TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2022) 46: 44-51

doi:10.3906/vet-2106-111

Molecular characterization of *Bacillus anthracis* isolates recovered from nomic and nonnomic hosts

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Received: 30.06.2021 • **Accepted/Published Online:** 26.11.2021 • **Final Version:** 23.02.2022

Abstract: Anthrax, which is primarily a disease of herbivores, is known to infect many animal species. In order to break the cycle of anthrax infection, it is important to identify the potential hosts or reservoirs of its causative agent. Therefore, this study aimed to characterize *Bacillus anthracis* isolates recovered from a variety of hosts and environments during anthrax sporadic in the Kars and Kayseri regions of Turkey. For this purpose, a total of 23 *B. anthracis* field isolates obtained from human, cattle, sheep, dog, horse, puma, soil, and fodder were used. In addition, the Sterne vaccine strain was included. All *B. anthracis* isolates were confirmed via molecular tests using plasmid-based PCR and 16S rRNA gene sequencing. All were found to be positive for protective antigen (PA) and capsule (Cap) genes. The presence of two specific 16S rRNA positions (1139 and 1148) with nucleotide mismatches was used to differentiate between *B. anthracis* species in the *Bacillus cereus* group, which has a relatively high (>99%) 16S rRNA sequence similarity. All of our isolates, including the Sterne vaccine strain, were classified in the same 16S rRNA type (Type 6) that has been reported previously as being the predominant type for *B. anthracis* isolates.

Key words: *Bacillus anthracis*, plasmid-based PCR, 16S rRNA sequencing, phylogeny

1. Introduction

Anthrax, caused by *Bacillus anthracis*, a Gram-positive spore-forming bacterium, is a sporadic disease of many warm-blooded animals such as dogs, cats, horses, and camels, and especially herbivores such as sheep and cattle [1]. There have been case reports of anthrax caused by feeding on the infected animal carcasses of Felidae such as lions, pumas, and leopards kept in zoos, and natural infections have also been observed in many amphibians, carnivores, poultry, and wild animal species [2,3]. The disease also causes various clinical presentations in humans by being transmitted through contact with infected animals, consumption of contaminated meat and meat products, and via the respiratory tract. Without treatment, anthrax acquired by inhalation is almost always fatal. In this regard, anthrax is a serious zoonotic disease [4].

Apart from its sporadic course, anthrax occasionally causes point outbreaks such as common source epidemics. The laboratory diagnosis of anthrax is generally carried out via isolation of the microorganism from tissue samples such as blood, spleen, and liver and identification by microscopic morphology, phage, and penicillin susceptibility [1,5]. PCR-based molecular methods provide more reliable results at the species level identification of *B. anthracis*. The genes responsible for the factors which mediate the virulence of *B. anthracis*, and which are located on two distinct plasmids (pXO1 and pXO2) are common targets used in the molecular diagnosis of anthrax [5,6]. 16S rRNA sequencing is more widely used in epidemiological studies. In addition, this method is used as a reliable method in taxonomic studies and for the identification of novel bacteria, as well as in the identification of *B. anthracis* from exceptional hosts [7–9].

Bacillus anthracis, which is placed in the *Bacillus cereus* group, has a very high level (>99) of DNA similarity with the other six species found in this group. Due to

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the limited availability of data in the early periods of sequencing analysis of the 16S rRNA gene and to this high degree of DNA homology, 16S rRNA typing was unable to differentiate this minimal diversity and identify closely related species. However, in particular, since the bioterrorism events that occurred in 2001 in the USA, a greater availability and accessibility of sequencing data has increased the importance of 16S rRNA sequencing as a diagnostic tool. Despite the relatively high level of DNA sequence similarity, 16S rRNA sequencing has provided precise species identification because of 13 nucleotide sequence differences. To date, almost all *B. anthracis* isolates have been included in the 16S rRNA Type 6 [7,9].

The current study aimed to characterize *B. anthracis* isolates obtained from animal, human, and environmental samples in the Kars and Kayseri regions of Turkey using plasmid-based PCR analysis and 16S rRNA sequencing.

2. Material and methods

Ethical approval of this study was obtained from the Kafkas University Animal Experiments Local Ethics Committee with an approval number of "2021/04".

