Iranian Journal of Microbiology
Volume 7 Number 3 (June 2015) 168-172

ORIGINAL ARTICLE

Genetic Characterization of *Bacillus anthracis* 17 JB strain

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Received: March 2015**, Accepted:** May 2015

ABSTRACT

Background and Objectives: *Bacillus anthracis* is one of the most homogenous bacteria ever described. Some level of diversity. *Bacillus anthracis* 17JB is a laboratory strain It is broadly used as a challenge strain in guinea pigs for potency test of anthrax vaccine.
Material and Methods: This work describes genetic characterization of *B. anthracis* 17 JB strain using the SNPs and MLVA genotyping.
Results and Conclusion: In SNPs typing, the originally French 17JB strain represented the A.Br. 008/009 subgroup. In Levy's genotyping method, 843, 451 and 864 bp long fragments were identified at AA03, AJ03 and AA07 loci, respectively. In the vaccine manufacturer perspective these findings are much valuable on their own account, but similar research is required to extend molecular knowledge of *B. anthracis* epidemiology in Persia.

Keywords: *Bacillus anthracis* 17JB, Genetic characterization, SNPs typing.

INTRODUCTION

In May 1881 Pasteur made history by his attenuated live anthrax vaccine trial in Pouilly-le-Fort, Paris (1). Pasteur developed a dual-shot vaccine schedule pertaining a first inoculation of *B. anthracis* cells prepared from cultures incubated at 42-43°C for 15-20 days (Pasteur I strain) followed by a second injection of cultured cells at 42-43°C for 10-12 days (Pasteur II strain) (2). In 1934 the veterinarian Max Sterne from the Onderstepoort veterinary laboratory, South Africa derived *B. anthracis* 34F2 from a virulent isolate blamed for severe outbreaks in South Africa (3). This was the most globally-approved anthrax spore vaccine strain ever achieved. Since 1938, *B. anthracis* 34F2 was used massively in preparation of anthrax vaccine and replaced almost all other vaccinal strains (3). Sterne also derived a challenge strain through continuous passage of Pasteur II strain in guinea pigs. This so-called "guinea pig challenge strain" is essentially virulent for guinea pigs but harmless to rabbits, domestic animals or human. This is now the standard challenge strain recommended by the World Organization for Animal Health (OIE) for potency test of anthrax spore vaccine (4).

In 2005, Levy introduced a three-locus MLVA typing system to genotype *B. anthracis* (5). His approach covered three new loci namely AA03, AJ03

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and AT07 that were not previously included in the MLVA genotyping system developed by Keim. Differentiation capability of Levy's system in *B. anthracis* sub-populations has been is left for further studies.

In 2007, van Erth showed the world population of *B. anthracis* can be divided into 12 lineages/groups based on combination of 13 specific canonical slowly evolving SNPs. Therefore by characterization of SNPs at C.Br.A1055, B.Br.KrugerB, B.Br.001/002, B.Br.CNEVA, A.Br.Ames, A.Br.001/002, A.Br. Aust94, A.Br.003/004, A.Br.Vollum, A.Br.005/006, A.Br.008/009, A.Br.WNA loci any given *B. anthracis* isolate can be assigned to one of the 12 mentioned group.

The earliest recorded evidences on existence of anthrax in Persia date back to 1860's when Persian wool came first in the Spears list of most noxiousness foreign wools causing anthrax (6). In the today's Iranian environment, anthrax vaccine is massively issued by veterinary officials. Over the last 12 years almost half a billion doses of live spore vaccine manufactured by Razi institute have been administrated in this country. In 1960's with fresh stocks of Sterne 34F2 and 17JB strains arrived from the Central Veterinary Laboratory, Weybridge, UK, Razi switched from old French strain(s) to these standard strains to manufacture the vaccine. The genomic structure of Razi *B. anthracis* Sterne 34F2 substrain was recently analyzed by MLVA genotyping system and published elsewhere, in the work presented here the genome of Razi *B. anthracis* 17JB substrain was subjected to van Erth's SNP and Levy's MLVA genotyping methods.

