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Research Article

The Clues of the Discovery of New Pathogens Eventuated by the Horizontal Gene Transfer Among the *Bacillus cereus* Group Members

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Abstract: Genes, which constitute the biological basis of heredity, are DNA segments that give various structural and functional properties to the organism they are found in. Genes have become differentiated and highly conserved among microorganisms that have completed their evolutionary development and completely distanced from each other. However, horizontal gene transfers (HGT) can occur between microorganisms even though they have high structural similarities in the environment, especially in soil. An example, HGT can occur between the members of the *Bacillus cereus* group, and thus other species can develop into new life-threatening pathotypes by taking some virulence structures of the most pathogenic species-*Bacillus anthracis*. This study aimed to investigate some virulence genes (protective antigen-PA and capsule-cap) of *B. anthracis* in *B. cereus* group members isolated from soil, which is known as the reservoir of this group of bacteria and can easily trigger the genetic material exchange. The study material has consisted of 15 soil samples taken from 5 different locations in the Kars region (Turkey) contaminated with cattle carcasses that died with suspected anthrax. *B. cereus* group bacteria were obtained from the soil by *in vitro* cultural method, and the agent identification was performed by phenotypic and molecular methods (PCR). As a result, a total of 1350 *Bacillus* species was identified from the soil, including 123 *B. anthracis*, 303 *B. mycoides*, 348 B. thuringiensis and 576 *B. cereus*. DNAs obtained from all isolates other than *B. anthracis* were pooled at a final concentration of 4 ng/mL and as an average of 10 extracts and analyzed by PCR for the relevant virulence genes (PA and cap). While the PA gene was found in 6 (4.88%) of 123 DNA pools, the cap gene was not detected in any of the pooled DNA. All *B. anthracis* isolates were evaluated as fully virulent strains as they did not lose the PA and cap genes. The DNA pooling method has been an obstacle to the selection of gene positivity individually, however, it roughly offered some clues indicating the HGT. Thus, further studies with sampling and methodological diversities are needed that will enable the proof of HGT, which mediates the emergence of new pathotypes among the *B. cereus* group members.

Keywords: *Bacillus cereus group, Bacillus anthracis, Horizontal gene transfer, PCR*

Bacillus cereus Grubu Üyeleri Arasında Yatay Gen Transferi İle Ortaya Çıkan Yeni Patojenlerin Keşfine İlişkin İpuçları

Öz: Kalıtımın biyolojik temelini oluşturan genler, bulundukları organizmaya çeşitli yapısal ve işlevsel özellikler kazandıran DNA parçalarıdır. Evrimsel gelişimini tamamlamış ve birbirinden tamamen uzaklaşmış mikroorganizmalar arasında genler farklılaşmış olup yüksek oranda korunaklıdır. Ancak çevrede, özellikle de toprakta yüksek yapısal benzerliklere sahip olsalar bile mikroorganizmalar arasında horizontal gen transferleri (HGT) meydana gelebilmektedir. Bunun bir örneği, *Bacillus cereus* grubunun üyeleri arasında meydana gelebilmekte ve bu nedenle diğer türler, grubun en patojen tür olan *Bacillus anthracis*'in bazı virülans yapılarını HGT ile alarak yaşamı tehdit eden yeni patotiplere dönüşebilmektedir. Bu çalışmada, bu bakteri grubunun rezervuarı olarak bilinen ve genetik materyal değişimini kolaylıkla tetikleyebilen topraktan izole edilen *B. cereus* grubu üyeleri arasında *B. anthracis*'in bazı virülans genlerinin (koruyucu antijen-PA ve kapsül-cap) araştırılması amaçlandı. Çalışmanın materyalini, Kars yöresinde şarbon şüphesiyle ölen sığır karkaslarıyla kontamine olmuş 5 farklı lokasyondan alınan 15 toprak örneği oluşturdu. *B. cereus* grubu bakteriler topraktan eldesi *in vitro* kültür yöntemiyle gerçekleştirildi ve etken tanımlaması fenotipik ve moleküler yöntemlerle (PCR) yapıldı. Çalışma sonucunda topraktan 123 *B. anthracis*, 303 *B. mycoides*, 348 B. thuringiensis ve 576 *B. cereus* olmak üzere toplam 1350 *Bacillus* türü tanımlandı. *B. anthracis* haricindeki tüm izolatlardan elde edilen DNA'lar, son konsantrasyonda 4 ng/mL ve ortalama 10 ekstrakt olacak şekilde havuzlandı ve ilgili virülans genleri (PA ve cap) yönünden PCR ile analiz edildi. Havuzlanan 123 DNA'nın 6 (%4.88)'sında PA geni saptanırken, DNA havuzlarının tümü cap geni yönünden negatifti. PA ve cap genlerini kaybetmemiş oluşuyla *B. anthracis* izolatlarının tümü tam virülent suşlar olarak değerlendirildi. DNA havuzlama yöntemi bireysel gen pozitifliğinin seçimine engel teşkil ederken, HGT'yi kabaca gösterecek bazı ipuçları sundu. Bu nedenle, *B. cereus* grup üyeleri arasında yeni patotiplerin ortaya çıkmasına aracılık eden HGT'nin kanıtlanmasını sağlayacak örneklem ve metodolojik çeşitlilik içeren ileri çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Bacillus cereus grup, Bacillus anthracis, Horizontal gen transfer, PCR*