2.1. *Bacillus anthracis* isolates

A total of 24 *B. anthracis* isolates were used in this study. Twenty-one of the *B. anthracis* isolates were obtained from anthrax cases that emerged in humans and animals in the Kars and Kayseri regions of Turkey. In addition, two isolates recovered from dead pumas in Kayseri Zoo [3] and the *B. anthracis* Sterne vaccine strain were included in the study. Detailed information about the *B. anthracis* isolates (n = 23) and the Sterne vaccine strain (n = 1) used in the current study is given in Table 1.

In the isolation of *B. anthracis* from clinical and environmental samples, a classic culture method was used, including inoculation of the samples onto 7% sheep blood agar (SBA) (CM0271, Thermo Fisher Sci., UK) and polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar (55678, Merck Millipore, Germany) followed by an incubation period at 37 °C for 24–48 h aerobically. Identification of the isolates grown was performed using phenotypic methods (microscopic and macroscopic morphology, penicillin (10 U, Oxoid, UK), and gamma phage (~10^9 PFU/mL) sensitivity) [5]. The puma isolates and the Sterne vaccine strain were resuscitated from the frozen stocks and plated on 7% sheep blood agar for fresh bacterial cultures.

2.2. DNA isolation

The genomic DNA extraction of 24 *B. anthracis* isolates was performed using a commercial extraction kit (56304, Qiagen, Germany) in accordance with the manufacturer's instructions.

2.3. Molecular characterization

2.3.1. PCR analysis of the protective antigen and capsule genes

Molecular characterization of the *B. anthracis* isolates was performed by PCR, which provides amplification of the protective antigen (PA) and capsule (Cap) genes [5]. Amplification of the PA gene was performed with primers PA5-5'-GAGGTAGAAGGATATACGGT-3' and PA8-5'-TCCTAACACTAACGAAGTCG-3' and of the Cap gene with Cap6-5'-TACTGACGAGGAGCAACCGA-3' and Cap103-5'-GGCTCAGTGTAACTCCTAAT-3', respectively. The PCR mix for each sample consisted of 25 µL of Taq PCR Master Mix Kit (Qiagen, UK), 5 µL of primer mix, 5 µL of template DNA, and 15 µL of deionized distilled water. Thermal cycling of PCR was set to 30 cycles consisting of initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s, extension at 72 °C for 40 s, and final elongation at 72 °C for 5 min. The amplified products were analysed in 1.5% horizontal gel electrophoresis (1640300, Bio-Rad, USA). The products of 596 bp and 1035 bp were evaluated as positive for PA and Cap genes, respectively. The *B. anthracis* Sterne strain ($pXO1^+$, $pXO2^-$) was used as the control microorganism in PCR.

2.3.2. 16S rRNA-based identification and phylogenetic analysis

The 16S rRNA gene was amplified and sequenced using the universal 27F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1492R (5'-ACGG(CT)TACCTTGTTACGACTT-3') primers [10]. The PCR reaction was generated for each sample in a volume of 50 µL with 25 µL of Taq PCR Master Mix Kit (Qiagen, UK), 5 µL of primer mix, 5 µL of template DNA, and 15 µL of dH_2O. Thermal cycling of PCR was set to 30 cycles consisting of initial denaturation at 95 °C for 5 min, followed by denaturation at 94 °C for 15 s, annealing at 59 °C for 30 s, extension at 72 °C for 45 s, and final elongation at 72 °C for 5 min. The amplified products were analysed in 1.5% horizontal gel electrophoresis, and products of 1465 bp for 16S rRNA gene were evaluated. The amplified products were sequenced using 27F and 1492R primers.

For the phylogenetic analysis, the chromatograms obtained were aligned and identified in the BLAST (Basic Local Alignment Search Tool) server database in the National Center for Biotechnology Information (NCBI). Base identification of chromatograms was also performed by visual inspection using Finch TV (version V1.4).

The genetic relationships between the 16S rRNA genes of 24 *B. anthracis* strains and representative strains from the *B. cereus* group (*Bacillus cereus* strain 2000031486 and *Bacillus thuringiensis* strain 2000031482) were inferred using the maximum likelihood method based on Kimura's two-parameter model with 1000 bootstrap using the MEGA X software (Version 10.2.2) [11-13].

Table 1. Phenotypic and molecular characteristics of *B. anthracis* isolates (n = 24) included in the study.

