The Examination of *Brucella* Cultures for Lysis by Phage

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SUMMARY

A total of 3919 brucella cultures was examined for lysis by five brucella phages, using a routine test dilution (R.T.D.) and $10,000 \times$ R.T.D. All cultures of *Brucella abortus* examined were lysed by all five phages at both dilutions. In addition, all cultures with the oxidative metabolic pattern characteristic of *B. abortus*, irrespective of their properties as determined by conventional typing methods, were lysed by both phage dilutions. Cultures of *B. suis* were not lysed by phages at R.T.D. but all showed lysis by phages at $10,000 \times$ R.T.D. Cultures of *B. melitensis* and those with the oxidative metabolic pattern characteristic of *B. melitensis*, irrespective of their properties as determined by conventional typing methods, were not lysed by phages at either dilution. All five phages used (Tb, 10/I, 24/II, 212/XV and 371/XXIX) displayed an identical host range; this was confirmed by neutralization tests with antiphage sera.

INTRODUCTION

In a previous publication (Morgan, Kay & Bradley, 1960) it was shown that a brucella phage obtained from the U.S.S.R. (Tbilisi or Tb phage) when used at a routine test dilution was able to lyse cultures of *Brucella abortus* but not of *B. suis*. Some of the *B. melitensis* cultures examined were lysed but others were not. Cultures of *B. melitensis* isolated abroad (U.S.A., Malta) were not lysed whilst cultures with the biochemical and serological properties of *B. melitensis* isolated from cattle in Great Britain were lysed. It was therefore possible to distinguish between these British cultures and the Mediterranean strains. Four new phages isolated in Poland (Parnas, 1961) have now become available to us. The purpose of the present paper is to report on the examination of these phages when tested with a larger collection of brucella cultures.

METHODS

Phages. In addition to the Soviet phage Tb, four other phages received from Professor J. Parnas (Lublin, Poland), were used; phages 10/I, 24/II and 371/XXIX had been isolated from carrier cultures of *Brucella abortus* and phage 212/XV from a culture of *B. suis*. Phage stocks were prepared by the double agar layer technique (Adams, 1959) with a suspension of organisms from a 24 hr. culture of *B. abortus* 544 (FAO/WHO reference strain) as propagating culture. The phages were suspended in phosphate buffer (pH 7.2) containing albumin (Fildes & Kay, 1955), the suspension centrifuged and filtered through Oxoid millipore (0.8μ diam.) filters and stored

at 4°. All the phages were used at a routine test dilution (R.T.D.) prepared in phosphate buffer containing albumin with *B. abortus* 544 as the indicating culture. The number of plaque-forming particles (pfp)/ml. in these preparations was assayed by the method of Adams (1959) with *B. abortus* 544. Counts were made after 48 hr. of incubation at 37° in air containing 10 % (v/v) added CO₂.

Partial purification of phage Tb was made by alternate low- and high-speed centrifugation. Phage was centrifuged at 100,000 *g* in a Spinco centrifuge and resuspended, after two such centrifugations, in albumin buffer.

An antiserum against phage Tb was prepared in rabbits by using as antigen a crude preparation containing 2.5×10^{10} pfp/ml. Four injections were given every other day, and a week after the last injection two more were given after an interval of 2 days. Blood was collected 10 days after the last injection and the sera Seitz-filtered and stored at 4°. Antisera were also prepared in rabbits against the partially purified phage Tb. Neutralization tests for assessing the K values were made by the method of Adams (1959).

Brucella cultures. Cultures recently isolated at this laboratory or sent here from various parts of Great Britain for routine typing constituted the majority of the cultures studied. Tests were made as soon as possible after isolation. Cultures received from other parts of the world were also studied. The type and countries of origin of these cultures are given later in Tables 1 and 4. All cultures were typed by conventional methods (as recommended by the World Health Organization Expert Committee on Brucellosis; Report, 1953); details of the technique and media have already been published (Morgan, 1961).

