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A Rapid Antimicrobial Susceptibility Test for *Bacillus anthracis*▽

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An effective public health response to a deliberate release of *Bacillus anthracis* will require a rapid distribution of antimicrobial agents for postexposure prophylaxis and treatment. However, conventional antimicrobial susceptibility testing for *B. anthracis* requires a 16- to 20-h incubation period. To reduce this time, we have combined a modified broth microdilution (BMD) susceptibility testing method with real-time quantitative PCR (qPCR). The growth or inhibition of growth of *B. anthracis* cells incubated in 2-fold dilutions of ciprofloxacin (CIP) (0.015 to 16 μg/ml) or doxycycline (DOX) (0.06 to 64 μg/ml) was determined by comparing the fluorescence threshold cycle (C_T) generated by target amplification from cells incubated with each drug concentration with the C_T of the no-drug (positive growth) control. This ΔC_T readily differentiated susceptible and nonsusceptible strains. Among susceptible strains, the median ΔC_T values were ≥7.51 cycles for CIP and ≥7.08 cycles for DOX when drug concentrations were at or above the CLSI breakpoint for susceptibility. For CIP- and DOX-nonsusceptible strains, the ΔC_T was <1.0 cycle at the breakpoint for susceptibility. When evaluated with 14 genetically and geographically diverse strains of *B. anthracis*, the rapid method provided the same susceptibility results as conventional methods but required less than 6 h, significantly decreasing the time required for the selection and distribution of appropriate medical countermeasures.

Bacillus anthracis, the etiological agent of anthrax, is generally susceptible to the antimicrobial agents currently approved for treatment or postexposure prophylaxis. The potential for the use of *B. anthracis* as an agent of bioterrorism has generated concern about the deliberate release of genetically engineered or *in vitro*-selected strains that are resistant to these antimicrobial agents. An effective public health response to such an event will rely on the rapid deployment of large quantities of medical countermeasures, including antimicrobial agents for prophylaxis and the treatment of potentially exposed or infected populations (35). However, the ultimate impact of this response depends on the rapid identification of the organism and the determination of its antimicrobial susceptibility profile. Without this information, there is the possibility of the use of ineffective antimicrobial agents, which would likely result in increased morbidity and mortality rates.

Conventional susceptibility testing of *B. anthracis* by broth microdilution, agar dilution, disk diffusion, or Etest requires an incubation time of 16 to 20 h (8). Recently, there have been numerous efforts to reduce the time required to detect resistance by using molecular methods such as PCR (2, 10, 20, 33) or microarrays (3, 22, 32, 38), both of which are designed to detect antimicrobial resistance genes or mutations associated with resistance. A basic prerequisite of this approach is that the gene or mutation responsible for resistance must be one that has been previously described, and the appropriate primers or probes must be included in the assay. The detection of a resistance gene sequence provides information only on the presence or absence of a specific DNA target, and a positive result does not necessarily correlate with the phenotypic expression of resistance. For example, there may be gene fragments, missing regulatory genes, insertions, deletions, or poorly executed laboratory engineering that may prevent the detected gene from being expressed, or, if expressed, the protein may be nonfunctional. In either case the isolate would remain susceptible to the antimicrobial agent. Consequently, a positive molecular test result could unnecessarily delay the use of an antimicrobial agent that would have provided effective treatment or postexposure prophylaxis. Alternatively, variations in resistance gene sequences may prevent the optimal hybridization of primers or probes. In this case the result would be a false-negative result, and ineffective antimicrobial agents may be distributed.

Real-time quantitative PCR (qPCR) by a fluorogenic 5′ nuclease assay (TaqMan) incorporates a fluorescently labeled probe that allows the quantification of template DNA based on an increase in fluorescence that is directly proportional to the amount of amplified product. Because the intensity of fluorescence is recorded at each cycle, this method monitors amplification in real time. The number of copies of template DNA can be estimated from the threshold cycle (C_T) value by comparing the C_T of a sample with a standard curve of fluorescence intensities derived from known quantities of the target sequence. Real-time PCR assays are rapid, extremely sensitive, and relatively simple to perform. Furthermore, qPCR can be utilized to determine the microbial gene target copy number in a sample (37). Relative increases in copy numbers over time can be utilized to evaluate the growth of an organism.

This report describes a rapid susceptibility assay for *B. anthracis* that is based on a comparison of real-time PCR results from cell lysates after the cells are incubated in the presence and absence of antibiotics. The assay was evaluated with a group of 14 genetically and geographically diverse strains of *B. anthracis* as well as nonsusceptible control strains.

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TABLE 1. *B. anthracis* strains used in this study

Strain	Alternate identification(s)	Origin	Genotype	Cluster	Reference
34F2	Sterne, Weybridge	South Africa	pXO1$^+$ pXO2−	A3.b	30
Sterne-402					This study
Sterne-411A2					This study
Sterne/pUTE29					19
Sterne-S1028					This study
A0193	K1256	United States	10	A1.a	17
A0293	2PT, K4241	Campania, Italy	20	A1.a	17
A0149	32, K9002	Yozgat, Turkey	23	A1.b	17
A0264	14, K7948	Sivas, Turkey	28	A1.b	17
A0419	H-1, K7038	South Korea	34	A3.a	17
A0188	93/37, K1285	Namibia	35	A3.a	17
A0376	BA1015, K4516	Maryland	51	A3.a	17
A0462	Ames, ASC159	Texas	62	A3.b	17
A0248	28, K2802	Ohio	68	A3	17
A0488	Vollum	United Kingdom	77	A4	17
A0465	RA3, K2762	France	80	B2	17
A0102	3, K2478	Mozambique	85	B1	17
240		Unknown	133	C	31

(This study was presented in part at the American Society for Microbiology Biodefense and Emerging Diseases Research Meeting, Baltimore, MD, 22 to 25 February 2009 [36].)

