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Mycoplasma californicum mastitis in ewes as an experimental model for antibiotic treatment

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SUMMARY

A strain of *Mycoplasma californicum* successfully infected an experimentally inoculated ovine mammary gland causing a severe mastitis. The condition lasted for about 25 days, and resulted in atrophy and loss of milk production in the gland. Four experimentally infected ewes, treated over a 3-day period with various regimes of the antibiotics oxytetracycline or tylosin during the acute stage of infection, successfully eliminated the infection. Two others similarly treated with combined intramammary and intramuscular tiamulin or with intramammary Bay Vp2674, did not eliminate the infection; but another ewe treated with intramuscular as well as intramammary Bay Vp2674, did resolve the infection. The two ewes that were unsuccessfully treated with antibiotics at the acute stage did respond to tylosin or oxytetracycline at a later stage of infection. Measurement of antibiotic concentrations demonstrated that the persistence of inhibitory levels in the milk varied between the antibiotics and were influenced by the extent of parenteral treatment.

INTRODUCTION

Mycoplasma californicum was first isolated from bovine mastitis cases in two herds in California (Jasper, 1977). Following the characterization and naming of this species (Jasper *et al.* 1981), it has been isolated in Hawaii (D. E. Jasper, personal communication), from two herd outbreaks in Northern Ireland (Mackie Ball & Logan, 1982; 1986), and from Czechoslovakia (Jurmanová, Hájková & Vévoda, 1983; Jurmanová *et al.* 1985).

Various attempts at antibiotic treatment of bovine mastitis caused by mycoplasma, particularly *M. bovis*, have met with limited success (Boughton, 1979; Jasper, 1981). Relapses following temporary clinical improvement have been attributed to local or systemic foci of infection (Jasper, 1977).

Although highly contagious, *M. californicum* infection appears to be confined to the mammary gland and has not been shown to spread to other organs. Because of the permanent damage caused by *M. californicum* infection resulting in a large drop of milk yield in infected glands (Mackie, Ball & Logan, 1982), treatment of established cases is impracticable. However, once infection by this organism has been recognized in a herd, therapeutic control of fresh acute cases before permanent damage occurs might be an economically sound approach. Some success with antibiotic treatment was reported on the farm where the first Northern Ireland outbreak occurred (Mackie, Ball & Logan, 1982).

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At this laboratory, experimentally produced mastitis in suckling ewes has been used as a model to determine the pathogenicity of ureaplasma strains (Ball & Mackie, 1984/5; 1985). The present study was designed to determine whether *M. californicum* infects the ovine gland, and whether such an infection could be used to evaluate antibiotic treatment.

MATERIALS AND METHODS

Mycoplasma strains

Two *M. californicum* isolates, 136 and 390, from a field outbreak in Northern Ireland (Mackie, Ball & Logan, 1986) were cloned three times as recommended by the Subcommittee on the Taxonomy of Mycoplasmatales (1972). Cultures of the tenth passage in artificial medium, grown in PPLO broth (Oxoid) supplemented with yeast extract (0·5%), inactivated pig serum (20%), ampicillin (100 μg/ml), bacitracin (100 μg/ml) and phenol red (0·0005%), pH 7·2, were used for the initial sheep inoculations. The titre of each inoculum was estimated by the transfer of measured volumes of duplicate tenfold broth dilutions to PPLO agar (Oxoid) with the above supplements. After incubation for 2–4 days at 37 °C, the mean of the colony count was expressed as colony forming units (c.f.u.) per ml.