Media. Serum glucose agar (Morgan, 1960) was used for the growth of cultures and as the basal medium for the propagation and plaque-counting of phage. For the double agar layer technique, the top layer consisted of nutrient agar + 0.5 % (w/v) glucose and 0.7 % (w/v) agar.

For routine typing, growth from 48 hr. slopes was suspended in buffered saline (pH 6.4) and a loopful streaked across well-dried serum glucose agar plates. When the five phages were used, three cultures were streaked; plates were dried with the lid partly open for 1 hr. at 37°. One drop (0.02 ml.) of each phage suspension at its routine test dilution (R.T.D.) was spotted on the cultures, allowed to dry and the plates incubated for 48 hr. in air + 10 % (v/v) CO₂.

RESULTS

Phage preparations containing between 2 and 6×10^{11} pfp/ml. were obtained. R.T.D. preparations contained approximately 1×10^7 pfp/ml. Plaques were not observed when *Brucella suis* 1330 or *B. melitensis* 16M were used as lawns; but lysis of *B. suis* though not of *B. melitensis* occurred at the highest dilutions of phage (10^{-1} and 10^{-2} with high titre stocks).

In Table 1 are given the results of testing with phage, at a routine test dilution, 3919 brucella cultures. Of 3775 cultures isolated in Britain, representing *Brucella abortus* type I, *B. abortus* type II (dye-sensitive), the variety which is *B. abortus* biochemically and culturally but *melitensis* serologically and the variety isolated in Great Britain from cattle which has the biochemical, cultural and serological properties of *B. melitensis*, 3736 cultures (98.8 %) were lysed by all the phages used.

Many of the cultures that were not lysed were rough; the remainder had been disposed of before tests for roughness were made.

Of 144 cultures received from abroad, all 23 strains of *Brucella abortus* III (thionin-resistant) were lysed and none of 22 cultures of *B. suis*. Fifteen of 37 cultures of *B. melitensis* were lysed by phage, thus resembling these cultures with

Table 1. *The lytic effect of brucella phage at its routine test dilution (R.T.D.) on 3919 brucella cultures*

Species or type	No. of cultures		Remarks
	Examined	Lysed	
<i>B. abortus</i> type I and type II	3454	3423	Britain
<i>B. abortus</i> type III (thionin-resistant)	23	23	Germany, Uganda and Turkey
<i>B. abortus</i> type III, but agglutinating in both monospecific sera	4	4	Turkey
Biochemically <i>B. abortus</i> /serologically <i>B. melitensis</i>	65	65	Includes 3 cultures that were dyesensitive/serologically <i>B. melitensis</i> (Britain)
Biochemically <i>B. melitensis</i> /serologically <i>B. abortus</i>	13	7	4 (Turkey), 1 (Germany) and 1 (Khartoum) lysed. 7 (Turkey) not lysed
<i>B. melitensis</i> isolated from cattle in Britain	256	248	Britain. The 8 not lysed were rough
<i>B. melitensis</i> (foreign)	37	15	14 (Germany), 1 (Uganda) lysed. 3 (Africa), 1 (Mexico), 1 (U.S.A.), 15 (Germany), 2 (Malta) not lysed
<i>B. melitensis</i> (foreign), but agglutinating in both monospecific sera	45	6	5 (Uganda), 1 (Turkey) lysed. 22 (Germany), 9 (Italy), and 8 (Turkey) not lysed
<i>B. suis</i> type I (4 cultures), <i>B. suis</i> type II (16 cultures), <i>B. suis</i> type III (2 cultures)	22	0	Denmark, U.S.A., Switzerland, Germany and Singapore

the biochemical and serological properties of *B. melitensis* isolated from cattle in this country. Six cultures that were culturally and biochemically *B. melitensis* but were *B. abortus* serologically were lysed by phage, as also were six cultures that were *B. melitensis* but which agglutinated in both 'abortus' and 'melitensis' monospecific sera.

In every case a culture was either lysed by all five phage preparations or by none; in no case was a culture lysed by one or two phages and not by the others.

