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Pentaplex real-time PCR for differential detection of *Yersinia pestis* and *Y. pseudotuberculosis* and application for testing fleas collected during plague epizootics

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Abstract

Upon acquiring two unique plasmids (pMT1 and pPCP1) and genome rearrangement during the evolution from *Yersinia pseudotuberculosis*, the plague causative agent *Y. pestis* is closely related to *Y. pseudotuberculosis* genetically but became highly virulent. We developed a pentaplex real-time PCR assay that not only detects both *Yersinia* species but also differentiates *Y. pestis* strains regarding their plasmid profiles. The five targets used were *Y. pestis*-specific *ypo2088*, *caf1*, and *pst* located on the chromosome, plasmids pMT1 and pPCP1, respectively; *Y. pseudotuberculosis*-specific chromosomal gene *opgG*; and 18S ribosomal RNA gene as an internal control for flea DNA. All targets showed 100% specificity and high sensitivity with limits of detection ranging from 1 fg to 100 fg, with *Y. pestis*-specific *pst* as the most sensitive target. Using the assay, *Y. pestis* strains were differentiated 100% by their known plasmid profiles. Testing *Y. pestis* and *Y. pseudotuberculosis*-spiked flea DNA showed there is no interference from flea DNA on the amplification of targeted genes. Finally, we applied the assay for testing 102 fleas collected from prairie dog burrows where prairie dog die-off was reported months before flea collection. All flea DNA was amplified by 18S rRNA; no *Y. pseudotuberculosis* was detected; one flea was positive for all *Y. pestis*-specific targets, confirming local *Y. pestis* transmission. Our results indicated the assay is sensitive and specific for the detection and differentiation of *Y. pestis* and *Y. pseudotuberculosis*. The assay can be used in field investigations for the rapid identification of the plague causative agent.

KEYWORDS

assay development, fleas, pentaplex real-time PCR, *Yersinia pestis*, *Yersinia pseudotuberculosis*

1 | INTRODUCTION

Yersinia pestis, a Tier 1 select agent, is the bacterial causative pathogen of a plague that infects rodents and can be transmitted to other mammals, including humans through flea biting (Perry & Fetherston, 1997; Stenseth et al., 2008). Plague causes serious illness or death if not treated promptly, especially if it develops into the pneumonic plague.

The plague has had a big impact on human history through three devastating pandemics (Perry & Fetherston, 1997) causing millions of deaths, as well as by locally endemic occurrence which results in hundreds to thousands of annual cases worldwide. Currently, the plague is endemic in Asia, Africa, and the Americas (Bertherat, 2016). Multiple recent outbreaks, including that in China, Democratic Republic of Congo, Madagascar, Peru, and Uganda (Abedi et al., 2018; Andrianaivoarimanana et al., 2019; Randremanana et al., 2019; Respicio-Kingry et al., 2016; Shi et al., 2018) warn us not to ignore this severe infectious disease.

Along with *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are the other two related species within the genus *Yersinia* that can cause diseases in both animals and humans (Perry & Fetherston, 1997). Nevertheless, symptoms of disease caused by enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* are vastly different from the plague. Belonging to the same genus, these organisms, especially *Y. pestis* and *Y. pseudotuberculosis*, are genetically closely related (Moore & Brubaker, 1975). Previous studies have indicated that *Y. pestis* has diverged recently from *Y. pseudotuberculosis* through events of genome reduction and gene gain; thus, the high genomic similarity were observed between the two species (Achtman et al., 1999; Brubaker, 2004; Chain et al., 2004; Hu et al., 1998; Moore & Brubaker, 1975; Parkhill et al., 2001). In the genetic structure, all three species possess a single circular chromosome and a common virulence plasmid termed pCD1, or pIB, or pYV in *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, respectively (Cornelis & Wolf-Watz, 1997; Iriarte & Cornelis, 1996; Perry & Fetherston, 1997). The most prominent genetic difference between *Y. pestis* and the enteropathogenic *Yersinia* species is the presence of pPCP1 and pMT1, two newly acquired plasmids in most but not all strains of *Y. pestis* (Ben-Gurion & Shafferman, 1981; Ferber & Brubaker, 1981; Filippov, Oleinikov, Motin, Protsenko, & Smirnov, 1995; Filippov, Solodovnikov, & Protsenko, 1990; Hu et al., 1998; Portnoy & Falkow, 1981). These plasmids contain different genes that play important roles regarding pathogenesis. The plasmid pCD1 contains the highly conserved type three secretion system (T3SS) injectosome and effector proteins Yops (Rajanna et al., 2010); pPCP1 contains *pla* gene and *pst* gene that encode plasminogen activator (PLA) protease and the pesticin protein (Bearden, Fetherston, & Perry, 1997); pMT1 contains *caf1* and *ymt*, encoding fraction 1 capsule antigen and murine toxin, respectively, as well as other putative virulence determinants (Hu et al., 1998).

Most *Y. pestis* strains isolated from humans, animals, or fleas contain all three typical plasmids. However, *Y. pestis* isolates lacking one or more plasmids may cause mild or even fatal disease, as previously been reported in South Africa and the United States (Beesley & Surgalla, 1970; Isaacson et al., 1973; Williams, Harrison, & Cavanaugh, 1975; Williams et al., 1978; Winter, Cherry, & Moody, 1960). Such variants have been reported infrequently. The natural occurrence of strains with atypical plasmid profiles might be greater than present data suggest.

