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Original Article

A Study on molecular characterization of Razi *Bacillus anthracis* Sterne 34F2 substrain in Iran

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ABSTRACT

Anthrax, a zoonotic disease caused by *Bacillus anthracis*, has affected humans since ancient times. For genomic characterization of Razi *B. anthracis* Sterne 34F2 substrain, single nucleotide polymorphism (SNP) genotyping method developed by Van Erth, variable-number tandem-repeat (VNTR)-8 analysis proposed by Keim, and multiple-locus VNTR analysis (MLVA)-3 introduced by Levy were employed. In the SNPs typing system, where the nucleotide content of the genome at 13 evolutionary canonical loci was collectively analyzed, the originally South African 34F2 substrain was categorized in the A.Br.001/002 subgroup. In the VNTR-8 analysis, fragments with lengths of 314, 229, 162, 580, 532, 158, and 137 bp were identified at the following loci: vrrA, vrrB1, vrrB2, vrrC1, vrrC2, CG3, and pxO1, respectively. In addition, application of Levy's MLVA-3 genotyping method revealed that the genome of this strain carried 941, 451, and 864 bp fragments at AA03, AJ03, and AA07 loci, respectively. The present findings are undoubtedly helpful in meeting the requirements set by the World Organization for Animal Health (OIE) and World Health Organization (WHO) for anthrax vaccine manufacturers including Razi Institute. However, further similar studies are required to promote the current epidemiological knowledge of anthrax in Iran.

Keywords: Iran, Anthrax, Max Sterne

INTRODUCTION

In an annual speech on June 5, 1880, Professor W. S. Greenfield from the Brown Animal Sanatory Institution in London remarked:

"...I think it must be allowed that they [Bacillus anthracis] are more irritant in their action and cause death more rapidly when directly inoculated in the fluid from an animal just dead of the disease than when cultivated…"

Greenfield pioneered in application of attenuated *Bacillus anthracis* strains in field trials (Tigertt, 1980; Turnbull, 1991). In 1881, in an experiment in Paris, France, Pasteur, who was inspired by Greenfield's findings, successfully examined a live attenuated anthrax vaccine (Turnbull, 1991). However, the serious drawbacks, caused by the field application of Pasteur's anthrax vaccine protocol, intrigued other researchers to introduce more effective vaccine regimens, using less detrimental vaccine strains. In 1934 at the

Onderstepoort Veterinary Laboratory in South Africa, Max Sterne succeeded in deriving *B. anthracis* 34F2 substrain from a noxious and indigenous virulent strain (Turnbull, 1991). In the late 1930's, stocks of *B. anthracis* Sterne 34F2 were supplied to vaccine manufacturers around the world. With the exemption of Russia and Eastern European countries, where STI-1 (Feodorova et al., 2014) and 1190R-Stamatin (Popa et al., 2009) are commonly used, this strain has gradually replaced almost all other vaccine strains (Gilfoyle, 2006). In the 1860's, the dry, dusty, low-quality wool, imported from Iran to Britain, was recognized as a transmission route for a textile industry-related disease, known as woolsorter's disease (Xu et al., 2002). Consequently, Iranian wool came first in the famous Spears list of most noxious foreign wools causing anthrax (Legge, 1905). In Iran, experiments on the vaccination of farm animals against anthrax were initiated by Razi Institute under the supervision of Dr Louis Paul Delpi in the 1930's, using French, South African, Moroccan, and native strains of *B. anthracis* (Delpi, 1938). Approximately one million doses of Razi anthrax vaccine were supplied to veterinary centers across Iran in 1932; this number increased to 6 million doses in 1935 (Delpi, 1938). By 1973, production of anthrax vaccine at Razi Institute was based on the mass culture of a native C5 strain in a peptone-free agar medium, which was then replaced by N. Z. case medium. In 1978, a major change in vaccine manufacturing came to effect as the institute switched from C5 strain to Sterne 34F2, which was obtained from the Central Veterinary Laboratory (Weybridge, UK) in the 1960's. Moreover, in the late 2000's, fresh stocks of Sterne 34F2 seeds were re-supplied to Razi Institute by a Turkish collaborating center (Etlik). Since 1988 with a fluctuating trend, the annual manufacturing rate of anthrax vaccine in Iran has declined from a peak of 91 million doses (1996) to the current roughly stable 44 million doses (2014). Furthermore, Razi Institute remains the exclusive Iranian supplier of veterinary anthrax vaccine. From a genomic perspective, *B. anthracis* is one of the typical and highly homogenous bacteria. Nevertheless, some degrees of diversity have been detected in the genome of this bacterium through single nucleotide polymorphism (SNP) typing method, variable-number tandem-repeat (VNTR) analysis, and multiple-locus VNTR analysis (MLVA). In 2000, Keim and colleagues developed an eight-locus VNTR genotyping system for *B. anthracis* (Keim et al., 2000). By consensus, this genotyping method is now regarded as the standard method for strain typing in epidemiological studies on anthrax. In 2007, Van Erth suggested that the use of 13 slowly evolving canonical SNPs is sufficient for assigning any given *B. anthracis* isolate to one of the 12 lineages or groups, identified in the global population of *B. anthracis* (i.e., 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, and A.Br.WNA) (Van Ert et al., 2007). Today, the SNP genotyping method by Van Erth is recognized as a well-established and essential approach for molecular typing of *B. anthracis*. In 2005, Levy introduced a three-locus MLVA typing system to genotype *B. anthracis* (Levy et al., 2005). The introduced approach encompassed three new loci, i.e., AA03, AJ03, and AT07, which were not previously incorporated in the MLVA genotyping system by Keim. In fact, assessment of the diversity index of Levy's system in *B. anthracis* sub-populations requires further research. In the present study, the genomic characteristics of Razi *B. anthracis* 34F2 substrain were addressed through Van Erth's SNP, Keim's VNTR, and Levy's MLVA genotyping methods.

