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Detection of viable *Yersinia pestis* by fluorescence in situ hybridization using peptide nucleic acid probes

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ABSTRACT

A successful method has been developed for the detection of live *Yersinia pestis*, the plague bacillus, which incorporates nascent RNA synthesis. A fluorescent in situ hybridization (FISH) assay using peptide nucleic acid (PNA) probes was developed specifically to differentiate *Y. pestis* strains from closely related bacteria. PNA probes were chosen to target high copy mRNA of the *Y. pestis caf1* gene, encoding the Fraction 1 (F1) antigen, and 16S ribosomal RNA. Among *Yersinia* strains tested, PNA probes *Yp*-16S-426 and *Yp*-F1-55 exhibited binding specificities of 100% and 98%, respectively. *Y. pestis* grown in the presence of competing bacteria, as might be encountered when recovering *Y. pestis* from environmental surfaces in a post-release bioterrorism event, was recognized by PNA probes and neither hybridization nor fluorescence was inhibited by competing bacterial strains which exhibited faster growth rates. Using fluorescence microscopy, individual *Y. pestis* bacteria were clearly differentiated from competing bacteria with an average detection sensitivity of 7.9×10^3 cells by fluorescence microscopy. In the current system, this would require an average of 2.56×10^5 viable *Y. pestis* organisms be recovered from a post-release environmental sample in order to achieve the minimum threshold for detection. The PNA-FISH assays described in this study allow for the sensitive and specific detection of viable *Y. pestis* bacteria in a timely manner.

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1. Introduction

Yersinia pestis, the etiologic agent of plague, is recognized as a devastating bacterium that has affected the course of human civilization and continues to infect individuals in plague endemic regions throughout the world. The potential public health threat of *Y. pestis* is demonstrated by its global reach, as endemic foci can be found on all the inhabited continents except Australia (Perry and Fetherston, 1997). Naturally occurring *Y. pestis* infections have been reported in more than 200 species of wild rodents and over 220 species of fleas (Anisimov et al., 2004; Gage and Kosoy, 2005; Pollitzer, 1954a,b). The widespread prevalence of natural disease foci has raised concerns over the opportunity for bioterrorist manipulation of this disease agent, the potential for intentional release events, and the appropriate public health preparation and response to such events (Inglesby et al., 2000; Riedel, 2005). The most common route for human *Y. pestis* infection is through flea-borne transmission (Barnes, 1982). However, pneumonic plague, which can be transmitted from person to person through the spread of infectious respiratory droplets, does occur sporadically in developing countries (Begier et al., 2006) and an aerosolized preparation of *Y. pestis* would be the likely mechanism of delivery in the event of an intentional release (CDC, 1994; Inglesby et al., 2000; Riedel, 2005). Such a release of bacteria into a highly populated area potentially could result in thousands of primary pneumonic infections, increase the risk of person-to-person transmission, and generate public health concerns regarding post-release exposures and resumed habitation of the targeted location. Existing molecular detection methods such as *Y. pestis*-specific multiplex real-time PCR (rt-PCR) (Stewart et al., 2008; Tomaso et al., 2003; Woron et al., 2006), rt-PCR targeting multiple bioterrorism agents (Skottman et al., 2007; Varma-Basil et al., 2004) and *Y. pestis* F1 antigen based immunoassays (Chanteau et al., 2003; Chu, 2000) likely would be employed to identify the pathogen as well as points of release and the dispersal patterns of the bacterium following release. While robust and exquisitely sensitive, these rapid and often field deployable assays for the presumptive identification of *Y. pestis* are limited to the detection of DNA and protein signatures of the plague bacillus but cannot determine viability. Since isolation of *Y. pestis* requires incubation periods of 36–48 h or longer if commercially available selective media are used (Ber et al., 2003; Chu, 2000), a method that rapidly identifies viable bacteria would greatly assist in post-release recovery efforts.

To that end, we have designed a protocol that will amplify detection of live plague bacilli, testing for high copy RNA synthesis through fluorescence in situ hybridization (FISH) with fluorescein-tagged peptide

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nucleic acid (PNA) probes (Perry-O'Keefe et al., 2001) within 8 h, start to finish.

FISH assays are based on the binding of nucleic acid probes to specific regions of RNA (Amann et al., 1997). PNA molecules are DNA mimics, in which the negatively charged sugar-phosphate backbone of DNA is replaced by an achiral, neutral polyamide backbone composed of repetitive units of *N*-(2-aminoethyl) glycine to which the nucleobases are attached via methylene carbonyl linkers (Nielsen et al., 1991; Nielsen et al., 1994). PNA probes obey Watson–Crick base-pair rules (Egholm et al., 1993), are generally much shorter than nucleic acid probes due to their enhanced binding properties and stability, are not degraded by proteases or nucleases (Demidov et al., 1994), and are

Table 1
Organism, strains, and fluorescent in situ hybridization (PNA-FISH) results for PNA probes *Yp*-16S-426 (16S) and *Yp*-F1-55 (F1)

Organism	Strain	PNA-FISH[a]		F1 DFA[f]	*Y. pestis* biotype	Source
		16S	F1		Y. *pseudotuberculosis* serotype	
Y. pestis	CO05-2175-34	+	+	+	Orientalis	CDC-FTC[b]
Y. pestis	CO04-2349	+	+	+	Orientalis	CDC-FTC
Y. pestis	CO99-0296	+	+	+	Orientalis	CDC-FTC
Y. pestis	CO96-2375	+	+	+	Orientalis	CDC-FTC
Y. pestis	WY85-5562-753	+	+	+	Orientalis	CDC-FTC
Y. pestis	AZ94-666	+	+	+	Orientalis	CDC-FTC
Y. pestis	NM05-2428-58	+	+	+	Orientalis	CDC-FTC
Y. pestis	KS97-2100-363	+	+	+	Orientalis	CDC-FTC
Y. pestis	TX04-1175-122	+	+	+	Orientalis	CDC-FTC
Y. pestis	A1122	+	+	+	Orientalis	CDC-FTC
Y. pestis	YOSEMITE 104	+	+	+	Orientalis	CDC-FTC
Y. pestis	CALIFST 268-T	+	+	+	Orientalis	CDC-FTC
Y. pestis	P EXU2	+	+	+	Orientalis	CDC-FTC
Y. pestis	PB6	+	+[e]	−	Orientalis	CDC-FTC
Y. pestis	HARBIN 35	+	+	+	Medievalis	CDC-FTC
Y. pestis	KZ99-3787	+	+	+	Medievalis	CDC-FTC
Y. pestis	PY,H1,R3	+	+	+	Medievalis	CDC-FTC
Y. pestis	GA02-5370	+	+	+	Medievalis	CDC-FTC
Y. pestis	FN84-0042	+	+	+	Medievalis	CDC-FTC
Y. pestis	PKR-133	+	−	−	Medievalis	CDC-FTC
Y. pestis	Nepal 516	+	+	+	Antiqua	CDC-FTC
Y. pestis	15–38	+	+	+	Antiqua	CDC-FTC
Y. pestis	KZ99-3829	+	+	+	Antiqua	CDC-FTC
Y. pestis	TZ02-1459	+	+	+	Antiqua	CDC-FTC
Y. pestis	Pestoides B	+	+	+	Medievalis	CDC-FTC
Y. pestis	Pestoides D	+	+	+	Medievalis	CDC-FTC
Y. pestis	Pestoides G	+	+	+	Antiqua	CDC-FTC
Y. pestis	15–39 (Pestoides E)	+	+	+	Antiqua	CDC-FTC
Y. pseudotuberculosis	I-A	+	−	−	IA	CDC-FTC
Y. pseudotuberculosis	NJ01-0572	+	−	−	Unknown	CDC-FTC
Y. pseudotuberculosis	713429	+	−	−	III	CDC-FTC
Y. pseudotuberculosis	722080	+	−	−	IB	CDC-FTC
Y. pseudotuberculosis	730315	+	−	−	I	CDC-FTC
Y. pseudotuberculosis	760133	+	−	−	III	CDC-FTC
Y. pseudotuberculosis	760989	+	−	−	IA	CDC-FTC
Y. enterocolitica	WA7048 YM2	−	−	−		CDC-FTC
Y. enterocolitica	ATCC 23715	−	−	−		ATCC[c]
Y. ruckeri	ATCC 29473	−	−	−		ATCC
Y. intermedia	ATCC 29909	−	−	−		ATCC
Y. aldovae	ATCC 51336	−	−	−		ATCC
Y. rohdei	ATCC 43380	−	−	−		ATCC
Y. bercovieri	ATCC 43970	−	−	−		ATCC
Y. frederiksenii	ATCC 29912	−	−	−		ATCC
E. coli	DH5α	−	−	NA		CDC-FTC
E. coli	ATCC 25922	−	−	NA		ATCC
E. coli	ATCC 8729	−	−	NA		ATCC
Acinetobacter calcoaceticus	ATCC 33951	−	−	NA		ATCC
Acinetobacter lwoffi		−	−	NA		PVH[d]
Bacillus cereus	BAA-512	−	−	NA		ATCC
Bacillus sp.		−	−	NA		PVH
Proteus vulgaris		−	−	NA		PVH
Serratia marscesens	ATCC 8100	−	−	NA		ATCC
Staphylococcus aureus		−	−	NA		PVH
Streptococcus pneumoniae		−	−	NA		PVH
Salmonella typhi		−	−	NA		PVH
Pseudonomas aeruginosa		−	−	NA		PVH
Candida keifer	ATCC 2512	−	−	NA		ATCC