The extreme pathogenicity of *Y. pestis* makes accurate and rapid detection of this bacterium a priority. Traditional methods of detecting *Y. pestis* include standard microbiological techniques (Norkina et al., 1994) and immunofluorescent staining (Feodorova & Devdariani, 2000). However, these methods take a long time and are relatively insensitive. In contrast, PCR-based assays have the advantage of rapid detection with high sensitivity. Furthermore, PCR is often preferable with the select agent and biosafety concerns. PCR assays for *Y. pestis* detection are not uncommon. Several PCR assays have been developed (Campbell, Lowe, Walz, & Ezzell, 1993; Matero et al., 2009; Neubauer et al., 2000; Radnedge, Gamez-Chin, McCready, Worsham, & Andersen, 2001; Riehm et al., 2011; Stewart, Satterfield, Cohen, O'Neill, & Robison, 2008; Tomaso et al., 2003; Woron et al., 2006). The pPCP1-situated *pla* gene, known to be present in 150–200 copies per bacterium (Parkhill et al., 2001), has been widely used due to the very high sensitivity (Riehm et al., 2011; Stewart et al., 2008; Woron et al., 2006). Ideally, if we are dealing with typical *Y. pestis* strains that harbor all three plasmids, a *pla*-based assay could be reasonable. However, a good portion (~25%) of *Y. pestis* strains existing in nature are deficient in one or more of the three plasmids (Filippov et al., 1990; Stewart et al., 2008) due to various host growth temperatures, as well as other unknown factors (Iqbal, Chambers, Goode, Valdes, & Brubaker, 2000). As such, a plasmid-based assay may yield false-negative results. On the other hand, a *pla* assay may cause false-positive results because *pla* may also be present in other Enterobacteriaceae such as *Escherichia coli* and *Citrobacter koseri* (Armougom et al., 2016; Hänsch et al., 2015). Further, a homolog of *pla* has been reported in rodents of multiple species (Giles et al., 2016). In addition to plasmid-based genes, chromosomal genes, such as *entF*3, were also applied for *Y. pestis* detection (Woron et al., 2006). The problem with using a chromosomal gene alone is it may involve non-specific amplification due to the genetic relatedness of *Y. pestis* to the other *Yersinia* species. Amplification with a chromosomal gene may not indicate which species is present (Woron et al., 2006).

In the current study, we developed a pentaplex real-time PCR assay that includes three *Y. pestis*-specific targets, one *Y. pseudotuberculosis*-specific target and one internal control for flea DNA since the assay is intended to test field-collected fleas. The three *Y. pestis*-specific targets in the assay are pPCP1-situated *pst*, pMT1-situated *caf1*, and chromosomal gene *ypo2088*. *ypo*2088 codes for a putative methyltransferase and is considered *Y. pestis*-specific (Chain et al., 2004; Matero et al., 2009). The *Y. pseudotuberculosis*-specific gene used in the assay is chromosomal gene *opgG*. *Yersinia pestis* lost this gene during its speciation from *Y. pseudotuberculosis*; hence, *opgG* can differentiate *Y. pestis* strains and *Y. pseudotuberculosis* strains (Quintard et al., 2015). For the internal control of flea DNA,

the very conservative 18S ribosomal gene was used for a wide application for any flea species in general. The objective of this study was to allow accurate and rapid identification of all *Y. pestis* strains from field-collected fleas; to discriminate *Y. pestis* from *Y. pseudotuberculosis*; and to differentiate *Y. pestis* strains in regard to their plasmid profiles.

2 | EXPERIMENTAL PROCEDURES

2.1 | Gene selection, primer, and probe design

Five genes were selected for developing the pentaplex real-time assay: *Yersinia pst*, *caf1*, *ypo2088*, and *opgG*, and flea 18S rRNA. Sequences of primer and probe used in the assay were designed for the current study except for those for *caf1* which were adapted from assays published (Liu et al., 2016) (Table 1). The TaqMan primers/probes design was conducted with the PrimerQuest program, Integrated DNA Technologies, Inc., Coralville, Iowa, https://www.idtdna.com/SciTools. Also, the *pla* gene of *Y. pestis* was tested using previously published primers and probe (Riehm et al., 2011) for comparison and validation purposes.

2.2 | Template DNA

All *Y. pestis* and *Y. pseudotuberculosis* DNA templates used in the study were obtained from BEI Resources or the University of Texas Medical Branch (UTMB) unless otherwise stated (Table 2). *Yersinia pestis*: CO96-3188 DNA was obtained from CDC Bacterial Diseases Branch. CO96-3188 is a fully virulent (Pgm+, pMT1+, pCD1+, and pPCP1+) North American biovar Orientalis strain (Eisen et al., 2006; Engelthaler, Hinnebusch, Rittner, & Gage, 2000). For the other 16 *Y. pestis* strains, both pMT1 and pPCP1 were present in 11 strains; both pMT1 and pPCP1 were absent in one strain; pPCP1was absent in four more strains (Table 2). *Yersinia pseudotuberculosis* was represented by strain B15 and four other strains (Table 2). Two *Y. enterocolitica* strains were included for specificity checking (Table 2). Also, we included 12 non-*Yersinia* species representing five *Bartonella* spp. (*B. bovis*, *B. doshiae*, *B. elizabethae*, *B. henselae*, and *B. rochalimae*), *Rickettsia rickettsii*, *Anaplasma phagocytophilum*, three *Borrelia* spp. (*B. burgdorferi*, *B. miyamotoi*, *B. mayonii*), *Trypanosoma cruzi*, and *Leptospira interrogans* for specificity testing (Table 3). The reason we included these species is that the transmission route of these bacteria is similar to the *Yersinia* species, either by fleas or other blood-feeding vectors (Bai et al., 2017; Brook et al., 2015; Livengood,

