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Extended Multiple-Locus Variable-Number Tandem-repeat Analysis of *Bacillus anthracis* Strains Isolated in Poland

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Abstract

Twenty-one variable-number tandem-repeat (VNTR) marker loci were used for extended multiple locus VNTR analysis (MLVA) of 14 laboratory strains of *Bacillus anthracis* isolated in Poland and vaccine strain Sterne 34F2A. The extended MLVA (MLVA-21) distinguished six genotypes clustered in three main branches. Monomorphic branch 1 consisted of the vaccine strain and six isolates from distinct samples of a cow died from anthrax. This group also encompassed three haemolytic isolates of *B. anthracis*. Branches 2 and 3 were heterogeneous and consisted of five and three isolates of the phylogenetic lineages B2 and A1, respectively. MLVA-21 supported thesis on the anthrax agent heterogeneity in Poland. This study brought an additional evidence that haemolytic *B. anthracis* strains isolated in Poland are closely related to the vaccine strain Sterne 34F2 and may together constitute the same *sensu stricto* strain. No epidemiological link could be however traced between both the vaccine and the haemolytic strains.

Key words: *Bacillus anthracis*, genotyping, haemolysis, MLVA

Introduction

Bacillus anthracis, the etiological agent of anthrax, is a spore forming, gram positive bacterium causing disease primarily in wild animals and domestic livestock (Koehler, 2002). Humans are infected occasionally, however *B. anthracis* received notoriety for its use in the 2001 anthrax letter attack. This threat shown a necessity of developing molecular tools for anthrax identification and typing that could be applied for microbial forensics. Due to the highly monomorphic nature of *Bacillus anthracis* differentiation of strains from diverse origins has proven to be difficult (Brumlik *et al*., 2001).

The most commonly used tool for *B. anthracis* genotyping is multiple-locus variable-number tandem repeat analysis (MLVA) developed by Keim and co-workers (2000). This approach based on eight variable-number tandem-repeat (VNTR) marker loci (MLVA-8) was used for typing of *B. anthracis* isolates in several countries worldwide (for reference see Van Ert *et al.*, 2007) including Russia and Georgia reported elsewhere (Tsygankova *et al.*, 2003; Merabishvili *et al*., 2006). However, the MLVA-8 was found insufficient for molecular forensics approaches, since it was unable to discriminate two geographically close isolates in natural outbreaks (Lista *et al.*, 2006). In order to enhance the discriminatory power of the approach additional VNTR markers were proposed (Le Flèche *et al*., 2001). Finally 25 VNTR markers were selected for MLVA (Lista *et al.*, 2006; Ciammaruconi *et al.*, 2008). Application of the MLVA-25 for genotyping of the previously characterized by the MLVA-8 strains of *B. anthracis* in France (Fouet *et al.*, 2002) and Italy (Fasanella *et al.*, 2005) allowed to distinguish 37 new genotypes and proved a significant increase of discriminatory power (Lista *et al.*, 2006). These findings together prompted us to apply extended MLVA for sub-typing of Polish *B. anthracis* strains previously characterized by the MLVA-8 (Gierczyński *et al.*, 2004).

In the present report we show MLVA genotyping results obtained by use of 21 VNTR markers for typing of 14 strains of *B. anthracis* isolated in Poland. Notably, the presented genotypes are in agreement with the current nomenclature (Lista *et al.*, 2006).