Isolate characteristics				Phenotypic characterization			Molecular characterization			
Isolate number	Host	Location	Year of recovery	Morphology	Susceptibility		PCR analyses		16S rRNA sequence type	NCBI Accession number
					Gamma phage	Penicillin	PA	Cap		
BA1	Puma	Kayseri	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144224
BA2	Dog	Kars	2013	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144225
BA3	Human	Kars	2013	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144226
BA4	Sheep	Kars	2015	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144227
BA5	Sheep	Kars	2015	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144228
BA6	Cattle	Kars	2016	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144229
BA7	Cattle	Kars	2016	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144230
BA8	Cattle	Kayseri	2007	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144231
BA9	Cattle	Kayseri	2007	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144232
BA10	Soil	Kars	2012	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144233
BA11	Soil	Kars	2012	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144234
BA12	Soil	Kars	2012	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144235
BA13	Soil	Kars	2012	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144236
BA14	Horse	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144237
BA15	Sheep	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144238
BA16	Sheep	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144239
BA17	Horse	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144240
BA18	Bull	Kars	2019	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144241
BA19	Bull	Kars	2019	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144242
BA20	Bull	Kars	2019	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144243
BA21	Fodder	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144244
BA22	Sheep	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144245
BA23	Puma	Kayseri	2018	Gr (+) bacilli, rough colony	+	+	+	+	Type 6	MZ144246
BA24	Sterne	Kars	2018	Gr (+) bacilli, rough colony	+	+	+	-	Type 6	MZ144247

Gr (+): Gram-positive, +: positive result, -: negative result

3. Results

3.1. Characteristics of *Bacillus anthracis* isolates

All 23 field isolates and the Sterne vaccine strain were confirmed as *B. anthracis* based on the results of phenotypic identification characteristics such as rough colony morphology on SBA without haemolyses, Gram-positive bacillus morphology on microscopy, and susceptibility to penicillin and gamma phage (Table 1).

3.2. Molecular characterization findings

3.2.1. Presence of protective antigen and capsule genes

In PCR, which provides amplification of the PA and Cap genes and thus enables molecular identification of *B. anthracis* isolates, all of the clinical and environmental isolates were found to be fully virulent bacteria with the presence of both plasmids, with a product size of 596 bp for PA and 1035 bp for Cap, respectively (Figures 1A and 1B, Table 1). The Cap-PCR negativity of the Sterne vaccine strain, which was the control bacterium of the study, was confirmed as expected since capsule formation does not occur in this strain due to the lack of the related gene on the pXO2 plasmid [14].

3.2.2. 16S rRNA-PCR analysis findings and subgrouping

PCR analysis performed with the universal primers (27F and 1492R) showed the successful amplification of the 16S rRNA gene region with the presence of 1465 bp bands in all 24 isolates (Figure 1C).

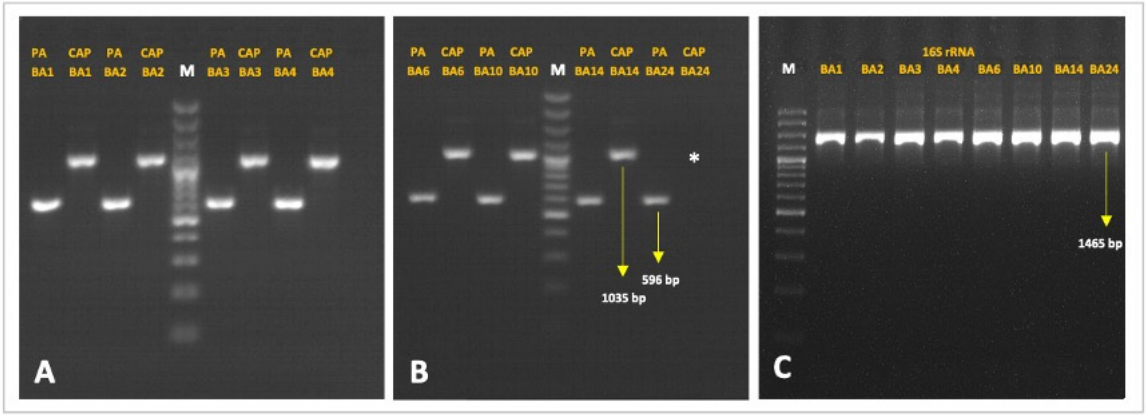

Figure 1. Agarose gel images of PCR products. **A, B:** The PA and Cap genes presence for *B. anthracis* isolates and Sterne vaccine strain. BA: *B. anthracis.* ***:** Negativity of the Cap region in the Sterne vaccine strain. **C:** The 16S rRNA-PCR results of *B. anthracis* isolates and Sterne vaccine strain. M: DNA marker GeneRuler 100 bp plus DNA ladder (SM0321, Thermo Fisher Sci., UK).