TABLE 1 Description of the genes selected for the pentaplex real-time PCR and the validation PCR, and the primers/probes sequences

Gene/location	Detection	Primer/probe sequences (5′-3′)	Product	Reference
pst/pPCP1	*Y. pestis* strains with pPCP1 plasmid presence	Forward: GCGAAGCAAACAGGATTTATTG Reverse: GAGGTGCTGTTCTCACTTTATC Probe: FAM-AGCCTCCTTCCCTCGAAGCATATAATACCC-BHQ−1	116 bp	This study
caf1/pMT1	*Y. pestis* strains with pMT1 plasmid presence	Forward: CCACTGCAACGGCAACTCTT Reverse: TGTAATTGGAGCGCCTTCCT Probe: QUAS705-TTGAACCAGCCCGCATCACTCTTACA-BHQ3	71 bp	Liu et al. (2016)
ypo2088/chromosome	All *Y. pestis* strains	Forward: TCGGCAACAGCTCAACACCT Reverse: ATGCATTGGACGGCATCACG Probe: CALRD610-CGCCCTCGAATCGCTGGCCAACTGC-BHQ2	107 bp	This study
opgG/chromosome	*Y. pseudotuberculosis* strains	Forward: ACGTGGGCGTGAATTCTCTCAA Reverse: GCCGTTGGGATCTCCACCAA Probe: QUAS670-CCTGCGCCCAAGCGCGTGGG-BHQ2	126 bp	This study
18S rRNA	Internal control for flea DNA	CAGATACCGCCCTAGTTC TAA Reverse: GTTTCAGCTTTGCAACCATAC Probe: HEX-TCATCGGAGGAACTTCGGCGGATC-BHQ−1	135 bp	This study
pla/pPCP1	*Y. pestis* strains with pPCP1 plasmid presence	Forward: Forward: GTAATAGGTTATAACCAGCGCTT Reverse: AGACTTTGGCATTAGGTGTG Probe: HEX-ATGCCATATATTGGACTTGCAGGCCAGT-BHQ1	232 bp	Rajanna et al. (2010) and Tomaso et al. (2003)

TABLE 2 Plasmid presence/absence in *Yersinia pestis* strains and PCR results in all strains of *Y. pestis*, *Y. pesudotuberculosis*, and *Y. enterocolitica* used to evaluate assay performance

			Plasmid presence		PCR			
ID	Strain	Source	pMT1	pPCP1	*ypo2088*	*caf1*	*pst*	*opgG*
Y. pestis								
NR-4709	KIM D23	BEI	Yes	No	pos	pos	neg	neg
NR-4713	A12 D6	BEI	Yes	No	pos	pos	neg	neg
NR-4706	KIM D2	BEI	Yes	Yes	pos	pos	pos	neg
NR-4717	Yokohama D11	BEI	Yes	Yes	pos	pos	pos	neg
NR-4719	Kimberley D13	BEI	Yes	Yes	pos	pos	pos	neg
NR-4727	K25 D80	BEI	Yes	Yes	pos	pos	pos	neg
NR-4705	KIM D19	BEI	Yes	Yes	pos	pos	pos	neg
NR-2645	KIM10+	BEI	Yes	No	pos	pos	neg	neg
NR-4708	KIM D22	BEI	Yes	No	pos	pos	neg	neg
NR-4714	Kuma D7	BEI	Yes	Yes	pos	pos	pos	neg
NR-4716	yokohama D10	BEI	Yes	Yes	pos	pos	pos	neg
NR-4718	Kimberley D12	BEI	Yes	Yes	pos	pos	pos	neg
NR-4721	K25 D72	BEI	Yes	Yes	pos	pos	pos	neg
NR-2718	PB6	BEI	No	No	pos	neg	neg	neg
NR-2719	Harbin 35	BEI	Yes	Yes	pos	pos	pos	neg
NR-2720	Nepal 516	BEI	Yes	Yes	pos	pos	pos	neg
Y. pseudotuberculosis					neg	neg	neg	pos
NR-4653	YPIII(p+)	BEI			neg	neg	neg	pos
NR-4651	IP2775	BEI			neg	neg	neg	pos
4284	4284	UTMB			neg	neg	neg	pos
6904	6904	UTMB			neg	neg	neg	pos
Y. enterocolitica								
NR-3064	Billups-1803-68	BEI			neg	neg	neg	neg
NR-3065	WA	BEI			neg	neg	neg	neg

TABLE 3 PCR results on non-*Yersinia* bacterial species for specificity testing of the pentaplex assay

Bacterial species	*YPO2088*	*caf1*	*pst*	*opgG*	18S rRNA
Anaplasma phagocytophilum	neg	neg	neg	neg	neg
Bartonella bovis	neg	neg	neg	neg	neg
Bartonella doshiae	neg	neg	neg	neg	neg
Bartonella elizabethae	neg	neg	neg	neg	neg
Bartonella henselae	neg	neg	neg	neg	neg
Bartonella rochalimae	neg	neg	neg	neg	neg
Borrelia bergdorferi	neg	neg	neg	neg	neg
Borrelia mayonii	neg	neg	neg	neg	neg
Borrelia miyamotoi	neg	neg	neg	neg	neg
Leptospira interrogans	neg	neg	neg	neg	neg
Rickettsia rickettsii	neg	neg	neg	neg	neg
Trypanosoma cruzi	neg	neg	neg	neg	neg

