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Presence of a dialysable fraction in normal bovine whey capable of killing several species of bovine mycoplasmas

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SUMMARY

Most normal bovine whey samples contain a fraction that survives heating at 56° C. for 30 min., passes through a dialysis membrane and kills a maximum of seven out of ten of the different bovine mycoplasma species tested. Some whey samples appear more active than others but not all affect the same strains of mycoplasma indicating some specificity in their action. Absorption of the active factor from whey by heterologous and homologous mycoplasmas and by erythrocytes was observed. Binding of the factor to mycoplasmas appears to be temperature-dependent and non-specific, but subsequent mycoplasmacidal action shows some specificity.

INTRODUCTION

Brownlie, Howard & Gourlay (1974*a*) reported that normal bovine whey was capable of killing ureaplasmas (T-mycoplasmas, Shepard *et al.* 1974). This activity of whey was not destroyed by 60° C. for 1 hr. but was lost after dialysis against phosphate buffered saline or barbitone buffer.

The purpose of the study reported here was to determine whether other mycoplasmas, representing ten species which have been isolated from cattle, were killed by the same or similar dialysable, heat stable substances. The possibility that other mycoplasmacidal factors might exist in whey was also examined.

METHODS

Media

The broth used to grow ureaplasmas and *Mycoplasma verecundum* (U3 broth) was similar to U2 broth (Howard & Gourlay, 1973) except that penicillin was replaced by ampicillin, 1 mg./ml. (Beecham Laboratories, Brentford, England), and magnesium sulphate 2·5 μg./ml. was added (Furness, 1973). Solid medium was similar to U3 broth but contained 20%, v/v, fetal calf serum, 0·8% Agarose; 0·05 M Hepes, 0·57 mM L-cysteine, and no phenol red or urea.

Glucose fermenting mycoplasmas, *M. agalactiae* var. *bovis* and *M. bovigenitalium* were grown in glucose serum (GS) broth and the corresponding solid medium (Gourlay & Leach, 1970) containing ampicillin (Andrews, Leach, Gourlay & Howard,

1973). Arginine metabolising mycoplasmas were grown in arginine broth and the corresponding solid medium (Gourlay, Mackenzie & Cooper, 1970).

Strains

Ureaplasma strains A417, T488, T95, B101 and Vic9 have been described previously (Brownlie *et al.* 1974*a*). The strains of *M. dispar*, *M. verecundum* and *Acholeplasma laidlawii* listed in table 1 have also been described previously (Howard, Gourlay & Collins, 1974; Gourlay & Wyld, 1972; Gourlay, Leach & Howard, 1974). *M. bovirhinis* strains 010C and PG43 were previously described (Howard & Gourlay, 1974); strains C56R and C155 were obtained from Dr Carmichael (Langer & Carmichael, 1963, strains 56R and 155) and purified as for the other strains (Howard & Gourlay, 1974). The remainder of the strains, listed in Table 1, were provided by Dr R. H. Leach, Mycoplasma Reference Laboratory, Colindale, London.

Assay of mycoplasmacidal activity

Whey was prepared as previously described (Brownlie *et al.* 1974*a*) and stored at −20° C. Each whey sample was from a different animal. Mycoplasma strains were screened for susceptibility to killing by whey by a modification of the method previously described (Brownlie *et al.* 1974*a*). One volume of broth culture of mycoplasmas, diluted in barbitone buffer (Oxoid complement fixation test diluent) to give about 10^6 viable organisms per ml., was added to nine volumes of whey. Incubation was at 37° C. for 4 hr. unless otherwise stated. The number of viable organisms present was determined as colony forming units (c.f.u.) before and after incubation.

Killing of mycoplasmas by whey is expressed as the decrease in $\log_{10}$ colony count (= $\log_{10}$ number of c.f.u. present at time 0 − $\log_{10}$ number of c.f.u. present after the stated length of exposure to whey). The control was barbitone buffer containing 2%, v/v, fetal calf serum or 0·1% bovine serum albumin (Armour, Eastbourne, fraction V). Most strains survived without decrease in the buffer for 4 hr. at 37° C. In some cases a decrease in c.f.u. of up to 0·5 $\log_{10}$ occurred. Any loss of viability in buffer is either stated in the text as it occurred or a corrected decrease in colony count was calculated (= decrease in $\log_{10}$ colony count in sample − decrease in $\log_{10}$ colony count in buffer).

