTTTCCGTGAGAAGACATCCGGCTCACGTTAT	Sequence-independent	Y.pes	pPCP	0.814 (8)	0.035
Y.pestisUPS_786	AATGATGAGCACTATATGAGAGATCTTACTTTCCGTGAGAAGACATCCGGCTCACGTTAT	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	Y.pes	pPCP	0.816 (16)	0.078
Y.pestisUPS_787	TAAATTCAGCGACTGGGTTCGGGCACATGATAATGATGAGCACTATATGAGAGATCTTAC	Sequence-independent and	Y.pes	pPCP	0.815 (8)	0.018
Y.pestisUPS_787	TAAATTCAGCGACTGGGTTCGGGCACATGATAATGATGAGCACTATATGAGAGATCTTAC	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	Y.pes	pPCP	0.722 (14)	0.164
Y.pestisUPS_788	AGCCCGACCACTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG	Sequence-independent	Y.pes	pPCP	0.823 (6)	0.025
Y.pestisUPS_789	TCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTC	Sequence-independent	Y.pes	pPCP	0.846 (8)	0.022
Y.pestisUPS_789	TCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTC	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	Y.pes	pPCP	0.746 (12)	0.156
Y.pestisUPS_790	AAAGGACAGCATTTGGTATCTGTGCTCCACTTAAGCCAGCTACCACAGGTTAGAAAGCCT	Sequence-independent	Y.pes	pPCP	0.793 (8)	0.051
Y.pestisUPS_791	AAGGAGTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAG	Sequence-independent	Y.pes	pPCP	0.847 (8)	0.030
Y.pestisUPS_791	AAGGAGTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAG	Y.pes/pPCP/8374/F and Y.pes/pPCP/8902/R	Y.pes	pPCP	0.784 (10)	0.185
Y.pestisUPS_792	TTTGTACCGAGAACCTTTCACGGTATCGGCATATGGCCTGGGTAACTCAGGTCCGTAAAC	Sequence-independent	Y.pes	pPCP	0.822 (8)	0.092
Y.pseudo_Owiz_224	TTTTGTTGTCTTAAGTTCGGCTGGTGTCAAAATGAGCAGGATTGGGCTTAACTATGATG	Sequence-independent	Y.pse	YPTB0244	0.714 (4)	0.015
Y.pseudo_Owiz_226	GGACGCTTTTAGCCGAACTCAACGAAAGCTTGGAAGGGTACACACCTGATTTATTT	Sequence-independent	Y.pse	YPTB0244	0.453 (4)	0.022
Y.pseudo_Owiz_227	TTGAGACTTCACCAAAAGGTGAGCATTTTGTTGTCTTAAGTTCGGCTGGTGTCAAAAT	Sequence-independent	Y.pse	YPTB0244	0.445 (4)	0.040
Y.pseudo_Owiz_229	TTTAGCGAAAGAACCATAGACCATGGGCCAACAGGAACGTTAGGTGTCTTAATTCT	Sequence-independent	Y.pse	YPTB0244	0.508 (4)	0.018
Y.pseudo_Owiz_234	GTTGATGATTATAGGTCCATTGATGATGAAGCCATCGAACGGGTAGCTTCTGATTGTAG	Sequence-independent	Y.pse	YPTB0245	0.821 (4)	0.013
Y.pseudo_Owiz_236	ATCAGCGTAAGCCGGAGTCGGTGAGGCTGTTGCCCAGATGTATTAATCTATTACT	Sequence-independent	Y.pse	YPTB0245	0.602 (4)	0.030
Y.pseudo_Owiz_237	CCAGAAAGACTAACCCAAATGCGAGAAGCTCGAGGGTTATCCAAAATAAACTTGGGTA	Sequence-independent	Y.pse	YPTB0245	0.597 (4)	0.058
Y.pseudo_Owiz_238	CAGTCTCGGCAAGGTTTTAAATTGCCCTGTGAGTTGGTTTACCAAAGTTGCTTATG	Sequence-independent	Y.pse	YPTB0245	0.636 (4)	0.067
Y.pseudo_Owiz_243	AATTTGCTGCCTGTTTTATGCTACCTGAAGAGGCTTTTTCTGCGGAATTACCCTCT	Sequence-independent	Y.pse	YPTB0245	0.721 (4)	0.057
Y.pseudo_Owiz_244	AGAGAAAATAATCCCGTTTTCTTTAGAACATTGTCGGCGACGGCCAAAGATTTGT	Sequence-independent	Y.pse	YPTB0245	0.541 (4)	0.024
Y.pseudo_Owiz_245	GGTGGCGTAAAGGTGAGCCCTTAGATGATCAGCGTAAGCCGGAGTCGGTGAGGCT	Sequence-independent	Y.pse	YPTB0245	0.795 (4)	0.020
Y.pseudo_Owiz_246	ATGAAGCCATCGAACGGGTAGCTTCTGATTGTAGAAAGAGATGGGGACTCGGTAT	Sequence-independent	Y.pse	YPTB0245	0.433 (4)	0.036
Yersinia_Owiz_271	CTGATTTGGCCTTGAACAAGGCAGACATGGCAGCGTTACAAAGTATTATTGACCGACTCA	Sequence-independent	Y.pse	YOPB	0.534 (4)	0.051
Yersinia_Owiz_272	ACTAAATTATTGGAGTCGGTCACCCGCGGCGCGGCAGGATCTCAACTGATATCAAATTAT	Sequence-independent	Y.pse	YOPB	0.460 (4)	0.014
Yersinia_Owiz_273	CATGGCAGCGTTACAAAGTATTATTGACCGACTCAAAGAAGAGTTATCCCATTTGTCAGA	Sequence-independent	Y.pse	YOPB	0.657 (4)	0.034
Yersinia_Owiz_274	CAAGAGAAAATCAAAGAAACAGAAGAGAATGCCAAGCAAGTCAAGAAATCCGGCATCGCA	Sequence-independent	Y.pse	YOPB	0.819 (4)	0.076