MATERIALS AND METHODS

Bacterial culture and extraction of the genomic material. By the use of a disposable syringe, 0.5 milliliter of content of an anthrax vaccine bottle (Razi Institute, Iran) was spread over a blood agar plate. The incubation phase continued for 15 h at 37 °C, and a loopful of bacterial colonies was transferred to a microtube, containing 400 µl of TE buffer (Tris HCl and 1.0 mM of EDTA; pH=8.0). To re-suspend the

bacterial load, the microtube was vortexed for a short period of time.

For thermal inactivation of the cellular suspension, the microtube was deeply submerged in a boiling water bath and rested for 20 min; afterwards, the suspension was removed and cooled down to room temperature. The cellular debris was cleared through centrifugation at 6,000 g for 2 min. To ensure the removal of any remaining bacterial spore, the extracted liquid passed through a 0.22 µM syringe filter. The cell lysate was transferred to a new microtube for storage at -20°C until further use.

Molecular experiments. The recently adopted simplified version of Van Erth's method suitable for use in traditional thermocyclers was used for SNP analysis (Najafi Olya et al., 2015). In the present study, VNTR analysis of *B. anthracis* 34F2 strain was conducted for the loci described by Keim and colleagues (Keim et al., 2000). Also, the original MLVA genotyping method proposed by Levy was employed with minor modifications (Levy et al., 2005; Seyyed-Mohammadi et al., 2015).

A Mastercycler (Eppendorf, Germany) was used to run polymerase chain reactions (PCRs) in 12 µl mixtures. For SNP experiments, individual reactions contained 0.5 µl of each primer, 0.6 µl of dimethyl sulfoxide (DMSO), 6 µl of PCR Master Mix, 0.4 µl of PCR water, and 4 µl of DNA template. The thermocycling conditions included denaturation at 95 °C for 5 min, followed by 30 cycles 95 °C for 30 s, 65 °C for 45 s, 72 °C for 45 s, and a final heating step at 72 °C for 10 min. For VNTR-8 experiments, 3 µl of DNA template was added to 6 µl of PCR Master Mix, 1.25 µl of each primer, and 0.5 µl of PCR water. The amplification process was initiated by raising the temperature to 94 °C for 5 min, followed by 35 cycles 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, and a final heating phase 72 °C for 10 min. For Levy's MLVA method, 2 µl of DNA template was added to 6 µl of PCR Master Mix, 0.5 µl of each primer, and 3 µl of PCR water. The amplification process was initiated by increasing the temperature to 94 °C for 5 min, followed by a heating cycle 94 °C for 1 min, 58°C for 30 s, and 72 °C for 1 min. This cycle was repeated 35 times and was complemented with a single final extension step 72 °C for 5 min.

In order to determine the size of PCR products, gel electrophoresis was performed, using 2% multi-purpose Agarose gel (Roche, USA), pre-stained with RedSafe (Intron, South Korea) solution; the amplification bands were visualized by ultraviolet transillumination. The exact size of amplification products was determined by sequencing the products (Macrogen Inc., South Korea). Moreover, Chromas Lite 2.1.1 software (available on www.technelysium. com.au) was used to edit the raw sequence chromatograms. The alignment of forward and reverse sequence strings was performed by Clustal X 2.1 software (available on www.clustal.org/clustal2/). This strategy was specifically used to identify the nucleotide of interest in the SNP analysis.