The tables represent the results of one assay performed in duplicate. Duplicate experiments gave almost identical results.

Absorption of whey

Mycoplasmas were grown in 200 ml. of GS broth, harvested by centrifugation, washed and resuspended in 2 ml. of barbitone buffer. The suspensions were divided into two and the pellets of organisms used to absorb 2 ml. volumes of whey for 2 hr. at 37° C. on two occasions. Micro-organisms were removed by centrifugation followed by successive filtration through 1200, 450 and 220 nm Millipore filters. Absorption of whey by two pellets of bovine erythrocytes was performed in the same manner.

Table 1. *Mycoplasmacidal activity of four bovine whey samples for 27 mycoplasma strains representing ureaplasmas and ten named species*

	Inoculum*	Decrease (corrected†) in $\log_{10}$ colony count after 4 hr. incubation with whey at 37° C. Whey sample 1	2	3	4
M. bovigenitalium M338/70	5·8	⩾ 2·7	⩾ 2·7	0·6	⩾ 2·7
M. bovigenitalium M991/70	7·4	1·7	0·6	0	0
Ureaplasma sp. Vic9	4·1	⩾ 2	⩾ 2	⩾ 2	⩾ 2
Ureaplasma sp. T488	3·9	⩾ 2·2	⩾ 2·2	1·2	⩾ 2·2
Ureaplasma sp. A417	3·8	⩾ 2·2	⩾ 2·2	0·2	⩾ 2·2
Ureaplasma sp. B101	3·8	⩾ 2	⩾ 2	0	⩾ 2
Ureaplasma sp. T95	4·9	⩾ 3·2	⩾ 3·2	0	⩾ 3·2
A. laidlawii BN1	5·1	⩾ 3·5	2·3	0	0
A. laidlawii 1307/68	5·5	⩾ 3·8	3·1	0	0
A. laidlawii 1305/68	5·5	1·7	2·6	0	0
A. laidlawii 011	5·6	0·4	0	0	0
M. agalactiae var. *bovis* M720/70	7·3	⩾ 4·2	2·4	0	0·8
M. agalactiae var. *bovis* NCTC10131	6·8	3·8	2·9	0	0·5
M. arginini M591/70	5·6	0·8	0·9	0	0
M. arginini M870/70	5·5	0	0·2	0	0
A. modicum PG49	5·3	0·5	0·4	0	0
M. bovoculi M165/69	5·4	1·0	0·7	0·6	⩾ 3·8
M. bovirhinis PG43	5·1	0	0	0	⩾ 3·5
M. bovirhinis 010C	3·8	0	0	0	⩾ 2·2
M. bovirhinis C56R	4·6	0	0	0	⩾ 3·0
M. bovirhinis C155	4·8	0	0	0	⩾ 3·2
M. dispar Vic12	3·8	0	0	0	⩾ 2·2
M. dispar 462/2	4·2	0	0	0	0·9
M. dispar F370	4·4	0	0	0	0·8
M. verecundum 107 (NCTC10145)	5·0	0	0	0	0
M. verecundum 108	4·8	0	0	0	0
M. alkalescens NCTC10135	5·9	0	0	0	0

* No. ($\log_{10}$) of viable organisms present in whey at 0 min.
† Corrected decrease = decrease in whey − decrease in control buffer.

RESULTS

Mycoplasmacidal activities of four bovine whey samples for twenty-seven mycoplasma strains

Whey samples from 4 cows were tested for their ability to kill 27 different mycoplasma strains representing ten different species of *Mycoplasma* and *Acholeplasma* and bovine ureaplasmas. The results are given in Table 1. *M. verecundum* strains 107 and 108 and *M. alkalescens* strain NCTC10135 were unaffected by any of the four wheys. All other strains were affected to some extent. Strains belonging to the same species reacted in essentially the same fashion, but there were quantitative differences in the extent of killing of strains of the same species with a given whey. *M. dispar* was killed only by whey 4, but strain Vic12 was killed to a greater extent

Table 2. *Effect of dialysis on the mycoplasmacidal action of whey*

Decrease (corrected) in $\log_{10}$ colony count after 4 hr. incubation in whey at 37° C.