MATERIALS AND METHODS

Bacterial strain and Culture. Inside a biosafety cabinet class II, content of an O-ring-equipped microtube holding *B. anthracis* 17JB spores in plain saline was used to inoculate a glass plate of blood-agar. The plate was incubated overnight at 37°C. A loopful of bacterial growth from the 14-hour incubated plate was transferred to a new microtube containing 400 µl of TE buffer (Tris-HCL plus 1.0 mM EDTA; pH 8.0). While re-capped, the microtube content was vortexed and transferred to a boiling waterbath where it was securely submerged for 20 min to heat-inactivate the bacilli. The boilate was centrifuged at 12,800 g for 5 min and the supernatant was passed through a syringe filter (0.22 µm).

For SNPs typing, the simplified version of the previously described van Erth strategy adopted by Najafi Olia and co-workers was used (7). This version is specifically suitable for traditional thermocyclers. For the MLVA genotyping, the original method developed by Levy with some modifications was employed (5).

All PCR reactions were performed in 12 µl mixtures in a Mastercycler (Eppendorf, Germany). For SNPs typing, each reaction contained 6 µl of PCR master mix, 0.5 µl each primer, 0.6 µl DMSO and 0.4 µl PCR water plus 4 µl DNA template. The mixtures were subjected to denaturation at 95°C for 5 min followed by 30 amplification cycles of 30 s at 95°, 45 s at 65°C and 45 s at 72°C with a final extension phase at 72°C for 10 min. For MLVA experiment, 2 µl DNA template was added to 6 µl of PCR master mix, 0.5 µl each primer and 3 µl PCR water. The amplification process was initiated by rising the temperature to 94°C lasting for 5 min. Each following temperature cycle was 94°C for 1 min, 58°C for 30 s, 72°C for 1 min with these triple steps repeated 35 times complemented with a single final extension step of 72°C for 5 min.

Correct amplification and relative size of PCR products was examined by gel electrophoresis on 2% multipurpose agarose gels stained with Redsafe which was followed by visualization under UV illumination. All the PCR products were sequenced at the collaborating laboratory (Macrogen, South Korea). The raw sequence chromatograms were edited by Chromas Lite software ver 2.1.1 (available on www.technelysium.com.au). Using Clustal X software ver 2.1 (available on www.clustal.org/clustal2/) the complimented forward and reverse sequence strings were aligned and the nucleotide of interest (SNP analysis) was identified. The Tandem Repeat Finder software ver 4.04 (available on http://tandem.bu.edu/trf/trf.html) was used to detect the unit repeats and copy number of them in MLVA analysis.

Molecular experiments. For SNPs typing, the simplified version of the previously described van Erth strategy adopted by Najafi Olia and co-workers was used (7) (Table 1). This version is specifically suitable for conventional thermocyclers. For the MLVA genotyping, the original method developed

by Levy with few modifications was employed (5).

PCR protocols. All PCR reactions were performed in 12 µl mixtures in a Mastercycler (Eppendorf, Germany). For SNPs typing, each reaction contained 6 µl of PCR master mix, 0.5 µl each primer (Macrogen®, South Korea), 0.6 µl DMSO and 0.4 µl PCR water plus 4 µl DNA template. The mixtures were subjected to denaturation at 95°C for 5 min followed by 30 amplification cycles of 30 s at 95°, 45 s at 65°C and 45 s at 72°C with a final extension phase at 72°C for 10 min. For MLVA experiment, 2 µl DNA template was added to 6 µl of PCR master mix, 0.5 µl each primer and 3 µl PCR water. The amplification process was initiated by rising the temperature to 94°C lasting for 5 min. Each following heating cycle consisted of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min with these triple steps repeated 35 times complemented with a single final extension step of 72°C for 5 min.