MATERIALS AND METHODS

Bacterial strains and culture methods. The *B. anthracis* strains used in this study (listed in Table 1) were provided by Alex Hoffmaster, Division of Food-Borne, Bacterial, and Mycotic Diseases, CDC, Atlanta, GA. The strains were selected to represent clusters of known genetic variability and diverse geographic regions as described previously (16, 17, 31). For conventional and rapid susceptibility testing, each strain was cultured overnight at 35°C in ambient air on Mueller-Hinton (MH) agar (BBL, Becton Dickinson, Sparks, MD) from glycerol stocks maintained at −70°C.

Ciprofloxacin (CIP)-nonsusceptible (NS) mutants and tetracycline (TET)-NS transformants of *B. anthracis* Sterne (30) were derived as control strains with permission from the CDC Institutional Biosafety Committee. CIP-NS mutants were generated by a three-step selection process with increasing concentrations of ciprofloxacin essentially as previously described (4, 5, 7, 11), and the relevant mutations were determined by DNA sequence analysis of the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* as described previously by Price et al. (24), with the exception of the *gyrA* and *parC* primers used for the amplification and sequence reactions, which were Ba-gyrA79F (5′-GCAATGAGTGTTATCGTATCTCG), Ba-gyrA389R (5′-GACATTCTTG CTTCTGTATAACGC), Ba-parC50F (5′-ACCGCTTTGCACGTTATAGTAA AT), and Ba-parC446R (5′-ACAACCGGTTCTTCACTAGTAT), selected with Oligo 7.0 software (Molecular Biology Insights, Inc., Cascade, CO). A TET-NS transformant of *B. anthracis* Sterne was generated by electroporation with pUTE29 as described previously by Koehler et al. (19). The MICs of CIP and doxycycline (DOX) for selected mutants (Sterne-402, Sterne-411A2, and Sterne-S1028) and the TET-NS transformant were determined by broth microdilution.

Conventional susceptibility testing. Susceptibility to CIP and DOX was determined by broth microdilution in cation-adjusted Mueller-Hinton broth (CAMHB) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (8), using 96-well plates prepared in-house or with JustOne strips from Trek Diagnostics (Cleveland, OH).

Cell lysis. To compare the efficiency of heat lysis with that of enzymatic lysis, a cell suspension of *B. anthracis* Sterne in CAMHB with TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (CAMHBT; Trek Diagnostics) with turbidity equal to a 0.5 McFarland standard (Microscan turbidity meter; Dade-Behring, West Sacramento, CA) was diluted 1:100 in the same broth and incubated for 4 h at 35°C.

For heat lysis, 100 μl of the broth culture was transferred to one well of a 96-well MicroAmp reaction plate (Applied Biosystems [ABI], Foster City, CA) and heated to 95°C for 15 min in a 9800 Fast thermal cycler (ABI). The resulting cell lysate was applied onto a 0.1-μm Durapore polyvinylidene difluoride (PVDF) spin filter (Millipore, Burlington, MA), and the cleared lysate was collected following microcentrifugation for 2 min at 5,000 × *g*.

For enzymatic lysis, 100 μl of the broth culture was mixed thoroughly with 100 μl of TL buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 4% Triton X-100) and filtered through a Durapore PVDF spin filter by centrifugation at 2,000 × *g* for 2 min to remove the medium and capture the cells on the filter. Ten units of purified PlyG lysin, (27) in 100 μl of lysis buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.5]) was added to the cells, and the cell suspension on the filter was incubated for 15 min at 35°C. The lysate was collected by centrifugation at 5,000 × *g* for 2 min. A 5-μl aliquot of the cleared cell lysate from either method was used as the qPCR template. Other methods evaluated for cell lysis included bead beating, essentially as described previously by Almeida et al. (1); heat lysis in the presence of 0.05 N NaOH (34); or heat lysis with detergent (SDS) (18). The purified PlyG lysin was a generous gift from Vincent Fischetti, Rockefeller University.

Rapid susceptibility test. Twelve-well strips that included 11 wells containing dried, 2-fold dilutions of CIP (0.015 to 16 μg/ml) or DOX (0.06 to 64 μg/ml) and 1 well without drug were designed and evaluated for this assay (JustOne strips; Trek Diagnostics). The inoculum was prepared by suspending cells from four to six *B. anthracis* colonies from a fresh culture grown overnight on MH agar in CAMHBT. The cell suspension, equivalent to a 0.5 McFarland turbidity standard, was then diluted 1:100 in the same medium. A 100-μl aliquot of the diluted cell suspension was added to each well of the JustOne strip and mixed to suspend the dried antibiotic, and the strips were incubated for 4 h at 35°C. After incubation, 100 μl of TL buffer was added to each well and mixed with the cells, and 170 μl was transferred onto a 96-well 0.1-μm Durapore filter unit (Millipore). The cells were captured on the filter by centrifugation at 500 × *g* for 2 min and resuspended in 75 μl of lysis buffer containing 10 units of PlyG. After incubation for 15 min at 35°C, the cleared lysates were collected by centrifugation through the same 0.1-μm filter plate for 5 min at 750 × *g*. Lysates were used immediately or stored at −20°C. Each sample was tested in triplicate, and each strain was tested in four independent experiments.

