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Assays for the rapid and specific identification of North American *Yersinia pestis* and the common laboratory strain CO92

Amy J. Vogler[1], **Elizabeth M. Driebe**[1], **Judy Lee**[1], **Raymond K. Auerbach**[1], **Christopher J. Allender**[1], **Miles Stanley**[1], **Kristy Kubota**[2], **Gary L. Andersen**[3], **Lyndsay Radnedge**[4], **Patricia L. Worsham**[5], **Paul Keim**[1], and **David M. Wagner**[1]

[1]Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ

[2]Centers for Disease Control and Prevention, Fort Collins, CO

[3]Lawrence Berkeley National Laboratory, Berkeley, CA

[4]Lawrence Livermore National Laboratory, Livermore, CA

[5]United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA

Abstract

We present TaqMan-minor groove binding (MGB) assays for an SNP that separates the Yersinia pestis strain CO92 from all other strains and for another SNP that separates North American strains from all other global strains.

Canonical SNPs (canSNPs) provide highly robust signatures for identifying specific phylogenetic clades of clonal pathogens, such as *Bacillus anthracis* (1,2). A similar approach should be generally applicable for other recently emerged clonal pathogens like *Yersinia pestis*. Canonical SNPs can be used to define any phylogenetic clade of interest, from a major phylogenetic division down to a single strain (1). The best examples are for *B. anthracis*, where a series of canSNPs have been identified and developed into assays. These include a species-specific canSNP separating *B. anthracis* from the closely related *B. cereus* and *B. thuringiensis* (3,4), a series of 13 canSNPs that define major phylogenetic lineages within *B. anthracis* (2), and a set of canSNPs specific for the Ames strain (5).

Although identification of potential canSNPs and their development into assays is relatively straightforward, extensive validation across large strain panels is essential to ensure that each potential canSNP is truly specific to the desired clade. However, once that task is accomplished, rapid diagnostic genotyping can take place with the validated assays. Canonical SNPs can also then be supplemented with other molecular markers, such as variable-number tandem repeats (VNTRs), in a nested hierarchical approach for highly accurate and highly discriminatory molecular genotyping (1).

Y. pestis, etiologic agent of plague, is a recently emerged pathogen that, historically, has been phenotypically classified into three biovars (6). Recently, a combination of several molecular typing schemes led to the reclassification of *Y. pestis* isolates into eight major populations (7). The most prevalent of these populations, identified as 1.ORI, exists

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The authors declare no competing interests.

throughout the world as a result of the third pandemic wave of plague that began in China in the 1800s (6,7). As the 1.ORI population is the most geographically widespread of the eight major populations, canSNPs that provide discrimination among phylogenetic lineages within this population would be especially useful. Here, we present two TaqMan-minor groove binding (MqGB) assays that provide discrimination among 1.ORI *Y. pestis* isolates: one assay distinguishes North American 1.ORI *Y. pestis* isolates from all other *Y. pestis* isolates (i.e., isolates from other regions of the world), and another assay distinguishes the 1.ORI strain CO92 from all other strains of *Y. pestis*.

Our CO92-specific SNP signature was among a set of SNPs identified by Achtman et al. (7), who suggested that one of the SNPs they identified (s34) might be specific to CO92. The specificity of this SNP to CO92 was further suggested by a comparison of the CO92 genome sequence with a gapped genome sequence from a second North American strain, FV-1 (8); this SNP is identified as SNP *r* in the latter publication. We confirmed the specificity of this SNP to CO92 using the assay and validation approach described below. Our North American-specific (NA-specific) SNP signature was among a set of SNPs identified by Chain et al. (9) by comparing the available whole genome sequences for *Y. pestis* strains CO92 (10), KIM (11), 91001 (12), Nepal516 (9), and Antiqua (9), and *Y. pseudotuberculosis* strain IP32953 (13). Among these genomes, Chain et al. (9) found 39 putative SNPs that were specific to CO92, which was the only North American strain included in their comparisons. We screened a subset of these SNPs across a small collection of global 1.ORI isolates, including a geographically diverse set of isolates from North America (data not shown); it is important to note that 1.ORI is the only major molecular group of *Y. pestis* found in North America. Based on the results of this screening, we identified a single SNP that appeared to be specific to North American *Y. pestis*. This SNP is located in locus YPO2853 at position 623 relative to the start of the gene (see Supplemental File 1 in Reference 9).