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Introduction

Horizontal gene transfer (HGT) is known as the process of transferring part or complete DNA from a donor to a recipient bacterium or host genome [1]. Gene transfer occurs more easily between bacteria or DNA sequences that are very close to each other or between different microorganisms with high structural similarity. Genetic material transfer can occur in natural conditions, especially in environments such as soil, and very rarely (10^{-7} - 10^{-9}), as well as through artificial techniques such as transfection, microinjection and vectorial processes, and the frequency of transmission can thus be increased (10^{-3} - 10^{-4}) [2]. As a result of HGT, the recipient bacterium turns positive in terms of the features encoded by the genes transferred by the donor bacterium, and thus new phenotypes and pathotypes can emerge. HGT can occur by three different mechanisms (transformation, conjugation and transduction) under normal conditions among the bacteria. HGT is more like the transfer of circular plasmids that can replicate independently of a pathogen's main genetic material (chromosomal DNA) [1,2].

Bacillaceae is a Gram-positive, aerobic and endospore-forming bacterial family commonly found in soil with approximately 94 different species. The family divides into 6 large groups according to their physiological, biochemical and morphological characteristics, and pathogenic species are found in the *Bacillus cereus* group. In this group, apart from *Bacillus anthracis*, there are some species such as *Bacillus cereus, Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* [3]. Members of the *B. cereus* group have a significant impact on human and animal health, agriculture and the food industry [4]. In addition, various enzymes and metabolites secreted by the *B. cereus* group can exhibit probiotic properties and are used in the destruction of environmental pollutants [5,6].

Bacillus anthracis, which is the agent of anthrax, differs from other species of the *B. cereus* group with its unique phenotypic (non-motility, presence of capsule, Gamma phage and penicillin susceptibility) and genotypic (presence of pXO1 and pXO2 plasmid) features [7]. However, this difference has recently come into question with the discovery of *B. cereus* and *B. thuringiensis* isolates that have genetic structures similar to the *B. anthracis* plasmids. Many of these mutant bacteria, called "*B. anthracis*-like organisms", exhibit potential or lethal pathogenic features and may lead to clinical pictures that do not comply with the defined classical anthrax [8-13]. Another risk of these mutant strains, most of which are detected from environmental sources such as soil, is that they have the potential to be used as biological agents instead of *B. anthracis*.

Soil is the main source of exposure to anthrax spores, for which the tolerable level in humans and many susceptible hosts is not yet known [14]. Considering that a cattle dying from anthrax sheds approximately 10^{12} spores [15], soil containing spore-laden untreated animal burials represents a significant accumulation of *B. anthracis* genetic material as well as being a serious source of infection. Genetic material transfer is much higher among the members of the *B. cereus* group compared to that in natural conditions (10^{-7} - 10^{-9}). Soil is seen as a favorite environment for the transfer of *B. anthracis* specific genetic structures with HGT alternatives and thus the evolution of *B. cereus* group bacteria and the emergence of new phenotypes [16].

