S5-4

Molecular Detection and Subtyping of *Bacillus anthracis* in Korea

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Abstract

Bacillus anthracis is causal agent of anthrax, a serious and often fatal infection of livestock and humans. It is considered one of the most potential biological weapons of mass destruction because of its highly pathogenic nature and spore-forming ability. *B. anthracis* is known to be one of the most monomorphic bacterial species, therefore only a few subtyping tools are now available.

For rapid and reliable detection of anthrax spores from environmental samples, specific primers and probes from *pag* gene and *cap* gene on the two plasmids, pXO1 and pXO2 as well as *sap* gene encoded on the chromosome were designed and evaluated by TaqMan real-time PCR. Serial dilutions of *B. anthracis* DNA and spore were detected up to a level of 0.1ng/μl and 10 spores/ml, respectively, at the correlation coefficient of 0.99 and soil sample with added spores was detected up to 10^4 spores/g soil within 3hrs by real-time PCR.

We have used eight-locus multilocus variable-number tandem repeat analysis(MLVA) typing technique for the characterization of genetic diversities and relationships within 60 Korean *B. anthracis* isolates. Korean isolates could be subtyped as 9 distinct MLVA genotypes including 7 new genotypes. Among sixteen strains from three outbreaks, three strains from Kyungjoo and one from Hongsung were genotype 32(cluster A3a) and 57(A3b), respectively, while twelve strains from Changnyung were identified as a new genotype showing a close relationship with genotype 85 in group B (cluster B1), which is known as a distinct branch restricted to southern Africa. Thirty-two strains, isolated from random soil samples collected in wide range of Korea during last 6 years, formed a distinct cluster of A4 and were subtyped into 6 new genotypes. These results suggest that Korean *B. anthracis* strains are from diverse geographical origin and some of outbreaks might be occurred by more recent contamination or dispersal of *B. anthracis*.

We also cloned and sequenced the genes of *pagA* and *gyrB* from Korean isolates to evaluate the potential of these genes in differentiating and identifying *B. anthracis* strains. When the entire *pagA* were sequenced and analyzed, we could identify more than twenty point mutations and the transitions in three nucleotide positions showing high frequency were used for major subtyping. All strains were included in the three types(type CCC, type TCT and type TTT) and phylogenic analysis by the *pagA* sequence variation well corresponded with MLVA subtyping. When *gyrB* sequences of 1,923 bp in chromosome of isolates were determined and aligned, the similarities in *gyrB* sequences

among all strains were higher than 99.8%, and in eleven major transition positions compared with other *Bacillus*, all *B. anthracis* strains tested had an identical sequence, suggesting *gyrB* could be used as a molecular diagnostic marker for rapid differentiation of *B. anthracis* from other related spore-forming *Bacillus* species.

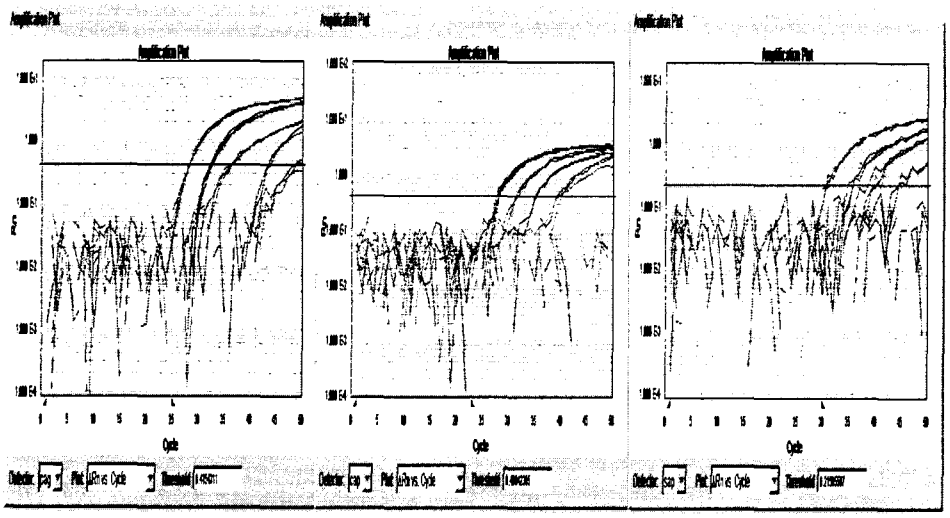

Fig 1. TaqMan real-time PCR of ten-fold serial dilutions of *Bacillus anthracis* spores added to soil sample. Serial dilutions of spores were run in (A) *pagA* (B) *cap* and (C) *sap* specific realtime PCR.

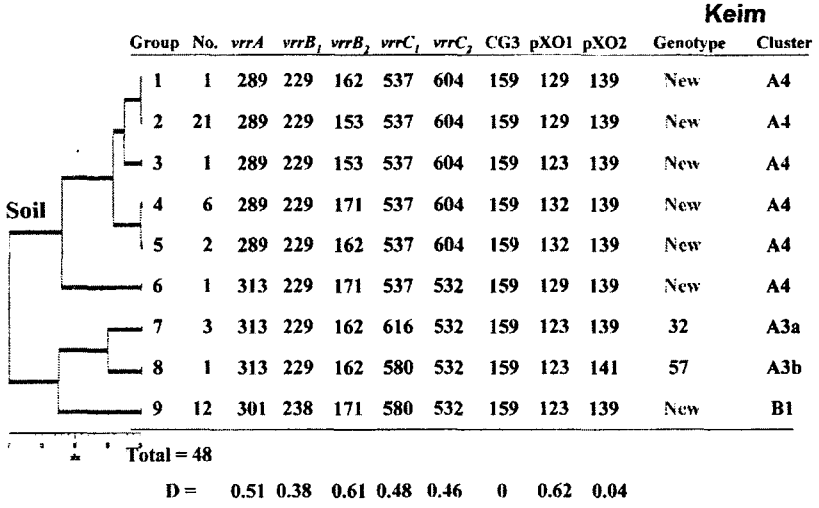

Figure 2. Genetic relationships among Korean *B. anthracis* isolates. Eight VNTR marker loci were used to estimate genetic relationships among the 48 *B. anthracis* isolates. The allele size at each VNTR locus is shown along with the number of isolates in each genotypic group. The D calculated for each of the eight marker is listed below the dendrogram.