We designed TaqMan-MGB allelic discrimination assays around both SNPs. The TaqMan-MGB primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA; Table 1). Real-time PCRs for the two assays were conducted in 10 μl reaction mixtures that contained 900 nM of both forward and reverse primers, 200 nM of the NA- or CO92-specific probe, 100 nM of the alternate probe, 1× AB TaqMan Universal PCR Master Mix with AmpErase UNG (Applied Biosystems), and 1 μl of template. In addition to these reagents, the NA-specific assay had 0.5 mM of additional magnesium chloride. Thermal cycling was performed on an Applied Biosystems 7900 HT sequence detection system (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min.

We validated the potential NA- specific and CO92-specific SNPs by screening them against two DNA panels. The worldwide (WW) panel contained 83 genetically and geographically diverse *Y. pestis* and *Y. pseudotuberculosis* isolates (Supplementary Table S1, available online at www.BioTechniques.com). The CO92 panel contained 20 CO92 variants and 13 genetic near-neighbors to CO92 (Supplementary Table S1). Three of the CO92 genetic near-neighbors (242, South Park, and CO96–2596–593) were determined by SNP analysis (Wagner and Vogler et al., unpublished data). The remaining 10 CO92 genetic near-neighbors were determined by multilocus VNTR analysis (14,15) of a large (700+) collection of North American *Y. pestis* isolates (Wagner and Vogler et al., unpublished data). DNA templates were diluted 1/10 for heat lysis preps (16), 1/100 for whole genome amplification preps (Qiagen, Valencia, CA, USA), or to ~1 ng/μl for genomic chloroform preps (17,18) before being screened against the TaqMan-MGB assays.

To test the sensitivity of the two assays, we screened them against a 10-fold dilution series of DNAs in triplicate, with template levels ranging from 1 ng to 10 fg. DNA from one North American isolate (FV-1) and one non-North American isolate (Nairobi) were used to test the NA-specific TaqMan-MGB assay. DNA from CO92 and one non-CO92 strain (FV-1) were used to test the CO92-specific TaqMan-MGB assay (Supplementary Table S1).

All of the North American isolates in the WW panel ($n = 23$) were shown to possess the NA-specific allele (A allele), whereas the rest of the isolates in the WW panel ($n = 60$) exhibited the alternate allele (G allele) (Figure 1A; Supplementary Table S1). All of the isolates in the CO92 panel also possessed the NA-specific allele (Supplementary Table S1). All of the CO92 isolates in both panels ($n = 20$) possessed the CO92-specific allele (C allele), whereas the remaining isolates in both panels ($n = 95$) exhibited the alternate allele (A allele) (Figure 1B; Supplementary Table S1). Both assays consistently detected and genotyped template levels as low as 100 fg for both alleles, with sporadic amplification for the 10 fg samples (Figure 2, A and B; not all data shown).

The real-time PCR assays presented here represent valuable tools for rapid genotyping of *Y. pestis* isolates. They illustrate the feasibility of the canSNP approach for another recently emerged pathogen, as first described for *B. anthracis* (1,2). In addition, they demonstrate that canSNPs can be either autapomorphic (CO92-specific) or synapomorphic (NA-specific). The NA-specific SNP identifies an important point of division among 1.ORI *Y. pestis* isolates and implies that *Y. pestis* populations in North America are monophyletic, which is consistent with suggestions that all extant North American populations originated from a single introduction event to native rodent species near San Francisco in 1908 (19). No doubt additional canSNPs will be discovered for other geographically diverse subpopulations of the global 1. ORI population as well as for the other major populations defined by Achtman et al. (7), all of which will greatly aid in molecular subtyping of worldwide *Y. pestis* isolates. The assays described here and future canSNP assays could then be coupled with the multiplelocus variable-number tandem repeat analysis (MLVA) system that already exists for *Y. pestis* (14,15) in a nested hierarchical approach (1) for highly accurate and yet highly discriminatory molecular genotyping of *Y. pestis*.