This study aimed to investigate *B. anthracis* specific virulence genes (protective antigen-*PA*, capsule-*cap)* by PCR in *B. cereus* group bacteria isolated from soil, which is a reservoir of these bacteria and highly promotes the horizontal gene transfer.

Material and Methods

Study Material

Soil contaminated with *B. anthracis* spores was used as study material. For this purpose, soils were taken from animal burials where cattle that died with suspected anthrax, soil ground areas contaminated with blood and body fluids, and restricted open pasture areas where their carcasses were left were sampled in Kars Province of Turkey. Totally, 500 g soil was taken from the upper 20 cm section (rhizosphere) of a 4 m^2 width area representing the spore contamination. These samples were used for the isolation of both *B. anthracis* and other members of the *B. cereus* group in order to demonstrate reciprocal gene transfer.

Bacterial Isolation

The isolation of *B. anthracis* and other *B. cereus* group bacteria from soil samples was carried out by modifying the method applied by the World Health Organization [17]. In this method, 40 g of soil sample was diluted with 200 mL of sterile distilled water in plastic cups, and left for 30 min at room temperature to obtain the supernatant by shaking gently for about 30 s. Then, 1 mL of supernatant was taken and heated in a water bath at 62.5-63°C for 15-20 min. Ten-fold serial dilutions of the heat-treated samples were prepared with distilled water, and 150 µL of the dilutions were plated onto 7% sheep blood agar (SBA) plates. Bacterial colonies formed after 24-48 h of incubation at 37°C in an aerobic environment were evaluated for specific agents, and the number of uniform colonies specific for each bacterium was noted for use in future spore and frequency calculations.

Phenotypic Identification

Non-hemolytic, 2-4 mm in diameter, white or grayish-white, medusa-head rough colonies on the SBA plates

were evaluated for *B. anthracis*. Techniques such as penicillin and Gamma phage sensitivity (sensitive to both), capsule presence/synthesis ability (azure-eosin staining and mucoid growth in bicarbonate agar medium), motility examination (nonmotile in semi-solid medium) were applied for identification of the suspected colonies [18,19]. In the identification of *B. cereus* and *B. thuringiensis*, phenotypic test features such as hemolysis on the SBA plates, resistance to penicillin and Gamma phage, lack of capsule and presence of motility were taken into account. The presence of insecticidal crystals formed during sporulation of *B. thuringiensis* was also investigated [20]. For this purpose, smears were prepared after incubation of *B. thuringiensis* suspected isolates in spore media at 37°C for 48 h. Smears stained with malachite green were analyzed by phase-contrast microscopy for the presence of parasporal crystal structure next to the bacterial spores. The identification of *B. mycoides* included typical rhizoid or hair-like colony production, as well as being immobile and encapsulated, and penicillin and Gamma phage resistance [21].

Molecular Identification

DNA extraction from *Bacillus* isolates was performed by boiling method. For this purpose, 1-2 copy of freshly cultured bacterial colonies were taken and diluted in 100 µL nuclease free water. The colonies were kept in the block heater at 95°C for 10 min, and then heat shock was applied by keeping them on ice blocks for 10 min. Supernatant, obtained by centrifugation at 7500 rpm for 10 min, was checked for sterility (no growth was observed after 48 h of incubation of 5 µL DNA plated on the SBA plates). For the confirmation of phenotypically identified *B. anthracis* isolates, PCR techniques with a couple of primer pairs, PA5/8 and CAP6/103, were used [18,19]. The PA5/8 primer pair targets the amplification of the *pag* gene which is originated from the pXO1 plasmid and encodes the protective antigen (PA). The CAP6/103 primer pair targets the amplification of the *capB* gene, which is one of the genes that originated from the pXO2 plasmid and encodes the capsule. *B. anthracis* vaccine strain (Sterne) containing *pag* gene and not *capB* gene was used as a control in both PCR. PCRs targeting the amplification of *gyr* gene were applied to identify *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates [22]. All PCRs were performed with the components and thermal cycles stated in *Table 1*.

