The isolation of *Bacillus anthracis* specific lytic bacteriophages from the burial sites of animals which have died of anthrax and host susceptibility

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Abstract
The aim of the this study was to isolate *Bacillus anthracis*-specific lytic bacteriophages from soil samples collected from the burial sites of animals that died from anthrax and stored by our department within the scope of previous studies and routine analyses and to determine the host susceptibility of the isolated phages. Eleven soil samples culture negative for *B. anthracis* were used for this. The logarithmic culture of *B. anthracis* Sterne 34F2 strain was used as host bacteria and the Gamma phage was used as reference phage. The host specificity of the phages obtained was determined using an in-house bacterial collection consisting of 112 virulent *B. anthracis* isolates (104 animal and 8 soil field isolates) and 5 reference strains of *Bacillus* group (*B. anthracis* Sterne 34F2, *B. megaterium* Pasteur Inst. 5117, *B. subtilis* ATCC 6633, *B. cereus* ATCC 11778, *B. thuringiensis* RSCC 380). As a result, 18 field phages were isolated specific to *B. anthracis*. Gamma phage and all the field phages showed complete lytic activity on *B. anthracis* Sterne 34F2 strain and 112 field *B. anthracis* strains; 16 of the field phages did not show any lytic activity on the *Bacillus* group strains other than the Sterne 34F2, while two phages coded SS and PB caused partial lysis on *B. megaterium* Pasteur Inst. 5117. This study is important as it involves the isolation of lytic phages with a narrow host specificity. Considering that lytic phages are more advantageous as therapeutic agents due to their self-replication and self-limiting properties that reveal automatic dosing patterns while lysing the host bacteria, the phages obtained from the current study may have the potential to be used as alternative diagnostic tools, therapeutic agents or environmentally friendly decontaminants in this regard.

Keywords: *Bacillus anthracis, bacteriophage, mitomycin C, soil*

INTRODUCTION

Bacillus anthracis is a Gram positive, spore forming bacterium which primarily infects ruminants but can affect any mammal, including humans (Mock and Fouet, 2001). The bacterium is soil borne and the causative agent of the anthrax disease (Hassim et al., 2020). The survival of the organism is due to its ability to form spores which render it resistant to the attentions of environmental insults such as bacteriophages (phages) (Titball et al.,1991). Bacteriophages represent one of the most abundent life forms on the planet and as the name suggests they prey on bacteria such as the vegetative form of *B. anthracis* (Hendrix et al., 1999). They are thought to play a key role in regulating the population density of specific bacterial species (Bohannan and Lenski, 2000; Thingstad, 2000; Weinbauer and Rassoulzadegan, 2004). This is due to the fact the most successful phages evolve to recognise a specific target on a specific bacteria. Thus it is perhaps not surprising that phages with the ability to infect *B. anthracis* are found in relatively high numbers in the soil surrounding the burial of an infected animal. This ability to specifically target and kill *B. anthacis* has prompted researchers to explore there potential as therapeutic agents and as a environmentally friendly decontaminants (Alisky et al., 1998; Barrow and Soothill, 1997; Brown and Cherry, 1955; Brown et al., 1958). Bacteriophages are significant tools used for diagnostic purposes in microbiology, as vectors in molecular biology and applications in phage therapy for decades (Letarov and Klikov, 2009). Phages are also used in the identification of *B. anthracis* (Fouts et al., 2006). So, the high specificity of *B. anthracis*-specific Gamma phage also forms the basis of a laboratory-based diagnostic test (Alisky et al., 1998; Barrow and Soothill, 1997).

