Phylogenetic Typing of *Bacillus anthracis* Isolated in Japan by Multiple Locus Variable-Number Tandem Repeats and the Comprehensive Single Nucleotide Polymorphism

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ABSTRACT. Twelve strains of *Bacillus anthracis* isolated in Japan were subjected to multiple locus variable-number tandem repeats analysis using 25 marker loci (MLVA25). The results showed that Japanese strains could be divided into two distinct genetic clusters, A3a and A3b. By using newly devised comprehensive single nucleotide polymorphisms (SNPs) analysis, Japanese strains were also divided into two groups. The results obtained by the MLVA25 and plasmids SNP analysis well coincided, indicating that both methods were highly sensitive to discriminate *B. anthracis* strains. These results suggested that MLVA25 had sufficient discrimination power to identify *B. anthracis* at the strain level, and that MLVA25 as well as comprehensive SNPs analysis could facilitate further studies of *B. anthracis* strains including Japanese and other Asian strains.

KEY WORDS: *Bacillus anthracis*, MLVA, phylogeny, SNP.

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Anthrax caused by *Bacillus anthracis*, spore forming agent, is one of the most severe zoonoses, which poses serious threats to both public and animal health. Although anthrax has been almost eradicated from Japan except some sporadic human [10], or animal cases [8, 9], there are growing concern over potential use of *B. anthracis* as a bioterrorism agent. In addition, various animal products imported from all over the world including those countries where anthrax is enzootic may become vehicles of anthrax from abroad as exemplified by an event recently reported in the UK [2]. It appears, therefore, important to have a sensitive and specific method for identifying *B. anthracis* isolated from humans and animals as well as from the environments at the strain level.

Since *B. anthracis* shows very limited genetic diversity [4], identification of *B. anthracis* strains requires methods which have high discrimination power. Multiple locus variable-number tandem repeats analysis (MLVA) has been used for identification of *B. anthracis* at the strain level. The number of target loci increased from eight (MLVA8) in the initial report [5] to twenty five (MLVA25) in the most recently published work [6]. Single nucleotide polymorphisms (SNPs) analysis also became a powerful genetic tool to differentiate *B. anthracis* strains [12]. A large numbers of canonical SNPs scattered throughout the whole genome were proved to be regarded as strain-specific markers that enables us to distinguish geographically and genetically

diverse isolates [14].

In this study, we have attempted to clarify the phylogenetic relationship of *B. anthracis* strains isolated in Japan with those from other parts of the world by MLVA25 as well as comprehensive SNP analysis. *B. anthracis* strains used in this study are listed in Table 1. These strains were kindly provided by several prefectural livestock hygiene service centers. BA102, 103, 108, 109 and 111 strains were isolated from dairy or beef cattle while BA104, 105 [1] and 107 [8] were isolated in the sporadic incidents of pigs which occurred in 1982. Neither the infectious routes nor sources have been identified. All the isolates were positive for 2 pathogenic plasmids: 181.7kb pXO1 encoding the protective antigen, lethal factor and edema factor [11] and 96.2kb pXO2 encoding the capsule operon [7]. They were also gamma phage sensitive, penicillin and ciprofloxacin sensitive, non-hemolytic on sheep blood agar (SBA) and non-motile.

Table 1. *Bacillus anthracis* strains isolated in Japan

Strain number	Isolated place	Isolated animal	Isolated year
BA102	Miyagi	dairy cattle	1983
BA103	Miyagi	beef cattle	1991
BA104	Shizuoka	pig	1982
BA105	Shizuoka	pig	1982
BA106	Okinawa	unknown	1956
BA107	Okinawa	pig	1982
BA108	Shiga	dairy cattle	1987
BA109	Mie	dairy cattle	1970
BA110	Mie	unknown	1967
BA111	Okayama	dairy cattle	1985
BA113	Okayama	human	unknown
BA115	Shizuoka	unknown	unknown

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Table 2. Genetical information of strains used for SNP analysis