The effect of using phage at R.T.D. and at 10,000 × R.T.D. was examined on a small number of cultures of each of the three main species; the results are in Table 2. Strains of *Brucella abortus* were lysed by both dilutions, *B. melitensis* by neither; the strains of *B. suis* showed complete lysis with 10,000 × R.T.D. but no lysis with R.T.D. With these *B. abortus* strains, resistant colonies developed in the area of lysis on incubation for four days but resistant colonies did not develop with *B. suis* strains.

To determine whether this apparent lysis of *Brucella suis* was caused by phage or by some toxic factor(s) present in the lysate, phage Tb was partially purified by differential centrifugation. The results (Table 3) show that the partially purified

phage containing 6.5×10^{11} pfp/ml. caused lysis of *B. abortus* and *B. suis* but not of *B. melitensis*. At the R.T.D., only *B. abortus* cultures were lysed. The supernatant fluid obtained after high-speed centrifugation caused no lysis of *B. suis* nor *B. melitensis* but caused lysis of *B. abortus* because of the presence of undeposited phage (45×10^8 pfp/ml.). Attempts to propagate phage Tb with *B. suis* as propagating culture (in solid and liquid media) were unsuccessful, the filtrate either gave no lysis of *B. abortus* or *B. suis* or only a very weak effect on *B. abortus* with the undiluted preparation.

Table 2. *The effect of using phage at routine test dilution and at 10,000 $\times$ routine test dilution*

Strain	Lysis by phage*									
	Tb		10/I		24/II		212/XV		371/XXIX	
	10,000 $\times$		10,000 $\times$		10,000 $\times$		10,000 $\times$		10,000 $\times$	
	R.T.D.	R.T.D.	R.T.D.	R.T.D.	R.T.D.	R.T.D.	R.T.D.	R.T.D.	R.T.D.	R.T.D.
<i>B. abortus</i> 544 (FAO/WHO reference strain)	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.
<i>B. abortus</i> S19	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.
<i>B. abortus</i> 61/1	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.	C.L.
<i>B. melitensis</i> 16M (FAO/WHO reference strain)	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.
<i>B. melitensis</i> 177	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.	N.L.
<i>B. suis</i> 1330 (FAO/WHO reference strain)	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.
<i>B. suis</i> 61/59 (type II)	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.
<i>B. suis</i> 61/178 (type II)	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.	N.L.	C.L.

* C.L. = Complete lysis; N.L. = no lysis.

Table 3. *The lytic effect of partially purified phage Tb*

Phage preparation	Species*			
	<i>B. abortus</i> 544	<i>B. melitensis</i> 16M	<i>B. suis</i> 1330	<i>B. suis</i> 20 other cultures
Phage Tb undiluted stock preparation (2.5×10^{11} pfp/ml.)	C.L.	N.L.	C.L.	C.L.
Phage Tb at routine test dilution (20×10^6 pfp/ml.)	C.L.	N.L.	N.L.	N.L.
Phage Tb partially purified by 2 centrifugations at 100,000g for 1 hour (6.5×10^{11} pfp/ml.)	C.L.	N.L.	C.L.	C.L.
Phage Tb partially purified R.T.D. (25×10^6 pfp/ml.)	C.L.	N.L.	N.L.	N.L.
Supernatant of phage Tb collected after centrifugation at 100,000g for 1 hr. (45×10^8 pfp/ml.)	C.L.	N.L.	N.L.	—

* C.L. = complete lysis; N.L. = no lysis; — = test not done.

The results obtained by using phage at the two concentrations on further cultures are presented in Table 4. Cultures that were lysed by phage at R.T.D. were also lysed by phage at $10,000 \times$ R.T.D. All the cultures of *Brucella suis* showed lysis by phage at $10,000 \times$ R.T.D. but not at R.T.D. On the other hand, of 31 cultures of *B. melitensis* which were not lysed at R.T.D. none was lysed at $10,000 \times$ R.T.D. These included *B. melitensis*, cultures with the biochemical and cultural characters of *B. melitensis* but which agglutinated in both monospecific *abortus* and *melitensis* sera as well as cultures that were biochemically *B. melitensis* but *abortus* serologically.