In vitro inhibition tests

A metabolic inhibition test (Taylor-Robinson *et al.* 1966) in PPLO medium in microtitre trays was used to measure the inhibitory effects of various antibiotics on the two cloned strains of *M. californicum* as described by Ball & McCaughey, (1986). The antibiotics are listed in Table 1. Bay Vp2674, a quinolin derivative, was obtained from Bayer Ltd., Wuppertal, Germany; flurofamide, a urease inhibitor, from Norwich Eaton Pharmaceuticals Inc., Norwich, New York; rosoxacin from Sterling Research Labs, Guildford, Surrey, England; and the remainder from Diamed Diagnostics Ltd., Merseyside, England. Freshly grown cultures of strains 136 and 390 at concentrations of $1{\cdot}7 \times 10^6$ and $3{\cdot}9 \times 10^7$ c.f.u./ml respectively, were the final addition to each test. During incubation at 37 °C, the minimal inhibitory concentrations were read immediately following the start of colour change indicating growth in the control wells without antibiotics. The inhibitory concentration was taken as the highest dilution in which growth was not observed.

To distinguish between mycoplasmastatic and cidal effects of the antibiotics, after 5 h incubation at 37 °C, mycoplasma death was determined by the transfer of 10 μl from the first six wells of each test row (32 μg–1 μg/ml antibiotic concentration) to 2 ml of fresh medium. The 1:200 dilutions reduced the antibiotics to concentrations (0·16–0·005 μg/ml) that were lower than the corresponding minimal inhibitory concentrations, allowing the growth of previously inhibited but still viable cells. The highest dilution failing to grow in the corresponding 1:200 dilution after 7 days incubation at 37 °C was taken as the measure of mycoplasmacidal activity.

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Sheep experiments

The experimental ewes were Suffolk crossbreds, all of which had lambed at least twice in previous years. They were housed in groups of two or three during the course of the experiment. All were suckling single lambs of at least 5 weeks of age. The lambs were removed from the ewes each night, from 16.00–10.00 h and the intramammary inoculation of *M. californicum* cultures, and the intramammary, intravenous or intramuscular administration of antibiotics were carried out during this period. One gland of the udder of each ewe was inoculated with *M. californicum*, the other was an uninoculated control.

Milk samples were collected daily at 09.30 h for at least 3 days before experimental inoculation and until at least 3 weeks after the elimination of mycoplasma infection. These samples were cultured for bacteria on blood agar and Edward's medium and for mycoplasma by titration in PPLO broth and agar as described above. The numbers of cells in the milk samples was measured, after fixing overnight with 10 % formalin, with a Coulter counter (FN Model). Additional milk and serum samples were collected during and for up to 7 days after treatment, and stored at −20 °C for antibiotic assay.

In an initial experiment, ('Experimental infection of sheep') to determine whether *M. californicum* could colonize the ovine mammary gland, three ewes were inoculated via the teat canal with 2 ml of broth culture, two with strain 390 ($0{\cdot}7 \times 10^8$ and $2{\cdot}2 \times 10^6$ c.f.u./ml) and one with strain 136 ($1{\cdot}1 \times 10^6$ c.f.u./ml).

In a second experiment ('Treatment of infection') seven ewes were inoculated with 2 ml *M. californicum* infected mastitic milk, taken from the ewe originally infected with strain 136, or from ewes subsequently infected from that milk. The milk inocula were used either fresh or after storage, at 4 °C overnight or at −70 °C for a longer period.

The ewes were each given a single antibiotic during the acute phase of infection, 3–5 days after intramammary inoculation when the milk cell count had risen to $> 10^7$ cells/ml and the *M. californicum* titre to $> 10^8$ c.f.u./ml. Two of the ewes which did not respond to the first treatment were given a second treatment with a different antibiotic, 11 and 12 days after inoculation.

The antibiotics used in the treatment were tiamulin (Dynamutilin, E. R. Squibb and Sons Ltd, Hounslow, Middx., England), Bay Vp2674 (Baytril, Bayer Ltd.), tylosin (Tylan, Elanco Products Ltd., Basingstoke, Hants, England) and oxytetracycline (Norbrook Labs (UK) Ltd, London) and the regimes are summarized in Table 2. They were based on a single daily intramammary administration (71–75 mg) over a 3-day period. This was either given alone or together with intramuscular or intravenous injection (10–20 mg/kg body weight) every 12 or 24 h over the same period.