NA — Not Applicable.
[a] +, PNA probe binding; −, no probe binding.
[b] Isolates from the reference collection of the Centers for Disease Control and Prevention, Fort Collins, CO.
[c] ATCC, American Type Culture Collection, Manassas, VA.
[d] Clinical isolates from Poudre Valley Hospital, Fort Collins, CO.
[e] Weakly positive.
[f] +, F1antigen present or −, absent, as detected using FITC-conjugated, anti-F1, rabbit polyclonal antibodies.

capable of more easily penetrating untreated bacterial cell walls than their negatively charged nucleic acid counterparts (Koppelhus and Nielsen, 2003). Having a neutral charge, PNA probes are less susceptible to fluctuations in ionic strength resulting in consistent hybridization performance (Nielsen et al., 1994). Moreover, when PNA probes encounter base-pair mismatches during hybridization, the melting temperature (*T*m) is noticeably reduced. As described by Jensen et al. (1997), *T*m values of PNA×RNA and to a lesser extent PNA×DNA duplexes are more sensitive to mismatches than DNA×DNA duplexes (Giesen et al., 1998; Takiya et al., 2004). Thus, PNA probes enable a higher level of sequence discrimination, to the level of single nucleotide mismatches, than DNA probes (Ray and Norden, 2000). This increased specificity, along with the stability of PNA oligomers and their strong binding affinity, further emphasizes their utility as highly efficient compounds for effective mRNA and rRNA detection (Nielsen et al., 1994). These characteristics help to define PNA-FISH as an ideal means for the *in vitro* detection of proliferating plague bacteria. Our results show that PNA-FISH is a specific and effective means for detection of viable *Y. pestis* and complements existing molecular and immunological techniques designed

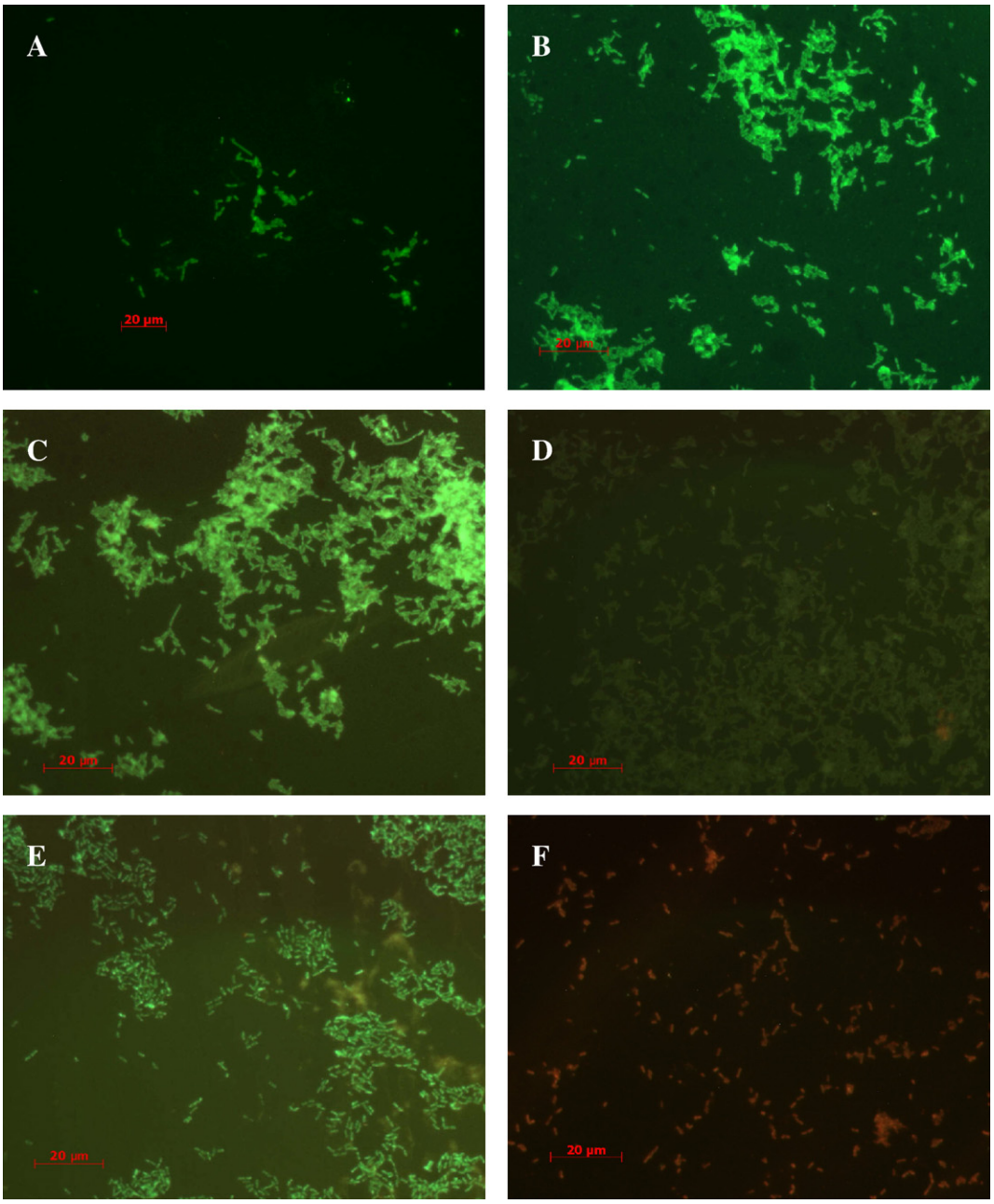

Fig. 1. PNA-FISH slide images of cells hybridized with FITC-conjugated 16SrRNA PNA probe *Yp*-16S-426 (Panels A–C) or *caf1* probe *Yp*-F1-55 (Panels D–F). Cells which retained apple green fluorescence when observed through the Texas Red dual band filter were scored as positive. Panel A, *Yersinia pestis* Nepal 516 imaged using a Texas Red dual band filter. Panels B and C, images of *Yersinia pseudotuberculosis* I-A observed using a FITC and Texas Red dual band filters, respectively. PNA-FISH images in Panels D and E compare *Y. pseudotuberculosis* I-A (negative) to *Y. pestis* WY85-5562-753 (positive), respectively, using cells grown at 35 °C, hybridized with PNA probe *Yp*-F1-55, and observed using the Texas Red dual band filter. Panel F, *Y. pestis* WY85-5562-753 grown at 25 °C and observed using the Texas Red dual band filter. Brown to reddish-brown cells (Panel F) signify the absence of detectable *caf1* mRNA due to growth at a *caf1* non-inducing temperature. All slides were observed under 630× total magnification and images were captured using a Zeiss Axiocam MRc5 scope-mounted digital camera. Scale bars within each panel are shown in red and indicate distance in microns (µm).

