Journal of Bacteriology and Mycology

Open Access

Austin Publishing Group

Special Article – Animal Brucellosis

Diversity of Phage-Host Specificity in *Brucella* Phage

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Received: March 24, 2017; **Accepted:** April 27, 2017; **Published:** May 04, 2017

Abstract

Bacteriophage typing of *Brucella* is accepted as an additional tool that can be used for the identification of bacterial species by the World Health Organization's Expert Committee of Brucellosis. Phage typing is based on the host specificity of bacteriophages. Few phage for bacterial species are currently validated for typing purposes. Tbilisi (Tb) is a unique phage isolated in the 1950s at the Eliava Institute of Bacteriophages, Microbiology, and Virology in Tbilisi, Georgia. This paper describes some historical studies of *Brucella* phage performed at the Eliava Institute, as well as a comparative characterization of several novel *Brucella* bacteriophages from the Institute's collection. Phage–host specificity was also examined using DNA restriction and is demonstrated by the efficient plating of bacteriophages grown on different *Brucella* hosts. The lytic reactions of the *Brucella* phage used in this study confirm the data that was also obtained by serological and molecular genotyping methods. Studies of the mechanisms of phage-host specificity support the use of phage typing schemes for the identification of bacterial strains of *Brucella*.

Keywords: Bacteriophage; Host-specificity; Efficiency of plating

Introduction

Brucellosis – a zoonotic disease and potential biological weapon - is caused by the bacterial genus of *Brucella*. The bacteria are transmitted from animals to humans by ingestion of infected food products, direct contact with an infected animal, or inhalation of aerosols. This disease continues to be a major public health concern worldwide and is a very common zoonotic infection throughout the world. Definitive diagnosis of this disease is based on culture (identification of the pathogen), serology, and molecular biological methods. Serological methods traditionally have been used to speciate *Brucella* isolates. Serotyping results should ideally be confirmed by molecular genotyping, a variety of which are available for this purpose: polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [1]; cytoplasmic protein-specific gene probe analysis [2]; Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) [3,4]; or typing with the *rpoB* gene coding the DNA-dependent RNA polymerase (RNAP) β subunit [5,6]. In addition to these molecular methods, phage typing is used as an additional confirmatory tool for the identification of *Brucella* species.

Phage typing using species-specific bacteriophages has been used to differentiate *Brucella* species for many years [7]; the phage Tb, Iz, Wb, Bk, and S708 are those most often used for typing purposes [7]. Despite their utility, the biology of these bacterial viruses is not well understood, particularly with respect to their interactions with host bacterial cells. *Brucella* phage were first isolated at the Tbilisi Institute of Vaccines and Sera (the prior name of the Eliava Institute of Bacteriophages, Microbiology, and Virology) in Georgia by Nemsadze, Popkhadze, and Kilasonidze in 1952 [8] and used to evaluate the activity of phage filtrates on museum cultures and freshly isolated cultures of *Brucella* on solid media.

Improved methods for the isolation and target-specific reinforcement of phage, together with parallel research on the antigenic structure of *Brucella*, led to the isolation of a considerable number of phage from various sources from the 1950s through the 1970s [9-14]. Twenty-three *Brucella* bacteriophages, which were stable at high concentrations [titer 10^{-4} – 10^{-9} by the method developed by Appelmans [15]] were isolated between 1955 and 1962 at the Eliava Institute. Seven of these phage were isolated from the environment, 15 from the blood of human brucellosis patients, and one from a person vaccinated with a live *Brucella* vaccine [16,17]. All 23 phage were specific to *B. abortus*, despite the detection of *B. melitensis* in the blood of three of the brucellosis patients from whom the phage were isolated [16].

Brucella phage typing has a long history [18-23]. The stable *Brucella* phage named Tb, was first isolated from manure in 1955 at the Tbilisi Institute of Vaccines and Sera [23]. Subsequently, this phage was approved by the International Subcommittee on the Taxonomy of *Brucella* as a reference phage for the diagnostics and differentiation of *Brucella* strains [25]. Tb phage have been studied and used for typing purposes by many scientists [25-28]. One series of experiments showed that Tb phage are specific to *B. abortus*; plaques were not observed on lawns of *B. suis*, although phage at high concentrations (10^4 x routine test dilution or greater) did cause inhibition of growth that resembled lysis. High concentrations of Tb phage have also been shown to inhibit the growth of *B. melitensis* [29].