Antibiotic assay

The antibiotics were quantitatively assayed in milk from the infected and control glands and from serum samples collected during treatment, by means of an agar plate diffusion method (Bennett *et al.* 1966). Samples and standard preparations were tested in duplicate on large plastic plates (Nunc, Kanstrup, Denmark) coated with agar seeded with test organism. Tiamulin was assayed with

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Micrococcus luteus (NCTC8340) on Antibiotic Medium No. 1 (Oxoid) with additional sodium chloride (5 g/1), pH 8·5; Bay Vp2674 with *Escherichia coli* (NCTC10418) on Antibiotic Medium No. 1 (Difco), pH 6·0; tylosin with *M. luteus* on Antibiotic Medium No. 1, pH 8·0; and oxytetracycline with *Bacillus cereus* (NCTC10320) on Antibiotic Medium No. 2 (Difco), pH 5·8. The plates were incubated at 30 °C for 18 h, the inhibition zone diameters measured, and the antibiotic concentrations of the samples determined.

RESULTS

In vitro inhibition tests

The inhibitory and cidal activities of the antibiotics against *M. californicum* are summarized in Table 1. Flurofamide did not show any activity. Small variations in activity against the two strains of *M. californicum* were observed in some others. The cidal concentrations in all were higher than the inhibitory levels.

Experimental infection of sheep

One of the ewes inoculated with a broth culture of strain 390 did not become infected. The milk cell count of the inoculated gland rose to 10^6 cells/ml for 1 day, and then returned to normal. *M. californicum* was not reisolated. The mammary cellular response of the other ewe inoculated with strain 390 was similar, but the organism was reisolated from 4–10 days post-inoculation (pi) during which time the cell count remained normal. The maximum mycoplasma titre of 4×10^4 c.f.u./ml was on day 5 pi. The ewe inoculated with a broth culture of strain 136 became infected and developed a severe mastitis. The cell count and mycoplasma titre of this animal during the infection are summarized in Fig. 1. The milk cell count rose rapidly to $> 10^7$ cells/ml and remained at this level until 25 days pi, 7 days after elimination of the organism, before rapidly returning to normal. The cell count in the control gland also rose and was $\geqslant 10^7$ cells/ml from day 3 to day 14 pi.

The ewe that did not become infected following inoculation with strain 390 was inoculated with 2 ml mastitic milk from the ewe that became infected with strain 136, into the previously uninoculated control gland (Fig. 2). This gland did become infected from days 1–16 pi but the mycoplasma titre did not rise above 4×10^4 c.f.u./ml (day 7 pi) and the milk cell count of the infected gland after initially rising to $> 10^7$ cells/ml the day after inoculation, fell to lower levels of $10^{5\cdot5}$–10^6 cells/ml for the duration of infection. The cells in the uninfected gland peaked to a lower level but rose from day 4 pi to $10^{6\cdot8}$ cells/ml on day 12 pi before returning to normal levels by day 18 pi.

Although not accurately measured, the milk content of the glands that recovered from infection was lower than in the controls, and the glands were noticeably indurated.

Treatment of infection

In the second experiment the ewes all became acutely infected from days 1 or 2 pi. Before the start of antibiotic treatment, the mycoplasma titre in the milk of the infected gland of each was $> 10^8$ c.f.u./ml (Table 2) and the milk cell count was $> 10^7$ cells/ml.

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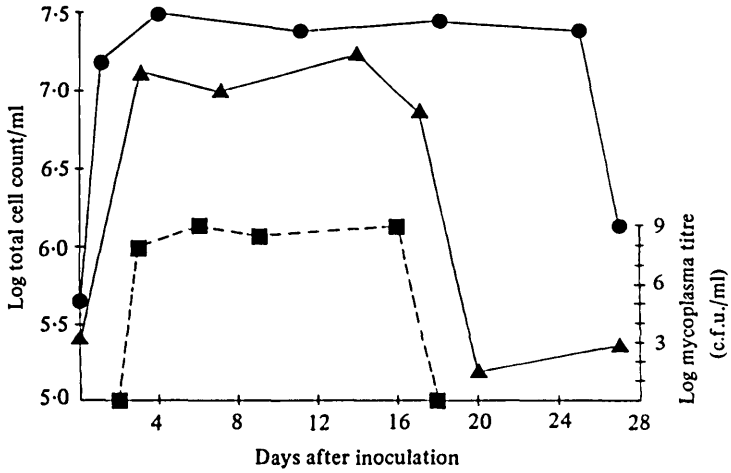