Table 2
PNA probe limits of detection (LOD) for *Y. pestis* strains and average fold increase in cell density compared to initial inoculum following exponential growth period for *Y. pestis* and non-*Y. pestis* strains

Strain	Mixed culture trial number (total number of trials)[a]	Average fold increase in cell density[b]	*Y. pestis* LOD[c]	
			Yp-16S-426	*Yp*-F1-55
Y. pestis A1122	1,2,7 (3)	6.6	3.16×10^4	3.16×10^3
Y. pestis Yosemite 104	4,6,12 (3)	7.7	2.67×10^3	2.67×10^3
Y. pestis Harbin 35	5,8,9 (3)	4.0	8.86×10^3	8.86×10^3
Y. pestis CALIFST 268-T	3,10,11 (3)	6.4	2.64×10^3	2.64×10^3
E. coli 8729	1–6,8 (7)	49.3	NA	NA
Pseudomonas aeruginosa	6,7,8 (3)	34.8	NA	NA
Acinetobacter calcoaceticus	3,4,5 (3)	27.9	NA	NA
Bacillus sp.	7,9–12 (5)	426.7	NA	NA
Salmonella typhi	2,9,10 (3)	47.5	NA	NA
Serratia marcescens	1,11,12 (3)	37.7	NA	NA

NA — Not applicable.
[a] For each mixed batch detection trial, one strain of *Y. pestis* was grown together with two non-*Y. pestis* strains. 12 independent trials were conducted and each strain was used in a minimum of three trials. The trial numbers (1–12) in which each strain was used are indicated.
[b] Average fold increase in cell density is defined as the average number of colony forming units (cfu) at the end point of each growth experiment divided by the average cfu for the initial inoculum of individually grown strains.
[c] Values represent cells per slide based on colony counts from 10-fold serial dilutions of individually grown *Y. pestis* strains subsequently used in mixed culture trials. LODs for each strain are from a single representative experiment.

to detect nucleic acid and protein signatures of this potential bioterrorism agent.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Bacterial and yeast stock strains were maintained at −80 °C in heart infusion broth (HIB) (Becton, Dickinson and Co., Sparks, MD) supplemented with 10% glycerol. Cultures were inoculated from frozen stock onto 6% sheep blood agar (SBA) (Centers for Disease Control and Prevention, Atlanta, GA) and grown at 35 °C for both the F1 and 16S PNA probe experiments (Table 1). Individual colonies were picked and resuspended in 0.5 mL HIB. A 50 µl aliquot of this suspension was added to 4 mL of fresh HIB and incubated at 35 °C and 175 rpm for 4.5 h. *Y. pestis* strain WY85-5562-753 was also grown in HIB at 25 °C and 175 rpm for 4.5 h as a negative control for F1 expression and F1 PNA probe binding (Fig. 1F). Bacterial strains were grown individually as well as in a mixed culture consisting of one *Y. pestis* strain and two non-*Y. pestis* strains. Colony counts and ratios of non-*Y. pestis* to *Y. pestis* strains for initial inocula and exponentially growing cultures were determined by plating 10-fold serial dilutions of individual bacterial strains on SBA plates and incubating at 35 °C for 24–48 h. Initial inocula for *Y. pestis* and non-*Y. pestis* averaged 2×10^6 cfu and 3.5×10^6 cfu per 50 µl aliquot, respectively. Average fold increase in bacterial cell density between initial inocula and exponentially growing cultures for *Y. pestis* strains and non-*Y. pestis* strains as well as the strains used in each mixed culture trial are shown in Table 2.

2.2. Cell viability determination

To assess the ability of probes *Yp*-16S-426 and *Yp*-F1-55 to report from viable versus non-viable target bacteria, *Y. pestis* strains A1122 and CO04-2349 were grown at 35° for 3.5 h then heat-killed at 55 °C for a period of 60 min. Aliquots of heat inactivated cells were subjected to PNA-FISH hybridization assays as described below. Viability of heat-killed *Y. pestis* was determined by plating on SBA and incubating at 35 °C for five days. Non-viability was confirmed after five days of incubation.

2.3. RNA target selection

Target sites chosen for PNA probe binding were the16S ribosomal RNA (16SrRNA) and *caf1* mRNA encoding the Fraction 1 (F1) capsular protein for *Yersinia* genus and species differentiation, respectively. BLAST searches of the non-redundant databases, as well as the completed and unfinished microbial genomes of the National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov/blastn) were performed to identify a conserved region of the *Yersinia* 16S rRNA gene sequences and a *Y. pestis* species-specific region of caf1 mRNA (Altschul et al., 1990; McGinnis and Madden, 2004). The 16S rRNA and *caf1* sequence target sequences, also were selected on the basis of their predicted secondary structure using the Mfold algorithm (*mfold* server: 1995–2005, Michael Zuker, Rensselaer Polytechnic Institute www.bioinfo.rpi.edu/applications/mfold/old/rna/). The target sequences were predicted to occur as part of putative RNA stem structures with minimal overlap with loops.

2.4. Selection of PNA probes

The PNA probes designed for this study were 15 and 17 nucleotides in length for *caf1* and 16S rRNA, respectively. PNA probes were chosen based on their predictability to preferentially bind to the RNA targets described above for the detection of *Y. pestis* at the genus and species levels with optimum sensitivity and specificity. The F1 antigen probe selection complements a region near the 5-prime end of the *caf1* mRNA sequence unique to the pMT plasmid of *Y. pestis*. The *caf1*gene encodes the 15.5 kDa F1 antigen whose expression is optimal at 36–37 °C and greatly reduced or undetectable at decreasing incubation temperatures from 33 °C to 25 °C (Brubaker, 1972; Pollitzer, 1954a,b; Simpson et al., 1990). For this reason, cultures used for FISH assays were incubated at 35–37 °C. The 16S rRNA probe was designed to preferentially bind to high copy 16S rRNA of *Y. pestis* and *Y. pseudotuberculosis* though homologous binding sites also were found in genome sequences of *Y. enterocolitica*, *Y. similis*, and *Y. frederiksenii*.

Sequence analysis was performed using DNAMAN version 5.2.10 (Lynnon BioSoft, Vaudreuil, Quebec) and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequences were chosen that were predicted to bind to *Yersinia* RNA with high specificity while meeting the requirements for a successful PNA probe. The latter considerations include nucleotide sequences having low self-complementary binding and minimal consecutive pyrimidine stretches. As with the RNA target sequences, uniqueness of the probes was determined using Genome BLAST searches with parameters modified for short input sequences. PNA probe sequences were used to query non-redundant sequence data from 988 finished and unfinished microbial genomes and 168 eukaryotic genomes (Altschul et al., 1990; McGinnis and Madden, 2004).

The specific probe sequences 5′ aatctgccgcattag 3′ and 5′ cgtattaaactcaaccc 3′ are reverse complements to the *Y. pestis caf1* mRNA and 16S rRNA targets, respectively. Fluorescent probes designated *Yp*-F1-55, and *Yp*-16S-426 refer to the target binding locations of the 5-prime end of probes F1 and 16S, respectively. Probes were synthesized using an Expedite 8909 Nucleic Acid Synthesis System (Biosynthesis Corp., Lewisville, TX). Fluorescein isothiocyanate (FITC) moieties were attached to the 5′ end of the synthetic oligonucleotides.

2.5. Y. pestis PNA-FISH

Exponentially growing bacterial cultures were serially diluted using either HIB or Tris-buffered saline (TBS, Becton Dickenson, Sparks, MD) and 20 µl aliquots were spotted onto 15 mm wells on a 75×25 mm glass slide (AdvanDX Inc Woburn, WA.). All glass slides were pre-treated with a 1% poly-L-Lysine (MP Biomedicals, Irvine, CA) solution overnight, air dried and stored at −20 °C. After drying, slides were flame fixed then submerged in 80% ethanol for 10 min and air

dried again. Cells were then covered with 25 µl of hybridization solution containing 900 mM NaCl, 10 mM $NaPO_4$, 1.3% polyethylene glycol, and 30% formamide (Hristova et al., Abstr. 100th Gen. Meeting Am. Soc. Microbiol.). Fluorescein-labeled PNA probe was added to the hybridization solution to a final concentration of 250 nM. Probe stocks were initially prepared at a concentration of 10 µM in sterile deionized water (dH_2OH_2O) and stored at 4 °C in the dark prior to mixing with hybridization buffer. Alternatively, 10 µM probe stocks were solubilized in 1% tri-fluoroacetic acid (TFA, Arcos Organics, Geel, Belgium). TFA probe stocks were stored for one week at 4 °C in the dark before using in hybridization assays, allowing for probes that may have aggregated to become fully soluble. Prior to addition to slides, hybridization buffer and probe were incubated together at 55 °C for 10 min. All steps were conducted with minimal light exposure to preserve the reporting integrity of the FITC conjugate. Coverslips were used to ensure even distribution of the hybridization/probe solution, prevent evaporation and to eliminate air bubbles. Covered slides were placed into a hybridization warmer (Slidemoat, Boekel, Feasterville, PA) and incubated for 2 h at 60 °C. Slides were washed once for 30 min at 55 °C with TBS with 0.1% Tween 20. Following the wash step, slides were rinsed gently with distilled water and dried for at least 30 min. Slides were examined through a 63× Achroplan objective (630× total magnification) attached to an Axioscope 2 Plus epi-fluorescent microscope (Zeiss, Gettingen, Germany) using either a FITC filter or Texas Red dual band filter (Zeiss). The FITC filter utilizes an excitation wavelength of 485±20 nm and an emission spectrum of 515–565 nm, whereas the Texas Red dual band filter uses excitation wavelengths of 485±20 nm and 578±14 nm emitting at 515–540 nm and 610 nm, respectively. Images were acquired using a Zeiss Axiocam MRc5 digital camera with measurements and analysis conducted using Axiovision 4.5 software (Zeiss). The limits of detection (LOD) for positive scoring in mixed culture dilutions were established when no fewer than three fluorescent plague bacilli per high-power field were observed through the FITC and Texas Red dual band filters in a minimum of three fields. At least fifteen 63×high-power fields were screened per slide.

