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# Antibiotic Susceptibility and Molecular Diversity of *Bacillus anthracis* Strains in Chad: Detection of a New Phylogenetic Subgroup

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**We genotyped 15 *Bacillus anthracis* isolates from Chad, Africa, using multiple-locus variable-number tandem repeat analysis and three additional direct-repeat markers. We identified two unique genotypes that represent a novel genetic lineage in the A cluster. Chadian isolates were susceptible to 11 antibiotics and free of 94 antibiotic resistance genes.**

*Bacillus anthracis*, the etiological agent of anthrax, is a sporulating bacterium causing disease primarily in herbivores in many countries of Southern Europe, South America, Asia, and Africa (22). In Chad, anthrax is hyperendemic in cattle (22), and human cases of intestinal and cutaneous anthrax are reported each year (World Anthrax Data Site [http://www.vetmed.lsu.edu/whocc/]). However, studies of the genetic diversity and antibiotic susceptibilities of *B. anthracis* in this country have not been conducted. Here, we used a previously reported eight-marker multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) method (13) and three additional direct-repeat (DR) markers (15) to examine the genetic diversity of this pathogen in Chad. We also evaluated the Chadian *B. anthracis* isolates for antibiotic resistance by using physiological and genetic methods.

Between 1996 and 2003, 174 blood samples were taken from the heart region or jugular vein of cattle carcasses in five southern prefectures in Chad. The samples were screened for *B. anthracis* by cultivation on tryptone soy agar plates containing 5% sheep blood. After incubation at 37°C for 48 h, cultures from 15 samples were identified as *B. anthracis* on the basis of colony morphology, absence of hemolysis, and susceptibility to *B. anthracis*-specific γ phage (2, 21). These cultures were sent to the Swiss Reference Centre (Bern, Switzerland) for further physiological and genetic testing.

Template DNA for PCR was obtained by cell lysis followed by filtration (18). PCRs were performed using *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland) and an annealing temperature of 55°C if not otherwise specified. We used PCR to screen the samples for the presence of *B. anthracis* chromosomal (Ba813) and plasmid (pXO1 and pXO2) sequences (see Table 1 for PCR targets and primer sequences). Eight VNTR markers, *vrrA*, $vrrB_1$, $vrrB_2$, $vrrC_1$, $vrrC_2$, CG3,

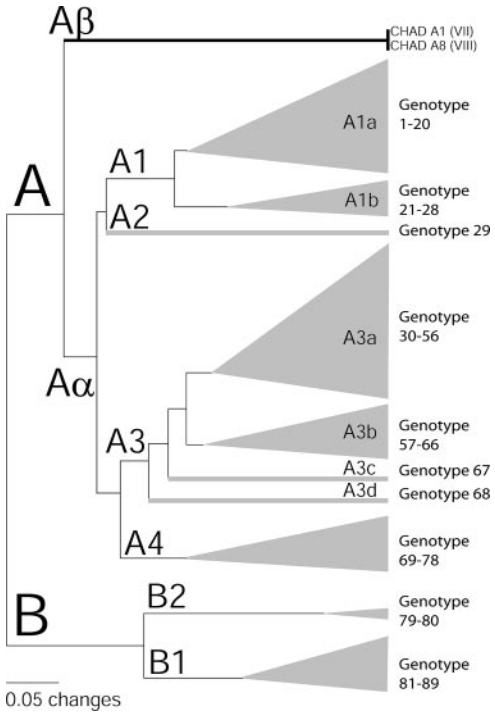


FIG. 1. Dendrogram based on MLVA of eight markers of Chadian *B. anthracis* strains and the diverse 88 genotypes reported by Keim et al. (13). The dendrogram was generated by clustering using the unweighted-pair group method with arithmetic means. Note that genotype 25 has been removed from the analysis since this strain no longer possesses the pXO2 plasmid, resulting in missing data in the MLVA.

