

1 **Rapid, specific detection and quantification of *Yersinia pestis***
2 **using a species-specific SNP in the ferric uptake regulator**
3 **gene (*furMAMA*)**

4

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17 Running head: Specific quantification of *Y. pestis* with TaqMAMA qPCR

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25

26 **Abstract**

27 *Yersinia pestis*, the causative agent of plague, is responsible for about 700
28 human cases of bubonic and pneumonic plague each year. Yet the disease is
29 far more prevalent within rodent reservoirs than in humans. One of the main
30 means of outbreak prevention is extensive wildlife surveillance, where
31 accurate and rapid detection is essential to prevent spillover into the human
32 population from which, it may otherwise spread more rapidly and over larger
33 distances. Moreover, detection and quantification of the agent aids in
34 investigative studies to understand aspects of the pathogen such as
35 transmission mechanics, pathology, contamination risk and more. Partially
36 based on a previously developed assay by Gabitzsch et al. 2008 we designed
37 a TaqMan® mismatch amplification mutation assay (TaqMAMA) where a
38 primer leverages a species-specific SNP in the chromosomal single copy
39 ferric uptake regulator gene of *Yersinia pestis*. The assay allows for specific,
40 rapid detection and quantification of *Yersinia pestis* using only a single
41 species-specific marker in a highly conserved virulence gene. This low-cost
42 and simple modification of an existing assay eliminates the need for running
43 multiple molecular markers for pathogen detection or performing time-
44 consuming culturing and counting of colonies for quantification.

45

46 **Keywords**

47 *Yersinia pestis*, TaqMAMA, quantification, qPCR, pathogen surveillance, SNP
48 based detection, private allele, bacterial detection.

49

50 **Background**

51 The causative agent of plague, *Yersinia pestis*, reached infamy after causing
52 three devastating human pandemics during recorded history. The disease
53 however, is mainly a vector-borne, wildlife disease, circulating within rodent
54 populations across the world, which occasionally spills over into human
55 populations. Today there are approximately 700 reported human cases of
56 plague worldwide each year with more than 100 deaths (World Health
57 Organization, 2017), most of which occur in Africa. Due to the pandemic
58 history of the pathogen and the continued potential for human outbreaks,
59 close surveillance of its presence in wildlife systems has been incorporated
60 into several local and national surveillance programs, some dating back to the
61 beginning of the 1900s (Melikishvili, 2006).

62

63 Plating of infected animal tissues or fleas and subsequent counting of
64 bacterial colonies is a well-established, sensitive and widely used method in
65 many surveillance programs and in diagnostics (Bevins et al., 2012). This is a
66 time-consuming method that exposes the workers to infectious material for
67 extended time. The culturing of *Y. pestis* has historically had challenges due
68 to the relatively slow growth of *Yersinia spp.* compared to the near
69 omnipresent contaminating microbes on traditional media (Dennis et al., 1999;
70 Gupta et al., 2015; Sarovich et al., 2010). To overcome some of these issues,
71 new and improved growth media with increased selectivity for *Y. pestis* have
72 been developed but still struggle with obstacles such as reduced recovery
73 rate of the pathogen and desired level of selectivity leading to underestimation
74 of bacterial numbers (Ber et al., 2003; Riehm et al., 2011; Sarovich et al.,

75 2010). As of today, culturing remains a gold standard for detection and in
76 particular quantification.

77

78 The recent development of immunoassay tests for rapid and easy detection in
79 the form of dipsticks have become preferential, particularly for quick
80 diagnostics in challenging field conditions in remote locations (Chanteau et
81 al., 2003; Simon et al., 2013). Yet immunoassays are typically not sensitive
82 enough to pick up cases of low-level infections or early on in infections where
83 only small amounts or no antibody is present (Andrianaivoarimanana et al.,
84 2012; Chanteau et al., 2003; Simon et al., 2013). Other limitations of these
85 assays are potential false negative results due to the lack of detection of
86 strains that have genetic deletions of the assays target (such as the F1
87 capsular antigen) (Anisimov et al., 2004; Eppinger et al., 2010; Perry and
88 Fetherston, 1997). They are also altogether unsuitable for quantification.