3. Results

3.1. Selection, optimization and performance of Y. pestis PNA probes

PNA probes *Yp*-16S-426 and *Yp*-F1-55 were selected using several criteria including low self-complementarity, secondary structure of the RNA target sequence, PNA probe-specific design criteria, and genome database homologies. As expected, BLAST searches revealed probe *Yp*-16S-426 to be 100% homologous to multiple copies of the 16S rRNA genes found in strains of *Y. pestis* and *Y. pseudotuberculosis* and single 16S rRNA gene copies in genomes of *Y. frederiksenii*, *Y. similis*, and *Y. enterocolitica* (data not shown). Surprisingly, the 16S PNA probe reported as negative for strains of *Y. frederiksenii* and *Y. enterocolitica* used in this study despite the presence of at least one homologous 16S rRNA gene copy in each of these species. Moreover, no other identical matches to *Yp*-16S-426 were noted and no matches to the remaining *Yersinia* panel strains were observed (Table 1). *Yersinia similis*, originally phenotyped as a strain of *Y. pseudotuberculosis* (Sprague et al., 2008), was not included in the panel. The probe sequence for *Yp*-F1-55 showed even greater specificity with significant matches only to the pMT plasmid of *Y. pestis*. Other 100% homologous matches to the F1 PNA probe including a hypothetical protein in the human colon bacterium, Bacteroides ovatum and a putative *N*-acetylglucosamine transferase of an *Acanthamoeba* endosymbiont were discounted since these organisms are unlikely to be encountered on surfaces following an intentional release event and the ability to cultivate them in HIB is also suspect.

Y. pestis strains were tested side by side with bacterial species that do not share the targeted RNA sequences (Table 1). Culture growth, hybridization, and wash conditions were optimized to obtain the best signal to noise ratio. Accordingly, FITC signal reporting was optimal when exponentially growing cultures were harvested and sampled at the 4 h and 6 h time points and was either noticeably reduced at the 2 h and 8 h sampling points or undetectable when tested at the 1 h and 16 h time points (data not shown). It has been reported that *Y. pestis* RNA turnover may slow beyond 5 h in this closed batch system as cells face growth restrictions, decreased availability of adenylates ATP and ADP, consumption of nutrients (*e.g.* calcium), and increased biomass pressures (Zahorchak et al., 1979). The 4.5 h incubation time falls within the period of logarithmic growth and before the restrictive controls of the closed batch system are believed to affect peak RNA production. This optimization was particularly important for mixed culture experiments described below using non-*Y. pestis* strains exhibiting significantly faster growth rates.

The stability of the PNA probes was determined by analyzing results from freshly reconstituted probe and probe that had been stored for eight months at 4 °C. Probes were reconstituted to a working concentration of 10 µM in dH_2O or 1% TFA. These probe stocks retained reporting integrity giving reproducible results with no apparent loss of performance between fresh and eight month old probes reconstituted in either diluent (data not shown). Though other buffer formulations were tested, PNA probes performed best when assayed in hybridization buffer containing 30% formamide as described above. Positive *Y. pestis* strains were easily distinguished from other bacteria by their ability to retain an apple green fluorescence when imaged with the Texas Red dual band filter. This was evident when testing individual (Fig. 1) as well as mixed cell cultures (Fig. 2).

In testing the *Yp*-16S-426 probe performance, 100% positive results were achieved for all panel strains expected to carry the targeted 16S rRNA region. This panel included twenty-eight strains of *Y. pestis* and seven strains of *Y. pseudotuberculosis* (Table 1.). The panel of *Y. pestis* strains represents all three classical biotypes of the bacterium, orientalis, medievalis and antiqua, believed to have originated during the three historical plague pandemics (Perry and Fetherston, 1997) while *Y. pseudotuberculosis* strains were chosen to represent a small subset of the different serotypes (Skurnik et al., 2000; Tsubokura and Aleksic, 1995) (Table 1). Probe *Yp*-16S-426 gave positive results for *Y. pestis* and *Y. pseudotuberculosis*, which both share identical 16S rRNA target sequences, but did not hybridize to *Y. enterocolitica* or to strains of other *Yersiniae* tested due to divergent 16S rRNA sequence homology in the target region (Trebesius et al., 1998) (Table 1).

The assessment of positive 16S PNA probe hybridization was based on those bacteria that emitted an apple green fluorescence when using both the FITC filter and Texas Red dual band filter (Fig. 1). Bacterial strains were reported as negative that emitted no fluorescence or rapidly diminishing yellow auto-fluorescence using the FITC filter and no fluorescent signal using the Texas Red dual band filter. For the sake of expedited and reliable results, the Texas Red dual band filter was used as the standard for determining positive and negative results (Table 1). Using this standard, *Y. pseudotuberculosis* reported positive for the *Yp*-16S-426 probe displaying an appearance much like *Y. pestis*. However, *Y. pestis* had a tendency to display bipolar fluorescence, while *Y. pseudotuberculosis* had a fluorescent signal that was more evenly distributed throughout the cell. While these slight differences were evident, they were not definitive enough to differentiate the species with a satisfactory level of confidence and thus, were not used as reporting criteria.

To differentiate *Y. pestis* from other *Yersiniae*, particularly *Y. pseudotuberculosis*, use of the *Yp*-F1-55 probe was necessary. A major genetic difference between *Y. pestis* and the enteropathogenic *Yersiniae* is the presence of the unique plasmids pPCP and pMT (Ferber and Brubaker, 1981; Portnoy and Falkow, 1981). Plasmid pMT encodes the murine toxin as well as the F1 capsular antigen (Cherepanov et al., 1991). The fluorescent reporting of *Yp*-F1-55 was similar to the reporting by *Yp*-16S-426 for all *Y. pestis* strains tested with the exception of *Y. pestis* strain PB6 which emitted a weak

