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Detection of a Yersinia pestis gene homologue in rodent samples

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Abstract

A homologue to a widely used genetic marker, *pla,* for *Yersinia pestis* has been identified in tissue samples of two species of rat (*Rattus rattus* and *Rattus norvegicus*) and of mice (*Mus musculus* and *Apodemus sylvaticus*) using a microarray based platform to screen for zoonotic pathogens of interest. Samples were from urban locations in the UK (Liverpool) and Canada (Vancouver). The results indicate the presence of an unknown bacterium that shares a homologue with *Yersinia pestis*.

Introduction

Yersinia pestis is the causative agent of plague in humans and, in the absence of antimicrobial therapy, the mortality rate can approach 100%. Future pandemics of *Y. pestis* are unlikely, as a result of improvements in living conditions and in public health, including improved rodent

control and antibiotics. However, a plague outbreak following the release of a biological weapon is a potential risk. The presence of *Y. pestis* in small rodent populations in which it is endemic (1-3) can cause human fatalities as a result of zoonotic transmission (4).

The Black rat (*Rattus rattus*) has been a major host of *Y. pestis* for centuries and can be a reservoir for numerous other pathogens. Although most mammalian species can be infected experimentally with *Y. pestis*, many species fail to develop the high bacteraemia that is necessary to infect the flea vectors. The majority of mammalian species are therefore likely to be dead end hosts (5).

Molecular methods, and in particular PCR, have been widely used to identify *Y. pestis* in tissue samples and the plasminogen activator/coagulase (*pla*) gene, located on the pPCP1 plasmid has been used as a target in many studies (1, 6-8). The *pla* gene is commonly used because it has a high copy number in *Y. pestis* (186 per bacterium) and can be detected relatively easily (9).

Materials and methods

The genomic sequence of the *pla* gene of *Y. pestis* was used to design oligonucleotide probes that were specific for *Y. pestis.* This work was part of a EU project (FP7 WildTech) to develop and use a microarray to detect zoonotic pathogens in rodent tissues. A sequence of the pPCP1 plasmid of the *Y. pestis* genome (CP000310.1) was obtained from the NCBI database for microarray probe design. Probes were designed using two publicly available software packages: OligoWiz (http://www.cbs.dtu.dk/services/OligoWiz/) and Unique Probe Selector (http://array.iis.sinica.edu.tw/ups/). All probes were checked for suitability using an *in silico* BLAST analysis. The results of the *in silico* analysis indicated that the probe sequences were specific to *Y. pestis* and no cross-hybridisation should occur with eukaryotic or prokaryotic

species. Primers were designed using the software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Additional probes, which were designed to hybridise with genomic regions common to all *Yersinia* species were included on the arrays. This approach was used so that in the presence of any of the three *Yersinia* species on the array (*Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis*), both sets of probes would hybridise, confirming the presence of the pathogen. Multiple probe sets would also be useful for pathogen discovery, detecting the presence of other *Yersinia* species not on the array, and for detecting sequence variants of known *Yersinia* species. The sequence of each oligonucleotide probe specific to *Y. pestis* is given in Table 1. During the confirmatory testing, both real-time PCR and end-point PCR were used. The primers used in standard end-point PCR and real-time PCR are shown in Table 2. These probes were evaluated thoroughly for specificity using reference samples of genomic DNA from *Y. pestis* NCTC5923 Type strain and non-related pathogens before screening took place. The microarray platform used was the ArrayStrip from Alere Technologies GmbH (Jena, Germany). During the evaluation stages of the project, reference samples of *Y. pestis* produced characteristic hybridisation patterns with both the *Y. pestis*-specific and the generic *Yersinia* probes.