Somewhat surprisingly only a relatively small number of phages such as AP50, Gamma, and Phi20 (Ackermann et al., 1994; Brown and Cherry, 1955; İnal and Karunakaran, 1996; Mc Cloy, 1951), Fah, Cherry, AP631, and Wbeta with the ability to lyse *B. anthracis* have been described to date (Li et al., 2024). However, in recent years, phages have also been isolated that are reported to represent new species related to the historical anthrax phages Gamma, Cherry and Fah, including *B. anthracis*-specific siphophage, J5a, F16Ba and z1a, and Wbeta, the phage of the genus Wbetavirus (Nakonieczna et al., 2022). The low number of *B. anthracis*-specific phage isolations may be partly due to the in part to the ability of phage to incorporate and be retained in the genome of infected bacterium a process which results in the bacterium gaining resistant to subsequent infection (İnal, 2003). The ability of bacteriophages such as Gamma and AP50c to infect *B. anthracis* strains with high specificity has been considered as influential factors in the development of bacterial detection tests (Forrest et al., 2023). Indeed

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analysis of the genome of over 300 isolates of *B. anthracis* have revealled the presence of at least 4 latent phages (Brown and Cherry, 1955; Fouts et al., 2006). Interestingly this is a reversable process in that treatment with a cell mitogen such as mitomycin C triggers the phage to break dormancy and re-establish its lytic life cycle (Bradley, 1964; Imaeda and Rieber, 1968).

To determine if we could recover novel *B. anthracis* lytic phages from soil taken from anthrax burial sites we mixed mitomycin C treated soil from various sites with logarithmic cultures of *B. anthracis* Sterne which served as a harmless indicator strain. The specificity of the resulting phages was then assessed against our in house collection of 112 virulent isolates of *B. anthracis* and 5 reference strains of *Bacillus* group.

MATERIALS AND METHODS

Bacterial Cultures

Host and reference strains: *B. anthracis* Sterne strain 34F2 as a host was obtained from The Ministry of Food, Agriculture and Livestock, Turkey. *B. cereus, B. megaterium, B. subtilis* and *B. thuringiensis* strains for susceptibility analysis were obtained from the culture collection of the Public Health Agency of Turkey in Ankara, Türkiye.

Bacterial isolates: A total of 112 fully virulent *B. anthracis* field isolates were included in this study. Eight of the isolates were obtained from soil and 104 isolates were obtained from cattle and sheep which belonged to the culture collection of the Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, Türkiye. Species-specific identification and virulence determination of the isolates were performed according to Büyük et al., (2015).

Bacteriophages

Reference Phage: Gamma phage, obtained from the Department of Microbiology and Clinical Microbiology, Medicine Faculty, Erciyes University, Türkiye, was used as a reference phage.

Soil samples for phage isolation and to check* B. anthracis *culture positivity: The soil samples used for phage isolation in this study were collected from the burial sites of an animal known to have died of anthrax and stored by our department within the scope of previous studies and routine analyses. For this purpose eleven soil samples were used. The same soils were subjected to cultural analysis for *B. anthracis* positivity as an alternative host for possible lytic or prophage induction other than the *B. anthracis* Sterne 34F2 host strain (Şahin et al., 2024).

Bacteriophage isolation from soil with and without mitomycin C: For phage isolation, the basic method was used, an enrichment procedure that has remained unchanged since its development by Felix d'Herelle (Van Twest and Kropinski, 2009). The mitomycin C induction method was also used in phage isolation procedures to obtain possible prophages from the Sterne strain used as host (Raya and Hebert, 2009). Five grams of soil was mixed with 5 ml Brain Heart Infusion (BHI) Broth (Sigma-Aldrich, USA) and incubated at 37°C for 2 hours in duplicate (one for isolation with mitomycin C and the other for without mitomycin C). For the mitomycin C batch, 1 µl of mitomycin C (2 mg/ml) (Kyowa Hakko Kogyo Corporation, Tokyo, Japan) was added to this culture and incubated for another hour under the same conditions. After incubation, 5 ml of logarithmic culture of *B. anthracis* Sterne 34F2 in BHI Broth was added to both series of cultures and incubated overnight at 37°C. The next day, the supernatants were filtered through a 0.22 µm pore diameter membrane filter. The filtrates were stored at +4°C until use.