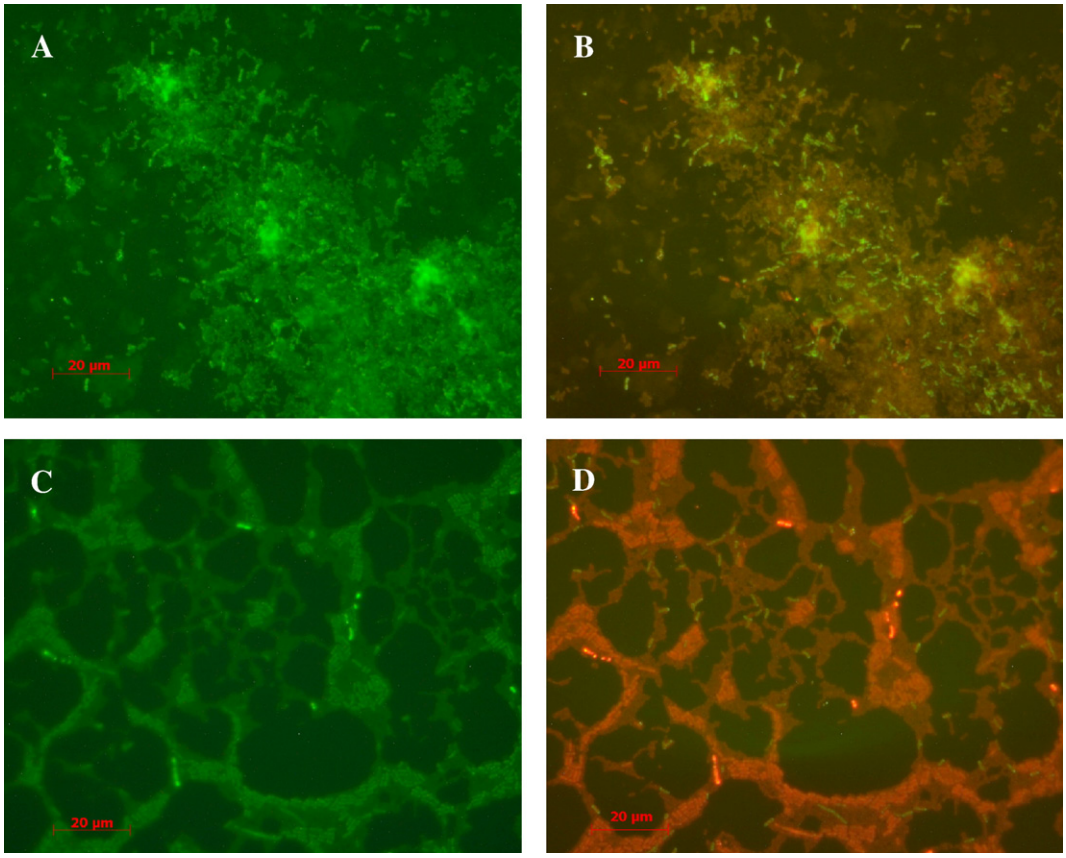

Fig. 2. PNA-FISH images of *Y. pestis* A1122, *Bacillus* sp., and *Pseudomonas aeruginosa* mixed culture (Panels A and B) using FITC-conjugated PNA probe *Yp*-16S-426. Panel A, mixed culture image using FITC filter; Panel B, mixed culture image using Texas Red dual band filter. Panels C and D, PNA-FISH images using probe *Yp*-F1-55 for detection of *Y. pestis* Harbin 35 in mixed culture with E. coli ATCC 25922 and *Bacillus* sp. and captured using FITC (Panel C) and Texas Red dual band (Panel D) filters. *Y. pestis* cells retain apple green fluorescence when imaged with the Texas Red dual filter in contrast to background strains which appear brown to reddish-brown in color (Panels B and D). Slides were observed at 630× total magnification and images were acquired using a Zeiss Axiocam MRc5 scope-mounted digital camera. Scale bars within each panel are shown in red and indicate distance in microns (µm).

positive signal despite the absence of the pMT plasmid in this strain. *Y. pestis* strain PKR-133, which is devoid of autonomous plasmids (Protsenko et al., 1991), reported negative for *Yp*-F1-55 as expected (Table 1). Unlike *Yp*-16S-426, however, *Y. pseudotuberculosis* did not bind *Yp*-F1-55 and was reported as negative (Fig. 1D). Moreover, *Y. pestis* strain WY85-5562-753 did not bind *Yp*-F1-55 when grown at 25 °C since F1 synthesis is abrogated at this temperature (Fig. 1F). Similarly, heat-killed preparation of *Y. pestis* A1122 and CO04-2349 were negative when probed with *Yp*-16S-426 and *Yp*-F1-55 (data not shown). Among all *Yersinia* strains and species tested (Table 1), specificity of PNA probe *Yp*-F1-55 was 98%. Interestingly, the signal strength emitted following hybridization with the *Yp*-F1-55 probe was superior when using 1% TFA probe stocks compared to 10 µM stocks in dH_2O. Diluting the *Yp*-F1-55 probe in hybridization buffer to a working concentration of 250 nM resulted in optimum signal to noise ratio and performed comparably to *Yp*-16S-426 which was reconstituted in water. Adding TFA to *Yp*-16S-426 had no affect on probe performance as was observed with *Yp*-F1-55. A likely explanation is that TFA conditions the probe by lowering the pH which enhances the solubility of PNA oligomers that may otherwise have a tendency to aggregate.

3.2. Sensitivity of PNA probes

To determine the number of plague bacilli needed for successful hybridization and reporting, cell cultures were decimally diluted and tested using optimum hybridization conditions described above. Sample dilutions were tested for ascertainable visual differentiation between positive signals, negative signals and possible false positive reporting from background. When the characteristic reporting signal of positive samples was no longer observed using the criteria established above, the limits of detection (LOD) were established. In some cases there appeared lone bacteria which fluoresced green when using the Texas Red dual band filter, a pre-requisite for positive signal reporting. However, in *Y. pestis* strain preparations, cells tended to cluster and report together under the PNA-FISH method (Fig. 1A). Samples were considered positive when three or more bacteria were visualized in the same high-power field within 20 µm of each other. While fluorescent background may be able to mimic the appearance of one or two bacteria together, groups of three or more bacteria are more clearly distinguishable from background. We therefore implemented a conservative requirement of three or more fluorescing bacteria within each of three or more 63× high-power field as a true positive signal when a minimum of fifteen fields were observed.

Using these criteria, the LOD for probe *Yp*-16S-426 ranged from 2.64×10^3–3.16×10^4 cells per slide (cps) with a mean of $1.14\times10^4\pm1.38\times10^4$ cps and the LOD for *Yp*-F1-55 ranged from 2.64×10^3–8.86×10^3 with a mean of $4.33\times10^3\pm3.03\times10^3$ cps for the four *Y. pestis* strains used in the mixed culture trials (Table 2). The average detection sensitivity for both probes among *Y. pestis* strains used in the mixed culture assays (Harbin 35, Calif. St. 268-T, Yosemite 104 and A1122) was $7.89\times10^3\pm9.97\times10^3$ cps. In practical terms, this would require the collection of 2.56×10^5 viable *Y. pestis* bacilli from a contaminated surface for inoculation into 4 mL of media in order to achieve the average LOD within 4.5 h.

3.3. PNA-FISH assays in mixed cell culture

Once PNA probe specificity was established for individual bacterial strains, mixed cell cultures were tested. Individual strains were grown

concurrently with mixed batch cultures to establish strain-specific growth rates over a 4.5 hour period (Table 2). The proliferation of cells during the 4.5 hour growth period was determined by plating serially diluted individual cultures (Table 2) on SBA and counting colony forming units (cfu). SBA provided consistent results and supported growth of a greater diversity of bacterial species when compared to other media tested (data not shown). It also allowed for the ability to morphologically differentiate between bacterial species that were grown and subsequently plated together. *Bacillus* sp. colonies, for example, formed halos that were distinguishable from E. coli colonies which grow at a similar rate. Attempts to count *Y. pestis* on mixed culture plates were hampered by the slow growth rate of *Y. pestis*; only pinpoint colonies were evident after 16 h of growth when compared to colony sizes of *Bacillus* sp. and E. coli. While *Y. pestis* counts were obtainable, the growth advantage of other bacteria raised concerns that *Y. pestis* bacterial counts were being underestimated. For this reason, cfu from individual cultures were used to determine cell count ratios from initial inocula and exponentially growing bacteria used in the mixed batch cultures.

Since mixed batches have more bacteria competing with each other for nutrients in a restricted volume, colony counts from individual strains are only an approximation of the ratio of different bacterial species in mixed cultures. While the growth effects on individual strains used in the mixed culture trials as a result of nutrient competition are unknown, it is reasonable to assume that within 4.5 h the faster growing non-*Y. pestis* bacterial strains are likely to encounter growth restrictions. It is also likely that the growth of *Y. pestis* strains will be slowed since competition for nutrients will presumably favor the faster growing strains. Though the initial inocula of *Y. pestis* and non-*Y. pestis* strains used in each mixed growth trial were approximately equal (averaging 2.0×10^6 and 3.5×10^6 cfu, respectively), competition for nutrients likely favored the faster growing non-*Y. pestis* strains in these experiments. Nonetheless, when examining assay results for the *Yp*-16S-426 and *Yp*-F1-55 PNA probes, both were found effective in reporting from targeted RNA in the presence of an average 7-fold over-abundance of competing RNA from each non-target strain excluding *Bacillus* sp. (average non-target to target ratio increases to 15.3 when including *Bacillus* sp.), based on colony count ratios of individually grown strains (Table 2; Fig. 2B and 2).

PNA probes were able to report with equal success for all *Y. pestis* and non-*Y. pestis* strain combinations tested. In all cases, the growth rate of *Y. pestis* strains was the slowest of all bacteria tested, averaging 0.14 (0.065 when *Bacillus* sp. is included in the growth rate calculation) the rate of other bacterial strains. The use of the Texas Red dual band filter allowed for *Y. pestis* cells to be distinguished easily from non-*Y. pestis* microbes whose combined prevalence was at least one order of magnitude higher. In the mixed population trials, *Y. pestis* bacteria were typically found in clusters ranging from 5 to 30 bacteria. These *Y. pestis* groupings were most often made up of bacteria aligned in a head to tail arrangement typical of the species. This presentation allowed for a quicker and more confident identification of positive cells targeted for detection.