Strain name	pXO1	pXO2	chromosome	SNP analysis ^{a)}	MLVA 25 ^{b)}	canSNP typing ^{c)}	Accession Number
A0193	+	+	+	A1a	A1a	A.Br.WNA	NZ_ABKF00000000
A0174	+	— ^{d)}	+	A1a			NZ_ABLT00000000
USA6153	+	+	+	A1a			NZ_AAER00000000
Australia 94	+	+	+	A3a	A3a	A.Br.Aust94	NZ_AAES00000000
BA104	This study	This study	N.A. ^{e)}	A3a	A3a		
A2012	+	+	+	A3b			NZ_AAAC00000000
Ames	—	—	+	A3b	A3b		NC_003997
Ames Ancestor	+	+	+	A3b			NC_007530
BA103	This study	This study	N.A.	A3b	A3b		
Sterne	+	—	+	A3b	A3b		NC_005945
A0389	+	+	+	A3b		A.Br.001/002	NZ_ABLB00000000
A0488	+	+	+	A4	A4		NZ_ABJC00000000
Vollum	+	+	+	A4	A4		NZ_AAEP00000000
A0442	+	+	+	B1		B.Br.Kruger	ABKG01000000
Kruger B	+	+	+	B1	B1	B.Br.Kruger	NZ_AAEO00000000
A0465	+	+	+	B2	B2	B.Br.CNEVA	NZ_ABLH00000000
CNEVA-9066	+	+	+	B2	B2	B.Br.CNEVA	NZ_AAEN00000000
A1055	—	+	+	C	C		NZ_AAEO00000000
Tsiankovskii-I	+	+	+	new			NZ_ABDN00000000

a) This study.

b) Referred by Lista *et al.* [6] and Fig. 1.c) Referred by Van Ert *et al.* [14].

d) Plasmid genome sequence is not available.

e) Not available.

Table 3. The observed and expected fragment sizes by fragment analysis and the number of repeats on 25 loci of MLVA in 12 *B. anthracis* strains isolated in Japan