89

90 In the era of sequencing and genomics and the power and relative ease of
91 PCR based methods, the gold standard of detection is shifting from culture-
92 based methods towards molecular tools. In this context, PCR based assays
93 are able to detect the pathogen at low quantities making them the most
94 appropriate choice for investigative studies where maximal sensitivity is
95 required. Yet specific detection of bacterial pathogens using PCR or
96 immunoassay tests must often navigate several major obstacles, many being
97 the products of bacterial evolution. Most pathogens evolve out of complexes
98 of related species, where mechanisms like horizontal gene transfer (HGT)
99 allows a constant exchange of genetic elements, often as plasmids or genetic

100 islands (Juhas, 2015). As a result of these evolutionary trends, specific
101 detection of the target of interest, as is the case for many pathogenic bacteria
102 including *Y. pestis* and *Y. pseudotuberculosis*, can be difficult (Achtman et al.,
103 1999; Easterday et al., 2005; U'Ren et al., 2005). For *Y. pestis*, its recent
104 evolution from *Y. pseudotuberculosis*, has been accomplished through a
105 process of functional gene loss and gain through HGT within
106 *Enterobacteriaceae*. This results in *Y. pestis* being, more or less, a clone of *Y.*
107 *pseudotuberculosis* with shared elements from other members of
108 *Enterobacteriaceae* (Achtman et al., 1999; Hinnebusch et al., 2016).

109

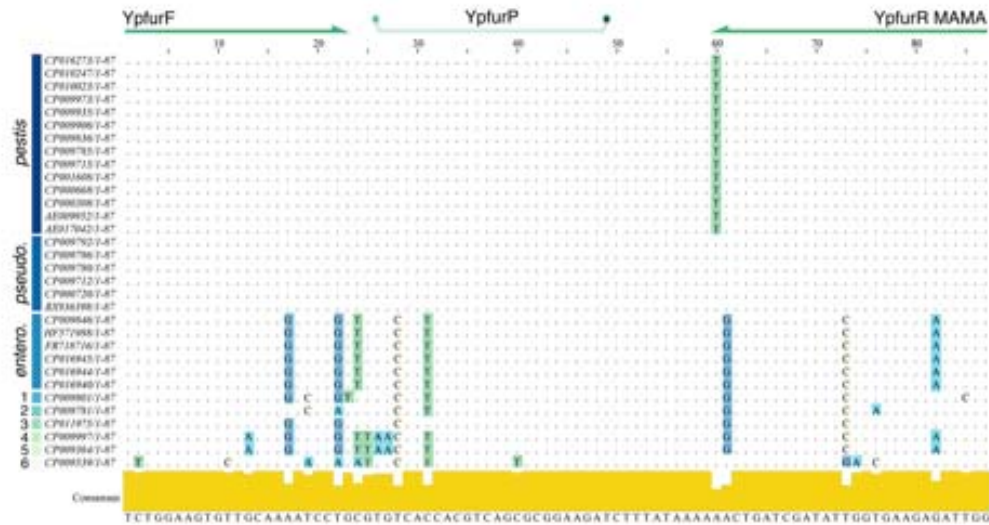
110 Historically, discrimination between *Y. pestis* and *Y. pseudotuberculosis* has
111 been challenging for methods targeting chromosomal sequences, due to the
112 high degree of genetic similarity between the two species (Califf et al., 2015;
113 Chain et al., 2004; Gabitzsch et al., 2008; Neubauer et al., 2000). A work-
114 around has been to use multiple assays to detect specific combinations and
115 variants of genes located on the chromosome and/or the plasmids of *Y. pestis*
116 (Stewart et al., 2008; Tomaso et al., 2003). Still, the problems with finding *Y.*
117 *pestis* specific genes on the main chromosome also extends to plasmid
118 targets, such as the pCD1 plasmid, a homologue of pYV1 found in *Y.*
119 *pseudotuberculosis* and *Y. enterocolitica* (Hu et al., 1998; Portnoy et al.,
120 1984).

121

122 The two most conventional targets for detection of *Y. pestis* both under
123 laboratory and field conditions are the Capsule antigen fraction 1 (*caf1*) gene
124 on the pMT1 plasmid and the plasminogen activator gene (*pla*) on the pPCP

