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The Isolation and Examination of the Host Specificity of Local Bacteriophages for Use Against *Brucella abortus*

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Abstract: Bacteriophages are viruses that infect bacteria. Although their effects on bacteria have been known for many years, the discovery of antibiotics has limited the widespread use of bacteriophages against bacterial infections. However, it is now essential to reconsider using phage therapies due to antimicrobial-resistant bacteria and uncontrolled bacterial zoonotic diseases becoming a global problem. This study aimed to isolate lytic phages against *B. abortus*, the most common agent that causes bovine brucellosis, which has become a worldwide animal and public health problem. This performed isolation is from cattle farms known to be previously or currently infected, and the study also evaluates the lytic effects of these phages on *B. abortus, B. melitensis, B. suis* biotypes, *B. ovis* and *B. neotomae* and *B. abortus* field isolates in our culture collection. In this process, seven local brucella-specific phages were identified by evaluating 112 samples via general phage isolation and purification. The lyticity of the isolated bacteriophages were analyzed with international reference: *B. abortus* (bv 1, 2, 3, 4, 5, 6, 9), *B. melitensis* (bv 1, 2, 3), *B. suis* (bv 1, 2, 3, 4, 5) biovars, *B. ovis, B. neotomae* and *B. abortus* field strains (n:20). It was found that 85% of *B. abortus* strains produced a lysis pattern like Tbilisi Φ through local phages. In terms of the lysis results, three different *B. abortus* specific phages were isolated (98 Φ, 104 Φ, and (P35, P70, P94/1, P94/2, P94/3) phages). It is thought that the applying cocktails prepared from these phages to fight against brucellosis will significantly contribute to controlling the disease. Since 15% of the field isolates were found to be rough strains, it is recommended that R/C Φ are included in the prepared cocktails.

Keywords: *Bacteriophage, Brucella abortus, Brucellosis, Phage therapy.*

Brucella abortus'a Karşı Yerel Bakteriyofaj İzolasyonu ve Konakçı Spesifitesinin Değerlendirilmesi

Özet: Bakteriyofajlar bakterileri enfekte eden viruslardır. Bakteriler üzerindeki etkileri uzun yıllardır bilinmekle birlikte bakteriyel enfeksiyonlara karşı yaygın olarak kullanımı antibiyotiklerin keşfi nedeniyle arka planda kalmıştır. Küresel bir sorun haline gelen antimikrobiyal dirençli bakteriler ve kontrol altına alınamayan bakteriyel zoonotik hastalıklar nedeniyle günümüzde faj terapileri bir tercihten ziyade zorunluluk haline gelmeye başlamıştır. Bu çalışmada, ülkemizde ve dünyanın birçok ülkesinde önemli bir hayvan ve halk sağlığı sorunu olan sığır brusellosisinin en yaygın etkeni olan *B. abortus*'a karşı daha önce ya da halen infekte olduğu bilinen sığır çiftliklerinden litik faj izolasyonunun yapılması ve bu fajların *B. abortus, B. melitensis B. suis* biyotiplerinde, *B. ovis* ve *B. neotomae'*de ve kültür koleksiyonumuzda bulunan *B. abortus* saha izolatlarında litik etkilerinin değerlendirilmesi amaçlanmıştır. Genel faj izolasyon ve saflaştırma yöntemleri uygulanarak 112 örnekten yedi adet yerel *Brucella* spp. spesifik faj tespit edildi. İzole edilen bakteriyofajlar, uluslararası referans *B. abortus* (bv 1,2,3,4,5,6,9), *B. melitensis* (bv 1,2,3), *B. suis* (bv 1,2,3,4,5) biyovarları, *B. ovis, B. neotomae ve B. abortus* saha suşları (n:20) ile litik etkinlikleri yönünden analiz edildi. *B. abortus* suşlarının %85'i yerel fajlarla Tbilisi Φ'na benzer lizis modeli meydana getirdi. Çalışma sonunda şekillenen lizis tablosu dikkate alındığında, 3 farklı *B. abortus* spesifik faj izolasyonunun yapıldığı anlaşıldı [98 Φ, 104 Φ, ve (P35, P70, P94/1, P94/2, P94/3) fajları]. Bu fajlardan hazırlanan faj karışımlarının brusellosis ile mücadelede uygulanmasının hastalığın kontrolüne önemli katkılar sağlayacağı düşünülmektedir. Çalışmada saha izolatlarının %15'i rough suşlar olarak tespit edildiğinden hazırlanan faj kokteyllerin içine R/C Φ'nın katılmasının da yerinde bir karar olacağı kanısına varıldı.