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TABLE 1. List of the oligonucleotide primers used for *B. anthracis* strain identification and differentiation

| Gene/locus | Primer | Oligonucleotide sequence (5′ to 3′) | Reference |
|---|---|---|---|
| *pag* (pXO1) | PA5 | TCCTAACACTAACGAAGTCG | 1, 22 |
| | PA8 | GAGGTAGAAGGATATACGGT | |
| *capB* (pXO2) | 1234 | CTGAGCCATTAATCGATATG | 12, 22 |
| | 1301 | TCCCACTTACGTAATCTGAG | |
| Ba813 | R1 | TTAATTCACTTGCAACTGATGGG | 17 |
| | R2 | AACGATAGCTCCTACATTTGGAG | |
| AA03 | AA032 | TTAGCGCCCCCTTGCGTTCC | 15 |
| | AA033 | TTAGCGCCCCTAGACCAATTGC | |
| AJ03 | AJ035 | AGCACCTCGTTCATGCTCATAACGG | 15 |
| | AJ036 | AGCACCTCGTCTACTTCATTTTGTGC | |
| AT07 | AT073 | CTCCTCAAATTACTAAAATGAAACC | 15 |
| | AT074 | TTGGCATAGACGTATATTGCGGTCC | |
| *vrrA* | *vrrA*-f1 | CACAACTACCACCGATGGCACA | 13 |
| | *vrrA*-r1 | GCGCGTTTCGTTTGATTCATAC | |
| $vrrB_1$ | $vrrB_1$-f1 | ATAGGTGGTTTTCCGCAAGTTATTC | 13 |
| | $vrrB_1$-r1 | GATGAGTTTGATAAAGAATAGCCTGTG | |
| $vrrB_2$ | $vrrB_2$-f1 | CACAGGCTATTCTTTATCAAACTCATC | 13 |
| | $vrrB_2$-r1 | CCCAAGGTGAAGATTGTTGTTGA | |
| $vrrC_1$ | $vrrC_1$-f1 | GAAGCAAGAAAGTGATGTAGTGGAC | 13 |
| | $vrrC_1$-r1 | CATTTCCTCAAGTGCTACAGGTTC | |
| $vrrC_2$ | $vrrC_2$-f1 | CCAGAAGAAGTGGAACCTGTAGCAC | 13 |
| | $vrrC_2$-r1 | GTCTTTCCATTAATCGCGCTCTATC | |
| CG3 | CG3-f1 | TGTCGTTTTACTTCTCTCTCCAATAC | 13 |
| | CG3-r1 | AGTCATTGTTCTGTATAAAGGGCAT | |
| pXO1-aat | pXO1-AAT-f3 | CAATTTATTAACGATCAGATTAAGTTCA | 13 |
| | pXO1-AAT-r3 | TCTAGAATTAGTTGCTTCATAATGGC | |
| pXO2-at | pXO2-AT-f1 | TCATCCTCTTTTAAGTCTTGGGT | 13 |
| | pXO2-AT-r1 | GTGTGATGAACTCCGACGACA | |

pXO1-aaT, and pXO2-at, were amplified by PCR according to the method of Keim et al. (13) and sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) with dRhodamine-labeled terminators. The sizes of the VNTR amplicons were compared to previously published allele sizes (13, 20) as well as to the 11 *B. anthracis* strain genome sequences from GenBank (http://www.ncbi.nlm.nih.gov).

Since comparisons of the sequence sizes to the fragment size data revealed minor size discrepancies (1 bp for *vrrA*, 3 bp for $vrrC_1$, and a previously reported 3-bp shift in the pXO1 size [P. Keim, personal communication] [6]), MLVA-8 fragment analysis of 88 diverse genotypes (13) and two Chadian isolates (isolate ChadA1, group VII, and isolate ChadA8, group VIII) was performed in the Keim genetics laboratory (Northern Arizona University) to ensure the comparability and accuracy of the raw VNTR scores from the Chadian strains with reference to those from the genotypes published by Keim et al. in 2000 (13) (Fig. 1).