125 plasmid. *Pla* is one of the most widely used markers for PCR based detection
126 of *Y. pestis* in ancient human remains, animals and environmental samples
127 due to it being present on the pPCP plasmid which is present in multiple
128 copies in the bacteria and its presumed high specificity for *Y. pestis* (Harbeck
129 et al., 2013; Stewart et al., 2008; Tomaso et al., 2008). However, recently it
130 has been established that the gene is present and highly conserved in closely
131 related bacterial species, and consequently is no longer considered a specific
132 marker for *Y. pestis* (Armougom et al., 2016; Hänsch et al., 2015). Generally,
133 a minimum of one other independent molecular marker is required to
134 positively determine presence of the bacteria; these include genes *caf1* and
135 Yersinia murine toxin (*ymt*) from the pMT1 plasmid, Low calcium response V
136 antigen (*lcrV*) from the pCD1 plasmid, pesticin gene on pPCP or
137 chromosomal targets such as 16S or Inner membrane protein *yihN* (*yihN*)
138 gene either in separate or multiplex real-time PCR assays (Iqbal et al., 2000;
139 Stewart et al., 2008; Tomaso et al., 2008; 2003; Woron et al., 2006).
140 Furthermore plasmids are subject to variation in copy number during certain
141 conditions, such as during an infection (Wang et al., 2016). Parkhill et al.
142 showed that *Y. pestis* on average contain about 200 copies of the pPCP1
143 plasmid (Parkhill et al., 2001), which is partly the reason for the preference of
144 *pla* as target for detection as the gene is present in high copy number, yet the
145 variability in copy number is a severe drawback for quantifying *Y. pestis*
146 concentrations (Stewart et al., 2008; Tomaso et al., 2003).
147
148 By combining the established TaqMan® assay with a mismatch amplification
149 mutation assay (TaqMAMA) (Cha et al., 1992), Glaab and Skopek (Glaab and

150 Skopek, 1999) developed a powerful and rapid method of discriminating
151 specific single nucleotide alleles in real-time providing a valuable tool for
152 several fields of science dealing with discrimination between highly similar
153 targets (Achtman et al., 1999; Chain et al., 2004; Lärkeryd et al., 2014).
154 TaqMAMA assays have successfully been designed to specifically amplify,
155 detect and quantify a particular allele when it is paramount to distinguish
156 between highly similar DNA sequences. Most of these have been developed
157 in cancer research (Abbaszadegan et al., 2009; Cha et al., 1992), but also for
158 work in bioforensics or surveillance when positive detection and discrimination
159 of a pathogen from close genetic neighbors is vital (Achtman, 2008;
160 Easterday et al., 2005). Previously, Gabitzsch et al. (Gabitzsch et al., 2008)
161 developed a TaqMan® assay to detect the ferric uptake regulator (*fur*) gene
162 for the specific quantification of *Y. pestis*, yet has become outmoded as new
163 sequences have become available showing the gene is highly conserved in
164 other *Yersinia* and more widely occurs within *Enterobacteriaceae*.
165 Fortuitously, a 100% *Y. pestis*-specific SNP exists within this gene inward
166 from the priming site of the reverse primer (*YpfurR*) from the Gabitzsch assay
167 (Fig. 1). Here we leverage this species-specific SNP to provide a simple and
168 cheap augmentation to the original assay creating the only single target assay
169 for *Y. pestis*, which is quantitative, sensitive and rapid as well as, species-
170 specific.



171

172 FIG 1. Nucleotide alignment of the 87 bp PCR fragment, targeted by the assay, of the *fur*
173 gene of a representative selection of *Yersinia* spp. Only the nucleotide differences between
174 the strains and the generated consensus is shown. Alignment was generated through an
175 initial BLAST search in Geneious, names (NCBI accession numbers) and sequences were
176 subsequently extracted and entered into Jalview where an alignment was automatically
177 performed to generate the consensus sequence and bar plot. The location of the forward and
178 reverse primers and the probe are indicated on the top of the alignment. *pestis*: a selection of
179 *Y. pestis* sequences covering several biovars and geographic locations, *pseudo*.: a selection
180 of *Y. pseudotuberculosis* sequences, *entero*.: a selection of *Y. enterocolitica* sequences, 1: *Y.*
181 *intermedia*, 2: *Y. aldovae*, 3: *Y. aleksiciae*, 4: *Y. kristensenii*, 5: *Y. frederiksenii*, 6: *Y. ruckeri*.

182

183 Results

184 *In silico* screen of SNP, a private allele for *Y. pestis*

185 The ubiquitous presence of the *Y. pestis* allele was confirmed by both
186 megaBLAST and BLASTn searches (NCBI) of the *fur* gene sequence from
187 CO92 and the PCR fragment. In both cases the allele was conserved in all
188 981 published *fur* gene sequences for *Y. pestis* (a global representation),
189 while the alternate allele was present in all 853 available *fur* gene sequences

