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Molecular Epidemiology of *Bacillus anthracis*: Determining the Correct Origin▽

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We analyzed and compared strains of *Bacillus anthracis* isolated from husbandry and industrial anthrax cases in Switzerland between 1952 and 1981 with published data using multiple-locus variable-number tandem repeat analysis. Strains isolated from autochthonous cases of anthrax in cattle belong to genotype B2, together with strains from continental Europe, while human *B. anthracis* strains clustered with genotype A4. These strains could be traced back to outbreaks of human anthrax that occurred between 1978 and 1981 in a factory processing cashmere wool from the Indian subcontinent. We interpret the worldwide occurrence of *B. anthracis* strains of cluster A4 to be due to the extensive global trade of untreated cashmere wool during the last century.

Bacillus anthracis, the causative agent of anthrax, is a gram-positive and spore-forming bacterium. Anthrax is mainly a herbivore disease, but human cases occur, primarily as a professional disease principally among breeders and veterinarians (9). Because of its history as an agent of biological warfare and its worldwide dissemination, it is essential to accurately subtype *B. anthracis* in order to react with appropriate measures in case of suspicious events. Nowadays, canonical single-nucleotide polymorphism markers (canSNPs) represent a fast way to discriminate among the major *B. anthracis* sublineages (13), while *m*ultiple-*l*ocus *v*ariable-*n*umber *t*andem *r*epeat (VNTR) *a*nalysis (MLVA) is the most suitable technique to differentiate and subtype *B. anthracis* strains (7). Comparison of MLVA data with published ones should help to trace back the geographical origin of the strains (3, 4, 5, 6, 8, 12). In Switzerland, anthrax was eradicated in cattle in the 1960s by strictly prohibiting the burial of dead animals or slaughtered waste and requiring the burning of the carcasses of animals that died from diseases. However, between 1978 and 1981, small outbreaks of human anthrax occurred in a plant which processed synthetic fibers and goat wool from the Indian subcontinent. In this study, we analyzed and compared Swiss isolates of *B. anthracis* from husbandry and industrial anthrax cases with the published data from a worldwide collection of *B. anthracis* strains using MLVA (6).

Swiss *B. anthracis* isolates (Table 1) were identified as described previously (11, 14). They were susceptible to the antibiotics most commonly used to treat gram-positive bacteria as determined by broth dilution (Table 1) (2, 8), except strain JF3852, which showed resistance to trimethoprim-sulfamethoxazole. Thirteen canSNPs (A.Br.001, A.Br.002, A.Br.003, A.Br.004, A.Br.006, A.Br.007, A.Br.008, A.Br.009, B.Br.001, B.Br.002, B.Br.003, B.Br.004, and A/B. Br.001) and eight VNTR markers for *B. anthracis* (VrrA, VrrB1, VrrB2, VrrC1, VrrC2, GC3, pXO1, and pXO2) were amplified by PCR using filtered lysates as template DNA (8) and sequenced as described elsewhere (6, 13). The canSNP sequences were compared to published data (13). Swiss strains belong to three major sublineages, A.Br.Aust94, A.Br.Vollum, and B.Br.CNEVA, according to the classification of Van Ert and colleagues (13). Strains JF3852, JF3854, JF3887, and JF3888, isolated from clinical cases in cattle between 1952 and 1962, have the same canSNP profile as the sublineage B.Br.CNEVA (with the following canSNP characteristics: A.Br.001, T; A.Br.002, G; A.Br.003, A; A.Br.004, T; A.Br.006, C; A.Br.007, T; A.Br.008, T; A.Br.009, A; B.Br.001, T; B.Br.002, G; B.Br.003, A; B.Br.004, C; and A/B.Br.001, A), while the cattle strain JF3853 belongs to the sublineage A.Br.Aust94 (with the following canSNP characteristics: A.Br.001, T; A.Br.002, G; A.Br.003, G; A.Br.004, C; A.Br.006, A; A.Br.007, T; A.Br.008, T; A.Br.009, A; B.Br.001, T; B.Br.002, G; B.Br.003, G; B.Br.004, T; and A/B.Br.001, A). All the other strains isolated from human clinical cases, goat hairs, and air filters during small iterative outbreaks of anthrax in a wool processing factory in the late 1970s and early 1980s belong to the sublineage A.Br.Vollum (with the following canSNP characteristics: A.Br.001, T; A.Br.002, G; A.Br.003, A; A.Br.004, T; A.Br.006, A; A.Br.007, C; A.Br.008, T; A.Br.009, A; B.Br.001, T; B.Br.002, G; B.Br.003, G; B.Br.004, T; and A/B.Br.001, A).