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Experimental

Material and Methods

Bacterial strains. Fourteen clinical isolates of *Bacillus anthracis* (Table I) collected in Poland (Fig. 1) and vaccine strain Sterne 34F2A were tested by extended MLVA. Altogether 15 laboratory-strains were genotyped. Among them, eleven (Table I) were described previously (Gierczyński *et al.*, 2004). Two novel strains BL7W and BL9W which originated from the same cattle as the formerly reported strains BL8 and BL8G (see Table I) were identified in the present study as pXO2 deficient *B. anthracis* by the methods described previously (Gierczyński *et al.*, 2004). Similarly to BL8 and BL8G, two phenotypic variants: W (white) and G (grey) have been distinguished in BL7 and BL9. The G variants of BL7 (BL7G) and BL9 (BL9G) grown forming grey colonies with the β-haemolysis zone when cultured on Columbia blood agar (Oxoid). Colonies of the W variants of BL7 (BL7W) and BL9 (BL7W) were white with no detectable haemolytic activity. Altogether, four novel laboratory-strains: BL7W, BL7G, BL9W and BL9G were tested in this study. All the tested laboratory-strains were shown to carry the *B. anthracis* specific nonsense mutation in the *plcR* gene (Gierczyński *et al.*, 2007).

Extended MLVA. Genotyping was performed as described previously (Gierczyński *et al.*, 2004) using panel of 23 VNTR markers: *vrrA*, *vrrB1*, *vrrB2*, *vrrC1*, *vrrC2*, CG3, pXO1-*aat* (Keim *et al.*, 2000) and Ceb-Bams: 1, 3, 5, 15, 21, 22, 23, 24, 25, 28, 30, 31, (Le Flèche *et al.*, 2001), together with Ceb-Bams: 34, 44, 51, 53 (Lista *et al.*, 2006). Briefly, each the VNTR marker was amplified separately by PCR in 25 µl reaction mixture containing 1U of *Taq* DNA recombinant polymerase (Fermentas, Lithuania) and 1×PCR buffer supplied with the polymerase, 2 mM $MgCl_2$, 10 pmol of each the appropriate primer (Keim *et al.*,

Fig. 1. The geographic distribution of origins of the *B. anthracis* strains tested in this study

Table I
Characteristics of tested strains of *B. anthracis* shown in order reflecting the genetic relatedness determined by the MLVA-21

Strain	Year	Material	Place of isolation	Haemolytic activity	MLVA-8 genotype*
Sterne 34F2	NA†	NA	NA	–	Related to 61
BL7W	1988	Spleen	Rajgród	–	NT‡
BL7G§	1988	NA	NA	+	NT
BL8W	1988	Blood	Rajgród	–	Related to 61
BL8G§	1988	NA	NA	+	Related to 61
BL9W	1988	Nasal fluid	Rajgród	–	NT
BL9G§	1988	NA	NA	+	NT
BL1	1993	Blood	Łomża	–	Novel B2-1
BL5	1988	Blood	Rajgród	–	Novel B2-1
BL10	1993	No data	Łomża	–	Novel B2-2
BL11	1993	Spleen	Łomża	–	Novel B2-2
BL12	1993	blood	Łomża	–	Novel B2-2
BL3	1947	No data	Jasło	–	Novel A1.a-1
BL4	1953	No data	Przemyśl	–	13
BL6	1996	Blood	Kolno	–	Novel A1.a-2
Total:15					

* the MLVA-8 (Keim *et al*., 2000) genotypes determined formerly (Gierczyński *et al.*, 2004).
† NA, not applicable.
‡ NT, not tested in the former study (Gierczyński *et al.*, 2004).
§ atypical variant of *B. anthracis* isolated in laboratory from the above parental isolate.