Table 1. Details of primers used and the expected size of PCR products along with nucleotide composition at the 13 examined SNPs loci based on the *B. anthracis* Sterne 34F2 vaccine strain genome. NA= not applicable

	Locus	Primers (5'-3) This study	Amplicon size (bp) in *B. anthracis* Sterne34F2 (Location in the reference genome)	SNP base in *B. anthracis* 34F2 (location in the reference genome)	Reference
SNP genotyping	A.Br.001	f) TTA CAG TGC CGC CAA AGA CA r) CCC ACT CAG TCG GGA TTT TCA	608 (181772-182380)	T (182107)	(7, 11)
	A.Br.002	f) TAG AGA TGT GGT CGC GAA GT r) AGC TTC AAA GAG TCC CAC CA	669 (947306-947975)	A^{comp} (947657)	(7, 11)
	A.Br.003	f) TCG TCA AGG AAT CGG ACG TT r) CGA GCT TCT TCC CAC ACA CT	614 (1493020-1493634)	G^{comp} (1493231)	(7, 11)
	A.Br.004	f) AAT AAG TGG CGC TGC CGT AT r) CAG ATG GAT CGC GTT TGC AC	557 (3601025-3601582)	C (3601360)	(7, 11)
	A.Br.006	f) ATG GAT GAA AAT GAT CAG CCG C r) CAG CAA TCT CCC CTT TCC GA	513 (162236-162749)	A (162510)	(7, 11)
	A.Br.007	f) TAG TAC CGC AAG CGG AAG AG r) TGT CAT CGG CGA CTT GTT CT	540 (266092-266632)	T (266452)	(7, 11)
	A.Br.008	f) CGC CAA ACG ATG CAA ACT CA r) ACC ATC GAT TGG CTG AA	458 (3947469-3947927)	T^{comp} (3947747)	(7, 11)
	A.Br.009	f) TCC CCT AAT GGA ATA CGC GG r) GCG CTT CGA ATT GGT GAT CG	545 (2590034-2590579)	A (2590283)	(7, 11)
	B.Br.001	f) GTT CTG GTG CTG CAT TTG GTA r) ACG CTT CAT CCG TAA ATC CCA G	599 (1455055-1455654)	T^{comp} (1455347)	(7, 11)
	B.Br.002	f) AAC GAA GGG GAC AGT GGA AG r) TCC CGT TGT AAG GGG AAA GAT	610 (1056274-1056884)	G^{comp} (100979)	(7, 11)
	B.Br.003	f) TTT CCG TAT GGC TGT GTT TGG r) CCA AAT GAA CCA CCA GCC CA	479 (1494037-1494516)	G (1376553)	(7, 11)
	B.Br.004	f) GTT TAT GCC GTG AGA GGA GGT r) AAC ACC CTT CGG AAT ACG GG	619 (69578-70197)	T (69953)	(7, 11)
	A/B.Br.001	f) TGG GCG TCG TTA CAA CTT CT r) CCA GCA AGC GAT ATA CCG GA	599 (3698200-3698770)	A (3698581)	(7, 11)
MLVA (Levy) genotyping	AA03	f) TTA GCG CCC CCT TGC GTT CC r) TTA GCG CCC CTA GAC CAA TTG C	931	NA	(5)
	AJ03	f) AGC ACC TCG TTC ATG CTC ATA ACG G r) AGC ACC TCG TCT ACT TCA TTT TGT GC	451	NA	(5)
	AT07	f) CTC CTC AAA TTA CTA AAA TGA AAC C r) TTG GCA TAG ACG TAT ATT GCG GTC C	864	NA	(5)

Correct amplification and relative size of PCR products was examined by gel electrophoresis on 2% multipurpose agarose gels stained with Redsafe which was followed by visualization under UV illumination.

Sequencing of amplification products. All PCR products were sequenced at the collaborating laboratory (Macrogen®, South Korea). The raw sequence chromatograms were edited by Chromas Lite software ver 2.1.1 (available on www.technelysium.com.au). Using Clustal X software ver 2.1 (available on www.clustal.org/clustal2/) the complimented forward and reverse sequence strings were aligned and the nucleotide of interest (SNP analysis) was identified. The Tandem Repeat Finder software ver 4.04 (available on http://tandem.bu.edu/trf/trf.html) was used to detect the unit repeats and copy number of them in MLVA analysis.