	Whey sample							
	1		2		3		4	
	Untreated	Dialysed	Untreated	Dialysed	Untreated	Dialysed	Untreated	Dialysed
M. bovigenitalium M338/70	⩾ 1·9	0	⩾ 1·9	0	0·2	0	⩾ 1·9	0
Ureaplasma sp. Vic9	⩾ 2·6	0	⩾ 2·6	0	⩾ 2·6	0·1	⩾ 2·6	1·0
A. laidlawii BN1	⩾ 3·6	0·6	⩾ 3·6	0·3	0	0·1	0	0
M. agalactiae var. *bovis* M720/70	⩾ 3·5	0	1·3	0	0	0	0·3	0
M. bovoculi M165/69	1·0	0	0·7	0	0·6	0·2	⩾ 3·8	1·9
M. bovirhinis C56R	0·1	0	0·2	0	0·1	0	⩾ 3·1	⩾ 3·1
M. dispar Vic12	0	0	0	0	0·2	0·1	⩾ 1·7	0·6

than the other two. The four *M. bovirhinis* strains were all killed by whey sample 4 but not by the other three wheys. Of the four *A. laidlawii* strains examined three were killed by whey samples 1 and 2. Strain 011 was less sensitive to killing by whey 1 than the other three strains and unaffected by whey 2. All five ureaplasmas were killed by wheys 1, 2 and 4. Only strains Vic9 and T488 were affected by whey 3. Strain Vic9 was the most sensitive of the ureaplasmas to the action of the whey samples, confirming previous results (Brownlie *et al.* 1974*a*). Some variations between the *M. bovigenitalium*, *M. agalactiae* var. *bovis* and *M. arginini* strains was also noted.

Besides this variation in susceptibility of different mycoplasma strains and species, variations in whey samples from different animals were also evident. Whey sample 3 appeared least active. Wheys 1 and 2 acted on a similar range of species and strains. Whey 4 was the only sample observed to be active against *M. dispar* and *M. bovirhinis*, but it was not active against certain of the strains killed by wheys 1 and 2.

Effect of dialysis and heat on the mycoplasmacidal action of whey

Previous work (Brownlie *et al.* 1974*a*) indicated that the mycoplasmacidal action of whey for ureaplasmas was lost after dialysis but was unaffected by heating at temperatures sufficient to destroy complement. Seven strains representing seven bovine species known to be affected by whey were tested against untreated whey and whey that had been dialysed against barbitone buffer overnight at 4° C. or heated at 56° C. for 30 min. (Table 2).

Heating had no apparent effect on the mycoplasmacidal activity of the wheys. However, after dialysis the mycoplasmacidal activity of whey samples 1, 2 and 3 for *A. laidlawii*, *M. bovoculi*, ureaplasma Vic9, *M. agalactiae* var. *bovis* and *M. bovigenitalium* was greatly reduced or lost. Whey sample 4 seemed distinct from the other three samples in that activity against *M. bovirhinis* was not lost after

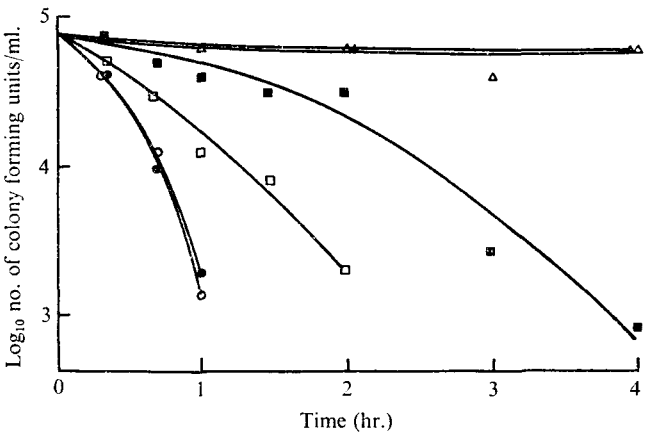

Fig. 1. Effect of dilution on the killing of ureaplasma Vic9 by whey. The concentrations of whey samples tested were: ○, neat; ●, $\frac{1}{2}$; □, $\frac{1}{4}$; ■, $\frac{1}{8}$; △, $\frac{1}{16}$. All dilutions were in barbitone buffer. The control ▲ was barbitone buffer containing 0·1 % bovine serum albumin.