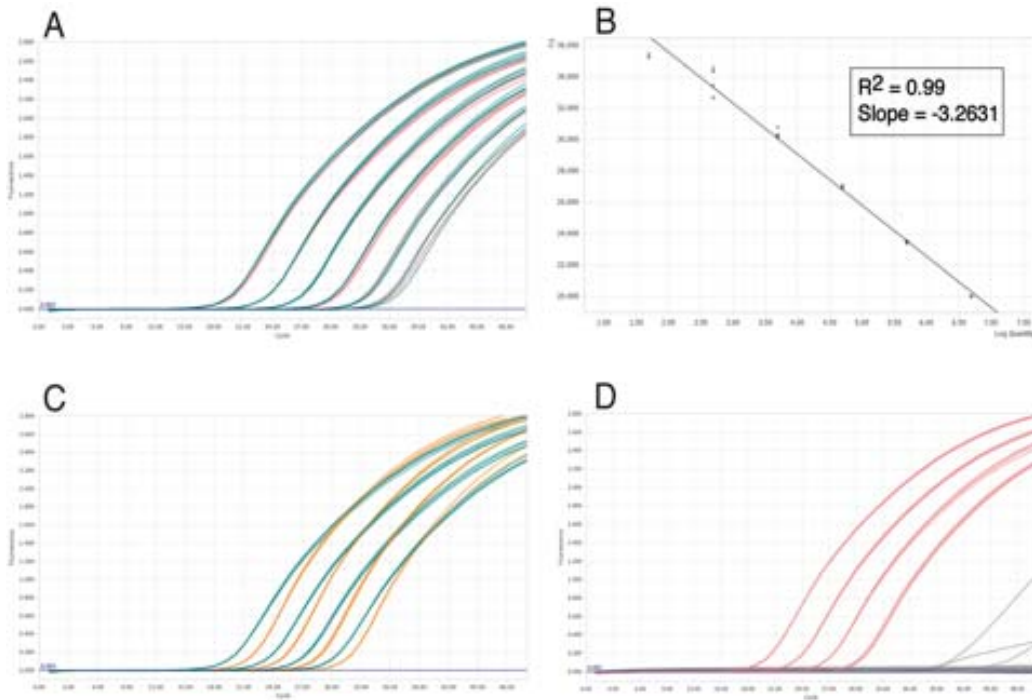
190 of *Y. pseudotuberculosis* and all 2950 available *fur* gene sequences of the
191 other members of the *Yersinia* genus (May 2018, Fig. 1). A BLASTn search of
192 the *fur* sequence of CO92 against the genome assembly of the strain used in
193 this study, Az-26 (1102) (not published) confirmed the presence of the *Y.*
194 *pestis* allele in the *fur* gene of this strain, as well as 100% identity with all
195 published *Y. pestis*. The SNP, as to date, is a private allele for *Y. pestis*.

196

197 **Specificity**

198 The specificity of the *fur*MAMA assay was assessed by analyzing serial 10-
199 fold dilutions of genomic DNA in quadruplicates from *Y. pestis* and *Y.*
200 *pseudotuberculosis* alongside the original assay from Gabitzsch et al.
201 (Gabitzsch et al., 2008). We found no difference in the amplification efficiency
202 between *Y. pestis* and *Y. pseudotuberculosis* when using the original reverse
203 primer from the original assay across all DNA concentrations (Fig. 2 (A)). Our
204 augmented assay successfully quantifies concentrations of *Y. pestis* ranging
205 from 5 ng (~100 000 000 genome copies) to 50 fg (~10 genome copies) with a
206 slight shift in amplification efficiency (higher Cq values) compared to when the
207 original reverse primer is used (Fig. 2 (C)). In contrast, we find amplification
208 failure in all dilutions of *Y. pseudotuberculosis* and were only able to achieve
209 false positives using extremely high concentrations of *Y. pseudotuberculosis*
210 template in the *fur*MAMA assay where we saw some weak cross-reactivity in
211 three of four replicates at 5 ng and one of the quadruplicates at 500 pg (Fig. 2
212 (D)). A combined 90 replicates consisting of 64 non-template controls (NTC),
213 and (assumed) negative tissue and soil samples (from non-endemic areas)
214 did not show any amplification.