Anahtar Kelimeler: *Bakteriyofaj, Brucella abortus, Brusellosis, Faj terapisi.*

Introduction

Bacteriophages, which are viruses that infect bacteria, are infectious particles that, like other viruses, have at least two components (nucleic acid and protein) (Campbell, 2003). Although their effects on bacteria were discovered as early as the 19th century, the discovery of antibiotics has reduced interest in bacteriophages (phages) in the scientific community (Jurač et al., 2019). However, the alarming increase in multi-antibiotic-resistant microorganisms has necessitated a renewed focus on phages (García et al., 2019; Ling et al., 2022). Phages, which significantly impact the biocontrol of microbial populations of bacteria in different environments, are naturally "green" applications as they only affect target microorganisms (Issabekov et al., 2022). There is also a great deal of potential for the use of phages in the treatment of bacterial infections, as well as in disinfection and the detection and categorizing of pathogens (Erdenliğ-Gürbilek et al., 2022; Issabekov et al., 2022; Phongtang et al., 2019; WOAH, 2022). The importance of phage therapies in the fight against bacterial diseases is increased by how bacteriophages demonstrate strong lytic effects, specifically against many target bacteria, and their ability to multiply exponentially in the infected environment (Aslam and Schooley, 2019).

Brucellosis, an important zoonotic bacterial disease, has become a global problem due to the difficulty in eradication. The problem's severity is apparent by the fact that the vast majority of animals globally are infected. It is, therefore, essential that the problem of brucellosis be tackled in whatever way possible, including the use of phage therapy (Khurana et al., 2021; Pappas et al., 2006) and, more specifically, through the targeted infection of *Brucella* bacteria with phages. It has been standard practice for many years to identify classical *Brucella* species that show host specificity (Flores et al., 2012; Projahn et al., 2020; WOAH, 2022). Furthermore, the self-replicating and self-limiting nature of these phages, as well as the fact that they do not harm regular flora, makes lytic bacteriophages a practical, reliable, and cost-effective alternative for brucellosis control (Mohan et al., 2020).

This study aimed to isolate local bacteriophages from samples taken from areas with a high probability of phage presence. These areas include litter material, fecal pits and sewage samples, and range soil from cattle farms. Current or defeated *Brucella* infection was detected, and the lytic effect of the isolated phages on the World Organization for Animal Health (WOAH) reference *Brucella abortus*, *B. melitensis*, *B. suis* biotypes, *B. ovis* and *B. neotomae* and *B. abortus* field isolates in our culture collection was determined.

Materials and Methods

Reference and field *Brucella* strains (Table 1) and phages used in the study were obtained from the laboratory strain collection of the Department of Microbiology, Faculty of Veterinary Medicine, Harran University. The materials used in the study consisted of samples taken from 112 different regions of 27 cattle farms. This study is not subject to HADYEK permission by Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Tablo 1. *B. abortus* field strains included in the study and their origin.

