Detection of a *Yersinia pestis* gene homologue in rodent samples

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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 for the *pla* gene of *Yersinia pestis,* so caution should be taken when using this gene as a diagnostic marker.

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25

26   Abstract

27   A homologue to a widely used genetic marker, *pla,* for *Yersinia pestis* has been identified in

28   tissue samples of two species of rat (*Rattus rattus* and *Rattus norvegicus*) and of mice (*Mus*

29   *musculus* and *Apodemus sylvaticus*) using a microarray based platform to screen for zoonotic

30   pathogens of interest. Samples were from urban locations in the UK (Liverpool) and Canada

31   (Vancouver). The results indicate the presence of an unknown bacterium that shares a

32   homologue for the *pla* gene of *Yersinia pestis* so caution should be taken when using this gene as

33   a diagnostic marker.

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35   Introduction

36   *Yersinia pestis* is the causative agent of plague in humans and, in the absence of antimicrobial

37   therapy, the mortality rate can approach 100%. In large parts of the world the threat from *Y.*

38   *pestis* has declined substantially over time as a result of improvements in living conditions and in

39   public health, including improved rodent control and antibiotics. However, a plague outbreak

40   following the release of a biological weapon is a potential risk. The presence of *Y. pestis* in small

41   rodent populations in which it is endemic (Ziwa et al., 2013, Eppinger et al., 2009, Biggins and

42   Kosoy, 2001) can cause human fatalities as a result of zoonotic transmission (ProMED-mail,

43   2014).

44   The Black rat (*Rattus rattus*) has been a major host of *Y. pestis* for centuries and can be a

45   reservoir for numerous other pathogens. Although most mammalian species can be infected

46 experimentally with *Y. pestis*, many species fail to develop the high bacteraemia that is necessary

47 to infect the flea vectors. The majority of mammalian species are therefore likely to be dead end

48 hosts (Eisen and Gage, 2009).

49 Molecular methods, and in particular PCR, have been widely used to identify *Y. pestis* in

50 tissue samples and the plasminogen activator/coagulase (*pla*) gene, located on the pPCP1

51 plasmid has been used as a target in many studies (Loïez et al., 2003, Raoult et al., 2000, Zhang

52 et al., 2013, Ziwa et al., 2013). The *pla* gene is commonly used because it has a high copy

53 number in *Y. pestis* (186 per bacterium) and can be detected relatively easily (Parkhill et al.,

54 2001). The PcP plasmid, and the *pla* gene in particular, is involved in transmission of *Y. pestis*

55 (Broekhuijsen et al., 2003). The protein encoded by the *pla* gene induces fibrinolysis and

56 degrades the extracellular matrix and basement membranes, these activities are thought to disrupt

57 the host's ability to contain the bacteria (Sebbane et al., 2006). The acquisition by *Y. pestis* of

58 PcP, and of another virulence plasmid, the pMT, is thought to have contributed to the

59 evolutionary transformation of *Y. pestis* from the mainly gut-associated *Yersinia enterocolitica*,

60 into a highly host-adapted mammalian blood-borne pathogen (Eppinger et al., 2010).

61 Results and Discussion

62 Probes specific to *Y. pestis* hybridised with samples from a subset of each of the rodent

63 species tested (12/33 *R. rattus*, 48/834 *R. norvegicus,* 3/163 *A. sylvaticus*, 2/35 *M. musculus*)

64 giving a total of 65/1065 samples (6.1%) which tested positive on the array. However, none of

65 the generic *Yersinia* probes hybridised in those samples for which a positive signal was recorded

66 for the *Y. pestis* specific probes.

67 Further testing was then carried out at the University of Nottingham, including real-time PCR

68 which targeted another region of the *Y. pestis* genome, the *caf1* gene, for which primers used

69   were identified from the literature (Janse et al., 2010). A subset (23 samples) of the array-

70   positive samples was tested further with primers for *pla* and *caf1*. Of these samples, 12 were

71   positive for the *pla* gene and all were negative for the *caf1* gene. A total of 30 array-positive

72   samples were also sent to colleagues in Berlin for further analysis by in solution-based sequence

73   hybridisation, as described previously (Tsangaras et al., 2014). Briefly a DNA extract from the

74   samples was fragmented and an aliquot was used to produce illumina libraries following a

75   custom protocol (Meyer and Kircher, 2010). PCR amplicons from *Y. pestis* genes were used to

76   enrich specific target DNA sequences in the rodent samples, the genes and primers used to make

77   the baits are shown in Table 3. The enriched samples were then sequenced using an illumina

78   Miseq. The results indicated that the majority of reads aligned with the rat genome, and only a

79   small number of reads aligned with the *pla* gene. No other reads mapped to any other gene from

80   *Y. pestis*.