215



216

217 FIG 2. Real-time plots indicating the amplification efficiency and specificity of the original fur
218 assay vs the *fur*MAMA TaqMan® mismatch amplification mutation assay (TaqMAMA). (A)
219 Results of quadruplicate analysis of the 10-fold serial dilutions of *Y. pestis* (blue/teal) and *Y.*
220 *pseudotuberculosis* (red) DNA (left to right: 5 ng – 50 fg) using the original *fur* assay. (B) The
221 standard curve plot of *Y. pestis* DNA with the *fur*MAMA assay; $R^2 = 0.99$, slope = -3.2631. (C)
222 Results of quadruplicate analysis of the 10-fold serial dilutions of *Y. pestis* with the original fur
223 assay (blue/teal) and the *fur*MAMA assay (orange). Only the first four dilutions are shown.
224 The average Cq values (quadruplicate analysis) in TE buffer were as follows: 5 ng, 17.87;
225 500 pg, 21.80; 50 pg, 25.33 and 5 pg, 28.64 for the original *fur* assay and 5ng, 19.98; 500 pg,
226 23.44; 50 pg, 26.96 and 5 pg, 30.36 for the *fur*MAMA assay (curves left to right). (D) Results
227 of quadruplicate analysis of the 10-fold dilutions of *Y. pseudotuberculosis* with the original *fur*
228 assay (red) and the *fur*MAMA assay (grey). Only the first four dilutions are shown. The
229 average Cq values (quadruplicate analysis) in TE buffer were as follows: 5 ng, 18.16; 500 pg,
230 21.75; 50 pg, 25.23 and 5 pg, 28.96 for the original *fur* assay while the dilutions show overall
231 amplification failure with the *fur*MAMA assay. For the 5 ng *Y. pseudotuberculosis* with
232 *fur*MAMA three of the four replicates amplified with the average Cq value of 40.15. For the

233 500 pg only one of the quadruplicates amplified and the Cq value was 43.88. No amplification
234 was seen for either the 50 pg or 5 pg dilutions.

235

236 **Specific quantification and sensitivity of the assay**

237 To evaluate the ability of specific quantification and the sensitivity of the
238 *furMAMA* assay, 10-fold serial dilutions of *Y. pestis* were analyzed. The assay
239 successfully amplified *Y. pestis* over a dynamic range of concentrations with a
240 correlation coefficient (R^2) of 0.99 (Fig. 2 (B)) and was able to consistently
241 quantify as little as 50 fg, which is equivalent to 10 bacterial genomes. The
242 assay showed sporadic amplification at levels of the 5 fg concentration (3 out
243 of 33 reactions).

244

245 **Detection by conventional PCR**

246 The *furMAMA* assay was also run as a standard PCR without probe to test its
247 applicability as a detection only assay for when quantification equipment is not
248 available. The primer pair successfully amplified *Y. pestis* and did not produce
249 a PCR product of the correct size for any of the quadruplicates of *Y.*
250 *pseudotuberculosis* (results not shown).

251

252 **Screening for inhibition by rodent tissue and soil extracts**

253 In order to assess possible effects of foreign DNA on the *furMAMA* assay we
254 ran target-spiked controls over a standard dilution range of concentrations
255 (5ng to 50fg) into DNAs extracted from rodent tissues and soils. *Y. pestis*
256 DNA was spiked into several types of DNA extracts including ear, tail, liver
257 and spleen from several rodent species including, great gerbil (*Rhombomys*

258 *opimus*), lab mouse (*Mus musculus*) and bank vole (*Myodes glareolus*) and
259 DNA from soil from a previous study (Turner et al., 2016). Regardless of
260 background DNA (tissue or soil) the assay still detects and quantifies across
261 the range tested without non-specific cross-reactivity. Environmental samples
262 such as soil extracts can show a high degree of inhibition depending on the
263 sample and extraction method used (Whitehouse and Hottel, 2007). Our
264 assay successfully amplified soil extracts spiked with *Y. pestis* although we
265 did observe an approximately 10-fold reduction in amplification efficiency that
266 persisted despite the addition of BSA to the reaction.

267

268 **Discussion**

269 Although many PCR-based assays exist for *Y. pestis*, none combines species
270 specificity with accurate quantification. The *fur*MAMA assay is designed to
271 specifically amplify a fragment of the *fur* gene in *Y. pestis* while preventing
272 amplification of the gene fragment in other members of the *Yersinia* genus
273 including its close genetic neighbor *Y. pseudotuberculosis* where the gene
274 fragment only differs by a single SNP. The SNP in the *fur* gene fragment
275 targeted by our *fur*MAMA assay is private to, and ubiquitous in all *Y. pestis*.
276 Specificity is acquired in a single assay through allele-specific amplification of
277 this SNP in a key virulence gene, *fur*, whose presence is found throughout
278 *Enterobacteriaceae* without the need for further molecular targets (Fig. 1).
279 Leveraging a specific SNP within a gene, as done here, is an efficient and
280 robust method for specific detection of a given bacterial pathogen and can aid
281 in pathogen detection in metagenomes using next generation sequencing
282 where close relatives are present in high numbers (Valseth et al., 2017). This

283 circumvents the present issues of specificity when targeting pathogens with
284 high degree of genetic similarity. A recent large-scale comparative study of *Y.*
285 *pestis* and *Y. pseudotuberculosis* genomes showed that fewer genes than
286 previously reported in smaller studies were unique to *Y. pestis* (Califf et al.,
287 2015). However, both specific combinations, variants of genes and SNPs, the
288 last example demonstrated here, will be unique to this slowly mutating
289 pathogen (Cui et al., 2013).