No	Strain	Host	Region	Material	Year
1	*B. abortus*	Cattle	Iğdır	Milk	2022
2	*B. abortus*	Cattle	Şanlıurfa/Akçakale	Milk	2022
3	*B. abortus*	Cattle	Diyarbakır	Fetus	2021
4	*B. abortus*	Cattle	Diyarbakır	Fetus	2021
5	*B. abortus*	Cattle	Şanlıurfa/Haliliye	Milk	2022
6	*B. abortus*	Cattle	Şanlıurfa/Viranşehir	Milk	2022
7	*B. abortus*	Cattle	İstanbul	Vaginal swap	2017
8	*B. abortus*	Cattle	Şanlıurfa/Viranşehir	Milk	2022
9	*B. abortus*	Cattle	Şanlıurfa/Viranşehir	Milk	2022
10	*B. abortus*	Cattle	Bursa	Vaginal swap	2022
11	*B. abortus*	Cattle	Iğdır	Vaginal swap	2022
12	*B. abortus*	Cattle	Iğdır	Milk	2022
13	*B. abortus*	Cattle	Konya	Milk	2022
14	*B. abortus*	Cattle	Bursa	Milk	2022
15	*B. abortus*	Cattle	Bursa	Milk	2022
16	*B. abortus*	Cattle	Bursa	Abomasal fluid	2022
17	*B. abortus*	Cattle	Bursa	Abomasal fluid	2022
18	*B. abortus*	Cattle	Bursa	Vajinal swap	2022
19	*B. abortus*	Cattle	İzmir	Milk	2022
20	*B. abortus*	Cattle	Kars	Abomasal fluid	2022

Bacteriophage isolation and proliferation: Phages were isolated from environmental samples from cattle farms where brucellosis had previously been detected. Phage isolation was performed in our laboratory through a modified method based on classical methods used for this purpose (Sambrook et al., 1989). In this process, fecal litter samples were diluted 1/10 with LB (Luria Bertani) broth, homogenized, and centrifuged at 6000 rpm for 20 minutes. The supernatant obtained was first passed through 0.45 µm filters, followed by 0.2 µm filters, and the sterilized filtrate was used as a phage source. *B. abortus* S19 vaccine strain, which has low virulence regarding laboratory personnel and environmental safety, was used as the host. A suspension of $1x10^9$ CFU bacteria per milliliter of the host strain was inoculated onto Tryptic Soy Agar (TSA). After the agar surface had dried, 250 µl of sterile phage filtrate was added and spread on the agar surface with a sterile loop. After the agar surface had again dried, the petri dishes were incubated at 37°C for one day. After incubation, passages were continued until significant lysis was observed in the petri dishes. Bacteria and phage combinations were collected from the surface of the lysis petri dishes with LB broth. The collected suspension was centrifuged at 6000 rpm for 20 minutes, and the supernatant was sterilized by first passing it through 0.45 µm filters, followed by 0.2 µm filters. The next stage was phage purification.

Purification of *Brucella* phages: In this stage, sterile phage filtrates were processed using an agar-overlay method to detect the phage plaques of bacteriophages. This method necessitated the preparation of 3 ml of soft agar (0.8 g agar, 2 g NaCl, 2 g Peptone, 150 µl $CaCl_2$), which was then poured into each TSA petri dish. The prepared agar was kept in a 40°C double-boiler to prevent solidification, and 150 µl of phage solution and 100 µl of *B. abortus* S19 bacterial suspension were added. The mixture was then immediately poured onto the TSA petri dish, spread, and allowed to freeze. Petri dishes were incubated overnight at 37°C, and the phage plaques were evaluated after incubation. The single plate isolation method was repeated three times in succession to obtain pure bacteriophages. For this purpose, phage plaques obtained by the double agar method were collected from singular sites into a sterile tube with a sterile pipette tip. Three ml of LB liquid medium and 250 µl of bacterial suspension containing $1x10^9$ CFU *B. abortus* S19 per milliliter were added. After waiting for 15 minutes for phage-bacteria adsorption, 10 ml of LB liquid medium was added. The mixture was then incubated overnight at 37°C, after which the mixture was centrifuged at 6000 rpm for 10 minutes, and the upper liquid phase was passed through a 0.22 µm filter to prepare a pure and sterile phage solution. The lytic effect of these phage solutions on *B. abortus*, *B. melitensis and B. ovis* species and biotypes was investigated. In addition, the lytic effect of Tbilisi Φ on *B. suis*, and *B. neotomae* species, which is known to occur on high titers, was also investigated. The isolated phages were additionally tested on different field *B. abortus* strains to detect possible differences. All phages isolated in the study were used in routine test dilution (RTD) (Alton et al., 1988), and ten-fold dilutions of phages were made for this purpose. The endpoint of complete lysis was determined as the routine test dilution of the relevant phage.