Strain name	VNTR locus name																									
	vrrA	vrrB1	vrrB2	vrrC1	vrrC2	CG3	pXO1	pXO2	Bams1	Bams3	Bams5	Bams13	Bams15	Bams21	Bams22	Bams23	Bams24	Bams25	Bams28	Bams30	Bams31	Bams34	Bams44	Bams51	Bams53	
Observed fragment sizes (bp)	BA102	307	223	158	569	524	153	123	136	488	576	378	775	610	663	717	637	596	391	495	728	767	148	408	496	234
	BA103	307	223	158	569	524	153	123	136	488	576	378	776	610	663	717	637	596	391	496	728	596	495	408	496	234
	BA104	307	223	158	604	524	153	123	155	317	606	378	839	601	663	717	637	596	391	495	728	695	379	408	496	234
	BA105	307	223	149	604	523	153	124	136	488	606	378	839	610	616	718	637	596	391	496	728	767	418	408	502	234
	BA106	307	222	158	604	523	153	124	136	488	606	378	839	610	616	718	637	596	391	496	728	767	418	408	502	234
	BA107	307	223	158	604	523	153	123	136	488	606	378	839	610	616	718	637	596	391	496	727	767	418	408	502	234
	BA108	307	222	158	569	523	153	126	133	296	576	339	866	610	663	717	637	596	391	493	674	642	495	408	496	234
	BA109	307	223	158	604	523	153	123	137	488	606	378	830	610	663	648	637	596	391	496	728	767	418	408	496	234
	BA110	307	222	158	604	523	153	124	137	488	606	378	830	610	663	648	637	596	391	496	728	767	418	408	496	234
	BA111	307	222	158	569	523	153	129	134	488	456	339	604	592	663	714	637	596	391	496	728	767	495	408	496	234
	BA113	307	223	158	604	523	153	124	137	488	606	378	866	619	663	717	637	596	391	496	728	767	418	408	496	234
	BA115	307	223	158	569	524	153	123	140	488	576	378	748	591	662	717	636	596	391	496	710	767	495	407	496	234
Expected fragment sizes (bp)	BA102	314	229	162	580	532	158	129	141	485	580	385	777	607	676	735	651	595	391	492	728	772	156	421	493	236
	BA103	314	229	162	580	532	158	129	141	485	580	385	778	607	676	735	651	595	391	493	728	601	503	421	493	236
	BA104	314	229	162	580	532	158	129	141	485	580	385	778	607	676	735	651	595	391	493	728	601	503	421	493	236
	BA105	314	229	162	615	532	158	129	160	314	610	385	841	598	676	735	651	595	391	492	728	700	387	421	493	236
	BA106	314	229	153	615	531	158	130	141	485	610	385	841	607	629	736	651	595	391	493	728	772	426	421	499	236
	BA107	314	228	162	615	531	158	130	141	485	610	385	841	607	629	736	651	595	391	493	728	772	426	421	499	236
	BA108	314	229	162	580	531	158	129	141	485	610	385	841	607	629	736	651	595	391	493	727	772	426	421	499	236
	BA109	314	228	162	615	531	158	132	138	293	580	346	868	607	676	735	651	595	391	490	674	647	503	421	493	236
	BA110	314	229	162	615	531	158	129	142	485	610	385	832	607	676	666	651	595	391	493	728	772	426	421	493	236
	BA111	314	228	162	615	531	158	130	142	485	610	385	832	607	676	666	651	595	391	493	728	772	426	421	493	236
	BA113	314	228	162	580	531	158	135	139	485	460	346	606	589	676	732	651	595	391	493	728	772	503	421	493	236
	BA115	314	229	162	615	531	158	130	142	485	610	385	868	616	676	735	651	595	391	493	728	772	426	421	493	236
Number of repeats	BA102	10	16	7	53	17	2	8	10	16	28	7	66	45	10	16	11	11	13	14	57	64	1 ^{a)}	8	9	8
	BA103	10	16	7	53	17	2	8	10	16	28	7	66	45	10	16	11	11	13	14	57	45 ^{a)}	9	8	9	8
	BA104	10	16	7	57	17	2	8	10 ^{a)}	8 ^{a)}	30	7	73	44	10	16	11	11	13	14	57	56	7	8	9	8
	BA105	10	16	6	57	17	2	8	10	16	30	7	73	45	9	16	11	11	13	14	57	64	8	8	9	8
	BA106	10	16	7	57	17	2	8	10	16	30	7	73	45	9	16	11	11	13	14	57	64	8	8	9	8
	BA107	10	16	7	57	17	2	8	10	16	30	7	73	45	9	16	11	11	13	14	57	64	8	8	9	8
	BA108	10	16	7	53	17	2	9	8	7 ^{a)}	28	6	76	45	10	16	11	11	13	14	51	50 ^{a)}	9	8	9	8
	BA109	10	16	7	57	17	2	8	10	16	30	7	72	45	10	14	11	11	13	14	57	64	8	8	9	8
	BA110	10	16	7	57	17	2	8	10	16	30	7	72	45	10	14	11	11	13	14	57	64	8	8	9	8
	BA111	10	16	7	53	17	2	10	9	16	20 ^{a)}	6	47 ^{a)}	43	10	16	11	11	13	14	57	64	9	8	9	8
	BA113	10	16	7	57	17	2	8	10	16	30	7	76	46	10	16	11	11	13	14	57	64	8	8	9	8
	BA115	10	16	7	53	17	2	8	12	16	28	7	63 ^{a)}	43	10	16	11	11	13	14	55 ^{a)}	64	9	8	9	8

a) new allele.

Fig. 1. Dendrogram constructed according to the MLVA25 of 51 *B. anthracis* strains including 12 Japanese isolates. The tree was drawn by UPGMA method using PHYLIP version 3.6 and Tree View versom1.6.6. "Cluster" refers to the clustering as proposed by Lista *et al.* [6]. The origin and strain (when known) names are indicated; ND, not defined.

Fig. 2. Unrooted phylogenetic tree of *B. anthracis* strains constructed based on SNPs found in the nucleotide sequences of pXO1 (A), pXO2 (B) and chromosomal DNA (C). The trees were drawn using two computer programs, Clustal X and FigTree. The BA103 and BA104 were included in the SNP analysis for the plasmid DNAs but not for the chromosomal DNAs. The clusters by MLVA25 are also shown.