Hutchinson, Thirumalapura, & Tewari, 2020; Pawelczyk, Asman, & Solarz, 2019) or by consumption of contaminated water (Casanovas-Massana, Pedra, Wunder, Begon, & Ko, 2018). All DNA from these non-*Yesinia* species was obtained from CDC Bacterial Diseases Branch. DNA extracted from *Xenopsylla cheopis* fleas raised at the CDC colony were used as internal controls in the experiment. Finally,

DNA extracted from alcohol-preserved field-collected fleas were tested to check whether 18S rRNA amplifies DNA of flea species other than *X. cheopis*, and if interference or inhibition would occur while applying the assay to field samples. These fleas were collected from guinea pigs (*Cavia porcellus*) in Peru and represent three species, including *Pulex* sp. (n = 6), *Ctenocephalides felis* (n = 5), and *Tiamastus cavicola* (n = 5). A previous study showed they were negative for *Bartonella* species (María et al., 2019).

2.3 | Optimization of primer concentration

DNA of *Y. pestis* wild-type strain CO96-3188, *Y. pseudotuberculosis* strain B15 and *Xenopsylla cheopis* fleas raised at the CDC colony were used for the optimization of the primer concentration and identification of the limit of detection (LOD) of each target. Three DNA concentrations (100 pg/µl, 10 pg/µl, 1 pg/µl) of each template were used to test the targets independently with the corresponding primers at different concentrations, for example, DNA of *Y. pestis* strain CO96-3188 for *pst*, *caf1*, and *ypo2088*; DNA of *Y. pseudotuberculosis* strain B15 for *opgG*; and DNA of *Xenopsylla cheopis* flea for 18S rRNA. The optimal primer concentrations were tested using a primer concentration matrix with final concentrations of 200 nM–1000 nM. PCR (25 µl) contained 12.5 µl of 2× PerfeCta MultiPlex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD), primers (forward and reverse), probes at a standard concentration of 200 nM, and 5 µl of DNA template. Real-time PCR was performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA) with the following conditions: 95°C for 2 min followed by 45 cycles of 95°C for 15 s and 64°C for 60 s. Distilled water was always included as a negative control. A primer concentration that generates a sigmoidal shape of the amplification curve with a relatively lower *Ct* value compared to other primer concentration is considered an optimized concentration.

2.4 | Sensitivity testing and specificity assessment

Sensitivity was estimated using the limit of detection (LOD). The LOD for each target was determined and verified by comparing the primer/probe in a single PCR and in a pentaplex PCR simultaneously using the optimized primer concentration by testing six replicates of each DNA template with the following dilutions: 2 pg, 1 pg, 0.5 pg, 0.2 pg, 0.1 pg, 50 fg, 20 fg, 10 fg, 5 fg, 2 fg, 1 fg, 0.5 fg, 0.2 fg, 0.1 fg, 0.05 fg, and 0.02 fg. The lowest dilution that was amplified in all six replicates in both single PCR and pentaplex PCR, with a threshold *Ct* value <40 in any of the reactions was considered the LOD. To validate the result, an identical set of real-time PCR was performed using the widely used *pla* gene (Riehm et al., 2011).

The specificity of each primer/probe set was assessed by testing high concentration (500 pg) of nucleic acid from *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica* strains, and other 12 non-*Yersinia* microorganisms (Table 3) by running a pentaplex real-time PCR using the optimized primer concentrations.

2.5 | Evaluation of the pentaplex assay

To evaluate whether the pentaplex assay is applicable for real sample testing, sixteen fleas collected from guinea pigs in Peru and were preserved in alcohol before DNA extraction were first tested for the presence of flea DNA and any other DNA using the pentaplex PCR assay; then, the flea DNA was spiked with DNA of *Y. pestis* strain CO96-3788 or *Y. pseudotuberculosis* strain B15. For comparison purposes, final DNA concentrations of *Y. pestis* strain or *Y. pseudotuberculosis* matched those used for sensitivity testing. Pentaplex PCR was performed to test the *Y. pestis*- and *Y. pseudotuberculosis*-spiked flea DNA separately to check whether the flea DNA and alcohol would interfere or inhibit the amplification. Six replicates were tested for each concentration of each spiked DNA.

2.6 | Differentiation of *Yersinia* species and elucidation of *Y. pestis* strains with different plasmid profiles

Genomic DNA of *Y. pestis* strains with different plasmid profiles, *Y. pseudotuberculosis* strains, and *Y. enterocolitica* strains were tested with all four sets of *Yersinia* primer/probe (*pst*, *caf1*, *ypo2088*, and *opgG*) with a correspondent optimized concentration of each primer in a multiplex PCR setting.

2.7 | Application of the assay for testing field-collected fleas

To evaluate the utility of the pentaplex real-time PCR assay, we tested fleas (n = 102) collected from burrows in a prairie dog colony in Converse County, Wyoming in July 2019. A prairie dog die-off was noticed in the area in February 2019 and plague activity was suspected. Collected fleas belonged to three species: *Oropsylla hirsuta* (n = 87), *O. tuberculata* (n = 14), and *Thrassiis fotus* (n = 1). The fleas were stored in 70% ethanol and kept at 4°C before testing. DNA extraction was prepared from individual fleas using a Kingfisher platform (Life Technologies, Inc., Grand Island, NY) following the instructions provided by the manufacturer.