The 16S rRNA sequence results of the *B. anthracis* isolates were found to be similar to those of many *Bacillus* strains including *B. cereus* strain MD152, *Bacillus tropicus* strain ISP161A, *B. anthracis* strain FDAARGOS-695, *B. thuringiensis* strain FDAARGOS-791, *Bacillus paramycoides* strain K7.2, *Bacillus nitratireducens* strain SKC/L-2, and *Bacillus albus* strain ROA098, with a similarity value of 99.93%. The 16S rRNA gene sequences belonging to the 24 *Bacillus* strains isolates were deposited in the GenBank database under accession numbers from MZ144224-MZ144247 (Table 1).

When the chromatograms were examined visually, it was found that dual peaks representing very weak R (A or G) and W (T or A) were seen in positions at 1139 and 1148, respectively (Figure 2). As a result of the 16S rRNA gene sequence analysis, our isolates were classified as 16S Type 6. When the phylogenetic analysis results were examined, it was seen that all of our isolates were in the same genogroup (Figure 3B).

4. Discussion

Anthrax is an infectious disease, primarily of herbivores. In a 1945-printed manual titled "Contagious and Infectious Diseases of Animals" the order of susceptibility of animals is: sheep, cattle, camels, and horses [15]; thus, these animal species can be considered "nomic hosts" in terms of the disease ecology [1]. Anthrax in humans, with its zoonotic aspect, is a reflection of the animal infection and ultimately humans can be included in this host scope (red circles in Figure 3A). However, anthrax has been scarcely reported in omnivores, carnivores, and many other vertebrate animal species with different nutritional habits or niches. Anthrax can occur as common source epidemics especially in domesticated or captive carnivores kept in zoos, rehabilitation centres, and wildlife shelters [2]. In this context, both domestic and wild carnivores such as cats, dogs, lions, wolves, and pumas can be described as "nonnomic hosts" (blue circles in Figure 3A). Alongside the high pathogenicity of the bacterium, the diversity of its nomic and nonnomic hosts and the surviving capacity in environmental sources such as soil are crucial factors for the continuity of the infection chain [16].

Anthrax in sheep and cattle is an apoplectic infection with a peracute course. The disease is endemic in Turkey and has been widely reported in areas where sheep and cattle breeding are intense, especially in the Eastern Anatolia, Southeastern Anatolia, and Central Anatolia Regions[1]. Although three of the cattle isolates and three of the sheep isolates obtained from two independent point epidemics in the Kars region (Figure 3B, blue and green, respectively) were definitively diagnosed as *B. anthracis* by amplification of the plasmid-based virulence genes, the 16S rRNA sequence analyses of the isolates were found to be insufficient to distinguish these isolates. On the other hand, all cattle and sheep isolates showed a 16S rRNA Type 6 profile and this profile had a great similarity to results of previously reported studies [7,9].

Anthrax in horses which are considered to be a nomic host has an acute course and causes death within 2–3 days following colic, diarrhoea, and massive oedema [17,18]. Since anthrax rarely occurs in horses, it may have been overlooked in the past. However, reports of the disease have increased recently, both in cases affecting horses alone and in epidemics involving other livestock [18]. In this study, it was shown that the *B. anthracis* strains recovered from sheep, horses, and horse fodder in a point epidemic in 2018 in the Kars region (green in Figure 3B)

[1] Word Organisation for Animal Health (OIE) (2021). World Animal Health Information System, Annual Animal Health Report in 2021 [online]. Website: https://wahis.oie.int/#/dashboards/country-or-disease-dashboard [accessed 28 June 2021].