MLVA25 was performed according to the method reported by Lista *et al.* with some modifications as mentioned below. Fluorescent dyes, 6-FAM, VIC and PET were used for primer labeling (Applied Biosystems Inc.). The bacterial cells cultured overnight at 37°C on SBA plates

(Eiken chemical, Tokyo, Japan) were lysed with 1 mg/ml each of lysozyme and achromopeptidase (WAKO chemical, Tokyo, Japan) at 37°C for 30 min followed by further digestion with 1 mg/ml proteinase K at 56°C overnight. Genomic DNA was extracted with phenol-chloroform and recovered

by ethanol precipitation. PCR amplification was performed with Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) using following condition; initial denaturation at 98°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 15 sec, extension at 72°C for 30 sec, and further extension at 72°C for 5 min. Annealing temperature was reduced to 50°C for Bams28, 30, 31 44 and 53 amplification. After capillary electrophoresis of the PCR products using the Applied Biosystems 3730 xl DNA Analyzer (Applied Biosystems Inc.), the fragment sizes were calculated by Peak Scanner software version 1.0 (Applied Biosystems Inc.) using GENESCAN 1200 LIZ Size standard (Applied Biosystems Inc.) as internal size standard. Since we do not have any *B. anthracis* strains used by Lista *et al.* in their MLVA25 analysis, the exact numbers of the tandem repeats of 25 loci of the BA103 strain were determined by the nucleotide sequencing. The number of repeats of the respective loci of other Japanese strains was deduced by the comparing the capillary electrophoregram with that of BA103.

By performing MLVA25, ten new alleles on 7 loci were identified (Table 3). The phylogenetic tree constructed with 25 VNTR loci by combining our data together with those reported by Lista *et al.* as well as those found in NCBI database (A0193, A0488 and A0465) revealed that Japanese isolates were divided into two distinct clusters. BA104, 105, 106, 107, 109, 110 and 113 were clustered in the A3a, together with the Australia94 strain, while 5 other strains were included in the A3b cluster with the Ames strain (Fig. 1).

The entire nucleotide sequences of pXO1 and pXO2 plasmids of BA103 and BA104 strains were determined by primer walking methods by 3730 xl DNA Analyzer with Big Dye Terminator v 3.1 Cycle sequencing kit (Applied Biosystems Inc.) with the PCR products, up to ~8 kb, amplified with Platinum Taq DNA polymerase High Fidelity (Invitrogen Japan, Tokyo, Japan). Obtained sequences were assembled and edited with Phred/Phrap/Consed [3]. SNPs on pXO1 and pXO2 plasmid DNA among *B. anthracis* strains shown in Table 2 were extracted by the SNPs Finder program [13]. All SNPs were concatenated to hypothetical nucleotide sequences for each strain, and the sequences were aligned using Clustal X version 1.83. An unrooted phylogenetic tree based on all SNPs information was drawn using FigTree version 1.1.2 (Fig. 2).

By sequencing the entire pXO1 and pXO2 DNA derived from BA103 and BA104 and comparing them with the nucleotide sequences of two plasmids of 17 other strains, 235 and 177 SNPs were identified on pXO1 and pXO2 plasmids, respectively (data not shown). The unrooted phylogenetic trees constructed by the extracted SNPs (Fig. 2) revealed that BA103 was clustered into the A3b together with the Ames Ancestor and A2012, while BA104 in the A3a with the Australia94. The result that phylogenetic relationships disclosed by the MLVA25 and SNP analysis were quite similar strongly suggested that the SNP analysis devised here as well as MLVA25 had very high resolution

power in terms of discrimination of *B. anthracis* strains. Furthermore, the results obtained by the SNP analysis using pXO1 or pXO2 sequence data alone coincided with that obtained by using chromosomal DNA sequences with 17 foreign strains from database (5684 SNPs, data not shown), suggesting that the identification of *B. anthracis* at the strain level would be feasible if the nucleotide sequence of either plasmid DNA is available (Fig. 2).

In this study, it was shown that either MLVA25 or SNP could be applied for the identification of *B. anthracis* at the strain level and that Japanese strains were grouped into the A3a and A3b cluster. Since the strains analyzed in this study were derived from regions or countries outside of Asia, it is necessary to include Asian strains in the phylogenetic analysis with high discrimination power such as MLVA25 or SNPs analysis to understand the possible routes of introduction of *B. anthracis* strains to Japan.