4. Discussion

Y. pestis can be identified by employing established microbiological, biochemical and immunological methods (Chu, 2000) or presumptively identified through the use of rapid and sensitive molecular and immunological techniques (Chanteau et al., 2003; Stewart et al., 2008; Tomaso et al., 2003; Woron et al., 2006) in order to detect and record its presence. The development of peptide nucleic acids is an emerging technology that utilizes a DNA mimic whose deoxyribose phosphate backbone has been replaced by a polyamide "peptide" backbone resulting in an oligonucleotide analogue with binding characteristics to DNA or RNA targets that are extraordinarily more stable than its conventional DNA counterpart (Nielsen et al., 1991, 1994). The use of PNA probes has benefited research pertaining to the surveillance of infectious diseases including recent successes for the detection of *Candida albicans* and *Staphylococcus aureus* in blood (Oliveira et al., 2002; Rigby et al., 2002), detection and enumeration of eubacteria, *S. aureus* and *P. aeruginosa* from South Florida beaches (Esiobu et al., 2004), and detection of *Legionellae* and *Mycobacterium avium* in biofilms (Lehtola et al., 2006; Wilks and Keevil, 2006). Due to its potential use as an agent of bioterrorism (Inglesby et al., 2000; Riedel, 2005), it is of utmost importance to be able to rapidly identify this bacterium and determine its viability in the environment (Riedel, 2005).

Currently, there is several detection methods employed for plague diagnosis. Multiplex rt-PCR assays provide a rapid and sensitive means of detecting DNA evidence of *Y. pestis* without culture isolation (Stewart et al., 2008; Tomaso et al., 2003; Woron et al., 2006). Similarly, rapid hand-held assays and immunofluorescence microscopy are rapid and moderately sensitive means for the detection of *Y. pestis* F1 antigen (Chanteau et al., 2003; Chu, 2000). However, despite the sensitivity and high through-put potential of these assays, none specifically test for *Y. pestis* viability. Determinations of viability currently rely on culture isolation which requires 24–48 h of incubation or longer if selective media or mouse inoculation is needed for recovery of pure culture. This is routinely followed by direct fluorescent antibody staining and specific bacteriophage lysis for presumptive and confirmatory identification of *Y. pestis*, respectively (Chu, 2000). When used in conjunction with or subsequent to more rapid diagnostic technologies, PNA probes serve as an additional tool for early *Y. pestis* detection by providing data on the presence and viability of plague bacilli following an intentional release event. Such knowledge on the viability of *Y. pestis* can be useful to those directing clean up and remediation efforts.

In the current investigation, a wide range of bacteria including pathogenic (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*) and non-pathogenic *Yersinia* species were analyzed to determine the sensitivity and specificity of PNA probes for the detection of *Y. pestis*. The pathogenic *Yersinia* species are closely related at the genetic level. DNA hybridization experiments of *Y. pseudotuberculosis* and *Y. enterocolitica* against *Y. pestis* revealed homologies of 83 and 23%, respectively (Moore and Brubaker, 1975). Moreover, 16S rRNA gene sequences are identical by BLAST analysis (http://ncbi.nlm.nih.gov/blastn) among *Y. pestis* strains for which sequence is available and are highly conserved among the *Yesinia* nomenspecies. While there is >97% identity among all *Yersiniae*, *Y. pestis* and *Y. pseudotuberculosis* share 100% identity at the nucleotide level (Kim et al., 2003; Ruppitsch et al., 2007; Trebesius et al., 1998). Nevertheless, 16S rRNA sequence analyses have demonstrated their applicability for identifying species-specific markers within the genus *Yersinia* (Ibrahim et al., 1993; Neubauer et al., 2000; Sprague and Neubauer, 2005). Furthermore, 16S rRNA targets have been utilized as sensitive genus and species-specific binding sites for the detection and enumeration of bacteria in a variety of PNA-FISH applications (Esiobu et al., 2004; Lehtola et al., 2005; Lehtola et al., 2006; Perry-O'Keefe et al., 2001; Wilks and Keevil, 2006).

Despite the notion that *Y. pestis* is thought to be a recently emerged clone of *Y. pseudotuberculosis* predicted to have evolved 1500–20,000 years ago (Achtman et al., 1999), PNA hybridization data presented here indicates that these species can be differentiated by the successful reporting of both the *Yp*-F1-55 and *Yp*-16S-426 probes. While the latter enabled discrimination of *Y. pestis* and *Y. pseudotuberculosis* from *Y. enterocolitica* and other *Yersiniae*, PNA probe *Yp*-F1-55 was uniquely specific for F1 antigen expressing strains of *Y. pestis*. The F1 protein is encoded by the *caf1* gene located on the ~100 kilobase (kb) pair pMT (pFra) plasmid, one of two endogenous plasmids unique to *Y. pestis* (Ferber and Brubaker, 1981; Portnoy and Falkow, 1981). It is part of a unique *Y. pestis* locus that spans 5.13 kb which encodes a chaperone, Caf1M, usher protein Caf1A, positive regulator caf1R, and capsular F1 antigen, Caf1 (Cherepanov et al., 1991; Hu et al., 1998). The F1 protein forms a gel-like, antiphagocytic capsule containing multimeric F1 aggregates at 37 °C but not 26 °C (Bennett and

Tornabene, 1974; Brubaker, 1972; Du et al., 2002; Pollitzer, 1954a,b). Temperature regulated expression of F1 was quantified recently by Motin et al. who reported a 100-fold increase in *caf1* expression in *Y. pestis* grown at 37 °C when compared to cells grown at 26 °C (Motin et al., 2004). Sequence analysis of *Y. pestis* isolates has shown that the *caf1* gene is highly conserved (>98% at the nucleotide level) (Chu, 2000) (Chu, unpublished data). Probe *Yp*-F1-55's specificity for *caf1* mRNA was demonstrated by its lack of hybridization to *Y. pestis* WY85-5562-753 cells grown at 25 °C (Fig. 1F) or heat-killed preparations of *Y. pestis* bacteria previously grown at 35 °C (data not shown).

Y. pestis strain PB6 was the only exception to the absolute specificity of probe *Yp*-F1-55. Reasons for the weakly positive response of PB6 are unclear as F1-specific direct fluorescent antibody tests and an in house *caf1*-targeted real-time PCR assay (Diagnostic and Reference Laboratory, Bacterial Diseases Branch, Division of Vector-Borne Infectious Diseases, CDC, Fort Collins, CO) are both negative for this strain (Table 1 and data not shown). One plausible explanation stems from the reported observation that the F1 capsule operon (*caf1*, *caf1A*, *caf1M* and *caf1R*) and the murine toxin locus, *ymt* have G+C content of 39.2% and 38.1%, respectively. The G+C content at these loci differs from the overall 50.1% G+C content of the plasmid and satisfies one of the criteria described for pathogenicity islands (Hacker et al., 1997; Lindler et al., 1998). Moreover, the pMT plasmid contains several complete and partial insertion sequence elements, bacteriophage integrases and ligases, and plasmid partitioning loci suspected to have originated through numerous bacteriophage or plasmid-mediated incomplete or aberrant transposition events (Hu et al., 1998; Lindler et al., 1998). Since integration of plasmid pMT into the *Y. pestis* chromosome has been reported previously, an occurrence that is probably mediated via homologous recombination of IS elements located on both the plasmid and chromosome (Protsenko et al., 1991), it is possible that pMT integration followed by an excision event occurred leaving behind a remnant of the *caf1* gene containing an homologous portion to probe *Yp*-F1-55.

PNA probes *Yp*-16S-426 and *Yp*-F1-55 demonstrated 100% and 98% specificity, respectively, among 56 strains tested. For method development, both probes were assayed simultaneously. However, an algorithm which utilizes the PNA probes sequentially (*Yp*-16S-426 followed by *Yp*-F1-55) is a reasonable approach for determining viable *Y. pestis* organisms in a large number of environmental samples following an intentional release event.

For differentiating *Y. pestis* from non-*Yersinia* species, mixed culture experiments were performed to mimic the potential diversity of bacteria and other microbes that would be inadvertently collected in an attempt to recover *Y. pestis*. In the current study, we conducted 12 mixed culture trials to simulate this prokaryotic diversity and to determine the detectability of *Y. pestis* RNA in a closed system where competition for micronutrients favors bacterial strains with faster growth rates. By limiting growth of the mixed cell closed batch system to 4.5 h, we attempted to control for peak RNA production from *Y. pestis* while minimizing growth limitations posed by the depletion of nutrients from competing non-*Y. pestis* strains. We determined that this experimental design was a reproducible and efficient means of RNA detection by PNA-FISH (Fig. 2).