Table 3. *Effect of temperature on the mycoplasmacidal action of bovine whey for ureaplasma Vic*9

Incubation temperature (° C.)	Decrease in log_{10} colony count at time (hr.)					
	$\frac{1}{3}$	$\frac{2}{3}$	1	2	3	4
40	⩾ 1·8	⩾ 1·8	⩾ 1·8	⩾ 1·8	⩾ 1·8	⩾ 1·8
37 with whey	0·7	⩾ 1·8	⩾ 1·8	⩾ 1·8	⩾ 1·8	⩾ 1·8
30 with whey	0·4	0·9	⩾ 1·8	⩾ 1·8	⩾ 1·8	⩾ 1·8
20 with whey	0·2	0·2	0·4	0·6	1·0	⩾ 1·8
* 40 in buffer	—	—	0·2	0·4	0·7	0·9

* No decrease in colony count was observed in buffer incubated at 37°, 30° and 20° C.

dialysis and some activity against the other species was retained. Thus the presence of heat stable, dialysable mycoplasmacidal activity was demonstrated in all four whey samples. In addition whey 4 apparently possessed non-dialysable mycoplasmacidal activity.

Whey 1 was dialysed against distilled water and the diffusate (i.e. material that had passed through the membrane) was freeze-dried. This whey fraction dissolved in barbitone buffer (10 mg./ml.) killed ureaplasma Vic9, *A. laidlawii* BNI, *M. bovigenitalium* M338/70 and *M. agalactiae* var. *bovis* M720/70 to the same extent as the original whey sample.

Examination of a further ten whey samples for mycoplasmacidal activity

In order to examine the frequency with which dialysable and non-dialysable mycoplasmacidal substances occurred in whey a further ten samples were tested against seven mycoplasma species. None of the ten whey samples affected *M. dispar* Vic12, *M. bovirhinis* C56R or *A. laidlawii* BN1.

The other four mycoplasmas tested, *M. bovigenitalium* M338/70, *M. agalactiae*

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Table 4. *Effect on mycoplasmacidal activity of absorbing whey sample* 1 *with mycoplasmas and bovine erythrocytes*

	Decrease (corrected) in $\log_{10}$ colony count of mycoplasma strain			
	BN1	M720/70	M338/70	Vic9*
Whey absorbed with:				
Nothing	⩾ 1·5	⩾ 3·1	⩾ 3·1	⩾ 1·7
A. laidlawii BN1	0	0	0	0
M. agalactiae var. *bovis* M720/70	0	1·1	⩾ 3·1	⩾ 1·7
M. bovigenitalium M338/70	0·8	1·6	2·2	1·2
M. dispar Vic12	1·1	0·8	⩾ 3·1	⩾ 1·7
Bovine erythrocytes	0·2	0·4	0·4	0

* *Ureaplasma* sp.

var. *bovis* M720/70, *M. bovoculi* M165/69 and *Ureaplasma* sp. Vic9 were killed by nine of the whey samples. Activity against these four strains was lost after dialysis but heating whey samples at 56° C. for 30 min. had no effect on mycoplasmacidal activity.

However, whey samples could not be classified simply as being of high or low activity since not all wheys were equally active against the same strains. For example whey 1 killed M338/70, Vic9 and BN1 but whey 5 killed M338/70 and Vic9. Whey sample 9 was more effective against M338/70 than Vic9 but whey sample 8 was more effective against Vic9 than M338/70. Thus variation occurred both in the general potency of the whey and activity against specific strains.