Phage typing has been used to confirm *Brucella* species. In two previous studies, 543 *Brucella* strains from different countries (former Soviet Union, United Kingdom, Poland, Germany, and South Africa) were identified [23,29]. Preliminary speciation was carried out using standard bacteriological and biochemical tests. The results of these studies indicated that 277 strains were *B. melitensis*, 62 strains were *B. abortus*, and 204 strains were *B. suis*. The results of the phage susceptibility testing demonstrated that 177 of the 277 *B. melitensis* strains in the Eliava Institute collection belonged to a single group according to the phage typing scheme described by Morgan

Citation: Antadze I, Dadunashvili M, Burbutashvili T, Gunia S, Balarjishvili N, Tevdoradze E, et al. Diversity of Phage-Host Specificity in *Brucella* Phage. J Bacteriol Mycol. 2017; 4(2): 1049.

[25]; a total of 99 non-Eliava strains also belonged to the same group. Seventy-three strains that were lysed by both dilutions of phage were identified as the *B. abortus* biotype V. All but five *B. abortus* strains were lysed by Tb phage; further investigation indicated that these five strains already contained temperate phage and were not lysed. Among the *B. suis* strains, 40 out of 204 were lysed by phage.

Currently, the Eliava Institute collection includes 44 *Brucella*-specific bacteriophages, including the Tb phage; one phage isolated from *B. canis*; four phage isolated from *B. ovis*; 19 phage isolated from *B. melitensis*; 17 phage isolated from *B. abortus*; and two phage isolated from *B. suis*. All of the phage in the collection are specific; the phage isolated from *B. abortus* and *B. melitensis* cause lysis only in *B. abortus* and a limited number of *B. melitensis* strains. Phages isolated from *B. ovis* and *B. canis* fully lyse *B. abortus* and partially lyse *B. melitensis* but do not lyse *B. ovis*, *B. suis*, or *B. canis*. The current paper describes features of a set of 10 bacteriophages that have been selected for typing purposes for various *Brucella* species. The bacteriophages outlined in this paper were characterized, including an analysis of their reproduction parameters and lytic specificity against various species of *Brucella*.

Materials and Methods

Some of the bacterial strains of *Brucella* used in the study were from the Eliava Institute's bacterial collection: *B. abortus* S19 vaccine strain; *B. abortus* 141, serotype I, originally isolated in Russia; *B. abortus* 544 serotype I; *B. abortus* 99 serotype V, was originally from the UK Weybridge collection; and *B. abortus* 64 serotype III, was isolated in Tbilisi. Among the *B. melitensis* strains in the Eliava museum, N7 was obtained from Saratov, Russia in 1963; and N16 was isolated in Moscow in 1962. The strain N110 was isolated from human synovial fluid in Tbilisi in 1942; N 237 and 238 were isolated from the blood of a brucellosis patient in Tbilisi in 1959. The strains N 71 m/z and 70 v/z were originally isolated from a patient in Bulgaria; 130 m/z was isolated from a patient in Germany; and 238 m/z and 254 m/z were originally isolated from a cow in England. Strain 63/9 was received from Almaty, Kazakhstan in 1979. Additional bacteriophages typing was conducted using bacterial strains that reside at the Louisiana State University (LSU) Ag Center and were as follows: *B. inopinata* BO1 from a breast cancer implant in a brucellosis patient [30]; *B. inopinata* BO2 from the lung biopsy of a patient with chronic pulmonary destructive pneumonia [31]; *Brucella* strain NF2653 from wild native rodents in Australia [32]; and SDRL an atypical *B. abortus* strain from a rat liver sample from San Diego, California [33]. The following *Brucella* phage were used for this study: Tb, originally isolated from manure; phage 1066 from *B. canis*; 281; 02 from *B. ovis*; 177; 110 from *B. melitensis*; V; 544; 141 from *B. abortus*; and 11sa from *B. suis*. All bacteriophages were propagated on two bacterial strains: *B. abortus* S19 and *B. abortus* 141 strains.