Real-time PCR. The primers and the fluorogenic probe for the *rpoB* gene of *B. anthracis* were designed by using Primer Express software, version 3.0 (ABI). The sequences of the oligonucleotide primers were forward primer 5′-CGTTTCTTCGATCCAAAGCGCTAT-3′ (positions 794 to 817), reverse primer 5′-CTAAAATTTCACCAGTTTCTGGATCT-3′ (positions 933 to 958), and probe 5′-ATTTAGCAAATGTAGGTCGCTACAAGATCAACAAGAAGT TA-3′ (positions 798 to 838). The fluorescent reporter dye on the 5′ end of the probe was 6-carboxyfluorescein (FAM), used in conjunction with a black hole quencher (BHQ). PCR was performed with the ABI 7500 Fast real-time PCR system. The 20-μl reaction mixtures consisted of 1.9 μM (each) forward and reverse primers, 380 nM probe, 50 nM the passive dye 5-carboxy-X-rhodamine (ROX), 1× (final concentration) TaqMan Fast Universal PCR master mix (purchased as 2× master mix; ABI), and 5 μl of cell lysate. The no-template control (NTC) reaction mixtures contained H_2O (CLSI type 1) in place of cell lysate.

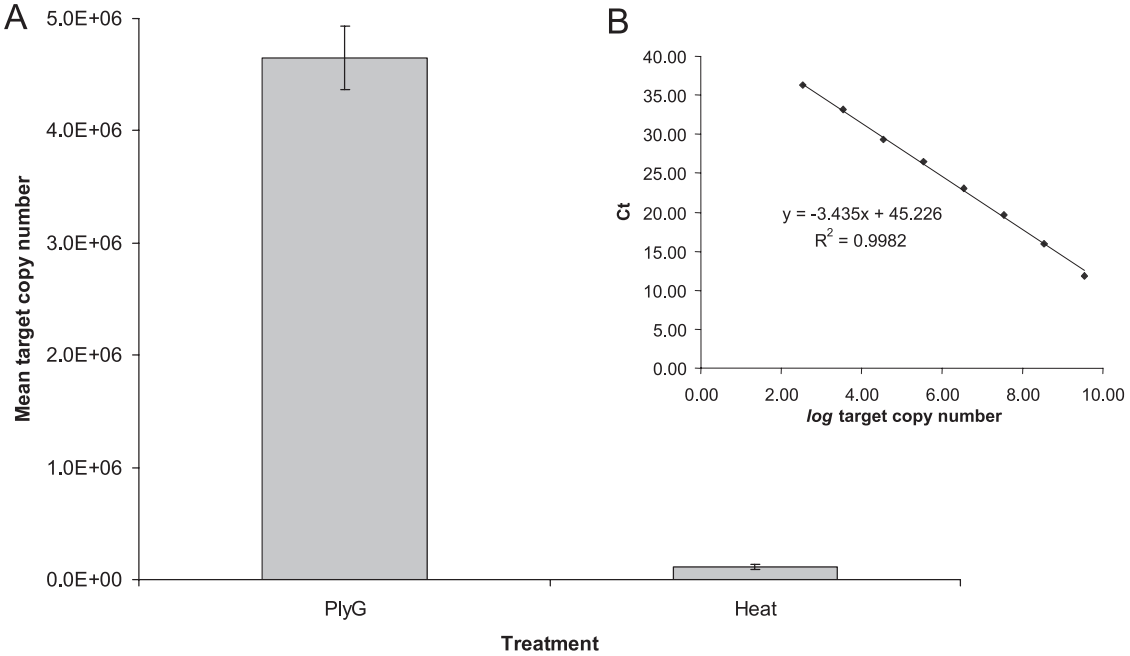

FIG. 1. (A) Comparison of target copy numbers released from *B. anthracis* cells following treatment with either the PlyG lysin or heat. Copy numbers are based on qPCR C_T values from each cell lysis method compared with a standard curve generated from dilutions of a known quantity of the target sequence. Bar graphs are based on three independent experiments. (B) Standard curve relating target copy number (*x* axis) and relative fluorescence (*y* axis) in qPCR.

qPCRs for each sample (representing one well in the strip) were run in triplicate. Thermocycler parameters were programmed for 95°C for 20 s followed by 45 cycles of 95°C for 3 s and 60°C for 20 s. Data collection and analysis were performed by using 7500 Fast real-time PCR system sequence detection software, v1.4 (ABI).

Estimation of *rpoB* target copy number. The C_T values obtained from qPCR assays were converted to estimated *rpoB* copy numbers by using a standard curve (Fig. 1B) generated from the C_T values of eight samples containing known concentrations of the target DNA sequence. Briefly, a 360-bp fragment containing the *rpoB* qPCR target was amplified from *B. anthracis* Sterne by using the following PCR primers designed to include BamHI and PstI restriction sites (underlined): forward primer 5′-AGGATCCGAGCGTCTACGTCCTGGTGA AC-3′ and reverse primer 5′-ACCTGCAGGCGCTCGCCTTCTGACTC-3′, respectively. The fragment was inserted into pUC19, which was then transformed into *Escherichia coli* DH10B electrocompetent cells (Invitrogen, Carlsbad, CA). Following plasmid isolation with the QIAprep plasmid miniprep kit (Qiagen, Valencia, CA), the plasmid concentration was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The number of *rpoB* gene fragment copies per pg of DNA was extrapolated from the calculated molecular mass of the *rpoB* gene target insert.

Statistical analysis. To account for skewness and reduce the influence of outliers in the distribution of ΔC_T values, the median and interquartile range for each strain and drug concentration were determined based on results from four independent experiments. The median and interquartile range of the distribution of ΔC_T values among all susceptible strains combined were also estimated at each drug concentration. All statistics were calculated by using SAS, v9.1.3 (SAS Institute Inc., Cary, NC).