Although canSNPs defining major phylogenetic divisions are most useful for general molecular subtyping, strain-specific canSNPs can be valuable as well in other settings. Common laboratory strains, such as CO92, are often used as reference and control strains. We therefore chose to develop a strain-specific canSNP for CO92, a common laboratory strain that was originally isolated from a human who became infected in Chaffee County, CO, in 1992 (10,20) and that has since been distributed to multiple laboratories. CO92 is now one of the most common laboratory strains used in the study of *Y. pestis* due to the whole genome sequence available for it (10) and its fully virulent status (21). A means of specifically and rapidly identifying such a commonly used strain could be useful for laboratories needing to differentiate this strain from others.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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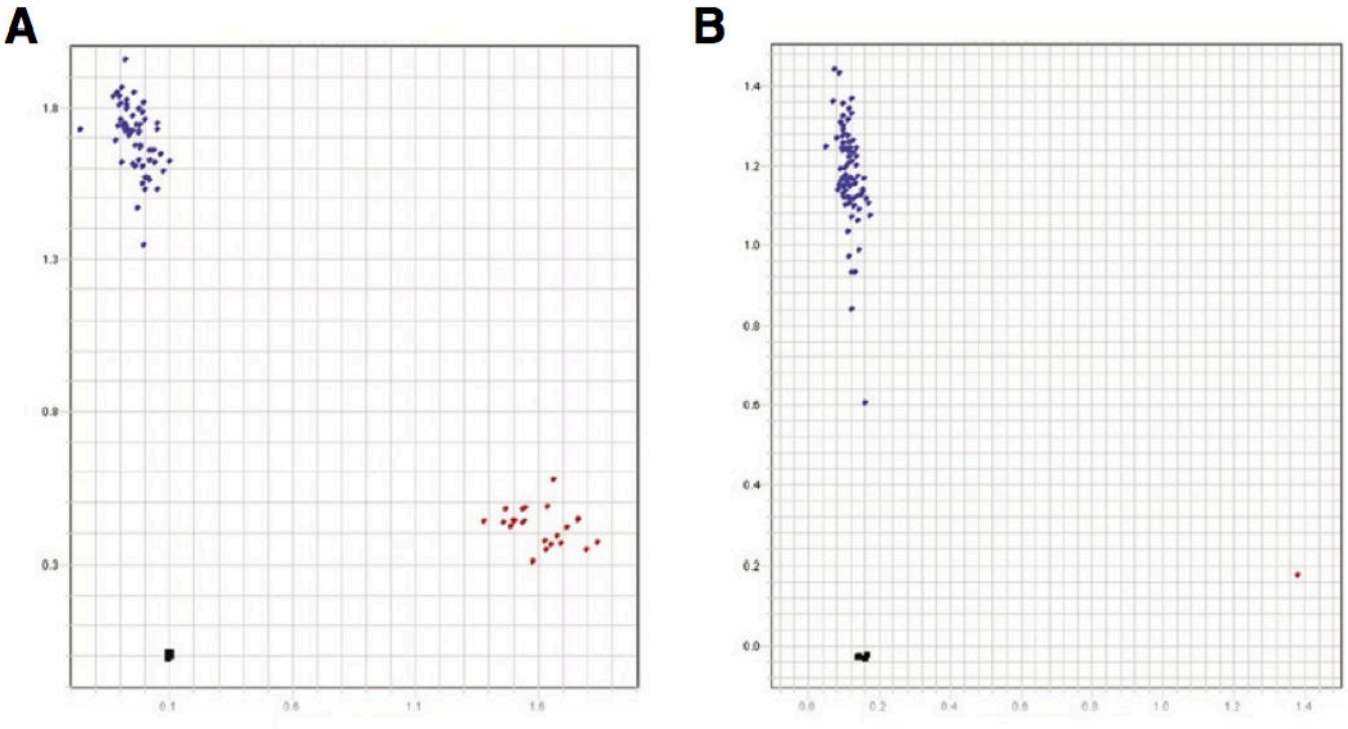

Figure 1. TaqMan end point allelic discrimination plots indicating specificity of two assays across a panel of 83 worldwide diverse isolates (23 from North America)

(A) North America (NA)-specific SNP assay. The dots along the *x*-axis (red) demonstrate the NA-specific allele ($n = 23$). The dots along the *y*-axis (blue) demonstrate the alternate allele ($n = 60$). Samples near the plot origin are negative no-template controls ($n = 3$). (B) C092-specific SNP assay. The dots along the *x*-axis (red) demonstrate the CO92-specific allele ($n = 1$). The dots along the *y*-axis (blue) demonstrate the alternate allele ($n = 82$). Samples near the plot origin are negative no-template controls ($n = 3$).