Four different rodent species (*R. rattus, R. norvegicus, Mus musculus* and *Apodemus sylvaticus*) were screened for a number of zoonotic pathogens. Tissue samples were obtained from Vancouver (Canada), Liverpool (UK), and Lyon (France) as part of other studies. Automated nucleic acid extraction was performed on the samples using the QIAcube (Qiagen, Hilden, Germany) and the kit (Cador Pathogen Mini Kit (Qiagen)). Liver, kidney and lung tissues were available from each rodent sampled from Vancouver and Lyon, and extracted nucleic acid from each tissue was pooled to make a single sample per individual animal which

was tested on the array. Only liver and kidney samples were available from the rodents sampled from Liverpool, and again, extracted nucleic acid was pooled to make a single sample.

Results

Probes specific to *Y. pestis* hybridised with samples from a subset of each of the rodent species tested (12/33 *R. rattus*, 48/834 *R. norvegicus,* 3/163 *A. sylvaticus*, 2/35 *M. musculus*) giving a total of 65/1065 samples (6.1%) which tested positive on the array. However, none of the generic *Yersinia* probes hybridised in those samples for which a positive signal was recorded for the *Y. pestis* specific probes.

Because bubonic plague is notifiable in the UK and Canada, all 65 suspected positive nucleic acid samples were subsequently sent to the Institut Pasteur, a WHO Reference Laboratory for *Y. pestis,* to undergo confirmatory testing, and F1 antigen dipstick testing and phage lysis were performed. The results from both methods indicated there was no *Y. pestis* present in any of the samples. Further testing was then carried out at the University of Nottingham, including real-time PCR which targeted another region of the *Y. pestis* genome, the *caf1* gene, for which primers used were identified from the literature (10). A subset (23 samples) of the array-positive samples was tested further with primers for *pla* and *caf1*. Of these samples, 12 were positive for the *pla* gene and all were negative for the *caf1* gene. A total of 30 array-positive samples were also sent to colleagues in Berlin for further analysis by in solution-based sequence hybridisation, as described previously (11). Briefly a DNA extract from the samples was fragmented and an aliquot was used to produce illumina libraries following a custom protocol (12). PCR amplicons from *Y. pestis* genes were used to enrich specific target DNA sequences in the rodent samples, the genes and primers used to make the baits are shown in Table 3. The enriched samples were

then sequenced using an illumina Miseq. The results of this test indicated that the pathogen was not present in the samples tested.

Discussion

Although a homologue to the *pla* gene has previously been reported in *R. rattus* and *R. norvegicus* from the Netherlands (13), this is the first time, to the author's knowledge, the first time that a homologue has been reported in *M. musculus* and *A. sylvaticus*. The potential discovery of a *pla* gene homologue in other rodent species, and on another continent than the species and locations in which it has previously been reported, suggests that the homologue could be more widely distributed than previously thought. These results suggest that markers other than the *pla* gene should be included to help avoid false positive results when screening for *Y. pestis*, as has been stressed by Janse et.al (13). It is not clear why the gene was present in a larger percentage of *R. rattus* samples than in the other species tested, but is something that would be of interest and should be investigated.

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155 **Table 1.** *Y. pestis* specific oligonucleotide probes used on the Alere ArrayStrip

Probe	Sequence (5'-3')	Pathogen	Gene target	Position*
Y.pestis_Owiz_117	TACAGATCATATCTCTCTTTTCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGG	*Y. pestis*	pPCP1	8781-8840
Y.pestis_Owiz_120	TGTTGTCCGCTAGGACGATGCGATTTCGGTTATTATTCAGAATGTCTTCGTTCTCTTTC	*Y. pestis*	pPCP1	6626-6684
Y.pestis_Owiz_121	TGTCCGGGAGTGCTAATGCAGCATCATCTCAGTTAATACCAAATATATCCCCTGACAGC	*Y. pestis*	pPCP1	7878-7936
Y.pestis_Owiz_127	GTGGAGATTCTGTCTCTATTGGCGGAGATGCTGCCGGTATTTCCAATAAAAATTATACTG	*Y. pestis*	pPCP1	8688-8747
Y.pestis_Owiz_129	GAATCGCGCCCGGATATGTTTTAACGCGATTTTCAGACTCAGACAAATTCAGCAGAAT	*Y. pestis*	pPCP1	9990-10047
Y.pestis_Owiz_147	TCGCTGGCTAAAAAGTACCATCCACATGCTCAACCCTATAACCTGTAGCTTACCCCAC	*Y. pestis*	pPCP1	9583-9640
YpestisUPS_785	AATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAGGCCAGTATC	*Y. pestis*	pPCP1	8392-8451