81   Although a homologue to the *pla* gene has previously been reported in bacteria found in *R.*

82   *rattus* and *R. norvegicus* from the Netherlands (Janse et al., 2013), this is the first time, to the

83   author's knowledge, that the bacterial homologue has been reported in *M. musculus* and *A.*

84   *sylvaticus*. The potential discovery of a *pla* gene homologue in other rodent species, and on

85   another continent than the species and locations in which it has previously been reported,

86   suggests that the homologue could be more widely distributed than previously thought and may

87   cause difficulties in accurate *Y. pestis* detection. The results found here supports other work

88   which suggests that markers other than the *pla* gene should be included to help avoid false

89   positive results when screening for *Y. pestis*, as has been stressed by Janse et.al (Janse et al.,

90   2013). This was recently confirmed by Hänsch et. al, as they found evidence that the pla gene is

91   present in some strains of *Escherichia coli* and *Citrobacter koseri* (Hänsch et al., 2015). It is not

92   clear why the homologue was present in a larger percentage of *R. rattus* samples than in the other

93   species tested; perhaps *R. rattus* carries more *E. coli* or *C. koseri*, but this is something that needs

94   to be investigated further.

95   Materials and Methods

96   This work was part of a EU project (FP7 WildTech) to develop and use a microarray to detect

97   zoonotic pathogens in rodent tissues. A sequence of the pPCP1 plasmid of the *Y. pestis* genome

98   (CP000310.1) was obtained from the NCBI database for microarray probe design. Probes were

99   designed using two publicly available software packages: OligoWiz

100 (http://www.cbs.dtu.dk/services/OligoWiz/) and Unique Probe Selector

101 (http://array.iis.sinica.edu.tw/ups/). All probes were checked for suitability using an *in silico*

102 BLAST analysis. The results of the *in silico* analysis at the time indicated that the probe

103 sequences were specific to *Y. pestis* and no cross-hybridisation should occur with eukaryotic or

104 prokaryotic species. Primers were designed using the software Primer3

105 (http://bioinfo.ut.ee/primer3-0.4.0/). The sequence of each oligonucleotide probe specific to *Y.*

106 *pestis* is given in Table 1. During the confirmatory testing, both real-time PCR and end-point

107 PCR were used. The primers used in standard end-point PCR and real-time PCR are shown in

108 Table 2. These probes were evaluated thoroughly for specificity using reference samples of

109 genomic DNA from *Y. pestis* NCTC5923 Type strain and non-related pathogens before

110 screening took place. The microarray platform used was the ArrayStrip from Alere Technologies

111 GmbH (Jena, Germany).

112   Four different rodent species (*R. rattus, R. norvegicus, Mus musculus* and *Apodemus*

113 *sylvaticus*) were screened for a number of zoonotic pathogens. Tissue samples were obtained

114 from Vancouver (Canada), Liverpool (UK), and Lyon (France) as part of other studies.

115   Automated nucleic acid extraction was performed on the samples using the QIAcube (Qiagen,

116   Hilden, Germany) and the kit (Cador Pathogen Mini Kit (Qiagen)). Liver, kidney and lung

117   tissues were available from each rodent sampled from Vancouver and Lyon, and extracted

118   nucleic acid from each tissue was pooled to make a single sample per individual animal which

119   was tested on the array. Only liver and kidney samples were available from the rodents sampled

120   from Liverpool, and again, extracted nucleic acid was pooled to make a single sample. Figure 1

121   depicts the sequence enrichment and microarray hybridisation used in this study.

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125   Key findings

126   1. Sequences homologous to the *pla* gene, which is present in *Y. pestis,* have been found in

127   samples from several rodent species, in the absence of *Y. pestis*.