Results

Seven phages were selected for evaluation due to the phage detection (Figure 1) and purification procedures using the *B. abortus* S19 vaccine strain from 112 samples taken from 27 cattle enterprises included in the study as hosts. Three of the selected phages were detected in samples from extremely different facilities of the same enterprise. The phages used in the study were coded as P35, P70, P94/1, P94/2, P94/3, P98, and P104.

Izatnagar (Iz) and Tbilisi (Tb) phages were used as reference control to evaluate the lytic effects of phages detected in and purified from the *B. abortus* S19 host. Reference strains used in the study were: *B. abortus* 544 (bv 1), *B. abortus* 86/8/59 (bv 2), *B. abortus* Tulya (bv 3), *B. abortus* 292 (bv 4), *B. abortus* B3196 (bv 5), *B. abortus* 870 (bv 6), *B. abortus* C68 (bv 9), *B. melitensis* 16M (bv 1), *B. melitensis* 63/9 (bv 2) *B. melitensis* Ether (bv 3), *B. suis* 1330 (bv 1), *B. suis* Thomsen (bv 2), *B. suis* 686 (bv 3), *B. suis* 40 (bv 4), *B. suis* 513 (bv 5), *B. ovis* 63/290, *B. neotomae* 5K33 strains and 20 strains of field *B. abortus* isolated from samples taken from eight different provinces (Table 1).

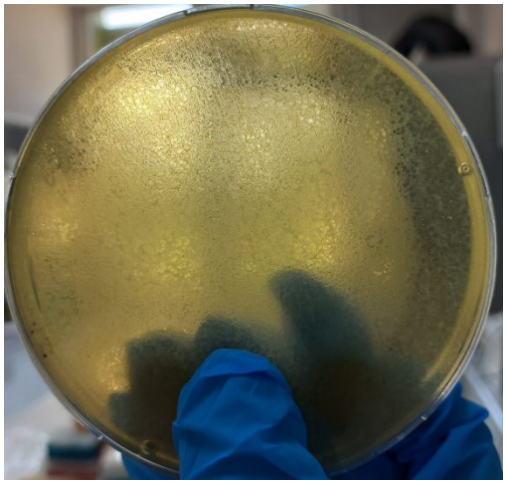

Figure 1. Densities of bacteriophage plaques.

At the end of 48 hours of incubation at 37°C in a microaerophilic atmosphere, local bacteriophages formed round and transparent lysis areas in 17 of the 20 *B. abortus* strains included in the study. All isolated phages produced the same lysis areas (Figure 2) as the Tb Φ in *B. abortus* strains (bv 1,2,3,3,4,4,5,6,9 and 17 field strains). The lysis induced by local and reference phages on the reference strains and field strains used are shown in Table 2. The reference and local phages also showed full lytic effects on *B. suis* bv 1, 2 strains and *B. neotomae*. However, *B. suis* bv 3 and *B. suis* bv 5 were not lysed by Tb, P98, and P104 phages, whereas they were fully lysed by Iz, P35, P70, P94/1, P94/2, and P94/3. *B. suis* bv 4 strain was lysed by all phages except P98 Φ. Local phages and Tb Φ did not produce lysis plaques

Table 2. Lytic effect of isolated *Brucella* phages and reference phages on *Brucella* strains. +: plaque formation, -: no lysis.