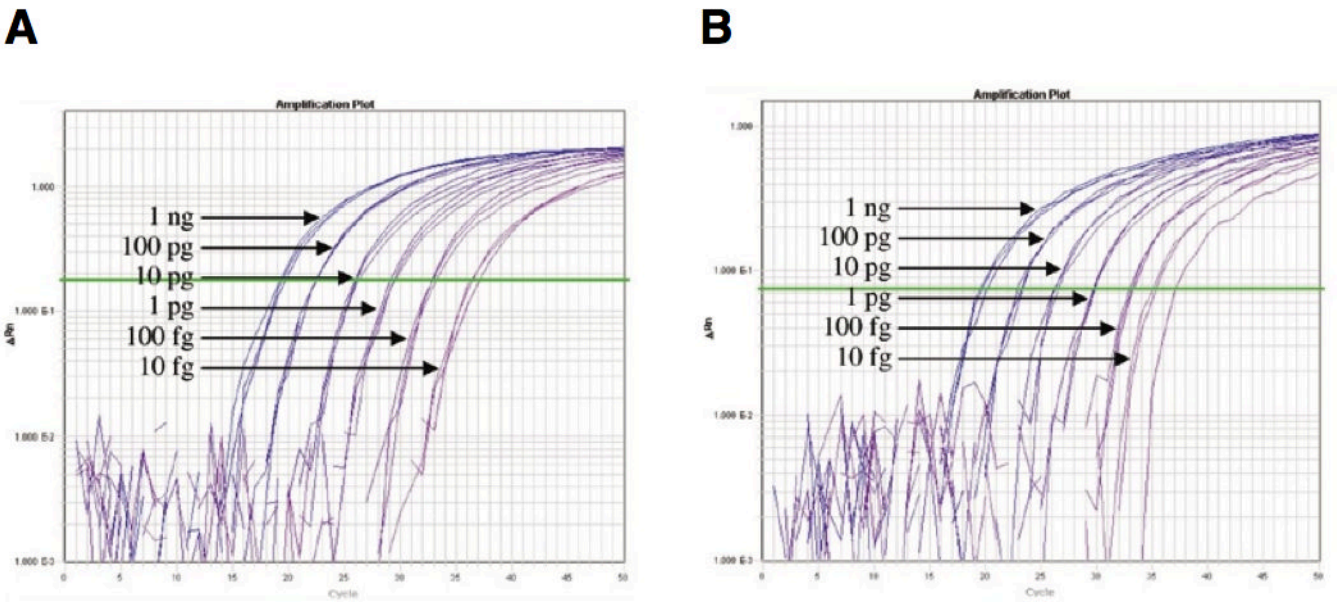

Figure 2. Results of triplicate analyses of 10-fold serial dilutions of DNA for two assays

(A) Amplification plot for the North American (NA)-specific SNP assay indicating cycle threshold values for 10-fold serial dilutions of DNA form isolate FV-1 (NA-specific allele). The average cycle threshold values for the alternate allele (isolate Nairobi) were similar (data not shown). (B) Amplification plot for the CO92-specific SNP assay indicating cycle threshold values for 10-fold serial dilutions of DNA from isolate CO92 (CO92-specific allele). The average cycle threshold values for the alternate allele (from isolate FV-1) were similar (data not shown).

Table 1

Primers and Probes Used in TaqMan-Minor Groove Binding (MGB) Allelic Discrimination Assays for the Specific Identification of *Yersinia pestis* from North America and the CO92 Strain

SNP	Forward Primer	Reverse Primer	MGB Probe 1—Specific Allele[a]	MGB Probe 2—Alternate Allele[a]
NA-specific[b]	5′-GCACCATTGACAGCCATATCA-3′	5′-TTTCGCCATCGAGTAATTCCA-3′	5′-VICAAATCTACaGCGGAAAC-3′	5′-6FAMTCTACgGCGGAAAC-3′
CO92-specific	5′-GGAATCATCGGTGCTTTGATATAA-3′	5′-GGGACACCAAGGCTTTTGAG-3′	5′-VICTTATCATCcCCCCAGAA-3′	5′-6FAMATCATCaCCCCAGAAA-3′

[a] Lowercase base indicates SNP

[b] NA: North America