Plaque assay

The lytic activity of the phage suspension was determined via plaque assay (Abshire et al., 2005) using Columbia agar (CA) (Sigma-Aldrich, US) and *B. anthracis* Sterne as a host bacteria. For the preparation of host bacterial inoculum, 5-6 colonies were selected from an overnight culture on CA and then the colonies transferred into 5 ml of 10 mM sterile PBS (pH 7.2). This inoculum was vortexed for at least 15 s at high speed to generate a smooth and uniform suspension and 100 µl of the inoculum was spreaded onto CA plates. Fifteen µl of phage suspension was inoculated on the CA plates and after fluid was allowed to absorb into agar, the plates were incubated at 37°C overnight. The cultures were inspected for plaque formation at intervals.

Phage amplification

In order to increase the concentration of *B. anthracis* specific field phages, equal volumes of phage filtrate and logarithmic culture of *B. anthracis* Sterne strain were mixed in a tube and incubated overnight at 37°C. Then, the culture was filtered through a 0.22 µm membrane filter and used as a phage suspension with increased concentration. The titre of the phage was determined using the plaque assay (Abshire et al., 2005). These procedures were repeated until a high titre phage suspension was obtained.

Determination of the routine test dilution (RTD) of phages

Turbidity of the overnight culture of *B. anthracis* Sterne strain was adjusted according to McFarland standard No 0.5 ($1.5x10^8$cfu/ml) in sterile isotonic peptone saline. Then, 0.2 ml of the suspension was spread on CA plates and kept in an incubator at 37°C for about 20 min. Ten-fold subdilutions of the phage were prepared in distilled water. Ten µl of each subdilution were dropped onto the inoculated plates and incubated at 37°C for 24 h. The next day, the most diluated one that provided complete clearance on the plates was evaluated as the RTD value of the phage (Kutter, 2009).

Host range analysis of phages

Phage host range is the taxonomic breadth of bacteria that a phage can successfully infect (Göller et al., 2021), characterized by the number of host subsets, species, and strains (Holtappels et al., 2023), and is a critical feature to understand in phage biology (Göller et al., 2021). Host range depends on as well as the genotype and morphology of the phage and bacterial host, the environment in which they interact (Hyman and Abedon, 2010). Phages vary considerably in their apparent host ranges. While

some of them infect a narrow subset of bacterial hosts, others affect a broad range of hosts (de Jonge et al., 2019). In the literature, the term "broad" host range is used to describe as well as phages that infect more than one bacterial species, it can also be used when a phage can reproduce in a wide range of strains within a species (Ross et al., 2016). Thus, the host range varies from wide to narrow depending on the genetic diversity of the challenged host (Göller et al., 2021).

In order to determine the host sensitivity of the phages obtained, 112 fully virulent *B. anthracis* field isolates, and reference strains including *B. anthracis* Sterne strain, *B. megaterium, B. cereus, B. subtilis* and *B. thuringiensis,* were used. The logarithmic cultures of all bacterial strains were prepared by incubating in 10 ml BHI broth at 37°C for 3-5 hours. Then, 0.2-0.3 ml of these cultures were inoculated into CA plates and spread over the entire surface of the medium and kept at 37°C until dry. Then, 10 µl of the phage with known RTDs were dropped onto the CA plates where bacteria were inoculated. Incubations were carried out overnight at 37°C. The presence of a clear zone at the point where the phage was released was evaluated as phage susceptibility. Gamma phage served as a control in this experiment (Corbel and Thomas, 1976).

RESULTS

As a result of the study, all soils used for phage isolation were negative for *B. anthracis* after the cultural analysis. A total of 18 lytic phages were isolated from all 11 soil samples with exactly the same origin and characteristics from both mitomycin C and non-mitomycin C batches. Thus, phage isolates did not differ in the mitomycin C addition set, which was considered as absence of prophage. A total of 4 phages were isolated from one soil sample taken from Dikme Village, 6 phages were isolated from three soil samples taken from Selim Centre, 2 phages were isolated from one soil sample taken from Benliahmet Village and 6 phages were isolated from 6 soil samples taken from Hanevler Village (Table 1). All of the phages obtained showed complete lysis effect on *B. anthracis* field isolates and the Sterne strain 34F2.