The colony forming units calculated for the competing strains were used to approximate the ratio of *Y. pestis* cells to non-*Y. pestis* cells. In analyzing hybridized slides from mixed cell growth trials (Fig. 2), it was evident that the visible cell ratios for *Y. pestis* and non-*Y. pestis* strains appeared to agree with the growth curves performed on these strains individually, showing a distinct proliferation advantage for the non-*Y. pestis* strains (Table 2). Although negative for fluorescence, competing bacteria are visible under FITC filters and appear as an overlying dense cellular mass versus the fluorescently positive *Y. pestis*. Clearly, the over-abundance of non-*Y. pestis* strains did not preclude hybridization to *Y. pestis* RNA and affect positive reporting from PNA probes in these potential mixed microbial community models.

Since *de novo caf1* mRNA synthesis must occur at temperatures above 33 °C in order for *Y. pestis* to be detected by the *Yp*-F1-55 probe (Perry and Fetherston, 1997), the current protocol allows one to assume a theoretical minimum recovery of as few as 6.94×10^4 viable cells (*Y. pestis* strain Yosemite 104) by sampling a contaminated surface or an average of 2.56×10^5 organisms for all *Y. pestis* strains tested in mixed culture based on an average 6-fold increase in cell density for *Y. pestis* strains over 4.5 h (Table 2). Though not part of the current assay, these LODs also assume that viable, surface-collected *Y. pestis* cells could be concentrated following the 4.5 h growth and recovery period and the entire sample resuspended in a volume sufficient for two PNA probe hybridization assays. This potential method modification reduces the theoretical minimum and average LOD to 8.67×10^2 and 3.2×10^3 organisms, respectively. Alternatively, methods for multiplexing different *Y. pestis*-specific PNA probes in a single hybridization assay to increase sensitivity and decrease growth and recovery time will also be explored.

5. Conclusion

In the current study, we have described the development and evaluation of PNA probes for the detection of viable *Y. pestis in vitro* and in the presence of competing bacteria. While this emerging technology should enhance the ability of microbiologists to positively identify viable *Y. pestis* bacilli in as little as 8h, it will be necessary to test this PNA-FISH method under real world conditions by sampling from a variety of different environmental surfaces and materials. Moreover, consideration will need to be given to the intentional release of *Y. pestis* cultured under conditions which inhibit the expression of *caf1* or the cultivation of a *Y. pestis* strain lacking the pMT plasmid or genetically engineered to abrogate expression of the F1 antigen. Since the current testing protocol for detection using probe *Yp*-F1-55 requires incubation of samples at 35°C prior to the PNA-FISH assay, detection of a *caf1* competent *Y. pestis* strain can be accomplished regardless of the initial growth temperature of the strain prior to release. However, detection of a *caf1* negative strain would be limited to the 16S PNA probe and points to the shortcomings of the current assay. Additional *Y. pestis*-specific probes that target genes that are constitutively expressed and chromosomally located are being developed to address this limitation. Furthermore, the collection and cultivation of environmental samples will likely result in the propagation of more than three bacterial strains, as described above in the controlled mixed culture PNA-FISH assays, as well as an abundance of other microbes. While the discriminatory power of PNA probes has been reported in the species-specific detection of microorganisms in similarly challenging samples such as beach sand, seawater, and potable-water biofilms (Esiobu et al., 2004; Lehtola et al., 2006), nevertheless, we will develop or test existing selective media to reduce the prevalence undesirable environmental contaminants and enhance the recovery and PNA-FISH detection of this potential biothreat agent.