Phage spot test

The phage spot test is a common tool to determine phage host specificity. The host range of each phage (phage specificity) was determined by spotting 10 µL of a phage suspension (~10^9 PFU/mL) in nutrient broth onto freshly prepared bacterial lawns and counting the plaques that appeared after 24 and 48 hours of incubation at 37°C. Visual characteristics of the phage plaques (PFU/mL) were also evaluated. The Routine Test Dilution (RTD) is defined as the highest dilution of the phage stock that will produce confluent lysis of a lawn inoculum of the propagating strain. The results of the phage spot test were determined from three sets of experiments after each of the 24 hour and 48 hour time points.

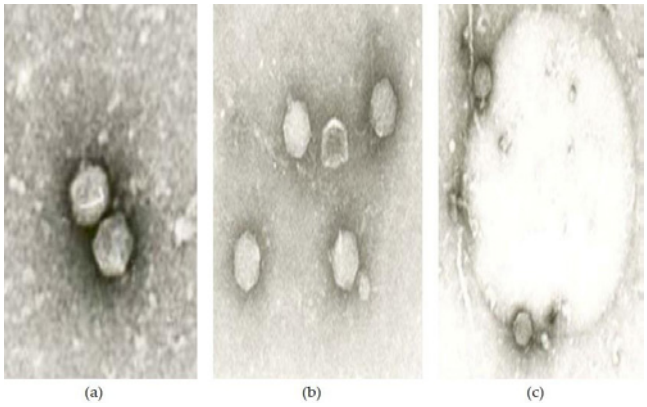

Figure 1: Electron micrographs of *Brucella* phage particles; (a) Phage 260 (magnification x 120,000); (b) phage X (magnification x 100,000), (c) Tb phage adsorbed on *Brucella* cell (magnification x 96,000).

Electron microscopy

The morphology of the phage particles was studied using an electron microscope JEM x100 (JEOL). Parlodion plates were overlaid with 1010 PFU/mL phage suspensions with uranyl acetate as a contrast agent.

Phage structural proteins

Phage suspensions (60 µL at 10^{10} to 10^{11} PFU/mL) were added to 13 µL of sample buffer containing 8% sodium dodecyl sulfate, 0.1% glycerol, and 0.5 % bromophenol blue; 5.0% β-mercaptoethanol was added to the solution prior to use. Samples were boiled for ten minutes and then loaded onto 10% polyacrylamide gels [34]. Electrophoresis was carried out at 60 V, 9 mA for 18 hours in tris-glycine buffer.

Phage biology study

Biological properties, mainly phage-host interaction parameters including adsorption, latent period, lysis time, and average burst size, were calculated by standard methodology [35].

DNA isolation and restriction

Phage DNA was isolated by standard phenol/chloroform deproteinization [36] and with QIAamp DNA mini kits (Qiagen). Several restriction endonucleases were used according to manufacturer's instructions (Biolab). Enzyme-restricted DNA fragments were subjected to electrophoresis on agarose gels. The gels were photographed with ultraviolet illumination.

Results and Discussion

For phage typing, ten bacteriophages (Tb, 141, 281, 544, 1066, 11sa, 02, 177, V, and 110) from the Eliava collection were selected based on the specificity of their lytic reaction on different *Brucella* species.

Morphology of phage

The size and shape of phage plaques varies considerably among *Brucella* phage [37-39]. In general, plaques range from 0.2 mm to

Table 1: Phage reproduction parameters on host bacteria.

Phage	Isolation source	Time of adsorption (min)	% of adsorption	Latent period (min)	Lysis time (min)	Average burst size (PFU/mL)
Tb	liquid manure	190	90	200	460	40-46
Tb*	liquid manure	180	81	210	460	50-53
110	*B. melitensis*	120	70	180-220	440	18-20
141	*B. abortus*	120	76	210-240	450	27-30
11sa	*B. suis*	120	53	180	410	28-30
1066	*B. canis*	150	98	180	425	110-120
02	*B. ovis*	120	82	240	400	36-40
544	*B. abortus*	90-100	82	270-300	480-490	30-35
281	*B. ovis*	90-100	70	250-280	450-460	60-65
177	*B. melitensis*	180	78	240	460	40-45
V	*B. abortus*	180	37	240	480	25-30

Note: Phage reproduction was studied on *B. abortus* 141; Tb phage was grown was on both *B. abortus* S19 (indicated by *) and 141 strains.

Table 2: Similarity in Hind III restriction of Georgian bacteriophages and Np bacteriophage.