128   2. PCR, microarray and sequencing data suggest that these sequences may be present in

129   environmental bacteria.

130   3. Caution is warranted in interpreting screening results for detection of Y. pestis when the pla

131   gene is used as the sole marker for the presence of the pathogen.

132

133   Acknowledgements

134   We would like to thank Chelsea Himsworth, Florence Ayral, and Kieran Pounder for providing

135   the tissue samples. The authors would also like to thank Brendan Wren from the London School

136   of Hygiene and Tropical Medicine for providing the *Y. pestis* DNA sample. Finally, the authors

137   would like to thank Tom Giles from the University of Nottingham's Advanced Data Analysis

138   Centre.

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201   **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	TCGCTGGCTAAAAGTACCATCCACATGCTCAACCCTATAACCTGTAGCTTACCCCAC	*Y. pestis*	pPCP1	9583-9640
YpestisUPS_785	AATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAGGCCAGTATCGCAT	*Y. pestis*	pPCP1	8392-8451
YpestisUPS_786	AATGATGAGCACTATATGAGAGATCTTACTTTCCGTGAGAAGACATCCGGCTCACGTTAT	*Y. pestis*	pPCP1	8510-8569
YpestisUPS_787	TAAATTCAGCGACTGGGTTCGGGCACATGATAATGATGAGCACTATATGAGAGATCTTAC	*Y. pestis*	pPCP1	8479-8538
Y.pestisUPS_788	AGCCCGACCACTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG	*Y. pestis*	pPCP1	4977-5036
YpestisUPS_789	TCATCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTC	*Y. pestis*	pPCP	8801-8860
YpestisUPS_790	AAAGGACAGCATTTGGTATCTGTGCTCCACTTAAGCCAGCTACCACAGGTTAGAAAGCCT	*Y. pestis*	pPCP	5129-5188
YpestisUPS_791	AAGGAGTGCGGGTAATAGGTTATAACCAGCGCTTTTCTATGCCATATATTGGACTTGCAG	*Y. pestis*	pPCP	8379-8438
YpestisUPS_792	TTTGTACCGAGAACCTTTCACGGTATCGGCATATGGCCTGGGTAACTCAGGTCCGTAAAC	*Y. pestis*	pPCP	9451-9510

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

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

Sequence (5'-3')	Reverse Primer	Sequence (5'-3')	Probe	Sequence (5'-3')
CCCGAAAGGAGTGCGGGTAA	Y.pes/pPCP/8902/R	CGCCCCGTCATTATGGTGAA	N/A	N/A
CCAGCCCGCATCACT	cafpri_r	ATCTGTAAAGTTAACAGATGTGCTAGT	Tqpro_caf	JOE-AGCGTACCAACAAGTAATTCTGTATCGATC BHQ1
AGACATCCGGCTCACGTTAT	Y.pes_pPCP_R	GAGTACCTCCTTTGCCCTCA	Y.pes_pPCP_Pr	FAM-CACCTAATGCCAAAGTCTTTGCGGA-TAMRA

204 ** The nucleotide position of the Y.pes_pPCP_F and Y.pes_pPCP_R primers based on the CP000310.1 *Yersinia pestis* Antiqua

205 plasmid pPCP

206 ***The nucleotide position of the cafpri_f and cafpri_r primers based on the KF682424.1 *Yersinia pestis* strain S1 plasmid pMT1

207 capsule protein F1 (*caf1*) gene

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

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Primer name	Primer Sequence	Target	Reference
F1	CAGTTCCGTTATCGCCATTGC	*caf1*	(Norkina et
F2	TATTGGTTAGATACGGTTACGGT		al., 1994)
Ypfur1	GAAGTGTTGCAAAATCCTGCG	*fur*	(Hinnebusch
Ypfur2	AGTGACCGTATAAATACAGGC		et al., 1998)
YPtoxU	AGGACCTAATATGGAGCATGAC	*Ymt*	(Riehm et
YPtoxUR	CGTGATTACCAGGTGCAACA		al., 2011)