3 | RESULTS

3.1 | Optimization of primer concentration

The amplification was grouped tightly by the concentration of DNA for each target (Figure 1). The higher the DNA concentration, the earlier the amplification started (lower *Ct* value). Primer concentrations

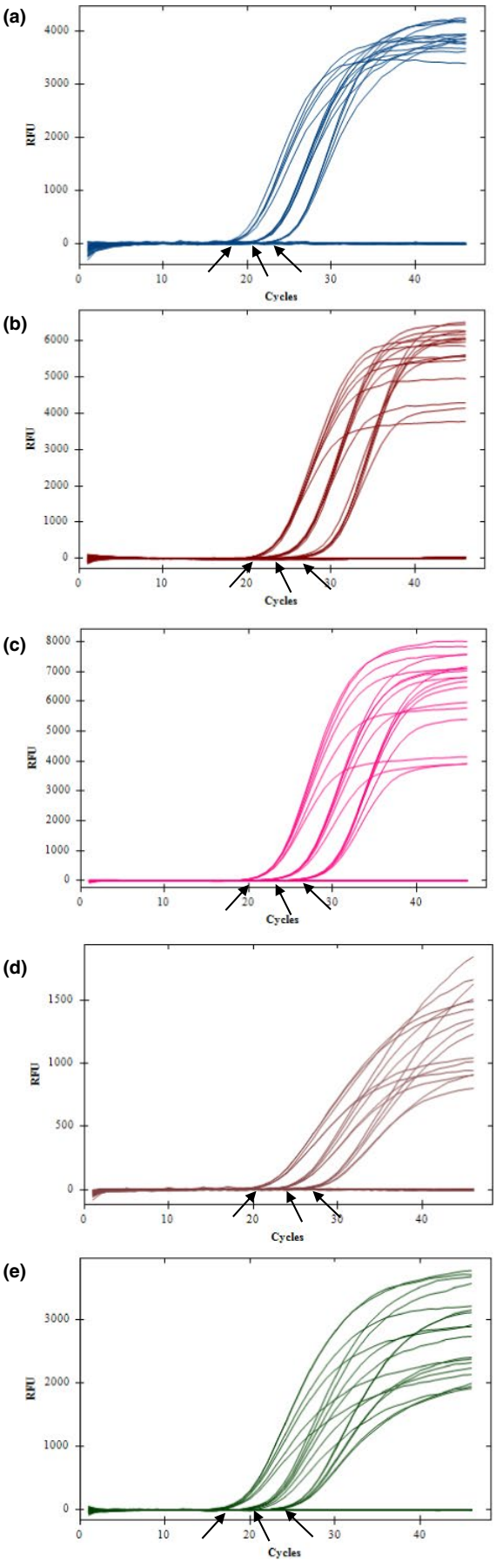

FIGURE 1 Amplification of DNA (100 pg/μl, 10 pg/μl, 1 pg/μl) with different primer concentrations ranging from 200 nM to 1000 nM. The amplification was grouped tightly by the concentration of DNA. The higher the DNA concentration, the earlier the amplification started (lower *Ct* value). Primer concentrations had no significant differences in the amplification efficiencies with a very small change of *Ct* value. The three arrows indicated the DNA concentration for each group was 100 pg/μl, 10 pg/μl, and 1 pg/μl from left to right. A. *pst*; B. *caf1*; C. *ypo2088*; D. *opgG*; E. 18S rRNA

TABLE 4 Optimization of primer/probe concentration and identification of limit of detection (LOD) of each gene used in the pentaplex assay

Gene	Primer concentration (nM)	Probe concentration (nM)	LOD (fg)
pst	200 nM	200 nM	1
caf1	200 nM	200 nM	50
ypo2088	200 nM	200 nM	100
opgG	600 nM	200 nM	5
18S rRNA	500 nM	200 nM	1

had no significant differences in the amplification efficiencies with the change of *Ct* value <1. For example, for *pst* gene, the *Ct* value ranged 22.14–22.82 with primer concentrations of 200 nM–1000 nM when the DNA concentration was 100 pg. Based on our criteria (generating a sigmoidal shape of the amplification curve with lower *Ct* value), the optimized primer concentration is 200 nM for all *Y. pestis*-associated targets (*pst*, *caf1*, and *ypo2088*) primers, 600 nM for *opgG* primers, and 500 nM for 18S rRNA primers (Table 4).

3.2 | Sensitivity and specificity

All targets showed extremely high sensitivity (Figure 2). Among the three *Y. pestis*-specific targets, *pst* was the most sensitive with a LOD of 1 fg per reaction, followed by *caf1* (LOD 50 fg) and *ypo2088* (LOD 100 fg) (Table 4). The *Y. pseudotuberculosis*-specific *opgG* also showed very high sensitivity, with a LOD of 5 fg per reaction; the internal control target 18S rRNA for flea DNA had a LOD of 1 fg. Standard curves showed the *Ct* values were correlated to the DNA concentration for each target (Figure 2).

To validate the above testing results, a PCR was performed using the widely used *pla* gene. The PCR detected 1 fg *Y. pestis* DNA as the LOD, which is identical to that of *pst*, suggesting that *pst* gene and *pla* gene are equivalent in terms of the sensitivity.