Similarity to Np phage	Bacteriophages
Similar DNA restriction	81, 2, 3, 5, 6, 27, 64, 43, 90, VII, VIII, XII, 12(B), 63, 110
Difference in restriction	Tb, 290, 100, BA, 248, 214, 19, 7sa, 141, 109, IV, OX, X, 273, 544, 281, 02, 1066, 177, VI, 11sa
Resistant to Hind III restriction	9, 147, 224, 239, 271

4 mm and are polymorphic on bacterial lawns; some of them are oval or perfectly round while others are more irregular. All *Brucella* phage from the Georgian collection, including the Tb phage, are morphologically identical and similar to other phage described to date [39-41]. They all have icosahedral heads (60-65 nm x 60-70 nm), short tails (14-20 nm; Figure 1), and belong to the Podoviridae family. Only minor differences are visible in the composition of structural proteins of *Brucella* phage

Phage reproduction parameters

The adsorption time of *Brucella* phages differ by host bacterial strain (*B. abortus* 141). Some phage (281, 544) adsorb on the host in less than two hours, while other phage require more time for adsorption (Table 1). Other parameters of reproduction on host bacterial cells for all the phage were prolonged too. The average burst size was comparatively low compared to other phage; the highest phage counts were recorded for phage 1066 (120-125 PFU/mL).

Comparative DNA restriction

DNA restriction analysis of *Brucella* phage genomes show that they only differ slightly from each other, indicating that they are highly conserved. DNA restriction results observed in this study correlate well with the results presented by Rigby et al. [39]; the six *Brucella* phage (including Tb) targeted in this study could not be differentiated by restriction digestion profiles produced by BglI, EcoRI, HindIII, or PvuII. Nepean phage (Np) DNA differed from Tb phage with restriction by PvuII, and BglII but only by one band. The authors explained such a difference in possible self-ligating cos ends in submolar fragments of phage, although further procedures to prevent self-ligation did not demonstrate the existence of cos sequences. A comparison of the phage isolated from Georgia to the Np phage (isolated from an atypical *B. abortus* strain from a cow in Ontario, Canada [39] by DNA HindIII restriction showed that

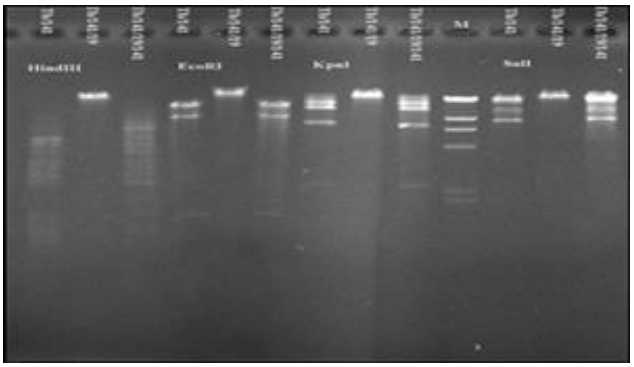

Figure 2: DNA restriction digestions of Tb phage cultivated on different host bacteria.

most of the phage from the Eliava Institute collection differ from both the Tb and the Np phage (Table 2). However, comparative restriction digestion of DNA from phage cultivated on various *Brucella* hosts indicated substantial differences. DNA from Tb phage cultivated on the vaccine strain *B. abortus* S19 was resistant to EcoRI digestion, while DNA from Tb phage grown on *B. abortus* 141 was susceptible to this enzyme. DNA restriction of phage 02 by EcoRV endonuclease also showed some differences: EcoRV digested DNA from phage 02 and from phage 1066 propagated on *B. abortus* S19, but EcoRV digestion did not cleave DNA extracted from phage 02 and from phage 1066 when they were cultivated on *B. abortus* 141. Further, KpnI did not digest 1066 phage DNA. The restriction digestion profiles by PvuII were identical for all phage. To examine these different DNA restriction patterns from phage propagated on different host bacteria, Tb phage that was initially cultivated on *B. abortus* 141 was propagated on *B. abortus* 19 and then *B. abortus* 141. DNA from all three phage preparations was then subjected to

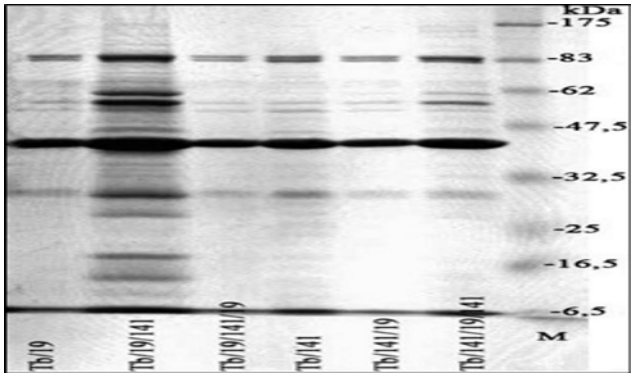

Figure 3: SDS-PAGE analysis of structural proteins of Tb phage propagated on different host bacteria.