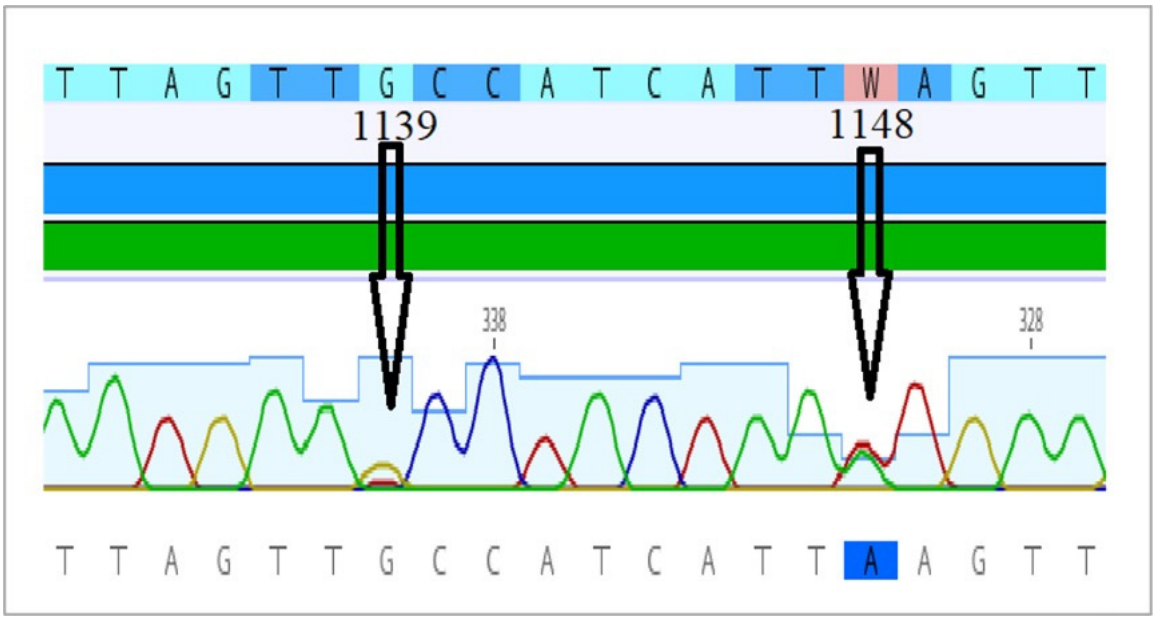

Figure 2. A representative chromatogram of the 16S rRNA gene sequencing. Since the *B. anthracis* genome includes more than one 16S rRNA gene, the *B. anthracis* isolates analysed in the current study had multiple peaks in single nucleotide positions.

exhibited a common 16S rRNA Type 6 profile (Table 1). This result revealed that the strains originated from the same ancestor (Figure 3B). Similarly, it has been reported that *B. anthracis* with the same genotype has been isolated from different hosts and sources [19,20].

Human anthrax cases are still being reported in Turkey, albeit at decreasing levels [4,21]. 16S rRNA sequence typing has been used for the typing of *B. anthracis* isolates obtained from different origins (animal, animal products, soil, and human), and all human isolates have been included in the Type 6 profile [7]. Similarly, in our study isolate BA3, recovered from a female patient with cutaneous anthrax in the Kars region in 2013, exhibited a 16S rRNA Type 6 profile (Table 1).

Dogs are more resistant to anthrax than other primary hosts. In many developing countries, the carcasses of animals suspected of anthrax are consumed, albeit reluctantly, by humans because of economic imperatives, and the offal of these animals are used to feed dogs [1,22]. The infection, which occurs as a result of heavy consumption of the offal of infected animals, ends in death in dogs following hypersalivation, massive oedema, toxaemia, and shock [23]. In this study, isolate BA2, obtained from a dog died after consumption of an infected cattle carcass in 2013, was found to have a 16S rRNA Type 6 profile. To the authors knowledge, it is the first reported genotype from the dog (Table 1).

Pumas, which are scavengers, are more resistant to anthrax due to their constant exposure to the bacterium through consuming infected animal carcasses and the immunity they acquire in this way [1,24]. Therefore, fatal infections are less reported in pumas in the wild [25]. Deaths associated with anthrax in these species have been reported as a result of the inadvertent feeding of animals with the meat and offal of infected animals, especially in areas such as zoos and shelters [2,3]. The number of such cases has steadily increased following the first report [26] in 1911 of anthrax that emerged as a result of consumption of infected meat in wild animals in captivity [2,3]. In this study, the two pumas from which the isolates BA1 and BA23 were taken, had been kept in the same cage and fed the same food at Kayseri Zoo before dying suddenly [3] (orange in Figure 3B). After the partial sequence analyses of the 16S rRNA gene, the puma isolates were found to have a Type 6 profile, similar to the profiles of other *B. anthracis* isolates in the study (Table 1).