	P35	P70	P94/1	P94/2	P94/3	P98	P104	Tb	Iz	R/C
***B. abortus* 544**	+	+	+	+	+	+	+	+	+	-
***B. abortus* 86/8/59**	+	+	+	+	+	+	+	+	+	-
***B. abortus* Tulya**	+	+	+	+	+	+	+	+	+	-
***B. abortus* 292**	+	+	+	+	+	+	+	+	+	-
***B. abortus* B3196**	+	+	+	+	+	+	+	+	+	-
***B. abortus* 870**	+	+	+	+	+	+	+	+	+	-
***B. abortus* C68**	+	+	+	+	+	+	+	+	+	-
***B. melitensis* 16M**	-	-	-	-	-	-	-	-	+	-
***B. melitensis* 63/9**	-	-	-	-	-	-	-	-	+	-
***B. melitensis* Ether**	-	-	-	-	-	-	-	-	+	-
***B. suis* 1330**	+	+	+	+	+	+	+	+	+	-
***B. suis* Thomsen**	+	+	+	+	+	+	+	+	+	-
***B. suis* 686**	+	+	+	+	+	-	-	-	+	-
***B. suis* 40**	+	+	+	+	+	-	+	+	+	-
***B. suis* 513**	+	+	+	+	+	-	-	-	+	-
***B. neotomae* 5K33**	+	+	+	+	+	+	+	+	+	-
***B. ovis* 63/290**	-	-	-	-	-	-	-	-	-	+
***B. abortus* field strains (1-10,12-15,17,19,20)**	+	+	+	+	+	+	+	+	+	-
***B. abortus* field strains (11,16,18)**	-	-	-	-	-	-	-	-	-	+

in *B. melitensis* (bv 1,2,3) strains. Similarly, the *B. ovis* strain was not lysed by any of the phages used in the study. While 3 of the *B. abortus* field strains were not lysed by any phage, they were lysed by R/C Φ, which is specific to rough strains.

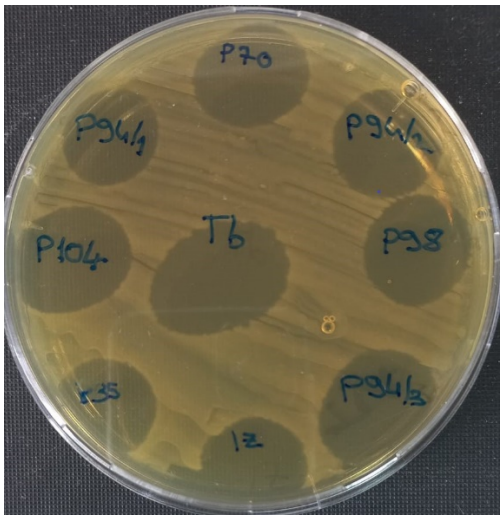

Figure 2. Analysis of local and reference phages via *B. abortus* field strain (No:2).

Discussion

The main causative agent of bovine brucellosis is biovars of *B. abortus*. This disease causes huge losses worldwide due to abortions, low yields, import restrictions, and public health problems (Khurana et al., 2021; WOAH, 2022). As the socio-economic effects of brucellosis are overwhelming, the need for practical and low-cost solutions has become imperative, especially in underdeveloped countries (Khurana et al., 2021; Prajapati et al., 2014). Phage therapies appear to be one such solution, as they are cost-effective and potentially adaptable to controlling bacterial diseases (Saxena, 2021). Support for the therapeutic efficacy of *Brucella* phages was provided by Prajapati et al. (2014), who found that phage therapy reduced colonization in mouse spleens. The researchers also reported that in cases where antibiotic treatment is not recommended in animals due to the expense, phage therapies could be effective in treating pregnant animals and those at risk of infection.

Lytic bacteriophages are unique microorganisms that have therapeutic and prophylactic properties (Issabekov et al., 2022), while bovine brucellosis is a bacterial disease that can potentially be controlled using lytic bacteriophages (Shaneen et al., 2021). The isolation of lytic phages that can be used against *B. abortus* has been performed in various studies conducted in many countries (Prajapati et al., 2014; Saxena, 2021; Shaneen et al., 2021). As far as can be ascertained, the present study is the first study in Turkey on the isolation of lytic phages to combat *B. abortus*. Unlike many other studies (Chachra et al., 2012; Gupta and Saxena, 2017; Shaneen et al., 2021), the current study isolated 7 local phages and evaluated the lytic effects of these phages on 20 *B. abortus* field strains isolated from samples taken from 8 different provinces, while referencing *B. abortus, B. suis* and *B. melitensis* biovars, and *B. neotomae* and *B. ovis* strains.