Table 3: Efficiency of plating of Tb phage on different host bacterial strains.

Phages cultivated on *B. abortus* strains	Bacterial strain of *B. abortus*			
	141	544	99	64
Tb/*B. abortus*141	1	0.9	0.9	0.9
Tb/ *B.abortus*141/B.a 99	1	0.9	1	0.7
Tb/*B.a.*141/*B.a.*99/B.a 141	1	1	0.9	0.8
Tb/B.a 141/B.a 64	1.2	0.9	1	1
Tb/*B.a.* 141/B.a 64/B.a 141	1	1.1	0.9	0.6
Tb/*B.a.* 99	1.1	0.9	1	1.3
Tb/ *B.a.* 99/ *B.a.* 141	1	1.2	1.3	1.3
Tb/ *B.a.* 99/ *B.a.* 141/ *B.a.* 99	0.9	1	1	0.9
Tb/ *B.a.* 99/ *B.a.* 64	1.2	1.1	1	1
ТБ/ *B.a.* 99/ *B.a.* 64/ *B.a.* 99	1	0.9	1	1
Tb/ *B.a.* 64	1.3	1	1.4	1
Tb/ *B.a.* 64/ *B.a.* 141	1	1.1	0.7	0.5
Tb/ *B.a.* 64/ *B.a.* 141/ *B.a.* 64	1.5	1.3	1.3	1
ТБ/ *B.a.* 64/ *B.a.* 99	1.2	1.3	1	1
ТБ/ *B.a.* 64/ *B.a.* 99/ *B.a.* 64	1.1	1.1	1	1
Tb/ *B.a.* 544	1.3	1	1.2	1
Tb/ *B.a.* 544/ *B.a.* 99	1.1	1	1	1
Tb / *B.a.* 544/ *B.a.* 99/ *B.a.* 544	0.8	1	1	1.1
Tb/ *B.a.*544/ *B.a.* 64	2.9	2.3	2	1
Tb/ *B.a.* 544/ *B.a.* 64/ *B.a.* 544	1.3	1	1.2	1

restriction analysis (Figure 2).

Restriction endonuclease KpnI did not digest the DNA from Tb phage propagated on *B. abortus* 19; the restriction profile of KpnI digestion from Tb phage DNA propagated on *B. abortus* 141, and the profile of Tb phage DNA propagated on *B. abortus* 19 and then on *B. abortus* 141 were similar except for two extra high molecular weight bands. Slight differences were observed in structural proteins of Tb phage cultivated on different hosts (Figure 3).

Efficiency of plating of phage propagated on different host bacteria

High Efficiency of Plating (EOP) of Tb phage propagated on various *B. abortus* strains has been demonstrated in several different studies [38-41]. In our investigations, the host specificity of Tb phage was evident in its EOP when grown on different host bacterial strains. A value more than 1 indicates a high infectivity of phage to the host bacteria. Phages were grown on one bacterial strain, then grown on a different bacterial strain, and then grown once more on the first bacterial strain. In general, the phages grown on different hosts revealed a high EOP on different bacterial strains, but *B. abortus* 141 might be considered the best host for the phages used in this study (Table 3).

