

Opinions

A New *Bacillus anthracis* Found in Wild Chimpanzees and a Gorilla from West and Central Africa

Fabian H. Leendertz, Saniye Yumlu, Georg Pauli, Christophe Boesch, Emmanuel Couacy-Hymann, Linda Vigilant, Sandra Junglen, Svenja Schenk, Heinz Ellerbrok*

Anthrax is one of the oldest known zoonotic diseases. Natural outbreaks threaten wildlife, livestock, and human health, and *Bacillus anthracis* has been used as a biological weapon [1,2]. Without exception, *B. anthracis* strains described to date show very little sequence diversity [2–4]. This has led to the conclusion that *B. anthracis* represents a clonal derivative of an ancestral member of the *Bacillus cereus* group that obtained pathogenic potential primarily through the acquisition of two *B. anthracis*-specific virulence plasmids, pXO1 and pXO2 [4,5]. Two major but closely related groups of *B. anthracis* strains (A and B) have been described by means of characterization of six genomic and two plasmid-encoded variable number tandem repeat regions (VNTRs) [6]. Group B strains are almost exclusively restricted to Southern Africa, while group A is found throughout the world and may have been spread by the domestication and international trade of livestock [7,8].

We determined that anthrax infection caused the sudden deaths of six wild chimpanzees in the Taï National Park, Côte d'Ivoire in 2001/2002 [9] and in three chimpanzees and one gorilla found dead more than 1,000 mi to the east, at the northern periphery of the Dja Reserve, Cameroon 2004/2005 [10]. In order to characterize the *B. anthracis* isolates from a totally new habitat that were responsible for the death of wild great apes that died in Côte d'Ivoire and Cameroon, we performed VNTR analyses on DNA isolated from tissue samples and bones of these great apes. Surprisingly, for the isolates from both outbreaks, three of the six genomic VNTRs were clearly different from those of any previously described strain, as is evident from the phylogenetic tree analysis. The strain from Côte d'Ivoire, named *B. anthracis* CI (Côte d'Ivoire), and the strain from Cameroon, named *B. anthracis* CA (Cameroon) are clearly distinct from group A and group B strains. They establish a separate branch with a new “forest anthrax cluster,” termed “F” (Figure 1) suggesting that *B. anthracis* is a far less homogeneous species than is currently believed. No differences were found among the six cases from Côte d'Ivoire, and also the four anthrax isolates from the three chimpanzees and the gorilla from Cameroon were identical.

Since these new *B. anthracis* strains were so clearly distinct from “classic” *B. anthracis*, we next examined the relationship of the new strains to other members of the *B. cereus* group. Therefore, we compared the sequences of two chromosomal genes, *gyrB* and *rpoB*, that typically differentiate *B. anthracis* from other closely related *Bacillus* species [11,12]. The *gyrB* sequences of the *B. anthracis* CI and CA were identical and in this analysis fell in a cluster of published *B. anthracis* strains that were well separated from all other members of the *B.*

cerus group and supported by a 100% bootstrap value (Figure 2A). While isolates CI and CA were also identical in the *rpoB* sequences, they fell outside the well-supported cluster of classic *B. anthracis* strains and instead clustered with *B. cereus* and *Bacillus thuringiensis* strains and most closely with a recently described atypical and pathogenic *B. thuringiensis* (72% bootstrap value) (Figure 2B).

The nucleotide differences found in the *rpoB* sequence from both strains prevent sensitive detection in *B. anthracis* specific real-time PCR assays designed for rapid detection and differentiation of *B. anthracis* from closely related *B. cereus* and *B. thuringiensis* strains [13–16]. Other PCR assays target segments of each of four recently identified *B. anthracis*-specific genomic insertions of 16.5-kb–45.5-kb regions (termed A, C, D, and E) not found in *B. cereus* strains [13,14]. All four PCR assays for these regions were negative for the CI and CA strains, while *B. anthracis* control isolates yielded the expected amplification products. We confirmed that the assay failure was due to the absence of the expected *B. anthracis* insertions in the CI and CA strains by designing specific primer pairs for each of the four loci, with a primer located 5' to the expected insert, either combined with a reverse primer specific for the insert sequence, resulting in an amplification product of approximately 200 bp in case the *B. anthracis*-specific insert was present, or with a reverse primer located 3' of the expected insert, resulting in a product of approximately 700 bp in case of absence of the *B. anthracis*-specific insert. In the event that the insertion was present, the latter primer combination would not have resulted in a