The sizes of the VNTR amplicons (Table 2) were compared to selected published allele sizes (6) as well as to the *B. anthracis* genome sequences from GenBank (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was derived from allelic profile data by the unweighted pair group method with arithmetic means with online software (http://pubmlst.org/) using the PHYLIP suite of programs (version 3.6; J. Felsenstein, University of Washington, Seattle, WA). The MLVA of the strains isolated in Switzerland showed that they belong to three distinct clusters according to their origin (Fig. 1). Strains JF3852, JF3854, JF3887, and JF3888 (Fig. 1) belong to cluster B2, together with strains from France, Croatia, Slovakia, and Poland (4, 6, 5). These strains represent autochthonous cases from cattle that died from anthrax, a disease that was common in most European countries in the first half of the 20th century,

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TABLE 1. Antibiotic susceptibility of *B. anthracis* strains from Switzerland

Strain	Origin/date of isolation	MIC (μg/ml)[a]																Reference(s) or source
		GEN	AMI	STR	ERY	CLI	SYN	TET	CHL	ENR	CEP	VAN	LNZ	PEN	AMC	NIT	SXT	
JF3783	Human anthrax, wool processing industry outbreak	≤2	≤16	≤4	1	≤0.5	2	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	1/19	Reference 10 and this study
JF3784	Air filter, wool processing industry outbreak	≤2	≤16	≤4	1	≤0.5	2	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	2/38	Reference 10 and this study
JF3785	Goat hair, wool processing industry outbreak	≤2	≤16	≤4	0.5	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	1/19	Reference 10 and this study
JF3786	Goat hair, wool processing industry outbreak	≤2	≤16	≤4	1	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	1/19	Reference 10 and this study
JF3787	Goat hair, wool processing industry outbreak	≤2	≤16	≤4	1	≤0.5	2	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	1/19	Reference 10 and this study
JF3788	Human anthrax, wool processing industry outbreak	≤2	≤16	≤4	1	≤0.5	2	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	1/19	Reference 10 and this study
JF3852	Cattle, Bern, Switzerland, 1953	≤2	≤16	≤4	2	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	>8/152	This study
JF3853	Cattle, central Switzerland, 1952	≤2	≤16	≤4	1	≤0.5	2	≤1	≤4	≤0.25	≤2	2	1	≤0.12	≤2/1	≤16	1/19	This study
JF3854	Cattle, central Switzerland, 1957	≤2	≤16	≤4	1	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	2/38	This study
JF3887	Cattle, eastern Switzerland, 1960	≤2	≤16	≤4	1	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	4/76	This study
JF3888	Cattle, eastern Switzerland, 1962	≤2	≤16	≤4	1	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	4/76	This study
JF3889	Eastern Switzerland, 1981	≤2	≤16	≤4	1	≤0.5	2	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	1/19	This study
NCTC8234	Vaccine strain, Sterne 34F2	≤2	≤16	≤4	1	≤0.5	1	≤1	≤4	≤0.25	≤2	≤1	1	≤0.12	≤2/1	≤16	4/76	NCTC

[a] AMI, amikacin; AMC, amoxicillin-clavulanic acid (ratio 2:1); CEP, cephalothin; CHL, chloramphenicol; CLI, clindamycin; ENR, enrofloxacin; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; NIT, nitrofurantoin; PEN, penicillin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole (ratio 1:19); SYN, quinupristin-dalfopristin; TET, tetracycline; VAN, vancomycin.

when carcasses of perished animals were buried. These strains therefore cluster in B2 with endemic, mostly bovine strains from central Europe. The bovine strain JF3853, from a 1952 case, which proved to belong to a separate canSNP sublineage, clusters with the A3a genotype group and could represent an imported infection (Fig. 1) (6).