All primer/probe sets showed 100% specificity to the targets accordingly. DNA of *Y. pestis* strain CO96-3188 was positive to *pst*, *caf1*, and *ypo*2088 but negative to *opgG* and 18S rRNA; DNA of *Y. pseudotuberculosis* strain B15 was positive to *opgG* but negative to all other genes; DNA of *X. cheopis* fleas was positive to 18S rRNA but negative to all other genes; no amplification to any of the genes was observed in *Y. enterocolitica* (Table 2) and any of the other 12 species of non-*Yersinia* microorganisms (Table 3).

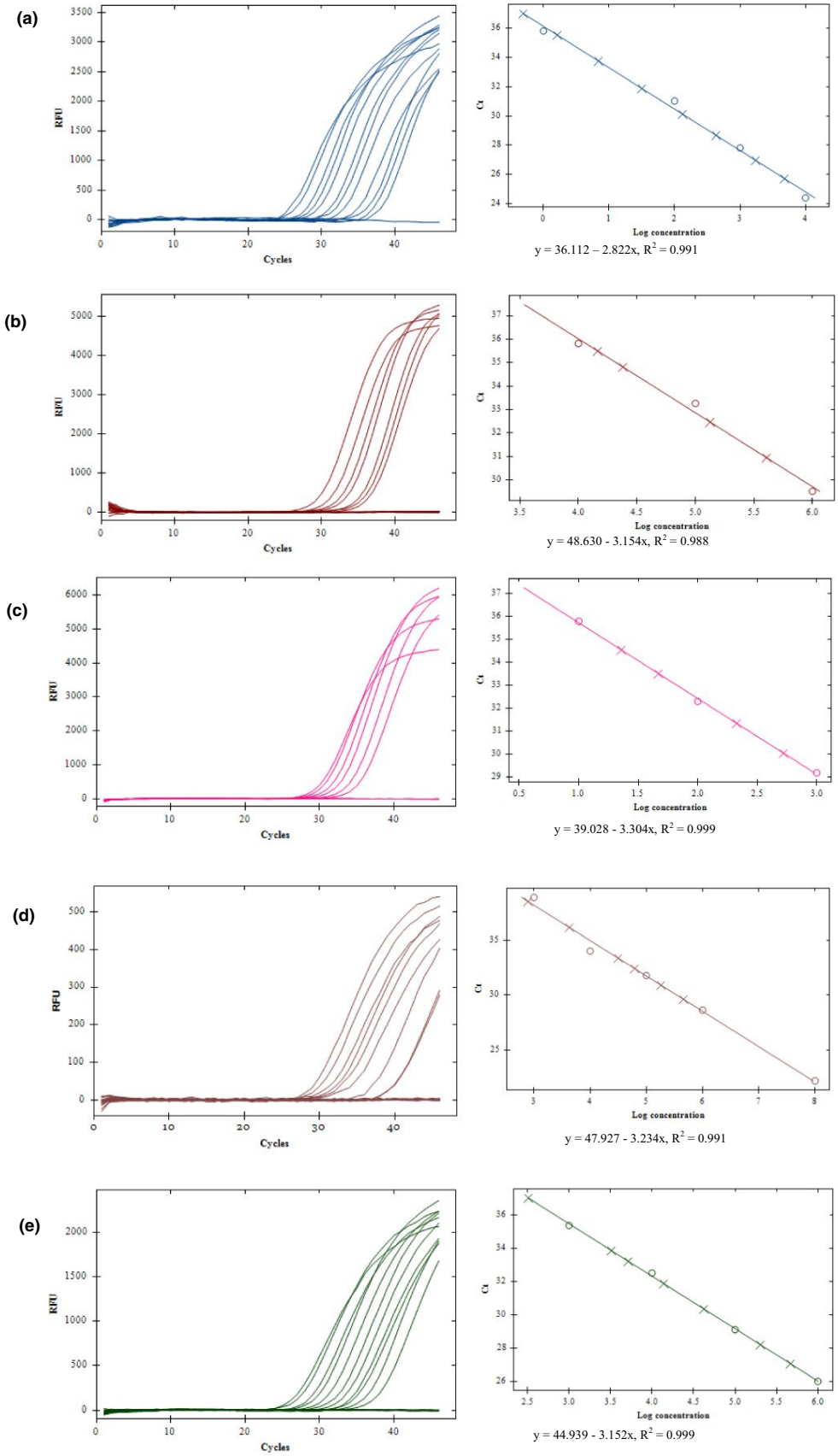

FIGURE 2 Sensitivity testing. Amplification (left panel) and standard curve (right panel) of each target with DNA dilutions 2 pg, 1 pg, 0.5 pg, 0.2 pg, 0.1 pg, 50 fg, 20 fg, 10 fg, 5 fg, 2 fg, 1 fg, 0.5 fg, 0.2 fg, 0.1 fg, 0.05 fg, and 0.02 fg. *Ct* values were correlated to the DNA concentration for each target. A. *pst*; B. *caf1*; C. *ypo2088*; D. *opgG*; E. 18S rRNA