Kinetics of killing by whey

Fig. 1 shows the kinetics of the killing of ureaplasma strain Vic9 by whey sample 1 undiluted and at various dilutions. The rate of killing became less as the dilutions of the whey was increased. No killing in 4 hr. was detected in whey diluted 1/16 in barbitone buffer. No killing occurred in barbitone buffer containing 0·1% bovine serum albumin in 4 hr. at 37° C. The kinetics of killing *A. laidlawii* BN1 was almost identical with that of the *Ureaplasma* sp.

Effect of varying the inoculum size

The effect of varying the inoculum size on the rate of killing of *A. laidlawii* BN1 by whey 1 was examined. Inocula of 10^4 to 10^7 were killed at the same rate and to the same extent during 4 hr. incubation at 37° C. When the inoculum was increased to give 10^8 organisms per ml. the rate and extent of killing was reduced.

Effect of varying incubation temperature on killing

The effect of varying the incubation temperature on the mycoplasmacidal action of whey sample 1 for ureaplasma Vic9 and *A. laidlawii* BN1 was examined. Very similar results were obtained with both strains. The results for strain Vic9

are given in Table 3. Over the range of temperatures tested killing was maximal at 40° C. However, at 40° C. both strains died in buffer. At 37° C. and below both survived in the buffer control. As the temperature was reduced so was the killing. Thus the mycoplasmacidal action of whey is a temperature dependent reaction.

Effect on mycoplasmacidal activity of absorption of whey with mycoplasmas and erythrocytes

The possibility that the mycoplasmacidal activity of whey might be specifically absorbable was examined. Whey sample 1 was absorbed with three strains of mycoplasmas representing different species that had been shown to be killed by this whey and also with *M. dispar* Vic12, which was not affected by this whey. The whey was also absorbed with bovine erythrocytes. Untreated and absorbed whey was then tested for mycoplasmacidal activity (Table 4). From this table it can be seen that absorption by homologous and heterologous mycoplasma strains occurred and also that *M. dispar* Vic12 absorbed activity. Furthermore, activity could be removed by erythrocytes.

A comparison of absorption by *A. laidlawii* at 37° C. and 4° C. showed that although efficient absorption occurred at 37° C. none was demonstrable at 4° C. Thus the attachment of the factor in whey to cells is dependent on temperature.

Killing of mycoplasmas by lysolecithin

Kaklamanis, Stavropoulos & Thomas (1971), suggested that the mycoplasmacidal action of tissue extracts was due to lysolecithin; subsequently, Mardh & Taylor-Robinson (1973) found that *Acholeplasma* were less susceptible to lysolecithin than *Mycoplasma*. The ability of lysolecithin (final concentrations in barbitone buffer of 50, 25, 10, 5, 1 and 0 μg./ml.) to kill ureaplasma strains A417 and B101, *M. bovirhinis* strains PG43 and C155, *M. dispar* strains 462/2 and Vic12 and *A. laidlawii* strains 1307/68 and BN1 was examined in a system identical with that used to test whey samples. All eight strains were killed ($\geqslant 3 \log_{10}$ reduction in viable count) by a lysolecithin concentration of 50 μg./ml. The ureaplasmas, *M. dispar* and *M. bovirhinis* were killed to the same extent by a lysolecithin concentration of 25 μg./ml. *A. laidlawii* was not affected by this concentration. None of the strains were affected by a lysolecithin concentration of 10 μg./ml. or less.

DISCUSSION

Whey samples from most cows are able to kill certain mycoplasmas including the proved natural pathogens *M. bovigenitalium* and *M. agalactiae* var. *bovis* (Gourlay, 1973; Fabricant, 1973) and *M. dispar* and ureaplasmas, known to cause clinical mastitis experimentally (Gourlay, Howard & Brownlie, 1972; Howard, Gourlay & Brownlie, 1973; Brownlie, Howard & Gourlay, 1974*b*). Strains of the same mycoplasma species are affected in the same general manner by whey. For example *M. dispar* and *M. bovirhinis* are, for the most part, unaffected by whey, whereas *M. bovigenitalium* and the ureaplasmas are usually susceptible. However, strain variation within a species of mycoplasma was evident.