TABLE 2. Sizes of VNTR amplicons of *B. anthracis* strains

Strain	Cluster	Amplicon size (bp)							
		vrrA	*vrrB1*	*vrrB2*	*vrrC1*	*vrrC2*	CG3	pXO1	pXO2
Sterne	A3b	314	229	162	580	532	158	132	0
Vollum	A4	290	229	153	535	604	158	135	139
Ames ancestor	A3b	314	229	153	580	532	158	126	141
Ames	A3b	314	229	153	580	532	158	126	141
SWI1	A4	314	229	162	535	604	158	132	139
SWI2	A4	314	229	162	535	604	158	138	141
PAK1	A2	314	193	162	517	604	158	135	137
PAK2	A4	314	229	162	535	532	158	135	139
PAK3	A4	326	229	162	535	604	158	144	139
JF3783	A4	314	229	162	535	604	158	138	141
JF3784	A4	314	229	162	535	604	158	135	139
JF3785	A4	314	229	162	535	604	158	135	141
JF3786	A4	314	229	162	535	604	158	135	141
JF3787	A4	314	229	162	535	604	158	135	141
JF3788	A4	314	229	162	535	604	158	138	141
JF3852	B2	302	220	144	580	532	158	132	135
JF3853	A3a	314	229	162	616	532	158	135	153
JF3854	B2	302	220	144	580	532	158	132	135
JF3887	B2	302	220	162	580	532	158	132	135
JF3888	B2	302	220	162	580	532	158	132	135
JF3889	A4	314	229	162	535	604	158	135	139
ChadA1	Aβ	290	229	171	616	604	158	123	133
IT	A1a	290	193	162	610	604	153	126	137
FRA	B2	302	220	162	580	532	158	132	137
POL1	B2	302	220	144	580	532	158	132	135
POL2	B2	302	220	144	580	532	158	132	133
USA	A1a	302	229	162	610	604	153	129	137
UK	A3a	314	229	162	610	532	158	138	149
India	A3a	326	229	162	610	532	158	129	145
TUR	A1b	314	229	153	610	604	158	120	137
NOR	B1	302	256	171	586	532	158	123	137

The third group of strains, isolated from wool processing factory outbreaks (10), represents a clonal population clustering with the A4 genotype (6). One strain, JF3889, isolated in Switzerland in 1981 but whose exact origin was unknown, clustered within the A4 branch, suggesting that it also originated from the outbreaks in the wool factory in the same year and hence can be attributed to the same series of outbreaks (Fig. 1). The factory handled mainly imported cashmere wool from Pakistan. In the classification of Keim and colleagues, the cluster also contains two strains reported as Swiss strains (SWI1 and SWI2) as well as strains from Pakistan (6). The MLVA profile of strain SWI2 is identical to the profiles of several strains from our collection that were isolated during the wool factory outbreak in 1981, while the profile of a strain analogous to SWI1 was not found in our collection. The minor variations found in the strains from the outbreaks of the wool factory from 1978 to 1981 are thought to be due to several different introductions from the same origin during a period of 3 years (10).

In the molecular epidemiological study of Keim et al. (6), which represents a world analysis of *B. anthracis* strains, cluster A4 is striking in that it contains genetically closely related strains from unrelated geographical areas. This cluster contains two strains (SWI1 and SWI2) that were supplied by a university laboratory of southern Germany and reposted as Swiss strains (6). In this respect, it is worthwhile to note that the wool factory that caused the outbreaks from 1978 to 1981 was located close to the German border. Diagnosis and identification of strains were done simultaneously in institutes of Swiss and German universities, which also shared strains (10). Cluster A4, as determined by Keim and colleagues, also con-