(*continued on next page*)

Table A.1 (*continued*)

Probe ID.	Probe sequence (5′-3′)	Amplification method	Pathogen tested	Gene target	Probe mean signal	Standard deviation
Yersinia_Owiz_292	TCGTCTCGGTGACGGGATTATGGCAGATATGTATACTTTAACGATTCGTGAAGCCGGTCA	Sequence-independent	Y.pes	YOPH	0.569 (4)	0.153
Yersinia_Owiz_292	TCGTCTCGGTCACGGGATTATGGCAGATATGTATACTTTAACGATTCGTGAAGCCGGTCA	Sequence-independent	Y.ent	YOPH	0.470 (4)	0.050
Yersinia_Owiz_292	TCGTCTCGGTCACGGGATTATGGCAGATATGTATACTTTAACGATTCGTCAAGCCGGTCA	Sequence-independent	Y.pse	YOPH	0.534 (4)	0.010
Yersinia_Owiz_293	CGCAATATGTATGAAGCAAAGGAAGTTCAGCGGTAGGAGATGACTCCAAATTACGGCCG	Sequence-independent	Y.pse	YOPH	0.682 (4)	0.025
YersiniaUPS_796	GTCGTGTCTCGGCATCATGGCTGCTACTTCTACGGTTGCTAGTGCTTTTTCTATAGCGAAA	Sequence-independent	Y.ent	pCD1	0.586 (4)	0.083
YersiniaUPS_796	GTCGTGTCTCGGCATCATGGCTGCTACTTCTACGGTTGCTAGTGCTTTTTCTATAGCGAAA	Sequence-independent	Y.pse	pCD1	0.643 (4)	0.038
YersiniaUPS_797	CAAGACACAGCCCAATTATCACGACCGGTTCACAGCTTGATGCCATCACTACAGAGAC	Sequence-independent	Y.ent	pCD1	0.780 (4)	0.026
YersiniaUPS_797	CAAGACACAGCCCAATTATCACGACCGGTTCACAGCTTGCCATCACTACAGAGAC	Sequence-independent	Y.pse	pCD1	0.854 (4)	0.041
YersiniaUPS_798	TTTATGCAAAGATGTCCTGCGCTTGATTGAACAATATGTTAGCAGTCATACTCACGCCATG	Sequence-independent	Y.pse	pCD1	0.538 (4)	0.107
YersiniaUPS_800	GCCAGGCAAGCTTATCTCGGTCACAGGTGCCAGAATTGATCAAACCGAGCCAGGGAATCA	Sequence-independent	Y.pse	pCD1	0.856 (4)	0.035
YersiniaUPS_801	TAACCCATGACCGCTCAACGCCAGTAACTGCGAAGTCTACTTCCCTACGTCGCAGACACCAG	Sequence-independent	Y.pse	pCD1	0.505 (4)	0.040

A table showing the oligonucleotide sequence of the probes designed for use on the array. The amplification method is given, be it sequence-independent amplification or sequence-specific amplification with unique primers. A cut-off point of 0.4 for the signal intensity was used. Reference material was only available for the pathogens listed in Table A.1, therefore the probes designed to hybridise with SEOV or HEV for example were not evaluated. The probe mean signal is given along with the number of probes that hybridised in parentheses. The legend for the pathogens in Table A.1 is as follows: C.jej = *C. jejuni*; CPXV = Cowpox virus; E.col = *E. coli*; LCMV = Lymphocytic Choriomeningitis; Lep = *L. interrogans*; Sal = *S. typhimurium*; T.gon = *T. gondii*; Y.ent = *Y. enterocolitica*; Y.pes = *Y. pestis*; Y.pse = *Y. pseudotuberculosis*; Yers = *Yersinia*.

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