	GCAT			
YpestisUPS_786	AATGATGAGCACTATATGAGAGATCTTACTTTCCGTGAGAAGACATCCGGCTCACG TTAT	*Y. pestis*	pPCP1	8510-8569
YpestisUPS_787	TAAATTCAGCGACTGGGTTCGGGCACATGATAATGATGAGCACTATATGAGAGATC TTAC	*Y. pestis*	pPCP1	8479-8538
Y.pestisUPS_788	AGCCCGACCACTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGA CACG	*Y. pestis*	pPCP1	4977-5036
YpestisUPS_789	TCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAAC CTTC	*Y. pestis*	pPCP	8801-8860
YpestisUPS_790	AAAGGACAGCATTTGGTATCTGTGCTCCACTTAAGCCAGCTACCACAGGTTAGAAA GCCT	*Y. pestis*	pPCP	5129-5188
YpestisUPS_791	AAGGAGTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTT GCAG	*Y. pestis*	pPCP	8379-8438
YpestisUPS_792	TTTGTACCGAGAACCTTTCACGGTATCGGCATATGGCCTGGGTAACTCAGGTCCGT AAAC	*Y. pestis*	pPCP	9451-9510

156 *The nucleotide position of each probe is based on the CP000310.1 *Yersinia pestis* Antiqua plasmid pPCP

157 **Table 2.** *Y. pestis* specific primers for standard end-point PCR and real-time PCR

rward imer	Sequence (5'-3')	Reverse Primer	Sequence (5'-3')	Probe	Sequence (5'-3')	Ge ne	Pos on
pes/pPCP/84/F	CCCGAAAGGAGTGCGGGTAA	Y.pes/pPCP/8902/R	CGCCCCGTCATTATGGTGAA	N/A	N/A	*pla*	837 890 **
fpri_f	CCAGCCCGCATCACT	cafpri_r	ATCTGTAAAGTTAACAGATGTGCTAGT	Tqpro_caf	JOE-AGCGTACCAACAAGTAATTCTGTATCGATG-BHQ1	*caf 1*	109 255 **
pes_pPCP_	AGACATCCGGCTCACGTTAT	Y.pes_pPCP_R	GAGTACCTCCTTTGCCCTCA	Y.pes_pPCP_Pr	FAM-CACCTAATGCCAAAGTCTTTGCGGA-TAMRA	*pla*	855 866 **

158 ** The nucleotide position of the Y.pes_pPCP_F and Y.pes_pPCP_R primers based on the CP000310.1 *Yersinia pestis* Antiqua
159 plasmid pPCP
160 ***The nucleotide position of the cafpri_f and cafpri_r primers based on the KF682424.1 *Yersinia pestis* strain S1 plasmid pMT1
161 capsule protein F1 (*caf1*) gene

162 **Table 3**. *Y. pestis* primers used to prepare baits for Illumina Miseq sequencing.

Primer name	Primer Sequence	Target	Reference
F1	CAGTTCCGTTATCGCCATTGC	*caf1*	(14)
F2	TATTGGTTAGATACGGTTACGGT		
Ypfur1	GAAGTGTTGCAAAATCCTGCG	*fur*	(15)
Ypfur2	AGTGACCGTATAAATACAGGC		
YPtoxU	AGGACCTAATATGGAGCATGAC	*Ymt*	(16)
YPtoxUR	CGTGATTACCAGGTGCAACA		