RESULTS

MICs by conventional susceptibility testing. CLSI breakpoints of ≤0.25 μg/ml CIP and ≤1.0 μg/ml DOX have been established to define the susceptibility of *B. anthracis* to these antimicrobial agents (8). However, the absence of naturally occurring CIP- or DOX-resistant strains prevents the definition of any other category. Therefore, MIC values can be interpreted only as susceptible or nonsusceptible. All strains of *B. anthracis* (Table 1) were susceptible to CIP (MIC range, 0.015 to 0.12 μg/ml) and DOX (MIC ≤ 0.06 μg/ml) by conventional broth microdilution (Tables 2 and 3).

CIP- and DOX-NS control strains. DNA sequence analysis of the QRDR of *gyrA*, *gyrB*, *parC*, and *parE* detected a single mutation in Sterne-402 that resulted in an amino acid change of Ser-85 to Leu in *gyrA* and a double mutation in Sterne-411A2 (Ser-85 to Leu in GyrA and Ser-81 to Phe in ParC). The MICs of CIP were 1 μg/ml for Sterne-402 and >16 μg/ml for Sterne-411A2.

The MIC of DOX was 16 μg/ml for the Sterne/pUTE29 transformant, and for the TET-selected mutant Sterne-S1028, the MIC of DOX was 1 μg/ml. The mutations that resulted in a decreased susceptibility of Sterne-S1028 to DOX were not identified. Mutant strains selected on CIP remained susceptible to DOX; the TET-NS strain Sterne/pUTE29 and the mutant strain selected on TET remained susceptible to CIP (Tables 2 and 3).

Cell lysis. A complete lysis of *B. anthracis* cells is necessary for accurate comparisons of cell growth in each well of the strip. We found that lysis using a standard heat lysis protocol, 95°C for 15 min (14, 15), gave inconsistent results between strains, and results were rarely reproducible for the same strain. No amplified product was detected after using the bead-beating method (data not shown). Heat lysis in the presence of either 0.05 N NaOH or detergents did not improve the reproducibility of target gene amplification from cell lysates compared with heat lysis alone (data not shown). Therefore, an alternative lysis method was evaluated by using purified PlyG lysin (27). qPCR results based on the amplification of the *rpoB* gene from lysates of *B. anthracis* Sterne prepared by either

TABLE 2. ΔC_T values[a] for *B. anthracis* isolates and control strains with ciprofloxacin[c]

Strain	ΔC_T with CIP at MIC (μg/ml) of:												MIC (μg/ml)
	0	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	
34F2	0	0.61	2.33	5.87	7.69	8.93	8.81	8.62	8.82	8.63	8.21	7.60	0.06
A0193	0	0.54	1.95	5.00	8.17	8.44	8.58	8.46	8.95	8.71	8.59	8.01	0.015
A0293	0	0.70	2.30	5.34	7.83	8.21	8.59	8.78	9.23	8.49	7.98	7.88	0.06
A0149	0	1.01	2.72	4.92	7.17	7.48	7.63	8.71	8.90	8.54	8.17	7.37	0.06
A0264	0	0.80	1.52	2.72	6.15	6.54	6.70	7.30	7.42	7.91	7.33	6.32	0.06
A0419	0	0.59	2.13	4.65	7.29	7.59	8.03	8.15	8.61	8.09	7.76	6.73	0.06
A0188	0	0.82	1.85	4.78	8.03	7.73	7.55	7.89	8.15	7.83	7.67	7.49	0.06
A0376	0	0.44	0.87	3.14	5.36	7.21	7.28	6.96	7.09	7.06	6.84	6.67	0.12
A0462 (Ames)	0	0.57	1.94	3.20	7.27	9.19	8.80	8.86	8.90	9.05	8.13	8.06	0.12
A0248	0	0.53	2.19	5.34	8.69	8.17	8.61	8.52	8.76	8.99	8.53	8.21	0.12
A0488 (Vollum)	0	0.66	2.53	5.07	8.60	8.54	8.74	8.48	8.75	8.35	8.24	7.98	0.06
A0465	0	0.72	2.94	4.99	7.12	7.55	7.26	7.76	7.07	7.27	7.34	7.03	0.03
A0102	0	0.88	4.18	5.59	6.69	7.64	8.31	8.22	8.89	9.27	8.98	9.00	0.06
240	0	0.23	0.61	1.99	4.89	6.16	6.94	7.05	7.25	7.41	7.36	7.36	0.06
Median[b]		0.63	2.02	4.89	7.31	7.73	7.97	8.11	8.49	8.18	7.83	7.51	
Lower quartile		0.48	1.57	3.28	6.45	7.23	7.49	7.57	7.54	7.50	7.22	6.88	
Upper quartile		0.82	2.83	5.34	8.32	8.52	8.64	8.71	8.99	9.13	8.55	8.32	
Control													
Sterne-402	0	0.07	0.10	0.06	0.12	0.19	1.07	2.84	5.67	6.87	7.57	7.86	1
Sterne-411A2	0	0.44	0.43	0.22	0.06	0.00	0.31	0.20	0.41	0.86	0.97	0.81	>16
Sterne-S1028	0	0.41	1.32	3.70	5.44	6.86	7.21	7.48	7.95	8.06	7.78	7.67	0.06
Sterne/pUTE29	0	0.41	1.97	4.58	6.83	8.64	8.74	8.73	8.58	8.35	8.01	7.71	0.06

[a] C_T values were calculated as follows: $C_{T\text{drug}} - C_{T\text{no drug}}$.
[b] For all susceptible strains.
[c] The breakpoint for susceptibility is ≤0.25 μg/ml CIP (8).

heat lysis or the PlyG lysin method (Fig. 1A, representing three independent experiments) indicated that at least 20-fold-more DNA was released by PlyG treatment. The estimated number of copies of the *rpoB* target sequence was calculated based on the standard curve shown in Fig. 1B.