2000; Le Flèche *et al.*, 2001; *Lista et al.*, 2006), 0.2 mM of each deoxynucleotide, and 2.5 µl of the template DNA solution (about 5–10 ng of genomic DNA) prepared as described previously (Gierczyński *et al*., 2004). Common thermal PCR-profile consisting of an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, and a final extension step at 72°C for 5 min was used for the all VNTR markers. To ascertain approximate length of the amplified VNTR markers, the PCR products were separated in 1,5% agarose gel (MP Biomedicals, Eschwege, Germany) in TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) at a constant voltage of 6 V/cm for three hours. DNA was visualized by a conventional ethidium bromide staining. Next, amplicons were grouped with respect to their size and subjected to denaturing gel electrophoresis (DGE) in adjacent lanes in a polyacrylamide gel as described previously (Gierczyński *et al.*, 2004). The amplicon-size determined by DGE was compared with the reference genotypes reported by Lista and colleagues (2006). Size of the MLVA markers obtained for the vaccine strain Sterne 34F2A was compared with the expected size deduced from the genome sequence of Sterne strain (GenBank accession no AE017225) [http://www.ncbi.nlm.nih.gov]. Selected amplicons were subjected to DNA sequencing that was carried out as described previously (Gierczyński *et al*., 2004). Both DNA strands were analysed. Tandem Repeats Finder (TRF) version 2.02 software (Benson, 1999) was used to determine a number of tandem repeats in each of the sequenced amplicons. The most restrictive parameters (2, 3, 5) of the TRF algorithm and alignment cost 150 were used. The number of repeats was converted to the marker size using conversion table described by Lista *et al.* (2006). The cluster analysis was performed using simple linkage agglomeration method of WinSTAT software version 2001.1. The analysis was performed in duplicate.

Results and Discussion

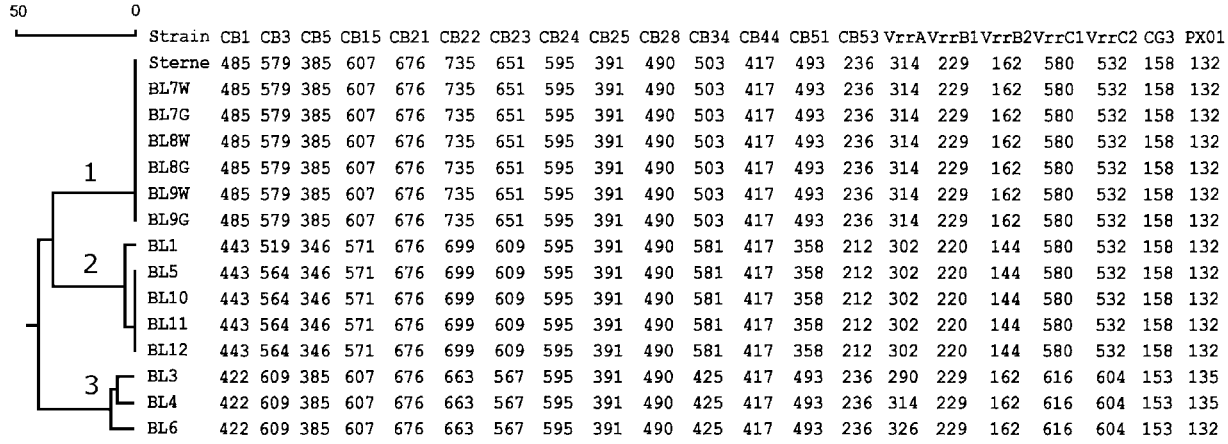
Extended MLVA approach optimization. The increase of a number of VNTR marker loci in MLVA was reported to enhance the genotyping resolution (Keim *et al.*, 2004). *B. anthracis* isolates that were indistinguishable by MLVA-8 could be effectively diversified when an extended panel of marker loci was used (Lista *et al.*, 2006). More recently, Van Ert and co-workers (2007) subtyped a large collection of *B. anthracis* strains from 42 countries by alternative panel of 15 VNTR markers. Number of distinguished genotypes increased from 89 to 221 for eight and 15 loci based MLVA, respectively (Van Ert *et al.*, 2007).

In this study, we applied the aforementioned strategy to gain a close insight into genetic relations of *B. anthracis* strains originated in Poland with a special reference to the haemolytic and non-haemolytic variants. In order to perform extended MLVA genotyping, 23 VNTR loci were initially selected from the original set of 25 marker loci used by Lista and colleagues (2006). Markers pXO2-*at* and Ceb-Bams 13 were excluded. The pXO2-*at* marker maps in virulence plasmid pXO2 that is absent in some of the tested strains. The latter marker yielded non-reproducible results during a pilot study.