PCR Analysis of the Obtained Isolates in Terms of the *Bacillus anthracis* Virulence Genes

Bacillus anthracis specific plasmid and virulence genes were investigated with the primer PA5/8 and Cap6/103 in *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates that were identified by phenotypic and molecular methods [18,19]. DNA extraction from each isolate was carried out separately by boiling method. After the DNA extraction, in order to reduce the heavy workload that will be encountered during the PCR processes, the "pooling method" was applied to the DNA extracts [23]. In this context, the concentrations of individual DNA extracts were measured with Microplate Spectrophotometer (BioTek, Take3 Mikro-Volume-Plate, SN243903). The concentration of the extracts was adjusted to 40-80 ng/µL with nuclease-free water. Then, 2 µL of each DNA extract was taken, with a final concentration of 4 ng/µL, and 123 DNA mixes were created in a total volume of 20 µL consisting of an average of 10 different DNA samples. While creating the DNA pools, care was taken to bring together the isolates in the same SBA plates. The individual DNAs of the isolates were stored in separate tubes at -20°C to be used in further extractions. PCR components and thermal cycle were created as in *Table 1*. Analysis of the PCR products was performed in horizontal gel electrophoresis containing 1.5% agarose.

Table 1. *Primers and PCR characteristics used for identification of the Bacillus cereus group members and virulence genes*

Species	Primer	Sequence (5'—3')	Target	PCR Component	Thermal Cycle	Amplicon (bp)	Reference
B. anthracis	PA8 PA5	GAGGTAGAAGGATATACGGT TCCTAACACTAACGAAGTCG	pXO1	• 2.5 µL PCR buffer (x10, with $MgCl_2$) • 0.5 µL dNTP (10 mM) • 1 µL primer F (10 pmol) • 1 µL primer R (10 pmol) • 0.5 µL Taq polymerase (5 U) • 2.5 µL template DNA (40-80 ng/µL) • Nuclease free water	**One cycle** • Denaturation at 94°C for 2 min **Thirty cycles** • Denaturation at 94°C for 1 min • Annealing at 55°C for 1 min • Elongation at 72°C for 1 min **One cycle** • Final elongation at 72°C for 5 min	596	[18,19]
	CAP6 CAP103	TACTGACGAGGAGCAACCGA GGCTCAGTGTAACTCCTAAT	pXO2			1035	
B. thuringiensis	BTJH-IF BTJH-R	GCTTACCAGGGAAATTGGCAG ATCAACGTCGGCGTCGG	*gyrB*		**One cycle** • Denaturation at 94°C for 5 min **Thirty cycles** • Denaturation at 94°C for 30 sec • Annealing at 63°C for 30 sec • Elongation at 72°C for 30 sec **One cycle** • Final elongation at 72°C for 5 min	299	[22]
B. cereus	BCJH-F BCJH-IR	TCATGAAGAGCCTGTGTACG CGACGTGTCAATTCACGCGC	gyrB			475	
B. mycoides	BMSH-F BMSH-R	TTTTAAGACTGCTCTAACACGTGTAAT TTCAATAGCAAAATCCCCACCAAT	gyrB			604	

Results

Isolation and Identification Findings of the *Bacillus cereus* Group Bacteria

In the study, a total of 15 soil samples were taken from 5 different locations in the Kars region contaminated with dead cattle carcasses with suspected anthrax. A total of 123 *B. anthracis* isolates were obtained from the soil samples, one representative of each SBA plate, after cultural examination. *B. anthracis* load in the soil samples ranged from $1.01x10^2$ to $1.24x10^8$ spores/gram *(Table 2)*. The organic matter contents of the soil ranged from 1.5% to 5.7%, and thus they were ranked as loamy sandy soil and clayey soil.

In order to detect possible horizontal gene transfer, all SBA plates showing *B. anthracis* colony morphology following the cultural analysis were evaluated. Isolate collections were created by collecting all of the colonies of individual *Bacillus* species on the SBA plates. The distribution of the collected isolates was as follows; 924 *B. cereus* and/or *B. thuringiensis* and 303 *B. mycoides* *(Table 2)*.