Minimum incubation time. To determine the minimum time of incubation required to provide consistent, reproducible results by qPCR, *B. anthracis* strains were incubated for 2.5 h to 6 h and analyzed at 30-min intervals. qPCR results from cells incubated for <4 h were ambiguous and not reproducible (data not shown). Incubation for ≥4 h provided consistent, reproducible results. Therefore, the rapid test was subsequently optimized by using a 4-h incubation time.

Detection of antimicrobial resistance based on the real-time PCR viability assay. PlyG cell lysates were prepared from the cells in each well of a 12-well strip after 4 h of incubation at 35°C. The qPCR results for CIP-susceptible *B. anthracis* Sterne (MIC = 0.06 μg/ml CIP) in Fig. 2A show that the amplified target decreased in quantity as the concentration of CIP approached the MIC (i.e., wells with 0.015 and 0.03 μg/ml CIP). At the MIC of ciprofloxacin (0.06 μg/ml CIP), the ΔC_T increased from 0.59 (at 0.015 μg/ml CIP) to 5.73, an estimated 40-fold decrease in copy number compared with that of the no-drug control. At concentrations above the MIC (≥0.12 μg/ml CIP), the ΔC_T continued to increase to a range of 7.54 to 9.42 cycles, representing a decrease in the target copy number of >150-fold compared with the sample from the growth control well. These data indicate that fewer copies of target DNA were available in wells with ≥0.06 μg/ml CIP, which in turn reflects an inhibition of cell growth and genome replication. Below the bar graphs that represent the estimated number of DNA copies in each sample, the corresponding ΔC_T values indicated that a ΔC_T of ≥5.73 cycles was observed for each sample when the concentration of CIP was at or above the MIC and that a ΔC_T of ≥8.08 cycles was observed when the concentration of CIP was above the breakpoint for susceptibility (0.25 μg/ml CIP).

In contrast, the growth of the CIP-NS double mutant of *B. anthracis*, Sterne-411A2 (MIC > 16 μg/ml CIP), was relatively uninhibited in all wells, as shown by the bar graphs in Fig. 2B, representing the number of copies of target DNA in each sample. The corresponding ΔC_T values (compared with the no-drug control) were 0.18 cycles at the breakpoint for susceptibility and 1.26 cycles at the highest concentration of CIP (16 μg/ml). These results indicated that the growth of the mutant strain was not inhibited when incubated in the presence of CIP at concentrations of up to 16 μg/ml and confirmed that the ΔC_T from qPCR is a reliable indicator of growth or inhibition of growth in the presence of antimicrobial agents.

Similar results were obtained when the Sterne strain was incubated in the presence of DOX (Fig. 2C). In this assay all concentrations of DOX (0.06 μg/ml to 64 μg/ml) were inhibitory, and the ΔC_T values for each well compared with the no-drug control were ≥7.29 cycles (decreased more than 100-fold). However, from lysates of the TET-NS strain Sterne/pUTE29 (Fig. 2D), the quantity of target DNA from the cells exposed to 0.06 to 1 μg/ml was decreased less than 2-fold compared with the no-drug control. At that point the number of copies began to decrease until the ΔC_T values reached 4.35 at 8 μg/ml DOX and 7.28 (or approximately 150-fold less) at

TABLE 3. ΔC_T values[a] for *B. anthracis* isolates and control strains with doxycycline[c]

Strain	ΔC_T with DOX (μg/ml) at MIC of:												MIC (μg/ml)
	0	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	
34F2	0	7.81	8.21	7.60	7.20	7.11	7.11	7.22	7.20	7.36	7.22	7.70	≤0.06
A0193	0	7.97	8.73	7.81	7.78	7.07	7.29	7.32	7.80	8.01	7.74	8.13	≤0.06
A0293	0	7.69	8.16	7.53	6.99	6.95	6.94	6.80	7.57	7.66	7.54	7.61	≤0.06
A0149	0	6.95	7.18	6.59	6.46	6.34	6.28	6.63	6.99	6.95	6.92	7.24	≤0.06
A0264	0	6.28	6.56	6.01	5.22	5.08	5.09	5.16	6.00	6.29	5.91	6.16	≤0.06
A0419	0	6.60	6.93	6.53	6.25	6.32	6.38	6.28	6.34	6.52	6.67	6.57	≤0.06
A0188	0	7.48	7.70	7.38	7.01	6.95	6.87	6.61	6.54	6.66	6.47	6.37	≤0.06
A0376	0	7.52	8.18	7.89	7.88	7.86	7.66	6.60	7.19	7.19	6.79	6.98	≤0.06
A0462 (Ames)	0	8.32	9.32	8.81	8.10	8.34	8.33	8.02	8.28	8.58	8.23	8.44	≤0.06
A0248	0	7.98	8.12	7.71	7.22	6.95	6.99	7.16	7.96	8.03	7.54	7.97	≤0.06
A0488 (Vollum)	0	8.82	9.70	8.77	8.53	8.79	9.27	7.87	8.08	8.39	8.51	8.60	≤0.06
A0465	0	7.29	7.19	6.77	6.37	6.37	6.88	6.82	7.63	7.46	7.21	7.25	≤.06
A0102	0	8.20	8.64	7.93	7.02	7.19	7.90	7.55	8.10	8.11	8.09	8.22	≤0.06
240	0	8.39	9.67	8.89	9.46	8.32	8.42	7.77	7.98	8.38	7.83	7.70	≤0.06
Median[b]		7.64	8.06	7.68	7.34	7.11	7.23	7.08	7.59	7.57	7.45	7.49	
Lower quartile		7.19	7.46	6.84	6.56	6.60	6.68	6.47	6.80	6.84	6.80	6.87	
Upper quartile		8.31	9.04	8.30	7.98	8.08	8.20	7.72	8.11	8.31	8.00	8.20	
Controls													
Sterne-402	0	7.73	8.23	8.39	8.35	8.22	8.44	8.12	7.69	7.91	8.13	8.26	≤0.06
Sterne-411A2	0	7.58	7.96	7.95	7.72	7.86	7.93	7.14	7.04	7.00	7.00	7.23	≤0.06
Sterne-S1028	0	0.65	1.64	2.46	3.82	5.45	6.61	7.24	7.68	8.00	6.52	7.07	1
Sterne/pUTE29	0	0.26	0.56	0.45	0.42	0.82	1.69	3.29	5.15	6.93	7.16	7.26	16