RESULTS

The nucleotide structure of the *B. anthracis* 17JB strain genome at the 13 SNPs were characterized as T, G, A, T, A, T, T, A, T, G, G, T and A at 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.Br003, B.Br004 and A/B.Br.001 respectively (Table 2). Consulting the standard SNP genotyping table of van Erth's analysis, it was learnt the Razi *B. anthracis* 17JB substrain matches with the A.Br. 008/009 group (Table 2).

In MLVA genotyping, at AA03, AJ03 and AA07 loci, three PCR products with 843, 451 and 864 bp length were detected respectively. In situ analysis of nucleotide structure of AA03, AJ03 and AA07 loci from the 17JB genome displayed 2.7 copies of a 88 bp TR, 2.8 copies of a 40 bp TR and 7.2 copies of a 39 bp TR at these loci, respectively.

Table 2. The SNPs arrangements at the 13 standard loci characterized by vanErth et al. representing the two important A.Br.001/002 and A.Br.008/009 lineage/group. The SNPs of difference between the two are underlined.

Lineage/Group (Strain)	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.Br003	B.Br004	A/B.Br.001
A.Br.001/002 (*B. anthracis* Sterne 34F2)	T	A	G	C	A	T	T	A	T	G	G	T	A
A.Br.008/009 (*B. anthracis* 17JB)	T	G	A	T	A	T	G	A	T	G	G	T	A

DISCUSSION

In the work presented here the genomic characteristics of the *B. anthracis* 17JB strain was analyzed. Bearing a French origin, 17JB was derived from Pasteur II strain comparing to the Sterne 34F2 that has a South African background (4). Application of the van Erth's SNP typing in the present work classified the 17 JB strain into the A.Br. 008/009 subgroup as reported previously elsewhere (8).

This subgroup of *B. anthracis,* also known as Trans-Eurasian group (TEA) is scattered throughout the world. In large part of Europe including Italy (9), Bulgaria (10), Hungary, Albania (11) and France (8) the TEA group is a well-established sub-population. Observations by MLVA analyses have acknowledged a remarkably larger genetic diversity within the isolates belonged to this subgroup (8) a likely reflection of ecologically well-establishment of this subgroup in the region (8). In Asia, 008/009 subgroup isolates are reported from China (12), Russia, Kazakhstan, the Caucasus (13) but are not specifically reported from Bangladesh (14), Korea (15) and Japan (16). Given the proximity of Iran to its northern neighboring former Soviet Union States, lack of reports on 008/009 subgroup existence in this Middle-Eastern country might be simply due to poor epidemiological work.

In MLVA genotyping, comparative analysis of nu-

cleotide structure of the three Levy's loci between Strene 34F2 and 17JB detected difference only at AA03 where Sterne 34F2 genome carries a longer segment (931 bp) compared to the 17Jb genome (843 bp). This observation might be helpful in laboratories where differentiation between the two strains is a serious challenge.

In order to extend the current epidemiological understanding of anthrax in Iran and global distribution of the pathogen, further molecular epidemiological studies are required to apply SNPs and MLVA typing systems on Iranian *B. anthracis* isolates.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge Razi Vaccine & Serum Research Institute (the Iranian Department of Agriculture) for financial support of this work (Research Grant No. 2-18-18-92107).

The authors are indebted to Professor Paul Keim from University of Northern Arizona, USA for comments and suggestions. Reza Banihashemi and Mohammad Sekhavati are thanked for their continuous technical assistance and providing the high quality genomic material required for the research. Sakineh S Mohammadi is a former MSc student in microbiology graduated from RVSRI in 2015.

Razi celebrates its 90th foundation anniversary in 2015, authors would like to congratulates their colleagues on the occasion of this joyful event.

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