Phage amplification and Determination of the routine test dilution (RTD) of phages

As a result of the study, it was observed that the RTD values of the phages obtained were between 10^{-1}-10^{-5} pfu/ml. Namely; RTD of HH phage was 10^{-5} pfu/ml; RTD of SO phage was 10^{-4} pfu/ml; RTD of MS, AU, FB, AG, AT, ET, DB, DK, DC and FR phages was 10^{-3} pfu/ml; RTD of SS, OC and HK phages was 10^{-2} pfu/ml; RTD of HG, BC and PB phages was 10^{-1} pfu/ml (Table 1).

Host range analysis of bacteriophages

Gamma phage used as reference phage and all 18 *B. anthracis* specific phages showed complete lytic activity on the *B. anthracis* Sterne 34F2 strain and 112 virulent *B. anthracis* field strains. While 16 phages did not show any lytic activity on the *Bacillus* group strains other than the Sterne 34F2 strains, two phages coded SS and PB caused partial lysis on *B. megaterium* Pasteur Inst. 5117. Again, Gamma phage did not show lytic activity on the these strains (Figure 1, Table 2).

Table 1. *Locations and years of soil sample collection and number of samples*

Location	Contamination date	Soil sample number	Phage number	Phage code	RTD of the phages (pfu/ml)
Selim center	2005	3	6	SS	10^{-2}
				MS	10^{-3}
				SO	10^{-4}
				AU	10^{-3}
				FB	10^{-3}
				ET	10^{-3}
Hanevler village	2009	6	6	OC	10^{-2}
				FR	10^{-3}
				HK	10^{-2}
				HG	10^{-1}
				BC	10^{-1}
				PB	10^{-1}
Dikme village	2010	1	4	HH	10^{-5}
				DB	10^{-3}
				DK	10^{-3}
				DC	10^{-3}
Benliahmet village	2010	1	2	AG	10^{-3}
				AT	10^{-3}

Table 2. *Bacillus spp. host range of various phages.*

Isolates	***B. anthracis* specific phages**																		
	γ	**HH**	**SS**	**MS**	**OC**	**SO**	**AU**	**FB**	**AG**	**AT**	**ET**	**DB**	**DK**	**DC**	**FB**	**HK**	**HG**	**BC**	**PB**
Reference strains																			
B. anthracis Sterne 34F2	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
B. megaterium Pasteur Inst. 5117	NL	NL	**PL**	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	**PL**
B. subtilis ATCC 6633	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
B. cereus ATCC 11778	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
B. thuringiensis RSCC 380	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
***B.anthracis* field strains**																			
Animal isolates (104 strains)	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
Soil isolates (8 strains)	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL

γ: Gamma, a *B. anthracis* diagnostic phage was obtained from the Department of Microbiology and Clinical Microbiology, Faculty of Medicine, University of Erciyes, Turkey. The remaining eighteen phages were obtained from soil. C: complete lysis, P: partial lysis, NL: not lysis