Three additional DR regions of the *B. anthracis* chromosome (markers AJ03, AA03, and AT07) were amplified by PCR as described previously (15), except that an annealing temperature of 45°C was used. The sequences for the different markers were compared to each other by multiple sequence alignment with ClustalW (http://www.ch.embnet.org) and the numbers of DRs determined (Table 2). The Sterne strain NCTC8234 was used as a control, and the positions of the DRs were matched to the complete nucleotide (nt) sequence of this strain (GenBank accession no. AE017225). In the Sterne strain, the AJ03 marker is characterized by a succession of two 40-bp DRs ($^{1092652}$CGAT-32 nt-GCGC$^{1092731}$), the AA03 by a succession of three slightly imperfect repeats ($^{1721256}$TTCA-61/62/63 nt-TTAG$^{1721502}$), and the AT07 marker by a succession of seven 39-bp DRs ($^{4234463}$ACTA-31 nt-TGAT$^{4234735}$).

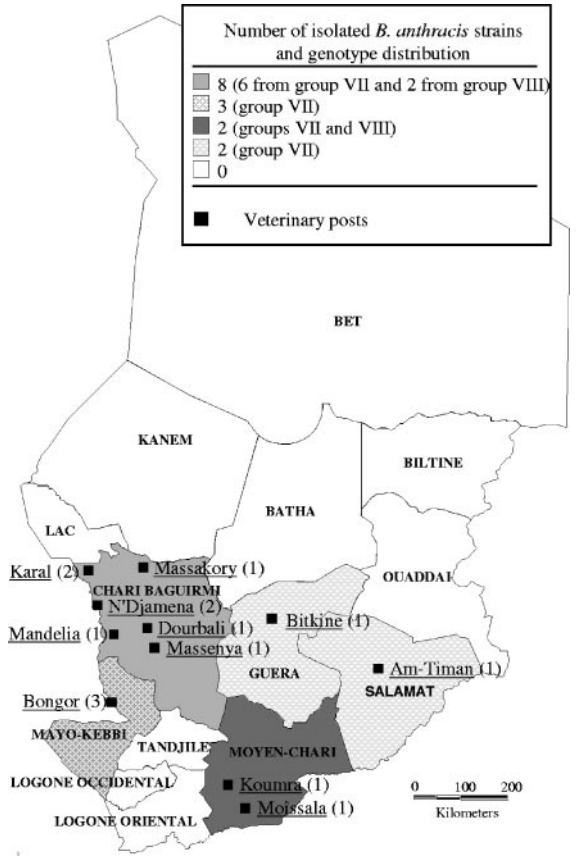


FIG. 2. Geographical and genetic distribution of *B. anthracis* in the different prefectures of Chad. Frequency of occurrence of *B. anthracis* in the different prefectures is indicated by different gray patterns. The numbers of strains isolated in the different town/village areas (■) appear in parentheses after the respective names. The distribution of the two different genotypic groups VII and VIII within the prefectures is indicated in parentheses in the figure panel.

TABLE 2. Genetic diversity and antibiotic susceptibilities of *B. anthracis* strains from different regions in Chad

| Strain(s) | Geographical origin | | Allele size (bp)[b] for indicated MVLA-8 marker | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Town/village | Prefecture (region[a]) | *vrrA* | $vrrB_1$ | $vrrB_2$ | $vrrC_1$ | $vrrC_2$ | CG3 | pXO1 | pXO2 |
| ChadA1 | N'Djamena | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA2 | N'Djamena | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA3 | Massakory | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA4 | Dourbali | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA5 | Karal | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA6 | Am-Timan | Salamat (CE) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA7 | Bongor | Mayo Kebbi (SW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA8 | Koumra | Moyen-Chari (SE) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA9 | Massenya | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA10 | Bitkine | Guera (C) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA11 | Mandelia | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA12 | Bongor | Mayo Kebbi (SW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA13 | Moïssala | Moyen-Chari (SE) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA14 | Bongor | Mayo Kebbi (SW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| ChadA15 | Karal | Chari Baguirmi (CW) | 290 | 229 | 171 | 616 | 604 | 158 | 123 | 133 |
| NCTC8234[f] | | | 314 (313) | 229 (229) | 162 (162) | 580 (583) | 532 (532) | 158 (158) | 132 (129) | |
| All Chadian strains | | | (289) | (229) | (171) | (613) | (604) | (158) | (120) | (133) |