[a] C_T values were calculated as follows: $C_{T\text{drug}} - C_{T\text{no drug}}$.
[b] For all susceptible strains.
[c] Breakpoint for susceptibility is ≤1 μg/ml DOX (8).

the MIC (16 μg/ml of DOX) for the transformed strain. Reduced susceptibility to DOX was also detectable in the mutant strain Sterne-S1028 (Table 3). In this case the ΔC_T was observed to gradually increase from 0.65 cycles (at 0.06 μg/ml DOX) to 5.45 cycles at the MIC (1 μg/ml DOX). For the same concentrations of DOX, the fully susceptible Sterne strain had ΔC_T values ranging from 7.11 to 8.21 cycles.

Having established that the ΔC_T reflects the difference in target copy number and, therefore, the growth or inhibition of growth of susceptible and nonsusceptible control strains, further evaluation of the rapid susceptibility test was performed by using strains of *B. anthracis* that were selected to represent known genetic and geographic diversity (Table 1). Median ΔC_T values of samples from each concentration of CIP or DOX in the JustOne strips are shown in Tables 2 and 3, respectively. Each strain was tested in four independent experiments, and within each experiment, every sample was tested in triplicate by qPCR. Median ΔC_T values varied as the concentration of CIP increased and approached the MIC for each strain (Table 2). These values ranged from 0.23 to 1.01 cycles at 0.015 μg/ml CIP and from 0.61 to 4.18 cycles when the concentration of CIP was 0.03 μg/ml. The highest MIC of CIP among the strains was 0.12 μg/ml CIP, and all median ΔC_T values were ≥4.89 cycles at this concentration. When CIP concentrations were above the breakpoint for susceptibility (0.25 μg/ml CIP), median ΔC_T values ranged from 7.51 to 8.49 cycles. The interquartile range of the median ΔC_T values indicated highly reproducible results among the susceptible strains (1.14 to 1.63 cycles) for all concentrations above the MIC of CIP.

The MIC of DOX for these strains was consistently ≤0.06 μg/ml; therefore, the median ΔC_T values from samples grown with the lowest concentration of DOX in the JustOne strip (0.06 μg/ml) were consistently higher than those for CIP. The overall median ΔC_T values for these susceptible strains ranged from 7.08 to 8.06 cycles, indicating the inhibition of growth by all concentrations of DOX that were tested. Interquartile ranges of the overall median ΔC_T values were highly consistent (1.12 to 1.58 cycles).

In contrast, qPCR data for the TET-NS strain Sterne/pUTE29 resulted in median ΔC_T values of 0.26 cycles from the 0.06-μg/ml DOX sample and 0.82 cycles at the breakpoint for susceptibility to DOX (1 μg/ml), and ΔC_T values did not exceed 5 cycles until the concentration of DOX reached 8 μg/ml, 1 dilution below the MIC. These data indicated that consistent and reproducible discrimination between susceptible and nonsusceptible strains could be determined based on ΔC_T data in this assay.

DISCUSSION

Although there have been no reports of naturally occurring resistance to CIP or DOX in *B. anthracis*, the *in vitro* selection of resistant mutants and cloning of resistance genes are relatively simple laboratory procedures. Previous reports of strains engineered for resistance to tetracyclines (23) or for multidrug resistance (MDR) (29) have generated concerns about the use of such strains in a deliberate release of *B. anthracis*. Also, the acquisition of resistance determinants by *B. anthracis* has been documented in experiments with the organism in the rhizosphere of grass plants (26). Therefore, the possibility of hori-