The whey samples were collected and stored without special precautions being taken to preserve complement. Mycoplasmacidal activity was not destroyed by heating at 56° C. for 30 min. and, with all but one whey, dialysis against barbitone buffer containing magnesium and calcium ions resulted in a substantial loss of activity. Thus the lethal action of whey does not involve complement components C'1, 2, 5, 8 or 9 all of which are heat labile (Muller-Eberhard *et al.* 1966).

Certain animals produce whey that is highly mycoplasmacidal whereas others produce whey with little activity. However, animal variation occurred both with respect to the general potency and potency for specific strains.

These results could be explained if animals produced either a single mycoplasmacidal factor in their whey able to affect a number of different strains, or several factors each with a limited range of activity.

If the killing of ureaplasmas is estimated by counting colony forming units instead of by colour change units more ureaplasmas appear susceptible to killing by whey than originally reported (compare the results in Table 1 with those of Brownlie *et al.* 1974*a*). However, it should be noted that the activity of whey sample 3, tested here, is very similar to that of the sample tested by these authors. Apparent differences between these results and those previously reported may be due in part to the different potencies of the whey samples tested. But the point made previously that differences between human and bovine strains may be quantitative rather than qualitative should be emphasized. Nevertheless, in a direct comparison of the two methods, killing appeared greater when counts were determined as colony forming units. Strain variation amongst the ureaplasmas is evident by either method and strain Vic9 is the most sensitive to killing by whey. Diluting samples in U3 broth instead of barbitone buffer for plating does not affect the decrease in colony count. The prolonged incubation in fluid medium that occurs when counting as colour change units may, therefore, result in the factor in whey eluting off the mycoplasmas and may indicate that the combination of the factor with mycoplasmas is a reversible reaction.

It proved possible to absorb the activity of whey with both heterologous and homologous mycoplasma species and also with erythrocytes. Thus a non-specific, possibly electrostatic, binding may be the mechanism by which the molecules attach to the mycoplasmas or erythrocytes. This would explain absorption of activity by heterologous strains and erythrocytes.

As the temperature of incubation of mycoplasmas with whey is reduced then so is the rate of killing. Absorption of the factor by mycoplasmas occurred at 37° C. but not at 4° C. Thus the reduced killing at lower temperatures may be due to less molecules being attached to the mycoplasmas. The binding of the cationic proteins, lysozyme and cytochrome c, and of cholesterol to *A. laidlawii* membranes has been shown to be reduced as incubation temperature is reduced (Razin, Rottem, Hasin & Gershfeld, 1973; Rottem, Hasin & Razin, 1973).

The finding that the mycoplasmacidal activity of whey is dialysable distinguishes it from antibody, known to kill or prevent the growth of mycoplasmas (Edward & Fitzgerald, 1954). Since the mycoplasmacidal activity of whey is heat stable and dialysable it is distinct from the inhibitory activity previously described for myco-

plasmas in serum and semen (Taylor-Robinson, Thomas & Dawson, 1969; Roberts, 1971). The mycoplasmacidal activity of this dialysable whey fraction is not confined to ureaplasmas, an action previously described (Brownlie *et al.* 1974*a*).

Kaklamanis *et al.* (1971) described a mycoplasmacidal factor in tissue extracts and suggested it was lysolecithin. Susceptibility of strains to killing by lysolecithin did not correlate with the susceptibility of strains to killing by whey. Therefore lysolecithin is apparently not involved.

The heat stability and dialysability of whey activity also distinguishes it from such antibacterial agents found in milk or other bovine secretions as lactenin (Wilson & Rosenblum 1952), the cationic protein fraction from bovine teat canal keratin and cervical mucus prepared by Hibbitt, Cole & Reiter (1969) and Brownlie & Hibbitt (1972) and lactoferrin (Oram & Reiter, 1968).

The mechanism by which the mycoplasmas are killed is not known. However, mere binding is not sufficient to kill the mycoplasmas since the active factor was absorbed by strains which were not affected by whey, and some subsequent action must be involved. It may be at this stage that the variable susceptibility of strains occurs.

It is possible that this factor is one of the animal's defence mechanisms against mycoplasma colonization and infection. The variation in synthesis of this factor may contribute to animal variation in susceptibility to mycoplasma infection.

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