[a] C, central; CW, central west; CE, central east; SW, southwest; SE, southeast.
[b] Values in parentheses indicate allele sizes determined following correction and normalization. The sizes of the alleles are based on their effective lengths in base pairs determined by sequence analysis, since discrepancies exist between the sizes determined by MLVA fragment analysis (14) and the real sizes of alleles *vrrA* (290 versus 289 bp for the Chadian strains and 314 versus 313 bp for the Sterne strain), $vrrC_1$ (616 versus 613 bp for the Chadian strains and 580 versus 583 bp for the Sterne strain), and pXO1 (123 versus 120 bp for the Chadian strains and 132 versus 129 bp for the Sterne strain).
[c] RAPD, random amplified polymorphic DNA.
[d] The groups are based on the numbers of DRs in markers AA03, AJ03, and AT07 according to the classification of Levy et al. (15); groups VII and VIII are new groups.
[e] MICs were determined in duplicate, and the MIC interpretive standards were those recommended in CLSI supplement M100-S15 (8). Antibiotics: GEN, gentamicin; ERY, erythromycin; CLI, clindamycin, SYN, quinupristin-dalfopristin; TET, tetracycline; CHL, chloramphenicol; CIP, ciprofloxacin; XNL, ceftiofur; VAN, vancomycin; PEN, penicillin; AMC, amoxicillin-clavulanic acid. The CLSI susceptibility breakpoints are as follows: for GEN, ≤4; for ERY, ≤0.5; for CLI, ≤0.5; for SYN, ≤1; for TET, ≤1; for CHL, ≤8; for CIP, ≤0.5; for VAN, ≤4; for PEN, ≤0.12; for AMC, ≤4/2. The CLSI susceptibility breakpoints for TET, CIP, and PEN are those recommended in CLSI supplement M100-S15 (8). For GEN, ERY, CLI, SYN, CHL, VAN, and AMC, *S. aureus* susceptibility breakpoints are suggested since no susceptibility breakpoints were available for *B. anthracis* in the CLSI supplement (3, 4, 16). No CLSI criteria for susceptibility were available for XNL. The CLSI resistance breakpoints are as follows: for GEN, ≥16; for ERY, ≥8; for CLI, ≥4; for SYN, ≥4; for CHL, ≥32; for VAN, ≥32; for PEN, ≥0.25; for AMC, ≥8/4. The resistance breakpoint for PEN is that recommended in CLSI supplement M100-S15 (8). For GEN, ERY, CLI, SYN, CHL, VAN, and AMC, *S. aureus* resistance breakpoints are suggested since no resistance breakpoints were available for *B. anthracis* in the CLSI supplement (3, 14, 16). No CLSI criteria for resistance were available for TET, CIP, or XNL.
[f] The vaccine strain Sterne 34F2 (NCTC8234) was used as a control.

The antibiotic susceptibilities of the Chadian *B. anthracis* isolates were measured in Müller-Hinton broth by using custom Sensititre susceptibility plates (Trek Diagnostics Systems, East-Grinstead, England, and MCS Diagnostics BV, JL Swalmen, The Netherlands), according to the CLSI guidelines (7). To exclude the possibility that Chadian *B. anthracis* isolates possessed antibiotic resistance genes that were not phenotypically expressed in vitro, we screened for the presence of 94 gram-positive bacterial antibiotic resistance genes using a microarray (18).