Smooth *B. abortus* strains are susceptible to Tbilisi, Izatnagar, Berkeley2, and Weybridge phages (Li et al., 2019). In our study, in which *B. abortus* S19, a smooth and vaccine strain that is relatively safer to field strains, was used as the host, seven phages were isolates performed from cattle facilities with a history of brucellosis. There are many studies

reporting successful isolation of lytic phages for use against *Brucella* bacteria, most of these phages isolated having lytic effects on *B. abortus*. These isolates are closely related to Tb Φ (Morris et al., 1973; Teydoradze et al., 2015). The lytic effects of Tb Φ, an international reference phage, on *B. abortus* strains is well known (Shaneen et al., 2021; WOAH, 2022). In the present study, seven local phage isolations and three different *B. abortus* specific phage isolations were performed compared to Tbilisi Φ in terms of host specificity. Of these, 98 Φ did not lyse *B. suis* biovars, except *B. suis* bv 1 and 2, while 104 Φ only lysed *B. suis* bv 1, 2 and 4 biovars. These two phages were thus considered to be two separate phages. Since the other test bacteriophages lysed all reference and test strains, except *B. ovis* and *B. melitensis* biovars and rough *B. abortus* strains, they are thought to be the same phage. (Table 2). *Brucella* phages are generally similar in morphology, antigen reactions, and various physicochemical properties (Flores et al., 2012).

Izatnagar Φ was found to be lytic in tests performed on many smooth *Brucella* species (WOAH, 2022). In the present study, Iz Φ showed lytic effects on the international reference smooth strains of *B. melitensis* (bv 1,2,3), *B. suis* (1,2,3,4,5), *B. abortus* (1,2,3,4,5,6,9) and *B. neotomae*. *B. ovis* and three rough *B. abortus* field strains were lysed as expected by R/C Φ, which is lytic for rough strains. In their study that successfully lysed *B. suis* 1330 with Tb and Iz phages, Hammerl et al. (2017) reported that the published data on the susceptibility of *B. suis* 1330 (bv 1) to Tb Φ is inconsistent. Similarly, in the present study, Tb and Iz phages caused *B. suis* 1330 to form lysis plaques, while complete lysis was also achieved with local phages. Hammerl et al. (2017) reported that *B. suis* 686, 40, and 513 strains were not lysed by Tb Φ. In this study, *B. suis* 686 and 513 were not lysed by Tb Φ and local isolates P98 and P104 phages while B. suis 40 strain produced phage plaques in all phages included in the study except 98Φ. The authors emphasize that there may be many reasons for the inconsistency of the results and recommend that the genomes of the examined phages should be sequenced. The present study focused on isolating local *Brucella* phages in the first stage, and thus represents the first important step for further analysis. Hence, there is a need for other studies analyzing the examined strains, which were phylogenetically isolated and evaluated as three different groups of *B. abortus* phages.

Conclusion

In this study, three different local phages to be used against *B. abortus* were isolated. In this era where the use of bacteriophages is becoming necessary in the fight against bacterial problems, isolations of local phages that can be used against bacterial agents are of critical importance. However, since phages are not only specific to a certain but also to multiple species and strains, it is thought that the use of bacteriophage cocktails in the fight against brucellosis will make significant contributions to the control of the disease. Since some *Brucella* isolates may, for various reasons, transform into rough colony morphologies, it was concluded that it is important for R/C Φ to be added to phage cocktails. It is felt that further molecular analyses on isolated *Brucella* phages will pave the way for more prophylactic and therapeutic use of phages in the fight against brucellosis.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

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

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Author Contributions

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Control/Supervision: SEG, AMS
Data Collection and / or Processing: SÖ, SEG, AMS
Analysis and / or Interpretation: SÖ, SEG, AMS
Literature Review: SÖ, SEG, AMS
Writing the Article SÖ, SEG, AMS
Critical Review: SEG

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