290

291 The original assay published by Gabitzsch et al. (Gabitzsch et al., 2008) was
292 developed to quantify *Y. pestis* in fleas for their work on flea transmission in a
293 laboratory experimental setup and not initially aimed at diagnostics or
294 quantification in field-collected samples. They tested the specificity by running
295 PCR on reference DNA from 28 species, including *E. coli* and other gram-
296 negative bacteria, and reported cross-reactivity for 6 *Yersinia* species,
297 including *Y. enterocolitica*. Surprisingly, they did not report cross-reactivity
298 with *Y. pseudotuberculosis*. BLAST of the derived *fur* gene fragment from the
299 Gabitzsch assay resulted in 99% overall homology with *Y. pseudotuberculosis*
300 sequence and high homology between other *Yersinia* spp. Indeed, we found
301 the original assay amplifies both *Y. pestis* and *Y. pseudotuberculosis* with
302 equal efficiency (Fig. 2 (A)). In contrast, the amplification failure of *Y.*
303 *pseudotuberculosis* with the YpfurR_MAMA primer establishes our assay's
304 ability to distinguish between the two highly genetically similar pathogens, as
305 well as the ability to quantify *Y. pestis* over a broad range of concentrations
306 (Fig. 2(D and C)). The *Y. pestis* SNP in the *fur* gene is universally present in
307 all available *Y. pestis* genomes, which limits the probability of false negative

308 results. Conversely, the absence of the mutation in all other *Yersinia* and
309 *Enterobacteriaceae* reduce the likelihood of false positive results. Our assay
310 presented false positives and very low-level cross-reactivity at the highest
311 concentration of *Y. pseudotuberculosis* DNA produced from whole genome
312 amplification (WGA). The lack of access to a larger collection of *Y.*
313 *pseudotuberculosis* prohibited us from further testing of cross-reactivity but
314 based on the specificity testing *in silico* and by targeting a conserved SNP in
315 the *Y. pestis* genome we are confident this will not be a major problem.

316

317 **Conclusions**

318 Our assay provides an alternative to the longstanding culturing method and
319 the immunoassays by providing a highly sensitive and rapid way of both
320 specifically detecting and quantifying *Y. pestis* in tissue samples. To our
321 knowledge, this is the first assay to target a species-specific SNP in a
322 chromosomal gene of *Y. pestis* with the aim of detecting and quantifying the
323 amount of the bacteria in tissue samples.

324

325 **Materials and Methods**

326 **Bacterial strains and DNA extractions**

327 *Y. pestis* strain Az-26 (1102) was isolated from an organ suspension
328 of *Meriones vinogradovi* in 1969, 1.5 km southeast of Sirab village in the
329 Nakhchivan Autonomous Republic of Azerbaijan. The culture has been stored
330 at the Republican Anti-Plague Station laboratory in Baku, Azerbaijan. The
331 culture became non-viable, yet it was still possible to isolate DNA from the

332 material in 2012 using Qiagen DNeasy® Blood and Tissue kit following
333 extraction protocol for gram-negative bacteria.
334 *Y. pseudotuberculosis* Type III strain was donated by Jack C. Leo and Dirk
335 Linke (University of Oslo). DNA was isolated from broth culture using Qiagen
336 Blood and Tissue kit following manufacturer's instructions (Qiagen Inc., USA).

337

338 **Rodent DNA**

339 No animals were killed for the purpose of this study. We procured samples of
340 rodent DNA for this study by reaching out to both internal and external
341 colleagues with existing DNA samples stored. The DNA samples stem from
342 unpublished work as detailed below.

343 The great gerbil DNA was provided by colleagues in China where the male
344 individual was captured as part of work carried out in the Junggar Basin of
345 Xinjiang Province, China (unpublished).

346 The C57BL6 mouse DNA stem from work conducted at the Institute of
347 Immunology at Oslo University Hospital, Rikshospitalet, Norway
348 (unpublished). The animal was originally obtained from Janvier labs
349 ([https://www.janvier-labs.com/rodent-research-models-services/research-](https://www.janvier-labs.com/rodent-research-models-services/research-models/per-species/inbred-mice/product/c57bl6jrx.html)
350 [models/per-species/inbred-mice/product/c57bl6jrx.html](https://www.janvier-labs.com/rodent-research-models-services/research-models/per-species/inbred-mice/product/c57bl6jrx.html)).