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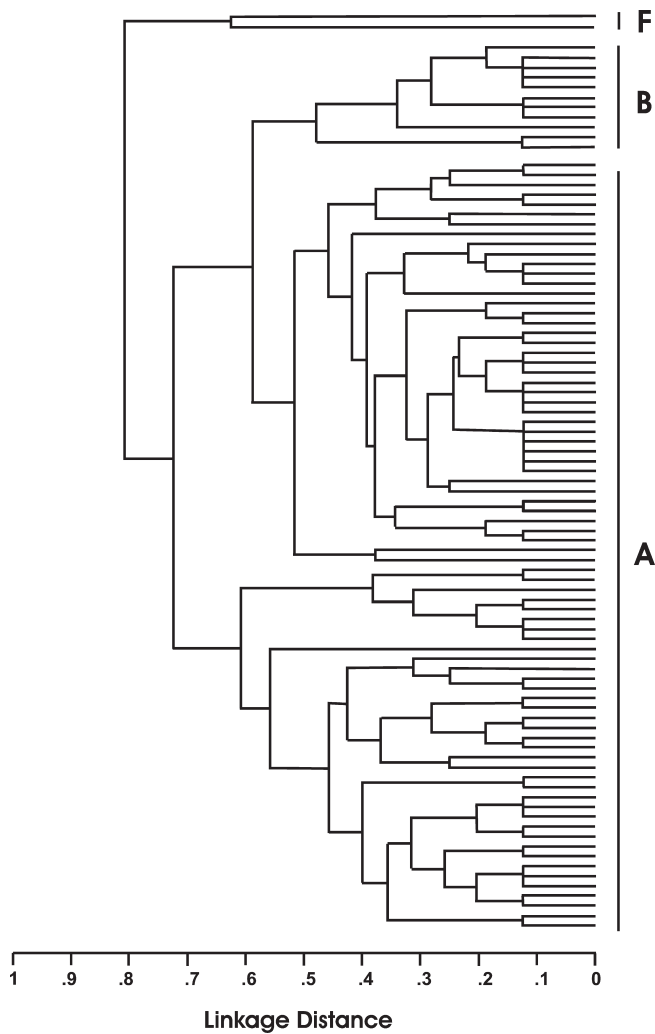
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Abbreviations: CA, Cameroon; CI, Côte d'Ivoire; VNTR, variable number tandem repeat regions

Fabian H. Leendertz, Saniye Yumlu, Georg Pauli, Sandra Junglen, Svenja Schenk, and Heinz Ellerbrok are at the Robert Koch-Institut, Center for Biological Safety, Berlin, Germany. Fabian H. Leendertz, Christophe Boesch, Linda Vigilant, Sandra Junglen, and Svenja Schenk are at the Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany. Fabian H. Leendertz is also with the Great Ape Health Monitoring Unit. Emmanuel Couacy-Hymann is at the Laboratoire National d'Appui au Développement Agricole and Laboratoire Central de Pathologie Animale, Bingerville, Côte d'Ivoire.