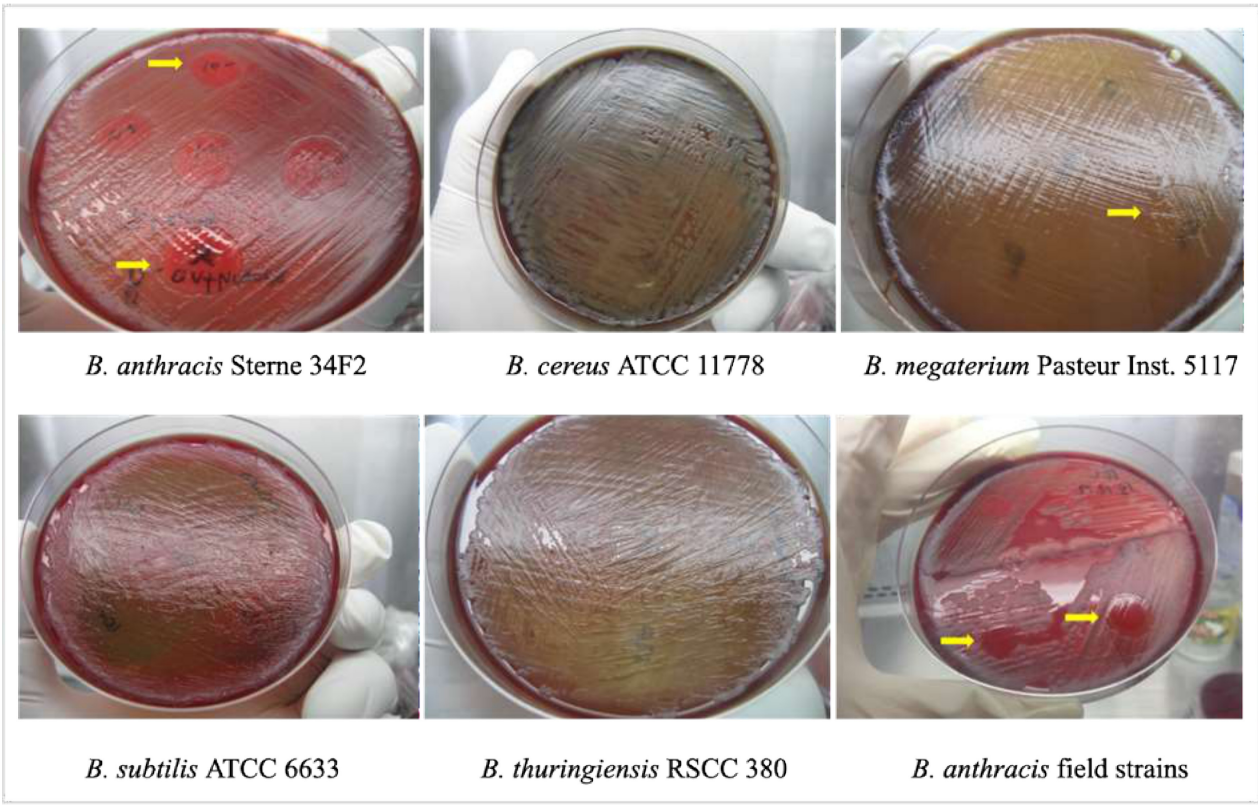

Figure 1. *Host range analysis of bacteriophages. The yellow arrows indicate complete lysis (B. anthracis Sterne 34F2 and B. anthracis field strains) or partial lysis (B. megaterium Pasteur Inst. 5117).*

DISCUSSION

B. anthracis, a Category A biological threat agent, is a member of the *B. cereus* sensu lato group. It is a zoonotic soil-borne bacterium. The presence of two large virulence plasmids encoding anti-host toxins and capsular structure of *B. anthracis* is associated with its emergence as an animal pathogen from the *B. cereus* group. Also, it is noteworthy that *B. anthracis* is distinguished from its closest relatives by the presence of integrated viral forms or prophages (Schuch and Fischetti, 2006). Bacteriophages are viruses belonging to the families *Siphoviridae, Podoviridae, Myoviridae* and *Tectiviridae* that infect bacteria. Bacteriophages have two replication strategies, lysogenic and lytic, with the lysogenic ones involving viral integration into the host genome, while the lytic ones activate the host machinery to replicate its progeny, leading to bacterial lysis. Bacteriophages are used as diagnostic tools in microbiology, vectors in molecular biology, phage therapy and more recently as an alternative disinfectant. *B. anthracis* bacteriophages, whose reports are increasing day by day (Gillis and Mahillon, 2014), are promising in these respects. In the present study, a total of 18 lytic phages specific to *B. anthracis* were isolated from all 11 soil samples taken from anthrax-infected animal burial sites. This positivity is not surprising in light of our further recognition of the diversity of bacteriophage species in the environment (10^8 different species) and their abundance in the biosphere (about 10^{31}). This situation, which is more likely in areas contaminated with host bacteria, can also be considered as an indirect indicator of *B. anthracis* contamination from another point of view.