Molecular Identification Findings

All 123 *B. anthracis* isolates phenotypically identified were confirmed by PCR with the presence of the *PA* and *cap* genes *(Fig. 1-B)*. Therefore, with the presence of these genes, which are critical for pathogenicity, all isolates were found to be fully virulent *B. anthracis* (without gene loss). As a result of PCR analysis of 924 isolates characterized phenotypically as *B. cereus* and/or *B. thuringiensis*, 567 were identified as *B. cereus* and 348 as *B. thuringiensis*. All 303 *B. mycoides* isolates phenotypically characterized were confirmed as *B. mycoides* by PCR *(Table 3)*.

PCR Findings of the *Bacillus* Isolates in Terms of the *Bacillus anthracis* Virulence Genes

As a result of PCR analysis with the PA5/8 primer of 123 DNA pools created from different *Bacillus* isolates, *B. anthracis* specific *PA* gene was found in 6 (4.88%) of the pools *(Fig. 1-A)*. The *PA* gene positive isolate combinations were as follows: *B. cereus* alone in 21 samples, *B. cereus* and *B. thuringiensis* in 28 samples, *B. cereus*, *B. mycoides* and *B. thuringiensis* in 10 samples. In the present study, in which *B. cereus* was detected to be significantly higher ($P<0.05$), the total distribution of these *PA* gene positive isolates was as follows; 39 *B. cereus*, 19 *B. thuringiensis* and 1 *B. mycoides*. As a result of PCR analysis with the Cap6/103, no *cap* gene was found in any of the DNA pools *(Table 4)*. Therefore, no *B. anthracis* specific *cap* gene transfer was observed among the *B. cereus*, *B. thuringiensis* and *B. mycoides* isolates. On the other hand, due to the presence of the *PA* and *cap* gene regions in all isolates, no simultaneous loss of related genes was observed in the *B. anthracis* isolates.

PCR was performed separately with the PA5/8 primers on all individual bacterial DNAs constituting each of

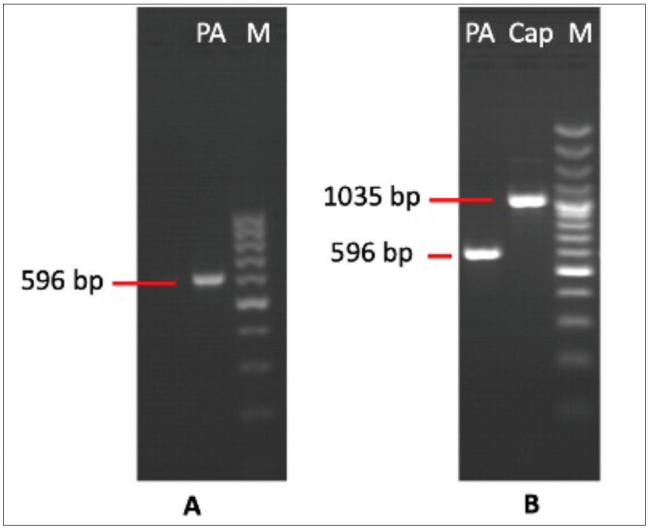

Fig 1. Gel electrophoresis image of amplified products of the PCRs. **A:** 596 bp size amplified product of the *PA* gene region detected in DNA pools from *Bacillus* isolates *(B. cereus*, *B. thuringiensis* and *B. mycoides)*, **B:** Amplified products of the *PA* (596 bp) and *cap* (1035 bp) genes of fully virulent *B. anthracis* isolates. PA: Protective antigen specific PCR, Cap: Capsule specific PCR, M: Marker: (DNA Marker 100bp Ladder, MBS657623, MyBioSource for *Fig. 1-A* and GeneRuler 100 bp Plus DNA ladder, SM0321, Thermo Fisher Sci for *Fig. 1-B*)

Table 2. *Sample locations and culture results of the soil in terms of the Bacillus cereus group bacteria*

Location	Contamination Date	Contaminant Type	*B. anthracis* Spore Load (spore/soil per gram)	Number of Soil Samples	Culture Results				
					Number of Isolates	Distribution of Isolates*			
						B. anthracis	*B. cereus*	*B. mycoides*	*B. thuringiensis*
Digor-Hanevler District	2009	Cattle	$1.01x10^2$	2	180	15	75	45	45
Selim-Center	2002	Cattle	$1.32x10^3$	4	360	30	155	80	95
Dikme Village	2010	Cattle	$1.24x10^8$	4	360	32	150	81	90
Çamurlu Village	2011	Cattle	$3.03x10^3$	2	180	20	76	39	46
Külveren Village	2011	Cattle	$3.46x10^4$	3	270	26	120	58	72
Total				15	1350	123	576	303	348