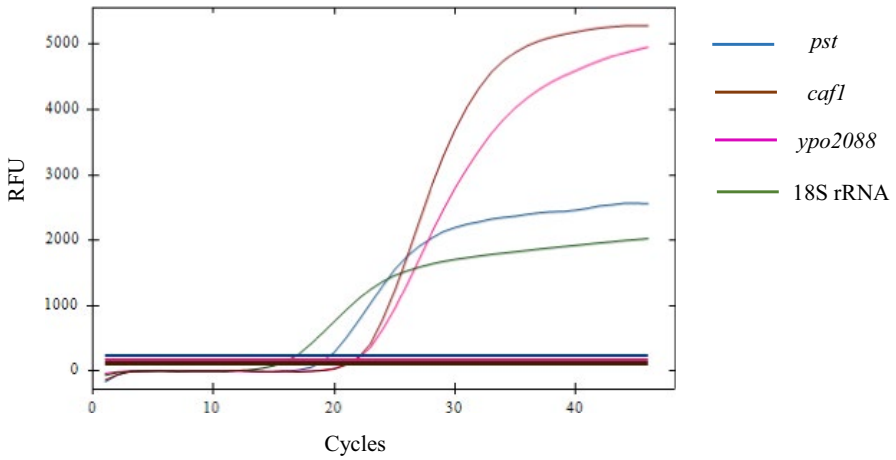

FIGURE 3 By performing the pentaplex real-time PCR, one *Oropsylla tuberculata* flea collected from prairie dog burrows after a prairie dog die-off in Wyoming in 2019 was positive to *pst*, *caf1*, *ypo2088*, and 18S rRNA, with *Ct* value 19.65, 21.08, 21.74, and 15.06, respectively, suggesting a local *Y. pestis* transmission

3.3 | Differentiation between *Y. pestis* strains and other *Yersinia* species

Testing of all *Yersinia* DNA (16 *Y. pestis*, 4 *Y. pseudotuberculosis*, and 2 *Y. enterocolitica*) with the four primers/probe sets of *Yersinia* targets demonstrated that the two *Y. enterocolitica* were negative to all targets; the four *Y. pseudotuberculosis* were positive to *Y. pseudotuberculosis*-specific *opgG* but to none of the *Y. pestis*-specific genes; the 16 *Y. pestis* DNA samples were positive to different *Y. pestis*-specific targets, which was associated with their plasmid profile (BEI resource): All strains were positive to the chromosomal gene *ypo2088*; the strain with pMT1 and pPCP1 absent was negative to both *caf1* and *pst*; four strains with pPCP1 absence were negative to *pst*. None of the *Y. pestis* strains were positive to *opgG* (Table 2).

3.4 | Pentaplex assay evaluation

Testing of 16 DNA of fleas collected from guinea pigs in Peru showed all fleas were amplified with 18S rRNA, with *Ct* values ranging 17.62–31.08 for the *Pulex* fleas; 20.54–34.33 for the *Ctenocephalides felis* fleas; and 21.13–31.05 for the *Tiamastus cavicola* fleas, showing that 18S rRNA worked equally for different flea species. All fleas were negative to *ypo2088*, *pst*, *caf1*, and *opgG*.

Testing of *Y. pestis* DNA-spiked flea DNA showed that *ypo2088*, *pst*, *caf1*, and 18S rRNA were amplified, with LOD 1 fg, 50 fg, 100 fg for *ypo2088*, *pst*, *caf1*, respectively, all of which were identical to the LOD observed in the sensitive testing. All DNA in this group was negative to *opgG*.

Testing of *Y. pseudotuberculosis* DNA-spiked flea DNA showed that *opgG* and 18S rRNA were amplified with LOD 5 fg for *opg*. The LOD was identical to that observed in the sensitive testing. All DNA in this group was negative to *ypo2088*, *pst*, and *caf1*.

3.5 | Detection of *Yersinia pestis* in field-collected fleas

By performing the pentaplex real-time PCR, all 102 fleas collected from prairie dog burrows several months after a prairie dog die-off was reported in Converse County, Wyoming in July 2019 were positive to 18S rRNA, with *Ct* value ranging 13.93–27.91. Among these, one *Oropsylla tuberculata* flea (*Ct* value 15.06 for 18S rRNA) was positive to all three *Y. pestis* genes, with *Ct* value 19.65, 21.08, and 21.74 for *pst*, *caf1*, and *ypo2088*, respectively (Figure 3). The other 101 fleas were negative to any of the three *Y. pestis* genes. No *Y. pseudotuberculosis* was detected in any fleas all of which was negative to *opgG*.

4 | DISCUSSION

It is of supreme importance using analytical assays for *Y. pestis* which can show the presence of various targets located on the chromosome and plasmids. A pentaplex real-time PCR assay developed in this study included three *Y. pestis*-specific genes (*pst*, *caf1*, and *ypo2088*), one *Y. pseudotuberculosis* gene (*opgG*), and 18S rRNA as an internal control of flea DNA if the assay is to be applied to test fleas. Multiplex PCR assays for *Y. pestis* are not uncommon (Stewart et al., 2008; Tomaso et al., 2003; Woron et al., 2006) but, to our knowledge, this is the first to integrate five biologically meaningful targets.

Many assays have used the *pla* gene that is located on the pPCP1 plasmid for the detection of *Y. pestis* due to its high sensitivity with the presence of high copy numbers (150–200 copies) (Parkhill et al., 2001). Finding a homolog of *pla* in other bacteria or rodents (Armougom et al., 2016; Giles et al., 2016; Hänsch et al., 2015) has raised a concern of false identification, particularly when considering the frequency of flea-feeding on rodent hosts. Instead of using *pla*, we used *pst* which is also located on pPCP1. Our

results demonstrated *pst* is equally as sensitive as the *pla* gene with the same LOD (1 fg per reaction). Such results suggest that *pst* is a plausible diagnostic marker to replace *pla*. Like *pla*, the pMT1-located *caf1* is another commonly used target for *Y. pestis* detection in multiple studies (Norkina et al., 1994; Stewart et al., 2008; Tomaso et al., 2003; Woron et al., 2006). Similar to other reports, our results also showed that *caf1* is highly sensitive, but somewhat less sensitive compared to *pst*. It is known that *caf1* is present in only about two copies per bacterium (Parkhill et al., 2001), explaining the differences in sensitivity between the two genes.