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References

Achtman, M., Zurth, K., Morelli, C., Torrea, G., Guiyoule, A., Carniel, E., 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia* pseudotuberculosis. Proc. Natl. Acad. Sci. USA. 96, 14043–14048.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Amann, R., Glockner, F.-O., Neef, A., 1997. Modern methods in subsurface microbiology: in situ identification of microorganisms with nucleic acid probes. FEMS Microbiol. Rev. 20, 191–200.
Anisimov, A.P., Lindler, L.E., Pier, G.B., 2004. Intraspecific diversity of *Yersinia pestis*. Clin. Microbiol. Rev. 17, 434–464.
Barnes, A.M., 1982. Surveillance and control of bubonic plague in the United States. Symp. Zool. Soc. Lond. 50, 237–270.
Begier, E.M., Asiki, G., Anywaine, Z., Yockey, B., Schriefer, M.E., Aleti, P., Ogen-Odoi, A., Staples, J.E., Sexton, C., Bearden, S.W., Kool, J.L., 2006. Pneumonic plague cluster, Uganda, 2004. Emerg. Infect. Dis. 12, 460–467.
Bennett, L.G., Tornabene, T.G., 1974. Characterization of the antigenic subunits of the envelope protein of *Yersinia pestis*. J. Bacteriol. 117, 48–55.
Ber, R., Mamroud, E., Aftalion, M., Tidhar, A., Gur, D., Flashner, Y., Cohen, S., 2003. Development of an improved selective agar medium for isolation of *Yersinia pestis*. Appl. Environ. Microbiol. 69, 5787–5792.
Brubaker, R.R., 1972. The genus *Yersinia*: biochemistry and genetics of virulence. Curr. Top. Microbiol. Immunol. 57, 111–158.
CDC, 1994. Update: human plague — India, 1994. Morbid. Mortal. Weekly Rep. 43, 761–762.
Chanteau, S., Rahalison, L., Ralafiarisoa, L., Foulon, J., Ratsitorahina, M., Ratsifasoamanana, L., Carniel, E., Nato, F., 2003. Development and testing of a rapid diagnostic test for bubonic and pneumonic plague. Lancet. 361, 211–216.
Cherepanov, P.A., Mikhailova, T.G., Karimova, G.A., Zakharova, N.M., Ershov, Y.V., Volkovoi, K.I., 1991. Cloning and detailed mapping of the Fra-Ymt region of the *Yersinia pestis* plasmid pFra. Mol. Genet. Mikrobiol. Virusol. 12, 19–26.
Chu, M.C., 2000. Laboratory manual of plague diagnostic tests. Centers for Disease Control and Prevention and World Health Organization, Geneva.
Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchard, O., Sonnichsen, S.H., Nielsen, P.E., 1994. Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. 48, 1310–1313.
Du, Y., Rosqvist, R., Forsberg, Å., 2002. Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis. Infect. Immun. 70, 1453–1460.
Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B., Nielsen, P.E., 1993. PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules. Nature. 365, 566–568.
Esiobu, N., Mohammed, R., Echeverry, A., Green, M., Bonilla, T., Hartz, A., McCorquodale, D., Rogerson, A., 2004. The application of peptide nucleic acid probes for rapid detection and enumeration of eubacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa* in recreational beaches of S. Florida. J. Microbiol. Methods. 57, 157–162.
Ferber, D.M., Brubaker, R.R., 1981. Plasmids in *Yersinia pestis*. Infect. Immun. 31, 839–841.
Gage, K.L., Kosoy, M.Y., 2005. Natural history of plague: perspectives from more than a century of research. Annu. Rev. Entomol. 50, 505–528.
Giesen, U., Kleider, W., Berding, C., Geiger, A., Ørum, H., Nielsen, P.E., 1998. A formula for thermal stability (Tm) prediction of PNA/DNA duplexes. Nucleic Acids Res. 26, 5004–5006.
Hacker, J., Blum-Oehler, G., Mühldorder, I., Tschäpe, H., 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. 23, 1089–1097.
Hu, P., Elliott, J., McCready, P., Skowronski, E., Garnes, J., Kobayashi, A., Brubaker, R.R., Garcia, E., 1998. Structural organization of virulence-associated plasmids of *Yersinia pestis*. J. Bacteriol. 180, 5192–5202.
Ibrahim, A., Goebel, B.M., Liesack, W., Griffiths, M., Stackebrandt, E., 1993. The phylogeny of the genus *Yersinia* based on 16S rDNA sequences. FEMS Microbiol. Lett. 114, 173–177.
Inglesby, T.V., Dennis, D.T., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Koerner, J.F., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Schoch-Spana, M., Tonat, K., 2000. Plague as a biological weapon: medical and public health management. J. Am. Med. Assoc. 283, 2281–2290.
Jensen, K.K., Orum, H., Nielsen, P.E., Norden, B., 1997. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. Biochemistry. 36, 5072–5077.
Kim, W., Song, M.O., Song, W., Kim, K.J., Chung, S.I., Choi, C.S., Park, Y.H., 2003. Comparison of 16S rDNA analysis and rep-PCR genomic fingerprinting for molecular identification of *Yersinia pseudotuberculosis*. Antonie Van Leeuwenhoek. 83, 125–133.
Koppelhus, U., Nielsen, P.E., 2003. Cellular delivery of peptide nucleic acid (PNA). Adv. Drug Deliv. Rev. 55, 267–280.
Lehtola, M.J., Loades, C.J., Keevil, C.W., 2005. Advantages of peptide nucleic acid oligonucleotides for sensitive site directed 16S rRNA fluorescence in situ hybridization (FISH) detection of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari*. J. Microbiol. Methods. 62, 211–219.
Lehtola, M.J., Torvinen, E., Miettinen, I.T., Keevil, C.W., 2006. Fluorescence in situ hybridization using peptide nucleic acid probes for rapid detection of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* in potable-water biofilms. Appl. Environ. Microbiol. 72, 848–853.
Lindler, L.E., Plano, G.V., Burland, V., Mayhew, G.F., Blattner, F.R., 1998. Complete DNA sequence and detailed analysis of the *Yersinia pestis* KIM5 plasmid encoding murine toxin and capsular antigen. Infect. Immun. 66, 5731–5742.
McGinnis, S., Madden, T.L., 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 32, W20–25.
Moore, R.L., Brubaker, R.R., 1975. Hybridization of deoxyribonucleotide sequences of *Yersinia enterocolitica* and other selected members of *Enterobacteriaceae*. Int. J. Syst. Bacteriol. 25, 336–339.
Motin, V.L., Georgescu, A.M., Fitch, J.P., Gu, P.P., Nelson, D.O., Mabery, S.L., Garnham, J.B., Sokhansanj, B.A., Ott, L.L., Coleman, M.A., Elliott, J.M., Kegelmeyer, L.M., Wyrobek, A.J., Slezak, T.R., Brubaker, R.R., Garcia, E., 2004. Temporal global changes in gene expression during temperature transition in *Yersinia pestis*. J. Bacteriol. 186, 6298–6305.
Neubauer, H., Aleksic, S., Hensel, A., Finke, E.J., Meyer, H., 2000. *Yersinia enterocolitica* 16S rRNA gene types belong to the same genospecies but form three homology groups. Int. J. Med. Microbiol. 290, 61–64.
Nielsen, P.E., Egholm, M., Berg, R.H., Buchardt, O., 1991. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science. 254, 1497–1500.
Nielsen, P.E., Egholm, M., Buchardt, O., 1994. Peptide nucleic acid (PNA). A DNA mimic with a peptide backbone. Bioconjug. Chem. 5, 3–7.
Oliveira, K., Procop, G.W., Wilson, D., Coull, J., Stender, H., 2002. Rapid identification of Staphylococcus aureus directly from blood cultures by fluorescence in situ hybridization with peptide nucleic acid probes. J. Clin. Microbiol. 40, 247–251.
Perry-O'Keefe, H., Rigby, S., Oliveira, K., Sorensen, D., Stender, H., Coull, J., Hyldig-Nielsen, J.J., 2001. Identification of indicator microorganisms using a standardized PNA FISH method. J. Microbiol. Methods. 47, 281–292.
Perry, R.D., Fetherston, J.D., 1997. *Yersinia pestis* — etiologic agent of plague. Clin. Microbiol. Rev. 10, 35–66.
Pollitzer, R., 1954a. Plague. W.H.O. Monogr. Ser. 22, 1–698.
Pollitzer, R., 1954b. Plague. World Health Organization Monograph Series No. 22. World Health Organization, Geneva, Switzerland.
Portnoy, D.A., Falkow, S., 1981. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis*. J. Bacteriol. 148, 877–883.
Protsenko, O.A., Filippov, A.A., Kutyrev, V.V., 1991. Integration of the plasmid encoding the synthesis of capsular antigen and murine toxin into *Yersinia pestis* chromosome. Microb. Pathog. 11, 123–128.
Ray, A., Norden, B., 2000. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J. 14, 1041–1060.
Riedel, S., 2005. Plague: from natural disease to bioterrorism. Proc. (Baylor Univ. Med. Cent.). 18, 116–124.
Rigby, S., Procop, G.W., Haase, G., Wilson, D., Hall, G., Kurtzman, C., Oliveira, K., Von Oy, S., Hyldig-Nielsen, J.J., Coull, J., Stender, H., 2002. Fluorescence in situ hybridization with peptide nucleic acid probes for rapid identification of *Candida albicans* directly from blood culture bottles. J. Clin. Microbiol. 40, 2182–2186.
Ruppitsch, W., Stoger, A., Indra, A., Grif, K., Schabereiter-Gurtner, C., Hirschl, A., Allerberger, F., 2007. Suitability of partial 16S ribosomal RNA gene sequence analysis for the identification of dangerous bacterial pathogens. J. Appl. Microbiol. 102, 852–859.
Simpson, W.J., Thomas, R.E., Schwan, T.G., 1990. Recombinant capsular antigen (fraction 1) from *Yersinia pestis* induces a protective antibody response in BALB/c mice. Am. J. Trop. Med. Hyg. 43, 389–396.
Skottman, T., Piiparinen, H., Hyytiainen, H., Myllys, V., Skurnik, M., Nikkari, S., 2007. Simultaneous real-time PCR detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. Eur. J. Clin. Microbiol. Infect. Dis. 26, 207–211.
Skurnik, M., Peippo, A., Ervela, E., 2000. Characterization of the O-antigen gene clusters of *Yersinia pseudotuberculosis* and the cryptic O-antigen gene cluster of *Yersinia pestis* shows that the plague bacillus is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O:1b. Mol. Microbiol. 37, 316–330.
Sprague, L.D., Neubauer, H., 2005. *Yersinia aleksiciae* sp. nov. Int. J. Syst. Evol. Microbiol. 55, 831–835.
Sprague, L.D., Scholz, H.C., Amann, S., Busse, H.J., Neubauer, H., 2008. *Yersinia similis* sp. nov. Int. J. Syst. Evol. Microbiol. 58, 952–958.
Stewart, A., Satterfield, B., Cohen, M., O'Neill, K., Robison, R., 2008. A quadruplex real-time PCR assay for the detection of *Yersinia pestis* and its plasmids. J. Med. Microbiol. 57, 324–331.
Takiya, T., Seto, Y., Yasuda, H., Suzuki, T., Kawai, K., 2004. An empirical approach for thermal stability (Tm) prediction of PNA/DNA duplexes. Nucleic Acids Symp. Ser. (Oxf). 48, 131–132.
Tomaso, H., Reisinger, E.C., Al Dahouk, S., Frangoulidis, D., Rakin, A., Landt, O., Neubauer, H., 2003. Rapid detection of *Yersinia pestis* with multiplex real-time PCR assays using fluorescent hybridisation probes. FEMS Immunol. Med. Microbiol. 38, 117–126.
Trebesius, K., Harmsen, D., Rakin, A., Schmelz, J., Heesemann, J., 1998. Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. J. Clin. Microbiol. 36, 2557–2564.
Tsubokura, M., Aleksic, S., 1995. A simplified antigenic scheme for serotyping of *Yersinia pseudotuberculosis*: phenotypic characterization of reference strains and preparation of O and H factor sera. Contrib. Microbiol. Immunol. 13, 99–105.
Varma-Basil, M., El-Hajj, H., Marras, S.A.E., Hazbon, M.H., Mann, J.M., Connell, N.D., Kramer, F.R., Alland, D., 2004. Molecular beacons for multiplex detection of four bacterial bioterrorism agents. Clin. Chem. 50, 1060–1062.
Wilks, S.A., Keevil, C.W., 2006. Targeting species-specific low-affinity 16S rRNA binding sites by using peptide nucleic acids for detection of legionellae in biofilms. Appl. Environ. Microbiol. 72, 5453–5462.
Woron, A.M., Nazarian, E.J., Egan, C., McDonough, K.A., Cirino, N.M., Limberger, R.J., Musser, K.A., 2006. Development and evaluation of a 4-target multiplex real-time polymerase chain reaction assay for the detection and characterization of *Yersinia pestis*. Diagn. Microbiol. Infect. Dis. 56, 261–268.
Zahorchak, R.J., Charnetzky, W.T., Little, R.V., Brubaker, R.R., 1979. Consequences of Ca^{2+} deficiency on macromolecular synthesis and adenylate energy charge in *Yersinia pestis*. J. Bacteriol. 139, 792–799.