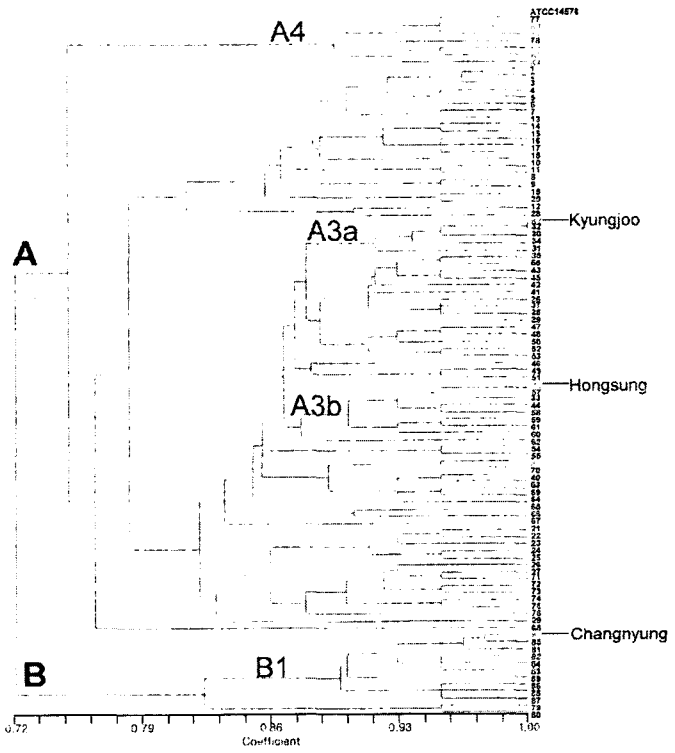

Figure 3. MLVA-based dendrogram performed on 9 genotypes(K1-K9) of Korea isolates and 89 genotypes obtained from Keim et al. study.

Table 1. Major mutation sites identified in *B. anthracis* strains used in this study.

	Nucleotide position [a]	Base change	Frepuency	Amino acid change
1	**1998**	**C ↔ T**	**27/48**	Synonymous
2	2885	T ↔ G	4/48	Val ↔ Gly(D2[b])
3	3100	A ↔ G	6/48	Ile ↔ Val(D2)
4	**3496**	**C ↔ T**	**23/48**	Pro ↔ Ser(D3)
5	**3602**	**C ↔ T**	**26/48**	Ala ↔ Val(D3)

[a] Nucleotide positions based on the sequence from sterne strain accession no. M22589.
[b] D, domain of the protective antigen protein.

Table 2. PA genotypes identified in this study

PA genotype I[a]	No. of strains (Standard and reference strains)	VNTR category	PA genotype II[b]	Mutation positions[c]					
				No. of strains	1	2	3	4	5
Type CCC	11 (Ames, sterne)	$VNTR_4$	type 1	10	C	T	A	C	C
			type 2	1	C	G	A	C	C
type TTT	23 (vollum ATCC 14578)	$VNTR_2$	type 3	21	T	T	A	T	T
			type 4	1	T	T	G	T	T
			type 5	1	T	G	A	T	T
Type TCT	14 (ATCC 14185 vaccine strain)	$VNTR_3$ or $VNTR_4$	type 6	7	T	T	A	C	T
			type 7	2	T	G	A	C	T
			type 8	5	T	T	G	C	T

[a] Designated by the 3 major nucleotides shown to vary. [b] Designated by the 5 nucleotides shown to vary.

[c] The same position as described in Table

Table 3. PA genotypes identified in this study

Bacillus species	Strains	No. of strains	Positions[a]										
			1 (329)	2 (426)	3 (499)	4 (501)	5 (544)	6 (546)	7 (557)	8 (781)	9 (782)	10 (811)	11 (1,13
anthracis	ATCC 14578(type strain)	1	G	A	C	A	C	G	T	T	C	A	A
anthracis	ATCC 14185, Ames, Sterne, KrugerB WesternNA, all isolates in korea	22	.[b]	.	.	.	.	.	.	.	.	.	.
anthracis	Pasteru #2H (AF090333)	1	A	.	.	.	.	.	.	.	.	.	.
cereus	JCM2152^T (AF090330)	1	A	.	.	.	.	A	.	.	T	.	.
cereus	H2 (AF136388), H6 (AF136389)	2	A	.	.	.	.	.	.	.	.	.	.
cereus	H16 (AF136387)	1	A	T	A	G	A	A	A	G	G	C	T
thuringiensis	(AF136390)	1	A	.	.	.	.	A	.	.	T	.	.
thuringiensis	IAM 12077^T	1	A	T	A	G	A	A	A	G	G	C	T
mycoides	(AF090332)	1	A	T	.	T	A	A	A	G	T	C	T

[a] Numbers refer to the number of positions where mismatches are found. Numbers in parentheses refer to positions from the start codon in *gyrB*.

[b] Nucleotides identical to those of B. anthracis are indicated by dot.

References

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