** This distribution was formed after the phenotypic analysis findings and the identification of the bacterial species was confirmed by PCR*

Table 3. *Phenotypic and molecular identification test findings of the Bacillus isolates*

Species	Phenotypic Identification Tests						PCR
	Colony Type on SBA	Hemolysis on SBA	Motility	Capsule	P	γ	
B. anthracis (n: 123)	Rough	Non-hemolytic	Nonmotile	+	+	+	*B. anthracis* (n: 123)
B. cereus and *B. thuringiensis* (n: 924)	Rough	*β- hemolytic*	Nonmotile	-	-	-	*B. cereus* (n: 576) *B. thuringiensis* (n: 348)
B. mycoides (n: 303)	Rhizoid	*β*- hemolytic	Nonmotile	-	-	-	*B. mycoides* (n: 303)

SBA: *%7 sheep blood agar,* ***P:*** *Penicillin sensitivity,* ***γ:*** *Gamma phage sensitivity*

Table 4. *DNA samples that were detected positive in terms of the presence of the Bacillus anthracis plasmid and virulence genes*

Number of DNA Pool	Number of Isolates			PCR Findings		P-value
	B. cereus	*B. mycoides*	*B. thuringiensis*	PA5/8	Cap6/103	
Pool 4	3	-	5	Positive	Negative	P<0.05
Pool 9	11	-	-	Positive	Negative	
Pool 11	3	-	7	Positive	Negative	
Pool 12	10	-	-	Positive	Negative	
Pool 26	3	1	6	Positive	Negative	
Pool 28	9	-	1	Positive	Negative	
Total	39	1	19			

the 6 DNA pools in which the *PA* gene was detected. However, all individual DNAs were found to be negative for the presence of the *PA* gene.

Discussion

Toxins [a cell-binding protein known as protective antigen (PA), enzymes known as edema factor (EF) and lethal factor (LF)] and capsule are the main virulence factors in the pathogenesis of *B. anthracis* [24]. The genetic materials originating from these virulence factors are plasmids pXO1 and pXO2, respectively. Although the horizontal transfer of these plasmids is greater in clinical specimens [9,12,25], few have been reported in environmental isolates [26,27]. Hu et al.[26] found the horizontal transfer rate of the pXO1 and pXO2 plasmids to be 6.6% and 7.7%, respectively. Simultaneous transfer of both plasmids among the environmental isolates was determined at a rate of 0.46% [26]. Compared to this [26], Cooper et al.[27] reported a lower rate (1.75%) of pXO2 loss among the *B. anthracis* isolates obtained from soil, but on the other hand, no gene gain was reported among the *B. cereus* group members. In many studies, compared to the pXO1 plasmid, the loss of the pXO2 plasmid was higher due to its sensitivity to the environment or fragility to the laboratory storage [18,26-31].

In the trace of *B. anthracis* plasmids, the PA gene, which provides the integration of the pXO1 plasmid originated toxins (EF and LF) into the host cell, and the pXO2 originated *cap* genes confer the antiphagocytic ability to the bacteria, are frequently investigated. For this purpose, primer pair PA5/8 for the amplification of *PA* and primer pair Cap6/103 for the amplification of the region between *cap B* and *cap C* gene, are widely used [18,27,32]. In this study, *PA* gene positivity was obtained in 6 (4.88%) of a total of 123 DNA pools, and these findings are quite similar to the rates found in *B. cereus* group isolates obtained from the environmental sources by Hu et al.[26]. However, since the *PA* gene positivity was detected only in DNA pools and not in the individual DNAs in the present study, the bacterial origin of the HGT and the *PA* gene prevalence specific to bacterial species could not be determined. Nevertheless, *B. cereus* and *B. thuringiensis* appear to be *Bacillus* species with the easier horizontal gene transfer capability (P<0.05). On the other hand, the absence of loss of these genes in *B. anthracis* isolates obtained from the same SBA plates containing *PA* gene positive isolates was interpreted as the presence of more than one copy of the relevant genes in donor bacteria *(B. anthracis)* [33] and as the absence of simultaneous gene exchange among the *B. cereus* group members.