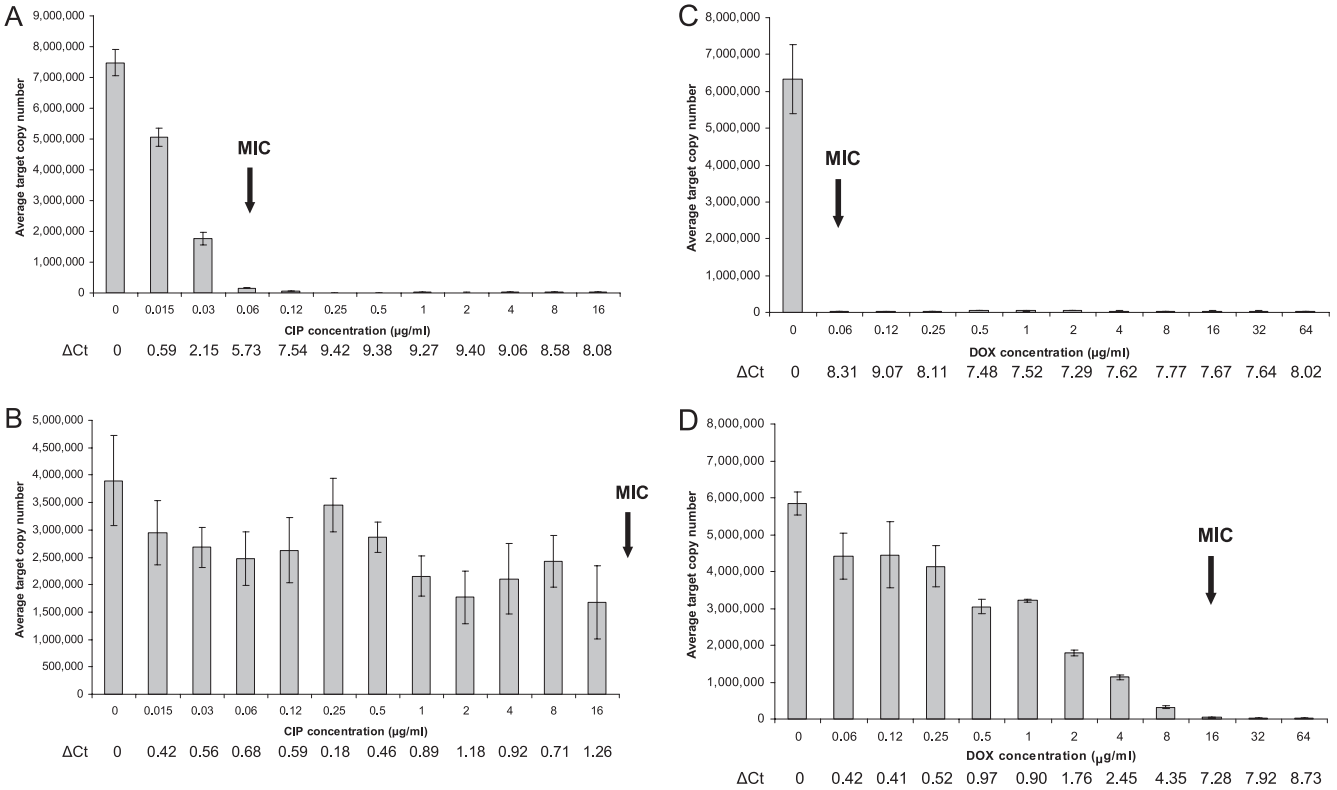

FIG. 2. qPCR results for susceptible and resistant strains following 4 h of incubation in the presence of CIP or DOX. (A) *B. anthracis* Sterne in CIP. (B) CIP-resistant double-mutant strain Sterne-411A2 in CIP. (C) *B. anthracis* Sterne in DOX. (D) DOX-resistant Sterne/pUTE29 in DOX. Bar graphs representing the target copy numbers in each sample of DNA are derived by comparing the fluorescence of each sample with a standard curve generated with known concentrations of the target sequence (Fig. 1B). The ΔC_T below each bar graph compares the C_T for each sample with the C_T observed when the strain was grown without CIP (A and B) or without DOX (C and D).

zontal gene transfer from coexisting soil-dwelling bacteria that harbor many resistance genes cannot be excluded (9), and rapid methods for detecting resistance are essential for public health preparedness and response.

The method described in this study is based on a method described previously by Rolain et al. (25), in which a LightCycler assay (Roche Biochemicals, Mannheim, Germany) was used to estimate the growth and antibiotic susceptibilities of various Gram-positive and Gram-negative species. In agreement with that report, we found that a 4-h incubation time was sufficient for discrimination between the growth and inhibition of growth of *B. anthracis*, as was reported for other rapidly growing Gram-positive organisms. However, efforts to adapt the described qPCR method to *B. anthracis* presented numerous challenges.

The ideal antimicrobial susceptibility testing method for the rapid analysis of strains isolated during an outbreak of disease should facilitate the simultaneous processing of multiple samples. The purification of template DNA from cells in each well of the 12-well strip for each strain was considered to be too time-consuming, so the method was modified to use simple cell lysates. Also, since a comparison of target concentrations by qPCR will provide reliable, reproducible results only if efficient cell lysis is achieved for each sample, numerous lysis methods were investigated to optimize this critical step. The cell wall of *B. anthracis* is known to be resistant to lysozyme and partially resistant to mutanolysin (39). Heat lysis is routinely used to release DNA from *B. anthracis*, and this lysis method is usually sufficient to provide enough DNA to detect the presence of *B. anthracis* by PCR. However, we found that the heat method was not efficient, nor was it quantitatively reproducible, even for a single strain. The reproducible, complete lysis of *B. anthracis* isolates was ultimately achieved with a purified *Bacillus* phage lysin (27). The optimized lysis procedure included three essential steps: (i) suspending *B. anthracis* cells in a buffer containing Triton X-100, (ii) capture of the bacterial cells on a 0.1-μm Durapore PVDF filter, and (iii) enzymatic breach of the cell wall with a purified lysin. Triton X-100 was required to disrupt tenacious clumps of cells that prevented the access of the lysin enzyme to all cells. The removal of the culture medium while capturing the cells on the filter allowed efficient lysis in an optimized buffer. After the lysis reaction, the lysate was collected by centrifugation through the 0.1-μm Durapore filter. This step was essential to remove any spores that may have been present, ensuring that the lysate would be noninfectious and safe to handle in a biosafety level 2 (BSL-2) environment. Additional time was saved because each step could be performed with an 8- or 12-multichannel pipette using a 96-well plate format, including the use of a 0.1-μm PVDF filter plate that was developed by Millipore for this project.