351 The bank vole DNA belongs to the EcoTick-project on tick-/rodent-borne
352 diseases conducted at the University of Oslo (unpublished).

353

354 **Whole genome amplification (WGA) and standard curves**

355 Whole genome amplification was performed on both bacterial strains using
356 the REPLI-g Mini Kit (Qiagen) following the manufacturer's instructions for

357 subsequent creation of a dynamic range of DNA starting at high
358 concentrations for standard curves. DNA concentrations after WGA were
359 determined using the Qubit dsDNA BR Assay kit (molecular probes, Life
360 Technologies) and the Qubit 2.0 fluorometer. The standard curves were
361 generated from that starting concentration as serial 10-fold dilutions in TE
362 buffer in the following concentrations: 5 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg
363 and 5 fg.

364

365 **TaqMAMA Primers and Probe design**

366 Forward primer and probe were identical to oligos designed by Gabitzsch et
367 al. (Gabitzsch et al., 2008) while the reverse primer was modified to a MAMA
368 primer to specifically amplify *Y. pestis*. This required the ultimate 3' base to be
369 complementary to the *Y. pestis* SNP while the penultimate 3' base was
370 designed to mismatch with the shared sequence between *Y. pestis* and *Y.*
371 *pseudotuberculosis* (underlined in Table 2). Several reverse MAMA primers
372 were designed with different nucleotide mismatches at the 3' penultimate
373 position. These were tested for specificity and amplification efficiency before
374 ultimately choosing the YpfurR_MAMA primer.

375

376 **Table 1. List of Primers and probe sequences assayed in qPCR and PCR**
377 **reactions**

Primer/Probe	Sequence (5'–3')	Product (bp)*
YpfurF	TCTGGAAGTGGTGCAAAATCCTG	
YpfurP	FAM-TGTCACCACGTCAGCGCGGAAGAT-BAMQ1	
YpfurR	AAGCCAATCTCTTCACCAATATCG	90

YpfurR_MAMA	CCAATCTCTTCACCAATATCGATCAGAA	87
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378 * The resulting PCR product size when combining the YpfurF (forward primer) with
379 the respective reverse primer.

380

381 **TaqMAMA PCR protocol**

382 For all experiments, PCR was conducted in 96-well plates (Roche
383 LightCycler[®] 480 Multiwell Plate 96) using 10- μ L reactions that contained final
384 concentrations of 600 nM of both forward and reverse primers, 250 nM probe,
385 1x Express qPCR Supermix (Invitrogen, by Life Technologies)) and 1 μ L
386 template DNA. Real-time PCR was performed on a LightCycler[®] 96
387 Instrument (Roche) as a two-step PCR with the following conditions: 95 °C for
388 2 min and 50 cycles of 95 °C for 15 s and 64 °C for 60 s.

389

390 **Specificity tests**

391 Given limited access to a large strain collection of *Yersinia*, specificity was
392 largely assessed using bioinformatics (BLAST) of available sequence data
393 (see “Bioinformatics” below). Therefore, the specificity and kinetics of the
394 assay was done through analyzing genomic DNA from a representative of
395 both alleles (see Fig. 1); *Y. pestis* strain Az-26 (1102) and *Y.*
396 *pseudotuberculosis* Type III strain running both original reverse primer
397 (YpfurR) and the modified MAMA primer (YpfurR_MAMA) in separate, parallel
398 reactions over a dynamic range of DNA concentrations (5 ng to 5 fg).

399

400 **Sensitivity tests**

401 Sensitivity of the furMAMA assay was determined by analyzing serial 10-fold
402 dilutions of genomic DNA from the *Y. pestis* strain Az-26 (1102) ranging from
403 5 ng to 5 fg with the lowest dilution being equivalent to one bacterial genome.

404

405 **Testing applicability on tissue and environmental samples**

406 The applicability of the assay on rodent tissue samples was tested by running,
407 in parallel, DNA extractions of liver and spleen from a great gerbil
408 (*Rhombomys opimus*), DNA from the tail of a C57BL6 mouse (*Mus musculus*)
409 and DNA extracted from the ear of a Bank vole (*Myodes glareolus*) with and
410 without spike-in of *Y. pestis* DNA standard curve. Real-time PCR was
411 conducted in 96-well plates (Roche LightCycler® 480 Multi-well Plate 96)
412 using 10-µL reactions that contained final concentrations of 600 nM of both
413 forward and reverse primer, 250 nM probe, 1x Express qPCR Supermix
414 (Invitrogen, by Life Technologies)), 1 µL template DNA and for the spiked
415 samples an additional 1 µL DNA from one of the standard curve
416 concentrations.