* To whom correspondence should be addressed. E-mail: EllerbrokH@rki.de

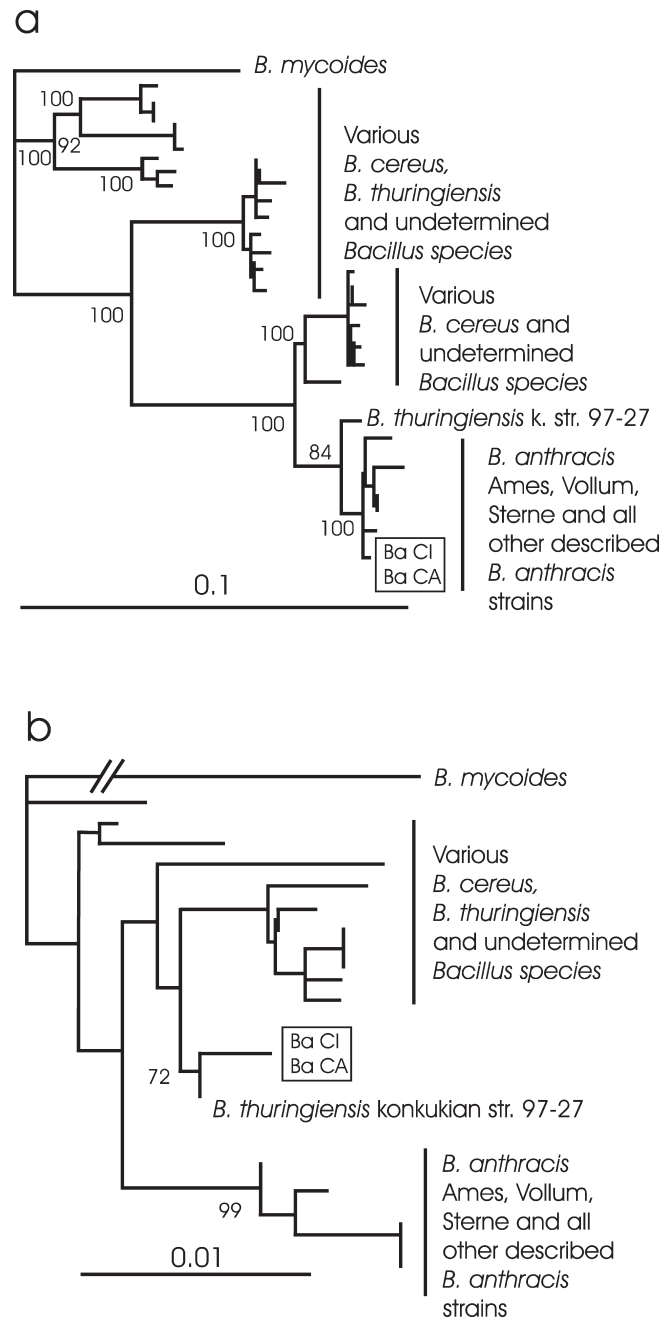


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Figure 1. Multilocus VNTR-Analysis-Based Dendrogram

Eight VNTR loci were analyzed following standard protocols [6]. The amplification products were analyzed by DNA sequencing and compared with 89 *B. anthracis* reference strains [4] processed with Sequence Type Analysis and Recombinational Tests (START) software (K. Jolley, University of Oxford). A, *B. anthracis* phylogenetic group A; B, *B. anthracis* phylogenetic group B [7]; F, the newly discovered *B. anthracis* group from sub-Saharan rainforests including the strains *B. anthracis* CI and CA.

product since the fragment was too long to be amplified under the conditions used. As expected, the CI and CA strains yielded products of about 700 bp for regions A, C, and D, and sequencing confirmed the absence of three insertions thought to be specific for *B. anthracis*. Repeated attempts with different primer sets to amplify segments of the E region from the CI and CA strains failed, although they produced products of expected sizes from *B. cereus*. This suggests that for the CI and CA strains, the sequence of this region differs from that of both previously described (“classic”) *B. anthracis* as well as from *B. cereus*. These data demonstrate that use of molecular assays relying upon chromosomal sequences for the identification of pathogenic *B. anthracis* may lead to misdiagnosis, and in the light of the potential use of *B. anthracis* as a biological weapon, it is important that diagnostic tests target various chromosomal sequences as well as both virulence plasmids pXO1 and pXO2 [16].



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Figure 2. Neighbour-Joining Phylogenetic Tree of the (A) *gyrB* (1,159-bp) and (B) *rpoB* (700-bp) Genes

The *B. anthracis* strains CI and CA are marked by the box. Analyses were carried out following the procedure described by others [11,12]. The trees were statistically evaluated with a bootstrap analysis with 1,000 bootstraps. Only relevant bootstrap values above 70% are shown for major branches.

Differences were also found in the sequence data from two genes (*pag* and *cya*) located on the *B. anthracis* plasmid pXO1. Previously, *pag* was shown to display limited sequence variation, with a total of only six single-nucleotide differences (three causing amino acid changes) found among 26 *pag* sequences [3]. Our sequence analysis of the *pag* toxin gene from the CI and CA strain identified three additional mutations, which result in unique amino acid changes (Table

1) in both new strains. For the strain *B. anthracis* CA, one additional but conservative mutation, not resulting in an amino acid change, was found. Analysis of 2,231 nucleotides of the *cya* toxin gene on plasmid pXO1 was only possible for the CI strain but identified four new nucleotide changes (three causing amino acid changes), compared with the six *cya* sequences from other *B. anthracis* strains.