PCR-amplicons of all the selected 23 MLVA markers were obtained for all the tested strains. The entire set of 23 markers was found to be useful for *B. anthracis* differentiation (data not shown). However, in case of loci Ceb-Bams 30 and 31 it was impossible to determine the exact size and a number of the repeats in amplicons above 700 bp. Since genotype accuracy is essential for epidemiological investigations and microbial forensics, loci Ceb-Bams 30 and 31 were excluded from the further analyses. Therefore, 21 VNTR markers were finally applied for MLVA (MLVA-21) to subtype strains of *B. anthracis* isolated in Poland. To assure genotyping accuracy and reproducibility required for inter-laboratory epidemiological and forensic analyses the DGE and the DNA sequencing were used to determine MLVA-21 genotypes. The MLVA-21 genotypes of the tested strains are shown in Fig. 2. Notably, MLVA-21 genotype of the vaccine strain Sterne 34F2 was identical to the genotype determined *in silico* from the Sterne genome sequence. Such consistency is a proof for the reproducibility of the MLVA-21 at inter-laboratory level.

B. anthracis diversity in Poland. Six the MLVA-21 genotypes of *B. anthracis* were distinguished in this study even though the relatively low number of tested strains. The clustering analysis generated dendrogram composed of three main branches (Fig. 2).

The monomorphous branch 1 consists of the vaccine strain Sterne 34F2A and 6 strains originated from distinct samples of a cow died from anthrax. Noteworthy, this group encompasses both the haemolytic and non-haemolytic variants. Thus, one can assume that the vaccine strain and the haemolytic *B. anthracis* variants BL7G, BL8G and BL9G are closely related. Accordingly to the nomenclature proposed by Struelens and colleagues (1996) all the branch 1 strains may be considered the *sensu stricto* strain that consists of isolates which are descendants of the common ancestor. The very high discriminatory power of the extended MLVA (Lista *et al*. 2006; Van Erth *et al*. 2007) and the fact that the Sterne genotype is relatively distant to genotypes of Polish strains reported elsewhere (Keim *et al.*, 2000; Van Ert *et al*., 2007) may suggest that the animal from which the haemolytic



Strain	CB1	CB3	CB5	CB15	CB21	CB22	CB23	CB24	CB25	CB28	CB34	CB44	CB51	CB53	VrrA	VrrB1	VrrB2	VrrC1	VrrC2	CG3	PX01
Sterne	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL7W	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL7G	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL8W	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL8G	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL9W	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL9G	485	579	385	607	676	735	651	595	391	490	503	417	493	236	314	229	162	580	532	158	132
BL1	443	519	346	571	676	699	609	595	391	490	581	417	358	212	302	220	144	580	532	158	132
BL5	443	564	346	571	676	699	609	595	391	490	581	417	358	212	302	220	144	580	532	158	132
BL10	443	564	346	571	676	699	609	595	391	490	581	417	358	212	302	220	144	580	532	158	132
BL11	443	564	346	571	676	699	609	595	391	490	581	417	358	212	302	220	144	580	532	158	132
BL12	443	564	346	571	676	699	609	595	391	490	581	417	358	212	302	220	144	580	532	158	132
BL3	422	609	385	607	676	663	567	595	391	490	425	417	493	236	290	229	162	616	604	153	135
BL4	422	609	385	607	676	663	567	595	391	490	425	417	493	236	314	229	162	616	604	153	135
BL6	422	609	385	607	676	663	567	595	391	490	425	417	493	236	326	229	162	616	604	153	132

Fig. 2. The genotyping tree of *B. anthracis* strains isolated in Poland based upon MLVA-21 genotypes. Size of the 21 MLVA markers determined by DNA sequencing is shown. CB is an abbreviation of Ceb-Bams markers (Le Flèche *et al.*, 2001). Sterne is the vaccine strain Sterne 34 F2

variants originated had been initially infected by Sterne 34F2A as a result of vaccination. However, an interview with local veterinary authorities and a farmer to whom the died cattle belonged has definitely excluded such vaccination or a contact with any other vaccinated animals. Therefore, in this particular case, source of the Sterne-like haemolytic *B. anthracis* strains in Poland remains obscure.