Environmental samples such as soil are important sources of infection. The vegetative bacilli, spread from dead animal carcasses, can sporulate in the rhizosphere of the soil in a short time and turns into long-lasting viable forms. These spore-laden areas are called "hot spot" areas for new contagions [1] and contain "incubator areas" which lead to *B. anthracis* vegetative expansion prior to the sporulation necessary for outbreaks (Figure 3A) [27]. Animals can acquire the spores from these areas during grazing. Such incubator areas may pose a risk of human infection, as well [28]. In our study, isolates BA10, BA11, BA12, and BA13, recovered from different areas

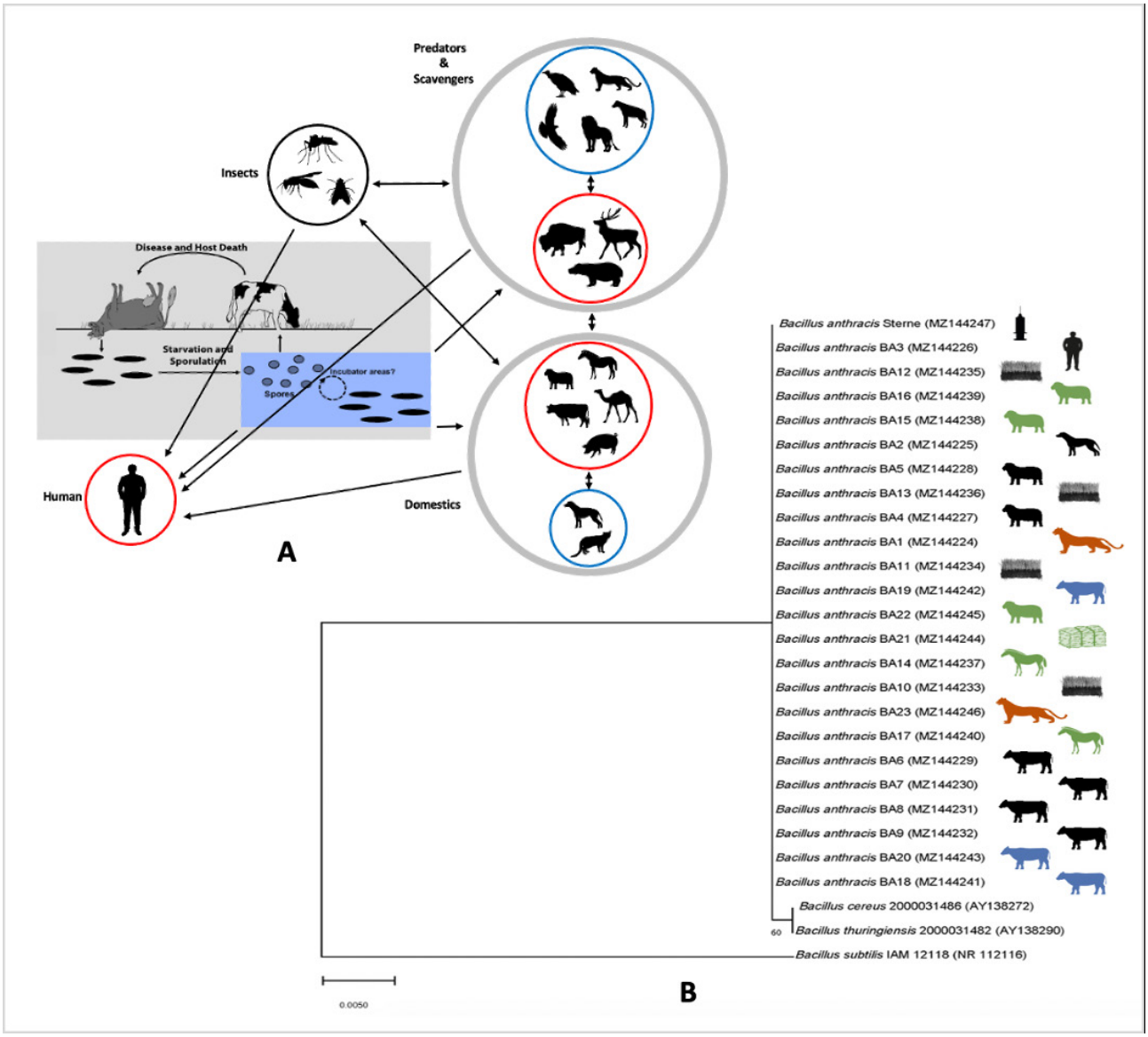

Figure 3. A: The lifecycle of *B. anthracis* in the ecosystem [1,16,27,31]. Red circles: Nomic hosts. Blue circles: Nonnomic hosts. Black circle: Insects. Cornflower blue: "Hot spot" areas which lead to new contagions. **B**: The phylogenetic tree of our *B. anthracis* isolates and the representative strains from the *B. cereus* group based on the 16S rRNA gene sequence. GenBank accession numbers for the 16S rRNA gene sequences are shown in parentheses. *Bacillus subtilis* IAM 12118 was used as an outgroup. Bar: 0.005 substitutions per nucleotide position.

contaminated with *B. anthracis* from infected animal carcasses in the Kars region in 2012, showed a uniform 16S rRNA Type 6 profile. In addition, an environmental isolate BA21 (from horse fodder) was from a point epidemic in the Kars region in 2018, in which 3 sheep and 2 horses died (green in Figure 3B), and 16S rRNA sequence type of BA21 was the same as those of the other isolates of this epidemic (Table 1). Despite the variability in location, year of recovery and materials sampled, a common genotype was detected in the environmental isolates.

The 16S rRNA gene in prokaryotic ribosome is a highly conserved region that provides valuable results in the identification and phylogenetic analysis of many bacteria [29]. The 16S rRNA sequence analysis results of our isolates were found to be similar to those of many *Bacillus* strains examined. As in previous reports, exact identification of our strains could not be performed [7,9,30] due to the relatively high level of similarity (>99%) in the 16S rRNA gene sequences of the *B. cereus* group. However, dual peaks on the chromatogram (Figure 2) in two specific 16S rRNA