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REFERENCES

1. Amano, H., Kajio, N., Mizoguchi, T., Ohmura, K., Sone, M. and Tsuchiya, Y. 1987. Pathological observation on intestinal anthrax in swine. *J. Jpn. Vet. Med. Assoc.* **40**: 124.
2. Anaraki, S., Addiman, S., Nixon, G., Krahé, D., Ghosh, R., Brooks, T., Lloyd, G., Spencer, R., Walsh, A., McCloskey, B. and Lightfoot, N. 2008. Investigations and control measures following a case of inhalation anthrax in East London in a drum maker and drummer, *Eurosurveillance* **13**.
3. Gordon, D., Abajian, C. and Green, P. 1998. Consed: a graphical tool for sequence finishing. *Genome Res.* **8**: 195–202.
4. Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S. E., Richmond, K., Adair, D. M., Hugh-Jones, M., Kuske, C. R. and Jackson, P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J. Bacteriol.* **179**: 818–824.
5. Keim, P., Price, L. B., Klevytska, A. M., Smith, K. L., Schupp, J. M., Okinaka, R., Jackson, P. J. and Hugh-Jones, M. E. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J. Bacteriol.* **182**: 2928–2936.
6. Lista, F., Faggioni, G., Valjevac, S., Ciammaruconi, A., Vaisaire, J., le Doujet, C., Gorge, O., De Santis, R., Carattoli, A., Ciervo, A., Fasanella, A., Orsini, F., D'Amelio, R., Pourcel, C., Cassone, A. and Vergnaud, G. 2006. Genotyping of *Bacillus anthracis* strains based on automated capillary 25-loci multiple locus variable-number tandem repeats analysis. *BMC Microbiol.* **6**: 33.
7. Makino, S., Uchida, I., Terakado, N., Sasakawa, C. and Yoshikawa, M. 1989. Molecular characterization and protein analysis of the cap region, which is essential for encapsulation

- in *Bacillus anthracis*. *J. Bacteriol.* **171**: 722–730.
8. Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan. 1982. Statistics on Animal Hygiene, pp. 14–15. *In*: Livestock Industry Bureau (ed.).
 9. Ministry of Agriculture, Forestry and Fisheries, Tokyo, Japan. 2000. Statistics on Animal Hygiene, pp. 16–17. *In*: Livestock Industry Bureau (ed.).
 10. Natori, N. 1995. A case of cutaneous anthrax. *Tohoku J. Exp. Med.* **176**: 187–190.
 11. Okinaka, R. T., Cloud, K., Hampton, O., Hoffmaster, A. R., Hill, K. K., Keim, P., Koehler, T. M., Lamke, G., Kumano, S., Mahillon, J., Manter, D., Martinez, Y., Ricke, D., Svensson, R. and Jackson, P. J. 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. *J. Bacteriol.* **181**: 6509–6515.
 12. Read, T. D., Salzberg, S. L., Pop, M., Shumway, M., Umayam, L., Jiang, L., Holtzapple, E., Busch, J. D., Smith, K. L., Schupp, J. M., Solomon, D., Keim, P. and Fraser, C. M. 2002. Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. *Science* **296**: 2028–2033.
 13. Song, J., Xu, Y., White, S., Miller, K. W. and Wolinsky, M. 2005. SNPsFinder--a web-based application for genome-wide discovery of single nucleotide polymorphisms in microbial genomes. *Bioinformatics* **21**: 2083–2084.
 14. Van Ert, M. N., Easterday, W. R., Huynh, L. Y., Okinaka, R. T., Hugh-Jones, M. E., Ravel, J., Zanecki, S. R., Pearson, T., Simonson, T. S, U'Ren, J. M., Kachur, S. M., Leadem-Dougherty, R. R., Rhoton, S. D., Zinser, G., Farlow, J., Coker, P. R., Smith, K. L., Wang, B., Kenefic, L. J., Fraser-Liggett, C. M., Wagner, D. M. and Keim, P. 2007. Global genetic population structure of *Bacillus anthracis*. *PLoS ONE* **2**: e461.