417 The ability of the assay to detect *Y. pestis* despite the inhibitory effects often
418 seen in environmental samples, was tested by using DNA extractions of soil
419 samples diluted 1:10. The soil samples were run in parallel with non-
420 acetylated Bovine Serum Albumin (New England Biolabs) added, with or
421 without DNA from the *Y. pestis* standard curve in the following manner 1) soil
422 + BSA, 2) soil + standard curve and 3) soil + standard curve + BSA. Real-time
423 PCR was conducted as above with the exception that the reactions containing
424 BSA did so in a final concentration of 1mg/ml.

425

426 **Detection using standard PCR**

427 The primers were also tested with standard PCR to determine their
428 applicability as a pure detection-based assay. PCR was conducted in 8-well
429 PCR strips in 10- μ L reactions that contained a final concentration of 600 nM
430 both forward and reverse primer, 1x Express qPCR Supermix and 1 μ L
431 template DNA. PCR was performed on a MJ Research PTC-200 Peltier
432 Thermal Cycler Instrument as a two-step PCR with the following conditions:
433 94 °C for 2 min and 40 cycles of 94 °C for 15 s and 64 °C for 60 s. Successful
434 amplification was confirmed by running a 3% agarose gel stained with GelRed
435 (Biotium) at 75 V for 1 hr and 20 min in TAE buffer.

436

437 **Bioinformatics**

438 Specificity of the SNP was assessed across available *Yersinia* and other
439 related *Enterobacteriaceae* genomes by performing both megaBLAST and
440 BLASTn searches of the PCR fragment and the whole *fur* gene from *Y. pestis*
441 CO92 (Accession no. NC_003143.1, locus_tag=YPO2634) as query in the
442 NCBI database using default parameters. BLAST hits were inspected to
443 confirm which allele was present in the sequence. Finally, the presence of the
444 *Y. pestis* allele in the strain used in this study (Az-26 (1102)) was confirmed
445 by a BLASTn search using the *fur* gene from *Y. pestis* CO92 as query against
446 a BLAST database generated from the Az-26 (1102) genome assembly. From
447 these data an alignment was made using representative sequences from
448 BLAST searches through Geneious (<https://www.geneious.com>) and aligned
449 using Jalview (Waterhouse et al., 2009).

450

451 **Abbreviations**

452 Fur gene: Ferric uptake regulator

453 BSA: Bovine serum albumin

454 Caf1: Capsule antigen fraction 1

455 Cq: Quantification cycle

456 HGT: Horizontal gene transfer

457 IcrV: Low calcium response V antigen

458 Pla: Plasminogen activator

459 SNP: Single nucleotide polymorphism

460 TAE: Tris-acetate-EDTA

461 TaqMAMA: TaqMan® mismatch amplification mutation assay

462 TE: Tris-EDTA

463 WGA: Whole genome amplification

464 yihN: Inner membrane protein yihN

465 Ymt: Yersinia murine toxin

466

467 **Declarations**

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479 **Authors contributions**

480 WRE and PN conceived and planned the experiments and analyzed the data.
481 PN carried out the experiments and performed bioinformatics. SG procured
482 and isolated the *Y. pestis* strain Az-26 (1102) for this study. The manuscript
483 was written with input from all authors. All authors read and approved the final
484 manuscript.

485 **Availability of data and materials**

486 The *Y. pestis* strain analyzed during this study (Az-26(1102)) is not publicly
487 available due to said strain being non-viable at isolation. The genome
488 sequence is available from the corresponding author upon reasonable
489 request.

490 **Consent for publication**

491 Not applicable.

492 **Competing interests:**

493 The authors declare that they have no competing interests.

494 **Ethics approval and consent to participate**

495 The DNA samples used in this study stems from existing samples stored by
496 internal and external colleagues and hence the appropriate approvals were
497 previously obtained as specified below.

498 The use of great gerbil tissue was approved by the Committee for Animal
499 Welfares of Xinjiang CDC, China.

500 The bank vole was captured as part of EcoTick-project on tick-/rodent-borne
501 diseases led by Atle Mysterud (University of Oslo) and permission for trapping
502 rodents were given by the Norwegian Environmental Agency (ref 2013/11201)
503 and hence conform to the Norwegian laws and regulations.

504 The C57BL6 mouse was used in a project at the Institute of Immunology at
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506 number 3775 from the Norwegian Food Safety Authority. This agency
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516

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