210 Primer name / Primer Sequence / Target / Reference
211 F1 / CAGTTCCGTTATCGCCATTGC / *caf1* / (Norkina et
212 F2 / TATTGGTTAGATACGGTTACGGT / al., 1994)
213 Ypfur1 / GAAGTGTTGCAAAATCCTGCG / *fur* / (Hinnebusch
214 Ypfur2 / AGTGACCGTATAAATACAGGC / et al., 1998)
215
216 YPtoxU / AGGACCTAATATGGAGCATGAC / *Ymt* / (Riehm et
217 YPtoxUR / CGTGATTACCAGGTGCAACA / al., 2011)
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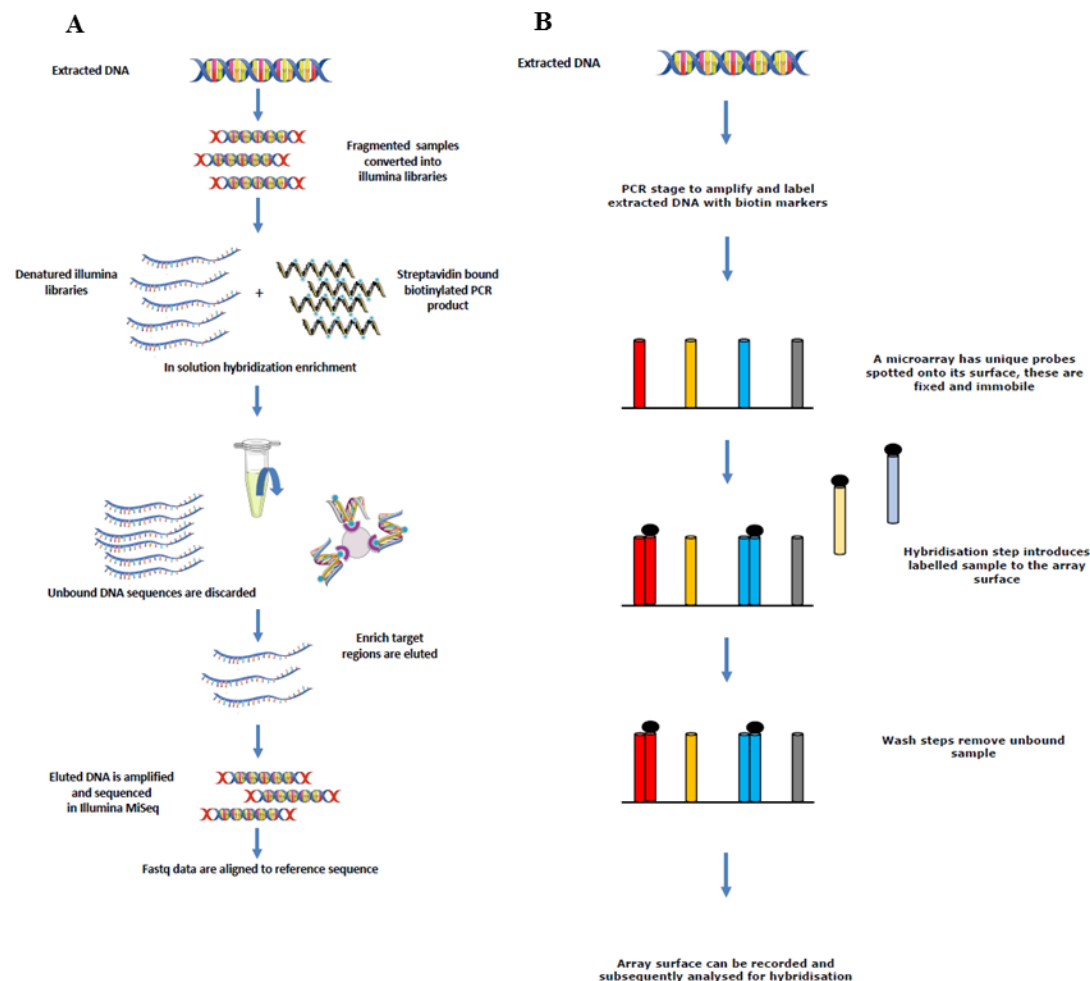
226 **Figure 1.** Sequence showing how extracted DNA is used for sequence enrichment capture (A)

227 and microarray hybridisation (B).

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