Evaluation of the assay with numerous isolates of *B. anthracis* was also essential. *B. anthracis* is considered to be geneti-

cally monomorphic or clonal in nature (12, 13, 17, 22). However, we observed numerous variations in growth characteristics among the various strains tested. Initial tests indicated that cell lysis performed directly in Mueller-Hinton broth was efficient for the Sterne strain, but this was not the case for several wild-type strains of *B. anthracis* that were tested. In addition, we observed an extended lag phase for several of the wild-type strains when colonies grown on tryptic soy agar (TSA) with sheep blood (SB) were used to prepare the inoculum for susceptibility tests in CAMHB. This lag time was detected as delayed qPCR signals from cell lysates prepared after 4 h of incubation in the no-drug control well (data not shown). Culture of the strains on Mueller-Hinton agar instead of TSA with SB eliminated the extended lag phase, suggesting that some strains of *B. anthracis* require more time than others to adjust to a change in nutrient composition. Also noted among the wild-type strains were colony types that varied in color, from gray to white, and in size and shape and differences in tenacity when touched with an inoculating loop. Phenotypic variation among strains of *B. anthracis* was reported previously (17, 28) and was even associated with differences in virulence (21). However, the genetic basis of such variation remains to be determined. Such observations suggest that the use of surrogate strains or species to develop assays for *B. anthracis* should be avoided.

Once the variables that affected optimal growth and lysis among the strains were identified and addressed, the evaluation of the assay was continued by using 14 geographically and genetically diverse strains of *B. anthracis*. When PCR results for these susceptible strains were compared with results for CIP-NS and DOX-NS derivatives of the Sterne strain, the observed C_T values allowed us to define a ΔC_T threshold (between the no-drug growth control and the antimicrobial drug dilution wells) that would consistently discriminate between susceptible and nonsusceptible isolates. Note that the ΔC_T must be derived only from a comparison of samples within an individual susceptibility test. Slight variations in the numbers of cells in each inoculum preparation, in the growth rates of each strain, and in pipetting procedures will introduce differences in the total numbers of cells after incubation. The resulting differences in the final concentrations of DNA template in the cell lysates are amplified by the sensitivity of qPCR. Therefore, the mean C_T value of either the no-drug control or samples from any of the antimicrobial drug dilution wells should not be compared to mean C_T values outside that individual test.

An accurate interpretation of susceptibility test results requires following standardized procedures as well as testing appropriate antimicrobial agents and the correct range of concentrations of antimicrobial agents. Procedures established by the CLSI (8) provide guidelines on specific parameters, such as the inoculum, incubation conditions, and composition of the medium, that are known to affect susceptibility test results. All guidelines specified for *B. anthracis* (except incubation time) were therefore incorporated in the development of this rapid susceptibility test. The interpretation of the results was simplified by the absence of any overlap between the median ΔC_T values from susceptible and nonsusceptible strains at the breakpoint for susceptibility.

The qPCR method of susceptibility testing has several advantages. In addition to decreasing the time required to obtain results, the use of qPCR eliminates the issues involved with the visual reading of susceptibility test results in a BSL-3 environment. By definition, the endpoint of conventional MIC testing is based on visible growth. In a BSL-3 laboratory the small amount of growth at the bottom of the wells of clear plastic 96-well plates or strips of wells (JustOne strips) is difficult to observe in the biosafety cabinet. In addition to the distance from the observer, the growth must be viewed through both the glass front of the cabinet and the plastic face shield of a power-assisted personal respirator (PAPR). Even without these conditions, susceptibility testing requires trained, experienced personnel. qPCR provides an alternative method for susceptibility testing with quantitative results that are easily interpreted. qPCR results from this assay for *B. anthracis* can be used as a rapid screen by performing the qPCR on the no-drug control and samples from drug concentrations at and just above the breakpoint for susceptibility.

Alternative methods developed for rapid assays include real-time PCR to detect specific resistance genes. This approach requires primers that are specific for each gene. However, there are now more than 40 tetracycline resistance genes that have been described (6), and most likely, there are many more that are as yet undiscovered. Without phenotypic data, the genetic approach has the potential to detect resistance genes that are not expressed, or, if expressed, the protein may not be functional. In either case the strain would be susceptible to DOX in spite of the positive PCR result. For ciprofloxacin, molecular analysis must be based on DNA sequence data to identify mutations in the DNA gyrase and topoisomerase IV genes.

Real-time PCR is used routinely by Laboratory Response Network (LRN) laboratories for the identification of biothreat agents. Therefore, the adaption of this PCR technology for susceptibility testing will provide a relatively simple assay using a technology that is both familiar and readily available in LRN reference laboratories. The use of the JustOne strips for the rapid susceptibility test also addresses the needs of LRN laboratories, as the strips are individually packaged and stable at room temperature for up to 2 years.

In this study, we have developed and evaluated an antimicrobial susceptibility test that has both conventional and molecular components. This test combines a broth microdilution susceptibility test (with a limited incubation time) and qPCR for the rapid analysis of growth (or inhibition of growth) at each concentration of the drug of interest. The method requires $\leq$6 h to complete, a relatively rapid time to results compared with conventional susceptibility tests for *B. anthracis*, which require 16 to 20 h of incubation time. This rapid method is now being adapted for use with other biothreat agents in ongoing efforts to reduce the time required to detect nonsusceptible strains of each species.

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