Table 4. *Comparison of the lytic effect of Brucella phage at routine test dilution and at $10,000 \times$ R.T.D.*

Particulars of lysis	Particulars of cultures
Lysed by phage at R.T.D. and at $10,000 \times$ R.T.D.	20 cultures of <i>B. abortus</i> (type I) 30 cultures of 'British' <i>B. melitensis</i> including strains from Germany, Italy and Uganda 18 cultures of <i>B. abortus</i> type III 4 cultures of <i>B. abortus</i> type III but agglutinating in both monospecific sera 3 cultures biochemically <i>B. abortus</i> but serologically <i>melitensis</i> 1 culture biochemically <i>B. melitensis</i> but agglutinating in both monospecific sera
Lysed by phage at $10,000 \times$ R.T.D. but not lysed at R.T.D.	22 cultures of <i>B. suis</i>
Not lysed by phage at either concentration	13 cultures of <i>B. melitensis</i> (including WHO/FAO reference culture 16M) and others from Malta, Mexico and Germany 15 cultures of <i>B. melitensis</i> but agglutinating in both monospecific sera (7 from Italy and 8 from Germany) 3 cultures of <i>B. melitensis</i> biochemically but <i>abortus</i> serologically (1 from Germany and 2 from Italy)

Table 5. *The neutralization of 5 Brucella phages by phage antiserum*

Phage	K value using phage antiserum produced against	
	Tb phage crude preparation	Tb phage, partially purified
Tb	492	165
10	485	163
24	502	144
212	640	157
371	587	145

The results of neutralization tests of the five phages by a phage antiserum produced in rabbits against crude and partially purified phage Tb are given in Table 5. The results clearly show that the five phages are very closely related antigenically.

DISCUSSION

By using the conventional methods of species differentiation in the genus *Brucella*, most cultures can be placed into one of the three species—*B. abortus*, *B. melitensis* or *B. suis*; but over the years, an increasing number of cultures has been found which show some properties of two species, e.g. those which are *B. abortus* biochemically but *B. melitensis* serologically; cultures which are *B. melitensis* biochemically but *B. abortus* serologically; cultures which are *B. melitensis* except that they produce H_2S or require added CO_2 for growth (Stableforth, 1959).

By studies of oxidative metabolism Meyer & Cameron (1961*a, b*) and Meyer (1961) showed that each of the species of *Brucella* showed a characteristic metabolic pattern and that biotypes within the species also gave the metabolic pattern characteristic of the species. Many of the cultures used in the present paper (*Brucella melitensis* from Britain as well as practically all the cultures received from abroad) have been examined for their oxidative metabolic patterns (Meyer, 1962; Meyer & Morgan, 1962). These studies showed that, without exception, all cultures with the oxidative metabolic pattern characteristic of *B. abortus*, irrespective of their biochemical and serological properties as determined by conventional methods, were lysed by phage at R.T.D. Cultures which showed the oxidative metabolic pattern of *B. melitensis*, irrespective of their serological properties, were not lysed by phage at R.T.D. There is, therefore, no correlation between susceptibility to lysis by phage and serological and biochemical properties as determined by conventional methods.

By using two dilutions of phages it was possible to divide the cultures into species corresponding with oxidative metabolic patterns. The apparent lysis of *Brucella suis* by concentrated phage has not been reported previously; that such lysis was not due to some toxic factors present in the lysate was shown by the results obtained by partially purifying the phage by centrifugation. Replication of phage in *B. suis* has not been observed and the apparent lytic effect of $10,000 \times$ R.T.D. may be due to lysis from without. The number of cultures of *B. suis* examined was, however, small and the observations need to be extended.

From the host range tested on a large number of cultures, all five phages studied were identical. This close similarity was confirmed by neutralization tests with phage antiserum.

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