The plasmid pXO2, which is smaller (96.2 kb) in size than pXO1 [34], is more common in transferability especially among the environmental isolates obtained from the soil [18,26,27]. High organic matter and exudate requirement for the conjugation, rhizosphere repellency for the transformation, and the presence of the specific phages for the transduction are the main factors affecting the gene acquisition pathways in the soil [35,36]. On the other hand, plasmid losses mostly depend on the soil type which differs according to matter, sand, silt and clay concentration [35-37].

As a matter of fact, Salgado et al.[37] obtained a *B. anthracis* strain with the pXO2 plasmid loss from a clay loam soil, while a *B. anthracis*-like organism with the pXO1 plasmid gain was obtained from a loamy sand soil. Contrary to the reports, none of the isolates were found positive for this plasmid in this study, even though the same gene losses have been found previously among the *B. anthracis* isolates obtained from the soil with divergent (1.5-5.7%) organic matter contents in the same study area [18,27]. It is still more likely that the pXO2 plasmid transfer may have been adversely affected this time by the factors such as the nutritional, biotic and abiotic structures of the soil which are assumed favorable for the HGT due to the substantial biosynthetic and energetic requirements of the conjugation, DNA uptake and lytic cycle. Furthermore, there is a biosynthetic gene operon *(capBCAD)* on the related plasmid that contains many genes encoding the capsule [34,38], and screening of only the *capB* and *capC* genes in this study may have been insufficient as an indicator of the HGT.

In large-scale studies, the "DNA pooling method" gives promising results in terms of reducing the workload and cost and adding practicality to the study [23]. It has even been reported that the "pooling method" is more useful in detecting gene alleles with low frequency [39,40]. In the "DNA pooling" process, the DNA concentration used, the methodology (double pooling, multiple pooling, etc.) and the order of the application (pooling before DNA extraction, pooling before PCR amplification, etc.) change the effectiveness of the method [23]. In addition to these advantages of the "DNA pooling method", there are also some disadvantages that reduce sensitivity and minimum detection limit of the method [41,42]. In this study, individual DNA extracts of average 10 isolates *(B. cereus, B. thuringiensis* and *B. mycoides)* were combined with "multiple-pooling" with a final concentration of 4 ng/µL and this process was done just before the PCR amplification [23]. One of the reasons for the PA gene positivity detected in pooled DNAs could not be detected in individual DNAs is thought to be the variational conditions listed above for the "pooling method". In addition, the use of DNA mixes of different isolates *(B. cereus*, *B. thuringiensis* and *B. mycoides)* taken simultaneously and randomly representing the same SBA plate may have caused the DNAs to act as complements to each other during the PCR amplification among the bacteria of the *B. cereus* group with very high DNA homology (>99%), and may have ultimately led to mismatch amplifications [43].

In conclusion, spore-contaminated soil representing the significant accumulation of the genetic material of *B. anthracis* appear to be favorite environments for transfer of plasmid or various virulence genes via HGT. As a matter of fact, in this study, which included PCR-based analytic methods, *B. anthracis* specific *PA* gene positivity was found to be 4.88% among the soil-borne *B. cereus* group bacteria *(B. cereus, B. thuringiensis* and *B. mycoides),* but this positivity did not be differentiated on an individual basis. Except for the *B. anthracis*, no horizontally transferred *cap* gene was found among the other *B. cereus* group members. By increasing the diversity of samples and methods that will enable the HGT evidence, such transfers of genetic material, which are seen as possible among the *B. cereus* group members and may mediate the emergence of new pathogens, will be able to detect more comprehensively.

Availability of Data and Materials

The datasets and analyses done during the current study are available from the corresponding author (F. Büyük) on reasonable request.

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Conflict of Interest

The authors did not report any conflict of interest related to this article.

Author Contributions

FB, ÖÇ, MRC and MŞ planned, designed, and supervised the research procedure, FB and MRC carried out the experiments and the analytic tests and all authors wrote the article.

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