The evergreen rainforest of Central and West Africa is a refuge for a number of animals and plant species and a high biodiversity has developed [17]. Thus there is a possibility that the *B. anthracis* detected in chimpanzees and gorilla is a “forest strain,” hidden for centuries or millennia in the rainforest of sub-Saharan Africa. Although it is tempting to conclude that modern human activities have caused anthrax emergence in wild apes, there are no data to support such a conclusion. Rather the description of this new group of *B. anthracis* underscores the need to collect baseline data on pathogens found in remote areas, such as the rainforests of Africa. In this respect, monitoring wild great apes, our closest relatives, for various pathogens may give important hints toward the presence of pathogens of importance for human health. In the light of increasing deforestation, disturbance of ecosystems, and bush-meat handling and consumption, these baseline data will contribute to the assessment of new pathogens which may spread to humans, such as shown for Ebola [18] and monkey pox [19] or other zoonotic diseases.

The detection of two closely related strains forming an unrecognized highly divergent group of *B. anthracis* at two different locations more than 1,000 mi apart was highly unexpected and implies distribution through Western and Central Africa. The presented genetic data suggest that both “classic” and the “forest anthrax” strains have a common ancestor with *B. cereus*, and that the forest strains diverged before the other *B. anthracis* strains acquired the characteristic *B. anthracis*-specific chromosomal insertions. From the data obtained, it is not possible to determine whether the plasmid pXO1 was already present in the CI and CA strains prior to divergence from the common *B. anthracis* ancestor, or whether the pXO1 plasmid was acquired independently in *B. anthracis* group F and “classic” *B. anthracis*. However, the recent description of a pathogenic *B.*

cereus strain containing a circular plasmid showing 99.6% similarity to the *B. anthracis* pXO1 [20] demonstrates that plasmid acquisition has occurred more than once within *B. cereus*.

Even though the anthrax described here shows several important differences from “classic” anthrax strains, the pathological and cytological findings reported in the anthrax victims of Taï National Park, Côte d'Ivoire, are in agreement with those reported in experimental infections of nonhuman primates with anthrax [9]. Unfortunately, no pathological and histological evaluation could be performed on the chimpanzees and gorilla carcasses found near the Dja Reserve in Cameroon since the carcasses were in an advanced stage of decomposition. However, in bone marrow, dental pulpa, and muscle tissue of these animals a high copy number of *B. anthracis*-specific *pag* and *capC* genes encoded by plasmids pXO1 and pXO2, respectively, and the chromosomal *rpoB* gene were detected, leading to the conclusion that these great apes also died of an acute *B. anthracis* infection [10]. So far we have found only great apes suffering from this new variant of the pathogen. However, this may be due to the fact that our research was focused only on great apes. In general, much mortality in wildlife but also in domestic animals and in humans in sub-Saharan Africa remains undiagnosed, and the pathogens responsible are frequently not found. The findings presented here show that a variety of new pathogens or variants of known pathogens have to be expected in these areas. It will be important to elucidate the capacity of classic microbiological tests to detect this new *B. anthracis*, and it would not be much of a surprise if anthrax cases caused by this pathogen go unrecognized. Therefore, studies will have to be undertaken to describe the distribution, biological features, and the pathology of these new *B. anthracis* forest strains. ■

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Table 1. Mutations Identified in the *pag* and *cya* Toxin Genes

Mutation ^a	Nucleotide Position ^b	Nucleotide Change	Amino Acid Change
1	2673	T → G	I → V
2	2743	G → C	E → Q
3	1999	T → C	S → P
4 ^c	1998	C → T	Synonymous
5 ^c	3602	C → T	A → V
6 ^d	3828	T → C	Synonymous
I	1022	C → T	Synonymous
II	1254	A → G	K → E
III	1375	T → C	I → T
IV	2502	T → C	V → A

^a1–5, *pag* of strains *B. anthracis* CI and CA; I–IV, *cya* of *B. anthracis* CI.

^bNucleotide positions are based on the 4,235-bp pXO1 sequence from the Sterne strain (accession number M22589) containing *pag* entirely (1–5) and on the *cya* sequence (accession number M 23179).

^cThese mutations have also been found in other *B. anthracis* strains [3].

^dThis mutation was only found in *B. anthracis* strain CA.

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