Lytic activity

Phage are used for typing at two concentrations, RTD and 10^4 x RTD, as recommended by the Subcommittee on the Taxonomy of *Brucella*. All smooth *B. abortus* strains are lysed by Tb phage at both concentrations. The set of ten phages used in this study were then used for typing 11 archival stains of *B. melitensis*. *B. melitensis* strains are generally resistant to Tb phage because these strains are smooth, although at high concentrations Tb phage may cause lysis. All smooth *B. suis* strains are resistant to Tb phage at RTD and are lysed at 10^4 x RTD. In this study, different bacteriophages at a high titer occasionally lysed the 11 archival strains of *B. melitensis* without infecting the organism (the phenomenon called "lysis from without" or soluble phage lysis effects) [42]. Semi-confluent lysis of strain N7 by phage Tb, 11sa, V, 177, 281, and 544 was observed; in addition the lysis of strain 16 by phage Tb, V, 110, 141, 177, 281, and 544. Bacterial strains 237, 238, 238 m/z, and 254 m/z were lysed at both dilutions and were therefore classified as *B. abortus* serotype V. Only one strain (63/9) was lysed by phage 1066 at both dilutions used in these experiments. Phage typing of the *Brucella* strains from the LSU AgCenter collection using ten phage cultivated on two hosts (*B. abortus* S19 and *B. abortus* 141) showed that *B. canis* and *B. ovis* are resistant to lysis by phage. The 10 phage used in this study also did not reveal any reaction against *B. inopinata* isolated from a patient with chronic lung infections; however, weak activity was observed against another strain of *B. inopinata* (Table 4). Several phages caused lysis of *B. suis*, though only in high concentrations that could be connected to lysis without infection, while *B. neotomae* was lysed by both concentrations of phage. In some cases, the susceptibility to a given phage varied depending on which host strains were used for cultivation. Phage 544 propagated on *B. abortus* 141 showed lytic reaction on strains *B. abortus* RB51 (with EOP $4x10^{-6}$) and non-typical *B. abortus* SDRL at both phage concentrations, while the same phage grown on *B. abortus* S19 did not lyse either of those strains. Phage 544 propagated on *B. abortus* 141 demonstrated low EOP on the phage-sensitive *B. abortus* RB51 strain. The same phage grown on *B. abortus* S19 revealed a high efficiency on sensitive strain *Brucella* F2653 but low efficiency on *B. neotomae* (Table 5). Phage 544 cultivated on *B. abortus* 141 did not lyse the *B. inopinata* BO1 strain. The same phage grown on *B. abortus* S19 showed semi-confluent lysis at RTD and no reaction at higher dilutions, which would be indicative of lysis without infection.

Discussion

Research of *Brucella* bacteriophages has a long history, although the mechanisms of virus interaction with the host bacteria *Brucella* – a slow-growing organism - remains unknown. Further, there is a

Table 4: Lysis of LSU AgCenter *Brucella* strains by bacteriophages.

Bacteriophages cultivated on different *B. abortus* strains	RTD	Bacterial strains						
		***B. abortus* 19**	***B. abortus* 19 (Shreveport)**	***B. abortus* RB51**	***B. inopinata* BO1**	***Brucella* species NF2653**	***B. neotomae***	***B. abortus* SDRL**
TB-19/TB-141	3 RTD	CL/CL	CL/CL	R/R	OL/OL	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	CL/CL	R/R
02-19/02-141	3RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	CL/CL	R/R
	RTD	SCL/CL	CL/CL	R/R	R/R	SCL/SCL	CL/CL	R/R
1066-19/ 1066-141	3 RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	SCL/SCL	CL/CL	R/R
281-19/281-141	3 RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	CL/CL	R/R
177-19/177-141	3 RTD	CL/CL	CL/CL	R/R	OL/OL	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	SCL/SCL	SCL/CL	R/R
544-19/544-141	3 RTD	CL/CL	CL/CL	R/SCL	SCL/R	CL/CL	CL/CL	R/SCL
	RTD	CL/CL	CL/CL	R/IPO4	R/R	CL/SCL	IPO100/SCL	R/IPO1
11sa-19/ 11sa-141	3 RTD	CL/CL	CL/CL	R/R	OL/OL	CL/SCL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	SCL/SCL	SCL/SCL	R/R
V-19/V-141	3 RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	CL/SCL	R/R	R/R
141-19/141-141	3 RTD	CL/CL	CL/CL	R/R	OL/OL	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	SCL/IPO100	R/R
110-19/110-141	3 RTD	CL/CL	CL/CL	R/R	OL/OL	CL/CL	CL/CL	R/R
	RTD	CL/CL	CL/CL	R/R	R/R	CL/CL	SCL/IPO500	R/R

Note: Bacteriophages were grown on two hosts: *B. abortus* S19 and *B. abortus* 141. 1˚ = phage at 1RTD; 2˚ = phage at 4 RTD; CL= Confluent Lysis; SCL = Semi-Confluent Lysis; OL = Opaque Lysis; IPO = Individual Plaques Opaque; R = Resistant.