Table 1. The SNP genotype of *B. anthracis* Sterne 34F2 and Razi substrains

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.Br.004	A/B.Br.001
A.Br.001/002 (*B. anthracis* Sterne 34F2, www.ncbi.nlm.nih.gov/genome/genomes/181:)	T	A	G	C	A	T	T	A	T	G	G	T	A
A.Br.001/002 (*B. anthracis Sterne 34F2,* Razi substrain)	T	A	G	C	A	T	T	A	T	G	G	T	A

RESULTS

Application of SNP genotyping on the genome of *B. anthracis* Sterne 34F2 led to the characterization of the nucleotide structure at 13 SNP loci, where T, A, G, C, A, T, T, A, T, G, G, T, and A were identified 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

1). As the standard SNP genotyping table of Van Erth's analysis revealed, Razi *B. anthracis* Sterne 34F2 belonged to the A.Br.001/002 subgroup (Van Ert et al., 2007). In VNTR-8 method, fragments with lengths of 314, 229, 162, 580, 532, 158, and 137 bp were identified at the following loci: vrrA, vrrB1, vrrB2, vrrC1, vrrC2, CG3, and pxO1, respectively (Table 2). Also, in Levy's MLVA genotyping method, three PCR products with lengths of 931, 451, and 864 bp were detected at AA03, AJ03, and AA07, respectively. Analysis of the nucleotide structure of AA03, AJ03, and AA07 displayed 3.7 copies of an 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).

DISCUSSION

B. anthracis 34F2 is an exotic laboratory strain in Iran, evolved from a South African ancestor, which is harmless to human and farm animals (Jula et al., 2011). As the SNPs genotyping method by Van Erth revealed, Razi *B. anthracis* 34F2 strain belonged to the A.Br.001/001 subgroup. This *B. anthracis* subgroup represents the lineage A of *B. anthracis* population, which was initially described by Keim and colleagues in 2000 (Keim et al., 2000). The Lineage A is the largest group of the three identified lineages (i.e., A, B, and C) in the global population of *B. anthracis*. Unlike less frequent isolates of B and C lineages, which are geographically more common in Africa and Asia, isolates from lineage A are spread across the world (Van Ert et al., 2007). Despite continuous research on the genetic population of *B. anthracis* isolates in Iran, circumstantial evidence supports the assumption that isolates from A.Br.001/001 subgroup are likely to be present in today's Iran, as traced in Southern Africa and Eastern Asia (Van Ert et al., 2007). In VNTR-8 genotyping method, Chinese and German substrains of *B. anthracis* Sterne 34F2 have been reported to carry VNTR genotypes different from the genotype of Razi substrain (Table 2). Since VNTR loci are classified as the most rapidly evolving genomic regions (Keim et al., 2000), it is not surprising that *B. anthracis* Sterne 34F2 substrains represent non-identical VNTR genotypes. Similarly, *Mycobacterium bovis* Calmette-Guérin (BCG) substrains from different manufacturing laboratories have been reported to contain different numbers of MLVA and/or *IS6110* copies (Behr and Small, 1999; Oettinger et al., 1999; Honda et al., 2006; Wang et al., 2012). In Levy's MLVA genotyping method, the nucleotide structure of AA03, AJ03, and AA07 loci did not vary among Razi Sterne 34F2 substrain and its whole genome-sequenced sister strains. However, the diversity index and differentiation power of these loci in indigenous Iranian *B. anthracis* isolates and the exotic Razi Sterne 34F2 substrain remain undetermined and require further analysis.

In conclusion, the present study described the genomic properties of Razi *B. anthracis* Sterne 34F2

Table 2. VNTR-MLVA analysis of the genomic structure of Razi *B. anthracis* Sterne 34F2 substrain in comparison with its Chinese and German sister substrains. NA= Not applicble

Strains	VNTR/MLVA PCR size (bp)										
	vrrA	vrrB1	vrrB2	vrrC1	vrrC2	CG3	pXO1	AA03	AJ03	AA07	References
B. anthracis Sterne 34F2 substrain (http://www.ncbi.nlm.nih.gov/genome/genomes/181)	314	229	162	580	532	158	137	941	851	864	-
B. anthracis Sterne 34F2 substrain (Razi substrain)	314	229	162	580	532	158	137	941	851	864	The present study
B. anthracis Sterne substrain (China)	313	229	162	583	532	158	129	NA	NA	NA	(Keim et al., 2000)
B. anthracis Sterne substrain (Germany)	313	229	162	583	532	158	129	NA	NA	NA	(Keim et al., 2000)

substrain. While SNPs analysis and MLVA findings presented the identical genomic structure of Razi substrain and its sister strains from other regions, Keim's VNTR-8 method reflected differences, which might have been induced by continuous lines of subcultures of these substrains at their maintaining laboratories.

Ethics

Hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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