Another feature that distinguishes *B. anthracis* from other members of the closely related *B. cereus* group, which exhibit a monomorphic genome, is the presence of lambdoid prophage on its chromosomes (Sozhamannan et al., 2006). Such prophages are integrated viral genomes located within bacterial chromosomes that remain dormant until triggered by various environmental stimuli. One of the most commonly used agents for prophage induction is mitomycin C, known for its ability to induce DNA damage (Chen et al., 2006). Indeed, induction with mitomycin C increased the excision frequency of one of the prophages possessed by *B. anthracis* by about 250-fold. This induction failure on the others was due to the defective nature of the prophages (Sozhamannan et al., 2006). Other mitomycin C-inducible progenies include *B. anthracis* Sterne-specific phage ϕ20, *B. thuringiensis*-specific SU-11, and *B. cereus*-specific Emet and Sole (Gillis and Mahillon, 2014). As in many studies (Braun et al., 2020; Davison et al., 2005; Forrest et al., 2023), *B. anthracis* Sterne 34F2 strain was used as the susceptible host in this study because the strain generally lacks inducible prophages, which are present in many *B. anthracis* strains and may hinder our identification procedure. However, just in case, mitomycin C induction was also tried for the induction of possible prophages of this host origin. A total of 18 lytic phages with exactly the same origin and characteristics were isolated from all 11 soil samples taken from both mitomycin C and non-mitomycin C batches, which was accepted as the absence of prophage.

To date, several *B. anthracis* phages have been characterized, including Gamma, Wbeta, Fah AP631 and Cherry. The Centres for Disease Control and Prevention (CDC) recommends Gamma phage susceptibility testing as a standard diagnostic test for suspected *B. anthracis*.

Although Gamma phage is 97% specific for *B. anthracis*, few strains of other *B. cereus* group members have shown sensitivity to this phage (Li et al., 2024). In China, the AP631 phage, which can lyse a small number of *B. cereus* strains, is used in the etiological diagnosis of anthrax (Liu et al., 2019). In the present study, Gamma phage used as reference phage and all 18 *B. anthracis* specific phages obtained showed full lytic activity on *B. anthracis* Sterne 34F2 strain and 112 virulent *B. anthracis* field strains. Phage resistance, which has been reported at low rates (Fulmer, 2003; Kolton et al., 2017), was not detected for either Gamma phage or field phages in this study. Although *B. anthracis* specific phages exhibit a rather narrow host range, several *B. cereus* strains (e.g. ATCC 4342) have been shown to be susceptible to infections by these phages (Abshire et al., 2005; Marston et al., 2006). However, most *B. thuringiensis* and *B. cereus* strains are known to be resistant to Gamma phage in particular. In the present study, Gamma phage did not show any lytic activity on *B. cereus* group members, while 2 (coded SS and PB) of the 18 *B. anthracis*-specific phages obtained caused partial lysis on *B. megaterium* Pasteur Inst. 5117 strain. This is in line with the idea that *B. anthracis* strains within the *B. cereus* group show little genetic diversity, probably as a result of their long dormancy in the soil as endospores, which limits the chances of finding new specific *B. anthracis*-specific phages, and that certain phages that infect *B. anthracis* may also infect *B. cereus* group members (Pilo and Firey, 2011).

CONCLUSION

In this study, isolation of *B. anthracis*-specific lytic phage was performed, which has the potential to be used in the ecological and epidemiological control, diagnosis and treatment of anthrax disease. The absolute effectiveness of these phages, whose ancestors are not yet known and not fully characterized, on reference and field strains where no resistance was observed is promising. The ability to obtain phages with this specificity by a simple and basic enrichment method is also valuable as an indirect indicator of *B. anthracis* from the environment.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k).

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Availability of data and materials: The data and materials of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

There is no conflict of interest between the authors.

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