Genetic Characterization of Bacillus anthracis 17 JB strain

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ABSTRACT

Background and Objectives: Bacillus anthracis is one of the most homogenous bacteria ever described. Some level of diversity. Bacillus anthracis 17 JB 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 17 JB 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 17 JB, 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 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 groups.

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°C, 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°C, 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* Sterne34F2 (location in the reference genome) | Reference |
|-------|----------------------------|---------------------------------------------------------------------------------|--------------------------------------------------------------------------------|------------|
| A.Br.001 | 0 TEA CAC TGCC CGGCAA AGA CA  0 GCC ACT CAG TGG GAA TT TCA | (181772-182380) 608 T | (182107) | (7, 11) |
| A.Br.002 | 0 TAG AGA TGGT CGG GAA GT  0 AGG TGC TCT CAG TCA CT | (947306-947975) 669 A | (947657) | (7, 11) |
| A.Br.003 | 0 TGCTCAAGG AAT CGG AC TT  0 GGA GCT TCT CAC ACA CT | (149302-1493634) 614 G | (1493231) | (7, 11) |
| A.Br.004 | 0 AAT AAG TGG GCG TGC CT AT  0 CAG AGG GAT GCC GTC AC | (360102-3601582) 557 C | (3601360) | (7, 11) |
| A.Br.006 | 0 CAG GTC AAT GAT CGG CCC G | (162236-162749) 513 A | (162510) | (7, 11) |
| A.Br.007 | 0 TAG TAC CCG AAC CGG AAG AG  0 TGC TCT CCC CTT TCC CT | (264892-266632) 540 T | (266452) | (7, 11) |
| A.Br.008 | 0 GGC CAA AGC ATG CAA ACT CA | (3947469-3947927) 458 T | (3947747) | (7, 11) |
| A.Br.009 | 0 GCC TCT AAT GGA ATAC GCC GG  0 GGC TCT CGA ATT GGT CAG | (259038-2590579) 545 A | (2590283) | (7, 11) |
| B.Br.001 | 0 GTT GTC GTC GTC CAT TGG GTA  0 AGC TCT CAT CCG TAA AAT CCC GAG | (1455055-1455654) 599 T | (1455347) | (7, 11) |
| B.Br.002 | 0 AAC GAC GAC GAC GAT GGA AG | (1058274-1058684) 610 G | (100979) | (7, 11) |
| B.Br.003 | 0 TT TCC CAG TAT GCT TGT TGG  0 CCA AAG GAC CCA CCC CA  0 AACC CTT CCG AAC ATG GAC G | (1490317-1494516) 479 G | (1376553) | (7, 11) |
| B.Br.004 | 0 GTT TAT GCC GTG AGA GAT GAG  0 AAC ACG TCT GGG AAT ACC G | (69978-70197) 619 T | (69953) | (7, 11) |
| A/B.Br.001 | 0 TGG GGC TGG TTA CAA CTT CT  0 CCA GCA GAG AAT CCG GA | (3698200-3698770) 599 A | (3698581) | (7, 11) |

**MLVA (Levy) genotyping**

| MLVA (Levy) genotyping | Primers (5'-3') | Amplicon size (bp) | Reference |
|-------------------------|-----------------|---------------------|------------|
| AA03 | 0 TGG CGG CCT TCC TCC CCC TT | 931 NA | (5) |
| AJ03 | 0 GGC ACC TCG TCT TCT ACA ACT TAG G  0 GGC ACC TCG TCT ACT TGT TGT GC | 451 NA | (5) |
| AT07 | 0 CTC TCT AAT TTA CAA AAG TTA AGC C  0 TGT GCA TAG AGC TAT AAT GGG GTG C | 864 NA | (5) |
DISCUSSION

In the work presented here the genomic characteristics of the \textit{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 17JB strain into the A.Br. 008/009 subgroup as reported previously elsewhere (8).

This subgroup of \textit{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 nucleotide structure of the 17JB strain genome at the 13 SNPs were characterized as T, G, A, T, A, 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.Br.003, B.Br.004 and A/B.Br.001 respectively (Table 2). Consulting the standard SNP genotyping table of van Erth's analysis, it was learnt the Razi \textit{B. anthracis} 17JB strain 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 | R.Br.001 | R.Br.002 | R.Br.003 | R.Br.004 | A.Br.001 |
|------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| A.Br.001/002 (\textit{B. anthracis Sterne 34F2}) | T | A | G | C | A | T | T | A | T | G | G | T | A |
| A.Br.008/009 (\textit{B. anthracis} 17JB) | T | G | A | T | A | T | G | A | T | G | G | T | A |

**RESULTS**

The nucleotide structure of the \textit{B. anthracis} 17JB strain genome at the 13 SNPs were characterized as T, G, A, T, A, 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.Br.003, B.Br.004 and A/B.Br.001 respectively (Table 2).
cleotide structure of the three Levy’s loci between Sterne 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.

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

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