Little is known about factors triggering haemolysis in *B. anthracis*. It was proposed (Mignot *et al.*, 2001; Slamti *et al*., 2004) that the haemolytic phenotype of *B. anthracis* could be an effect of the pleiotropic regulator PlcR activation as a result of reversion of the nonsense mutation in the *plcR* gene (Easterday *et al*., 2005). However, all the haemolytic variants of Polish *B. anthracis* were shown to carry the nonsense mutation in the *plcR* gene (Gierczyński *et al*., 2007). Further studies are therefore necessary to elucidate the haemolytic trait present in the tested strains.

Branch 2 of the MLVA-21 genotypes distinguished in this study contains five laboratory-strains which were previously identified as the phylogenetic *B. anthracis* lineage B2 (Gierczyński *et al.*, 2004). Three of them (BL10, BL11, BL12) were obtained from the same laboratory. The genotyping results of both the former and present study suggest that these laboratory-strains belong to the same clone. Notably, MLVA-21 differentiated strains BL1 and BL5 which were indistinguishable by MLVA-8. Both the strains were isolated in neighbouring districts of the North-eastern Poland in 5 years interval. This finding argues that MLVA-21 may be a useful tool for epidemiological investigations of natural anthrax outbreaks. Nevertheless, strains BL5 and BL10 could not be distinguished by MLVA-21. Both the strains differ only in pXO2-*aat* marker, that was not used in MLVA-21. This may argue for the importance of the plasmid-associated VNTR loci in *B. anthracis* genotyping. In the present study we have excluded pXO2-*aat* marker from MLVA-21 since our goal was to compare the Sterne related pXO2 deficient strains versus the other strains of *B. anthracis* from Poland.

The most heterogeneous branch 3 is composed of a cluster of strains BL3, BL4 and BL6 which were previously classified as belonging to the lineage A. This branch is topologically similar to the branch A1.a of the former study (Gierczyński *et al.*, 2004). Interestingly, in the present study BL3 and BL4 from the South-eastern Poland were clustered together while BL6 from the North-eastern region of the country was distantly related.

In the present study, we observed a relatively low diversity of the Ceb-Bams loci in tested strains belonging to the same evolutionary lineage. In fact, only a single difference for the Ceb-Bams 3 was found in five strains of the lineage B2 while the most heterogeneous strains of the A1 linage were indistinguishable by the Ceb-Bams markers used in this study. These findings are in contrast to those reported by Lista *et al*. (2006), who found significant improvement of the genotyping resolution by the Ceb-Bams markers. However, it should be taken into consideration that a relatively low number of strains was tested in our study. Foremost, some of the tested strains appeared to be epidemiologically linked or closely related.

Herein we shown extended MLVA genotypes of *B. antracis* in Poland. The most commonly used VNTR markers in *B. antracis* typing were analysed in this study to achieve conformity with the other reports on *B. anthracis* diversity in Europe (Keim *et al.*, 2000; Le Flèche *et al*., 2001; Lista *et al.*, 2006; Merabishvili *et al.*, 2006; Ciammaruconi *et al.*, 2008). In conclusion, this study support previous findings that *B. anthracis* in Poland is highly heterogeneous when compared to other European countries. When brought together genotypes distinguished in this study

and those published elsewhere (Gierczyński *et al.*, 2004; Keim *et al.*, 2000) at least seven genotypes could be found for *B. anthracis* in Poland. Moreover, an additional genotype represented by strain A00622POL can be deduced from data presented by Van Ert *et al*., (2007). Furthermore, comparative analysis of the MLVA-8 data presented by these authors led us to conclusion that strains BL1 and BL5 from our collection are highly related or even belong to the same clone as *B. anthracis* strains A1085POL and A1088POL from Poland, which were tested by Van Ert and colleagues (2007).

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