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Note

Estimated copy number of *Bacillus anthracis* plasmids pXO1 and pXO2 using digital PCR

Timothy Straub *, Cheryl Baird, Rachel A. Bartholomew, Heather Colburn, Derrick Seiner, Kristin Victry, Li Zhang, Cynthia J. Bruckner-Lea

Chemical and Biological Signature Sciences Group, National Security Directorate, Pacific Northwest National Laboratory, PO Box 999 MS P750, Richland, WA 99354, United States

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ABSTRACT

We evaluated digital PCR (dPCR) to directly enumerate plasmid and chromosome copies in three strains of *Bacillus anthracis*. Copy number estimates based on conventional quantitative PCR (qPCR) highlighted the variability of using qPCR to measure copy number whereas estimates based on direct sequencing are comparable to dPCR.

Digital PCR (dPCR) was first described by Vogelstein and Kinzler (1999). The theory behind digital PCR is to prepare a conventional quantitative PCR reaction (qPCR), and then divide it into several hundreds to thousands of PCR reactions, with each reaction containing several picoliters to nanoliters per reaction with ideally only one or no molecule in each reaction. Scoring the positive and negative reactions, it is then possible to get a direct measure of copy number of a target gene without the need for the internal controls or standards for quantitation and assessing reaction efficiency that are required for qPCR (Bhat et al., 2009; Burns et al., 2010; Corbisier et al., 2010; Day et al., 2012; Dube et al., 2008). Whereas recent dPCR citations focus on copy number variation in clinical samples (Azuara et al., 2012; Barrett et al., 2012; Schell et al., 2012), applications of dPCR for environmental biodetection are starting to appear. For example, Hoshino and Inagaki (2012) reported that dPCR performed better than qPCR for DNA recovered from soils. Their findings were possibly explained by significant dilution of humic acid in the individual sub-reactions in dPCR.

Employing dPCR, our aim was to characterize copy number variation between chromosome and plasmids pXO1 and pXO2 for several virulent *Bacillus anthracis* strains in a multiplexed digital PCR format. DNA stocks from 3 virulent strains of *B. anthracis* (Ames (MLVA subtype A3b), South Africa 1035 (MLVA subtype B1), and Canadian Bison (MLVA subtype A1a)) were obtained from the United States Critical Reagents Program via the NIH Biodefense and Emerging Infections Research Resources Repository (BEI), NIAID, and NIH (Manassas, VA). Concentrations of the stocks were measured using a double stranded DNA intercalating dye assay followed by quantification on a fluorescence plate reader per manufacturer's protocol (Quant-iT™ PicoGreen dsDNA kit, Life Technologies; Carlsbad, CA). *B. anthracis* working dilutions of 13.4, 6.7, 4.02, 2.68, 1.34, and 0.134 pg/μL were prepared as 50 μL aliquots in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5, (Teknova Hollister, CA)) and frozen at −20 °C until used. These dilutions were made to calculate PCR efficiency for qPCR and determine the optimal concentration for dPCR. Due to dead volume in dPCR, the effective quantities tested ranged from 6.2 pg in the reaction wells (for the 13.4 pg/μL concentration) down to 0.062 pg in the reaction wells. Primer and probe sequences for *B. anthracis* are described by Wielinga et al. (2011). Each gene (PL3, cya, and capB for chromosome, pXO1, and pXO2, respectively) occurs as a single copy as determined by BLAST. Both qPCR and dPCR were performed as multiplexed PCRs using ABI's Gene Expression Master Mix and primers (900 nM) and probes (250 nM). The fluor-quencher combinations were FAM-BHQ-1 (chromosome), JOE-BHQ-1 (pXO1), and Cy5-BHQ-2 (pXO2) (Integrated DNA Technologies, Coralville, IA). Copy number variation for qPCR involved verification that the efficiency of PCR was 90–100% for each gene within the multiplexed qPCR. Copy number for each gene was then determined based on each gene target's efficiency curve equation.