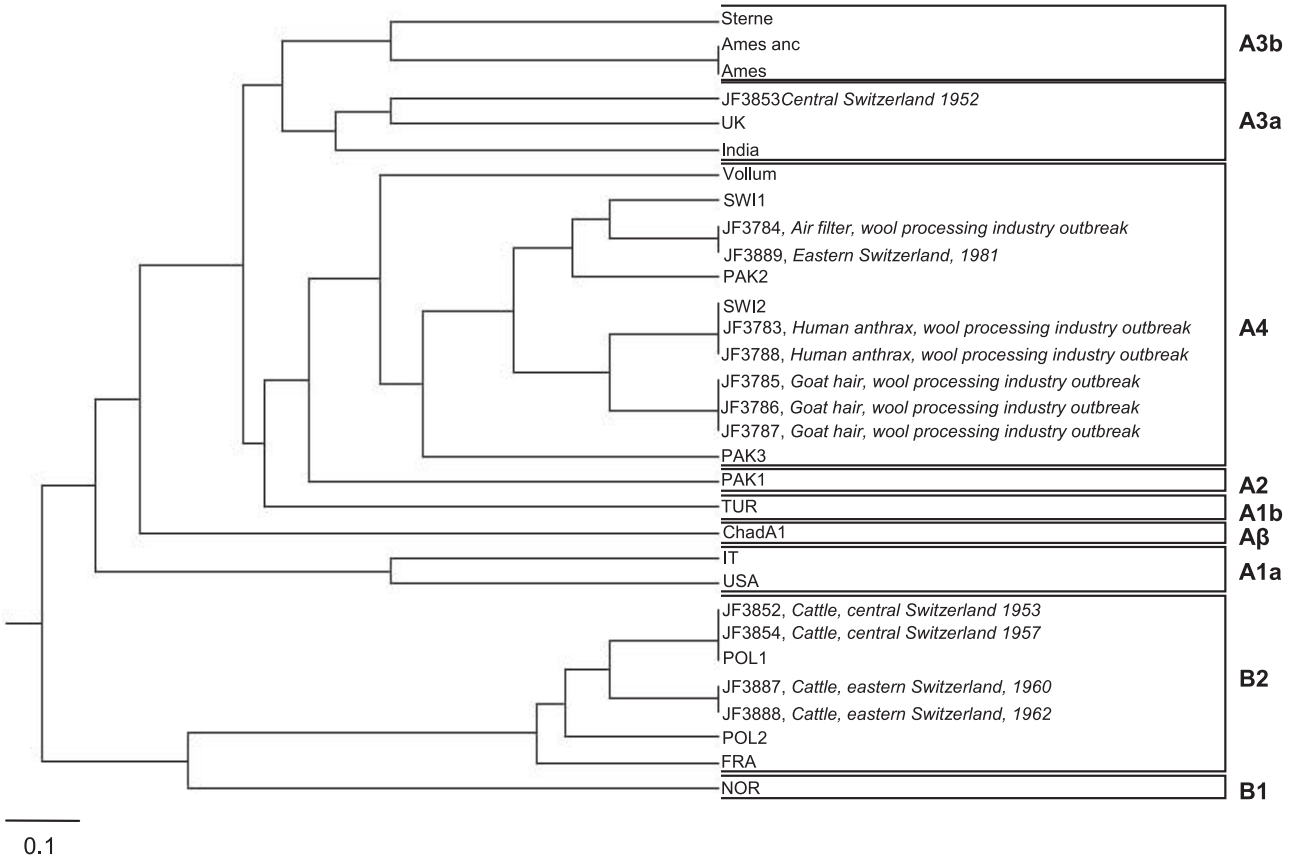

FIG. 1. Dendrogram based on MLVA of eight markers of Swiss *B. anthracis* strains and the genotypes reported by Keim and colleagues (6). The dendrogram was generated by clustering using the unweighted pair group method with arithmetic means. The genetic distance is presented as the absolute number of differences in marker alleles among genotypes.

tains two strains reported as German strains, one of which has a genotype identical to strain SWI2 and some of our strains from the wool factory outbreak (6). Therefore, we suppose that the two German strains belonging to cluster A4 originated from the same outbreaks from 1978 to 1981 that were caused by contaminated cashmere goat wool.

Cashmere goat wool was processed in many wool factories worldwide during the last century and frequently caused human anthrax among workers (1). Taking into account our results and the fact that cluster A4 also contains strains from Pakistan, we interpret the geographically broad distribution of *B. anthracis* strains belonging to the genetically very tight cluster A4 (6) to be due to ovine strains from the Indian subcontinent that spread by the trading of unprocessed wool over the whole globe, causing human anthrax cases in very distant locations.

Although MLVA is a powerful molecular epidemiological method for tracing back the origin of strains, reliable epidemiological data must be available in order to draw sound conclusions on the global spread of *B. anthracis*.

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