Table 5: Efficiency of plating of phage 544 with different hosts on different bacterial strains.

Bacterial strains	544 propagated on *B. abortus* 141 (titer $4.4x10^9$ PFU/mL)		544 propagated on *B. abortus* S19 (titer $1.2x10^{10}$ PFU/mL)	
	Titer	EOP	Titer	EOP
B. abortus RB51	$2x10^4$	$4x10^{-6}$	-	-
Brucella species NF2653			$6.9x10^9$	0.57
B. neotomae			$2x10^7$	$1x10^{-4}$
B. inopinata BO1			complete lysis in initial dilution	-

wealth of *Brucella* phage work in older scientific publications from the former Soviet Union that is still not available to the scientific society. In this paper, we tried to present some limited information about the *Brucella* phage studies from the old Soviet periodicals. *Brucella* bacteriophages are used to type various *Brucella* species; and when taken together with other identification methods, phage typing is a useful tool for such purposes. A set of typing phages and phage-typing schemes has existed for many years, but this study is an attempt to include more phages into typing schemes, providing a better understanding of phage biology and bacterial phage-host interactions.

Based on the historic scientific literature, phages against *Brucella* species are relatively homogenic. In this study, ten phages were selected for typing purposes. These phages exhibited differences in phage plaques on solid media, which was generally a characteristic feature for *Brucella* phages. All the phage particles have an icosahedral head and short tail. The reproduction cycle of these phages of these slowly growing bacteria is also very slow. Reproduction parameters for Tb phage were previously studied by Antadze and Popkhadze [38]. Each of the phage analyzed in this study demonstrated a slow growth cycle on host bacterial cells.

Phage host bacteria cell interaction mechanisms are not well determined for *Brucella* phages. Efficiency of plating, which is derived from the ratio of the phage titer on the target strains to the phage titer on the host strains, was examined for phages cultivated on different hosts. Phages were grown on one strain, then grown on a different strain, and then grown once more on the first strain. An EOP value greater than 1 indicates a high efficiency of cultivation. The efficiency of cultivation of Tb grown on different hosts is generally high, but Tb cultivated on *B. abortus* strain 64 and then *B. abortus* strain 544 showed higher EOP than that on other hosts. The EOP of bacteriophages cultivated on *B. abortus* strain S19 followed by *B.*

abortus strain 141 was greater than 1, indicating that phage have high efficiency on *B. abortus* 141.

A total of 11 archival bacterial strains of *B. melitensis* from the Eliava Institute were typed by ten bacteriophages in this study. The data show that four bacterial strains out of 11 were lysed by both dilutions (RTD and 4TD) of phages, therefore they were identified as *B. abortus* serotype V. Lytic reactions against *B. melitensis* were caused by the phenomenon characteristic of some temperate phages.

Brucella species are very similar [46]; the DNA restriction maps of different *Brucella* strains show that a 640-kb insertion was found in the small chromosome of *B. abortus* 544 biovar 1 [47]. Division of the genus into species and biovars is based solely upon a small number of metabolic traits, two lipopolysaccharide epitopes, and sensitivity to a set of phage that are host-range variants of the same ancestor [48]. Although phage susceptibility to different species is evident, it indicates the significance of phage typing as a tool for *Brucella* species differentiation. The sequence of the omp2 locus in different *Brucella* species supports the existence of species-specific lineages. A close relationship has been found between *B. melitensis* and *B. abortus*, while an extreme divergence of *B. ovis* and *B. neotomae* from the other species was observed. This divergence may be the result of a gene conversion for this locus in these two species [48]. In this study, we found that *B. neotomae* strains are only partially susceptible to Tb phage, producing a few isolated plaques at RTD. They are completely lysed by the phage at 10^4 x RTD. *B. ovis* and *B. canis* strains are invariably resistant to the phage at both dilutions.

It has been shown that cultivating bacteriophages through several rounds on different hosts will increase phage lytic activity; this is known as a way to adapt phage on various hosts. Even after adaptation, phages preserve their specificity against various species of *Brucella*. The phages in the study do not show any activity against *B. canis* and *B. ovis*; however, several phages caused lysis of *B. suis*, though only in high concentrations, which may be connected to the phenomenon of lysis without infection, while *B. neotomae* was lysed by both concentrations of phage.