Copy number determination using dPCR followed the example calculations of Whale et al. (2012) with the exception that "reference" was set to be the number of molecules for the chromosome

* Corresponding author at: Pacific Northwest National Laboratory, PO Box 999, MS P750, Richland, WA 99354, United States. Tel.: +1 509 375 1477.
E-mail address: Timothy.Straub@pnnl.gov (T. Straub).

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Table 1
Comparison of qPCR to dPCR to calculate ratio of pXO1:chromosome and pXO2: chromosome.

Strain	Ratio measurement	Coventional qPCR			Digital PCR		
		Average ratio	95% CI low	95% CI high	Average ratio	95% CI low	95% CI high
Ames	pXO1:chrom	0.54	0.45	0.80	3.82	3.50	4.17
	pXO2:chrom	1.23	1.03	1.46	2.37	2.17	2.59
1035	pXO1:chrom	0.59	0.20	1.76	3.06	2.82	3.32
	pXO2:chrom	1.00	0.44	2.31	2.00	1.84	2.17
Bison	pXO1:chrom	0.48	0.43	0.52	2.43	2.25	2.62
	pXO2:chrom	0.80	0.76	0.85	1.30	1.20	1.40

and "target" was either pXO1 or pXO2. Calculations are based on the assumption that the number of target molecules in each dPCR well follows the Poisson distribution (Dube et al., 2008). Digital PCR was performed using the BioMark™ HD system (Fluidigm, South San Francisco, CA). The 12.765 digital PCR card (12 samples, 765 reactions per sample) was used following the manufacturers' workflow.

Table 1 compares the results measuring the ratio of plasmids pXO1 to chromosome and pXO2 to chromosome, respectively, for conventional qPCR and dPCR. For the data in Table 1, approximately the same total DNA quantity per analysis method was used (6.7 pg for qPCR and 6.2 pg for dPCR), with three replicates of each sample. With one exception, the 95% confidence interval (CI) range of ratios of plasmid to chromosome based on qPCR was less than that based on dPCR. A review of the literature suggests that qPCR results vary widely for the quantitation of plasmids in *B. anthracis*. For example, Coker et al. (2003), reported ratios of up to 41 copies of plasmid pXO1 per cell. Pilo et al. (2011) reported 10–11 copies of pXO1 in their *B. anthracis* isolates. Both estimates for pXO1, based on qPCR, appear to be elevated. When directly sequenced, the estimates for pXO1 are approximately 3 copies per cell (Rasko et al., 2007; Ravel et al., 2009). However, for estimates of copy number for pXO2, both qPCR and direct sequencing estimate approximately 1–2 copies of this plasmid per cell (Coker et al., 2003; Pilo et al., 2011; Rasko et al., 2007; Ravel et al., 2009). Our dPCR results for Ames and 1035 support direct sequencing data in that there are likely 3–4 copies of pXO1 per cell for Ames and closer to 3 copies of pXO1 per cell for 1035. Our dPCR results for pXO2 for Ames and 1035 are congruent with direct sequencing. For qPCR, our results are within range of estimates for pXO2 copies. When measured by dPCR, results for the Canadian Bison strain were slightly different in that the ratio of pXO1:chromosome was 2:1 and for pXO2:chromosome, the ratio was 1:1.

Our application of dPCR typically involves evaluation of multiple next generation qPCR assays for dangerous pathogens. Nucleic acid panels for LOD and specificity testing can be scarce and we are limited to 10–100 pg of material per year. At very low sample concentrations, typical A_{260} and nucleic acid intercalating dye assays do not have the sensitivity required to accurately measure DNA concentration. Digital PCR offers an attractive, very sensitive, and rapid alternative that allows direct and accurate quantitation of target genes. In addition it provides a tool to characterize and verify the authenticity of isolates (e.g., pathogens with known lack of target genes and/or plasmids) that are used for inclusivity or exclusivity analysis.

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