We did not use any pCD1-located genes in this study. This is because pCD1 is possessed by all currently recognized pathogenic *Yersinia* species. Detection based on pCD1-located genes does not necessarily indicate the species of the presenting organism as previous studies have shown detection of *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* using pCD1-located *lcrV* or other targets (Stewart et al., 2008). Further, using a pCD1-located gene may not be as efficient compared to pPCP1- and pMT1-located genes because the pCD1 plasmid is absent in many more *Y. pestis* strains compared to pPCP1 and pMT1 plasmids in nature due to the intrinsic variability of pCD1 plasmids in *Y. pestis* (Stewart et al., 2008).

Unlike plasmids, chromosomal genes are not likely to be lost during laboratory cultivation or in nature; thus, chromosomal genes are more reliable targets than plasmid genes. Nevertheless, specific identification can be difficult if substantial genomic similarities are shared among multiple species. In the past, researchers have used the *inv* gene, the *entF3* gene, the *wzz* gene, and the 16S rRNA gene in the identification of *Y. pestis* (Matero et al., 2009; Neubauer et al., 2000; Tomaso et al., 2003; Woron et al., 2006). However, non-specific amplification has been observed with these genes. All of them not only amplify *Y. pestis*, but also *Y. pseudotuberculosis*. In our assay, we used *ypo2088* and *opgG*, two chromosomal genes that are designed for *Y. pestis* and *Y. pseudotuberculosis*, respectively. Our results demonstrated that all *Y. pestis* strains were detected by *ypo2088* and *Y. pseudotuberculosis* strains by *opgG*. Both *ypo2088* and *opgG* showed high sensitivity (LOD 100 fg and 5 fg respectively) with 100% specificity to their targeted *Yersinia* species. Because *opgG* has been lost in *Y. pestis*, this makes it ideal for the differentiation of *Y. pestis* and *Y. pseudotuberculosis*. With a combined analysis of *Y. pestis*-specific and *Y. pseudotuberculosis*-specific assays, any detection will be elucidated by the exclusion of one from the other. No amplification was observed with *Y. enterocolitica* to either gene. Also, several non-*Yersinia* microorganisms were tested using the assay for specificity assessment with no amplification observed in any of them. Our results demonstrated all primer/probe used in the assay was 100% specific.

Using the developed assay, we tested *Y. pestis* strains with known plasmid profiles (BEI Resource). All strains were successfully identified by *pst* and *caf1* in accordance with the presence of the pPCP1 plasmid and pMT1 plasmid with 100% specificity.

Laboratory-based PCR assays may struggle with different problems in reality, such as low concentration, and inhibition. To observe if that would be a case for our pentaplex assay, we spiked *Y. pestis* DNA and *Y. pseudotuberculosis* DNA into DNA of fleas that were collected from guinea pigs in Peru and preserved in alcohol before DNA extraction. The results showed the LOD of each primer/probe set on the *Y. pestis*- and *Y. pseudotuberculosis*-spiked flea DNA was the same as that observed in the sensitive testing, suggesting no interference or inhibition from fleas or alcohol on the amplifications.

We applied the assay to test 102 fleas that were collected from prairie dog burrows where prairie dog die-offs were reported 5 months before our field investigation. All fleas were successfully amplified with the 18S rRNA. One out of the 102 fleas was positive for *Y. pestis* by all three *Y. pestis*-specific genes (*pst*, *caf1*, and *ypo2088*). The result confirmed the local transmission of *Y. pestis* in the prairie dog colony. Since the flea collection did not occur until 5 months afterward the prairie dog die-off, the low number of positive samples may have suggested plague activity had diminished gradually after the epizootic peak. Flea numbers were noticeably lower compared to an earlier investigation conducted in the area (around 3 months after the die-off). This indicated that most fleas may have died of starvation. The extremely low number of positive fleas was reasonably expected (Tripp, Gage, Montenieri, & Antolin, 2009). Not surprisingly, no *Y. pseudotuberculosis* was detected in any of these fleas.

In conclusion, the pentaplex real-time PCR assay developed in this work is highly sensitive and 100% specific in the detection and differentiation of *Y. pestis* and *Y. pseudotuberculosis*. Further, the assay allows one for the elucidation of the presence/absence of *Y. pestis* pPCP1 plasmid and pMT1 plasmid in a particular strain, which can be applied to testing fleas and other field-collected materials when the plague is suspected.

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CONFLICT OF INTEREST

The conclusions, findings, and opinions expressed by authors contributing to this journal do not necessarily reflect the official position of the U.S. Department of Health and Human Services, the Public Health Service, the Centers for Disease Control and Prevention, or the authors' affiliated institutions.

AUTHOR CONTRIBUTIONS

Ying Bai: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (equal); supervision (lead); validation (lead); visualization (lead); writing-original draft (lead); writing-review and editing (lead). **Vladimir Motin:** Conceptualization (lead); methodology (lead); writing-review and editing (equal). **Russell E. Enscore:** Investigation (lead); writing-review and editing (supporting). **Lynn Osikowicz:** Investigation (equal). **Maria Rosales Rizzo:** Investigation (equal). **Andrias Hojgaard:** Methodology (equal). **Michael Kosoy:** Conceptualization (equal); writing-review and editing (equal). **Rebecca J. Eisen:** Writing-review and editing (equal).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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