Host specificity plays an important role in the biology of *Brucella* phage. DNA restriction of different bacteriophages against *Brucella* shows homogeneity of these bacterial viruses. Evident differences are shown in susceptibility to the restriction enzymes of phages grown on different hosts. Differences in DNA restriction patterns of various phage propagated on different hosts might be explained by the temperate nature of *Brucella* phage, particularly by possible horizontal gene transfer between host bacteria facilitated by bacteriophages. Since most *Brucella* bacteriophages are isolated from bacterial strains, there is a high probability of detecting phage genome fragments in the host. However, this hypothesis does not align with the results from previous experiments by Rigby et al. Southern blot analysis of 32P-labelled Tb DNA did not hybridize to any fragment of HindIII- or EcoRI-digested chromosomal DNA of *B. abortus* S19 or any other *Brucella* species [39].

Brucella phage described in different scientific publications are members of the same family Podoviridae. These phages have the same morphology (icosahedral head and short tail) and have similar DNA restriction profiles with only some minor differences (the extra bands visible in some patterns may be due to repetitions or shared base sequences that affect the locations of endonuclease recognition sites). Phage Tb produces divergent PCR profiles with various arbitrary primers compared to host strain *B. abortus* S19 fragments. The complete genome of this unique phage is already sequenced [44]; genome analysis will explain all functional peculiarities connected to phage-host interaction. The most notable differences among the *Brucella* phage reside in their host ranges and phage-specific lytic reactions to different bacterial strains [43]. In a previous study by Tevdoradze [49], the full genomic sequences of several of the bacteriophages used in this study were reported, revealing pronounced sequence homogeneity. Interestingly, fine-scale genetic variability of these phages grown on multiple hosts within a single *Brucella* species remains unknown; and the genomic changes, as a result of passaging, were observed in similar genes and predominantly occurred at identical sites in separate phages. This 2015 study also showed that there were multiple instances of 'within-sample' genetic heterogeneity observed often at shared genomics positions across phages. Our study supports the previous work and shows that bacteriophages propagated on two distinct host bacterial strains illustrates multiple common sequence variations which frequently display within-sample genetic heterogeneity [49].

The phage-host bacterial interaction study clearly demonstrated in *Brucella*-specific bacteriophages is an interesting issue of phage biology. Over the course of their evolution, bacteriophages have developed unique responses to their hosts; although the biological features of temperate phage are generally dependent on host bacteria. Using phage typing as an additional tool for the differentiation of *Brucella* species is still a reliable methodology and has been validated by scientists and medical workers worldwide. Identification of *Brucella* species based on phage-specific lytic reactions can be used to confirm the results obtained by serological and molecular genotyping methods. The mechanisms of host specificity of phage, however, are not completely clear; and further investigation of phage-bacterial genomics is needed to elucidate *Brucella* phage biology and explain the unique interactions that have been observed between these bacteria and their viruses.

Acknowledgments

This research was made possible by financial support provided by the U.S. Department of Defense (DoD), Defense Threat Reduction Agency (DTRA), through the Cooperative Biological Engagement Program's (CBEP) Cooperative Biological Research Project (CBR) GG-17, in Georgia. The findings, opinions and views expressed herein belong to the authors and do not reflect an official position of any organization listed. The authors are grateful to the US Centers for Disease Control and Prevention for supplying the atypical *Brucella* strains to the LSU Ag Center. The authors also thank Attimo Research and Development, and Sonya Narodny for editorial assistance.

Author Contributions

R.O. and M.K. conceived and designed the experiments; I.A., M.D., T.B., S.G., N.B., E.T., T.P., and M.K. performed the experiments; I.A., M.D., T.B., S.G., N.B., E.T., T.P., R.O., S.H., P.E., and M.K. analyzed the data; S.H., P.E., and M.K. contributed reagents/materials/analysis tools; R.O., S.H., P.E., and M.K. wrote the paper."

Conflicts of Interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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J Bacteriol Mycol - Volume 4 Issue 2 - 2017
ISSN : 2471-0172 | www.austinpublishinggroup.com

Citation: Antadze I, Dadunashvili M, Burbutashvili T, Gunia S, Balarjishvili N, Tevdoradze E, et al. Diversity of Phage-Host Specificity in *Brucella* Phage. J Bacteriol Mycol. 2017; 4(2): 1049.