PCR analysis revealed that the 15 isolates were *B. anthracis* and positive for the pXO1 and pXO2 plasmids. The fragment sizes and DNA sequences for the eight MLVA markers were identical in all Chadian strains. The effective sizes and the corrected, normalized scores for the eight MLVA alleles of the Chadian strains are listed in Table 2. Of note, this is the first report of a 133-bp allele in pXO2. Analysis by unweighted-pair group method using average linkages clustered samples into a new genetic group in the A branch according to Keim's classification system (Aβ) (Fig. 1) (13). We used the system proposed by Levy et al. (15) to classify the 15 *B. anthracis* isolates into two different, novel genotypic groups (VII and VIII) (Table 2). These groups are new since this combination of DRs within the three markers has yet to be reported for *B. anthracis*, and the five-DR pattern in the AT07 marker in all Chadian strains is unique. We found no obvious geographic clustering of group VII and VIII strains (Fig. 2).

The Chadian isolates were susceptible to the antibiotics tested but displayed decreased susceptibilities to ceftiofur, erythromycin, and the combination quinupristin-dalfopristin and a slightly higher MIC for clindamycin (MIC, 0.5 μg/ml) than the Sterne strain NCTC8234 (Table 2).

Microarray-based analysis revealed that the strains were free of all antibiotic resistance genes tested except the β-lactamase genes *bla1* and *bla2*, which are endogenous to *B. anthracis* but are not expressed (5).

This study is the first to describe the genetic and physiological attributes of *B. anthracis* strains in Chad. Certainly, this region of Africa has been underrepresented in previous studies of the global genetic diversity of *B. anthracis* (13). On a regional level, the identical MLVA genotypes of the Chadian strains indicate a high degree of genetic similarity among *B. anthracis* isolates within the country. This is supported by the existence of only two unique, closely related strains identified by the three additional DR markers (groups VII and VIII) and the absence of antibiotic resistance genes in all the tested isolates. Similarly, our data suggest a low level of phenotypic diversity as it relates to antibiotic resistance. The finding that

TABLE 2—*Continued*

| Cluster | No. of DRs for indicated RAPD[c] marker | | | Group[d] | Antibiotic MIC (μg/ml)[e] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AA03 | AJ03 | AT07 | | GEN | ERY | CLI | SYN | TET | CHL | CIP | XNL | VAN | PEN | AMC |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 2 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 2 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 1 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 2 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 4 | 5 | VIII | <1 | 1 | 0.5 | 2 | <1 | 8 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 2 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 4 | 5 | VIII | <1 | 1 | 0.5 | 1 | <1 | 8 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 4 | 5 | VIII | <1 | 1 | 0.5 | 1 | <1 | 8 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 1 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 0.5 | 0.5 | 1 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 2 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 1 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 0.5 | 0.5 | 1 | <1 | 8 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 1 | <1 | 8 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | 2 | 2 | 5 | VII | <1 | 1 | 0.5 | 1 | <1 | 4 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| | 3 | 2 | 7 | II | <1 | 1 | <0.25 | 1 | <1 | 8 | <0.25 | 8 | 2 | <0.12 | 2/1 |
| Aβ | | | | | | | | | | | | | | | |

Chadian isolates were susceptible to 11 antibiotics and free of antibiotic resistance genes is significant from a public health perspective and can be used for therapeutic measures in the treatment of people affected with *B. anthracis* in this country.

From a global perspective, our MLVA and DR analysis showed that the Chadian strains displayed unique genetic signatures and are not closely related to any of the worldwide *B. anthracis* strains analyzed to date (6, 9, 10, 11, 13, 19, 20). Indeed, the long branch lengths of the Aβ group indicate a considerable degree of evolutionary divergence for these two strains from the remaining *B. anthracis* isolates in the A lineage (Fig. 1). In summary, our results provide a first look at the genetic diversity and antibiotic resistance patterns of this pathogen in Chad and can be useful for future epidemiological analyses of anthrax outbreaks in this region.

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