positions (1139 and 1148) with nucleotide mismatches were used to identify *B. anthracis* when the consensus 16S rRNA gene sequence did not qualify for differentiation of the closely related *B. cereus* sensu lato strains. Nonetheless, 16S rRNA sequencing contributed to exact identification of the *B. anthracis* isolates that were already characterized by the plasmid-based PCR.

Despite the high degree of DNA homology of the species in the *B. cereus* group, different 16S rRNA sequence profiles specific to the members of this group have been reported. Sacchi et al. [7] reported a total of 11 genotypes including two (type 6 and 7) for *B. anthracis*, seven (type 2, 3, 5, 7, 9, 12 and 13) for *B. cereus*, two (type 4 and 10) for *B. thuringiensis*, and one (type 1) for *Bacillus mycoides.* Also, 28 types of 16S rRNA were reported from the *B. cereus* group in a subsequent study [9]. In the current study, based on the 16S rRNA gene sequence, our isolates were classified as 16S rRNA Type 6. When the phylogenetic analysis results were examined, it was seen that all of our isolates were in the same genogroup (Figure 3B). As reported previously, the capacity of phylogenetic analysis using the 16S rRNA sequence to discriminate among the members of the *B. cereus* group was very low [7,9].

In conclusion, plasmid-based PCR was found to be suitable for the confirmation of *B. anthracis* isolates that had been obtained from various sources and identified phenotypically. The 16S rRNA sequencing revealed that all of our isolates had a Type 6 profile, previously reported as the predominant type. However, the discriminatory power of the 16S rRNA-based phylogenetic analysis was not sufficient to differentiate between isolates from different geographies or to trace a common ancestor in point epidemics caused by *B. anthracis*. It is thought that complementary tests, such as multiple locus variable-number tandem repeat analysis (MLVA) or whole genome analysis, should be performed when further discrimination of the *B. anthracis* isolates is needed.

Acknowledgments
We are thankful for Prof. Dr. Atila Akça and Mrs. Catherine Akça (Kafkas University, Turkey) for their contributions on proofreading of the paper.

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