Fig. 1. Number of cells and *Mycoplasma californicum* titre in milk of ewe following intramammary inoculation with strain 136. ●—●, Cells in infected gland; ▲—▲, cells in control gland; ■—■, mycoplasma.

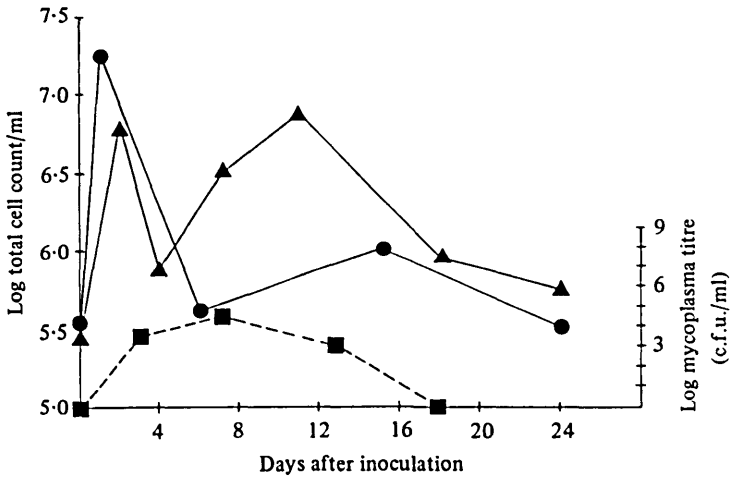

Fig. 2. Number of cells and *Mycoplasma californicum* titre in milk of ewe following intramammary inoculation with mastitic milk containing strain 136. ●—●, Cells in infected gland; ▲—▲ cells in control gland; ■—■, mycoplasma.

Table 1. *Inhibitory and cidal activity of antibiotics on* M. californicum *strains 136 & 390* in vitro

	Minimal inhibitory concentration (μg/ml) against strain		Cidal concentration (μg/ml) (after 5 h) against strain	
Antibiotic	136	390	136	390
Tylosin	⩽0·03	0·125	⩽2	8
Oxytetracycline	⩽0·03	⩽0·03	>32	32
Chlortetracycline	⩽0·03	⩽0·03	>32	>32
Tetracycline	⩽0·03	⩽0·03	8	16
Tiamulin	⩽0·03	0·25	>32	>32
Rosoxacin	2·0	0·25	>32	>32
Bay Vp2674	0·06	⩽0·03	16	16
Lincomycin	0·5	2·0	>32	>32
Flurofamide	>32	>32	>32	32

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Table 2. *Antibiotic treatment on* M. californicum *infected ovine mammary glands*

Ewe no.	Mycoplasma titre immediately before treatment (c.f.u./ml)	Duration of infection before treatment (days)	Antibiotic	Intramammary injections, at 24 h intervals (mg)	Additional injections (mg/kg)	Intervals between additional injections (h)
1	$0{\cdot}6\times10^9$	5	Tiamulin	3 × 75	3 × IM (15 mg)	24
2	$0{\cdot}9\times10^9$	5	Bay Vp2674	3 × 75	—	—
3	$0{\cdot}7\times10^9$	5	Bay Vp2674	3 × 75	3 × IM (10 mg)	24
2	$1{\cdot}6\times10^8$	11	Tylosin	3 × 75	3 × IM (10 mg)	24
4	$1{\cdot}0\times10^9$	4	Tylosin	3 × 75	6 × IM (10 mg)	12
5	$2{\cdot}2\times10^9$	3	Oxytetracycline	3 × 71	6 × IV (10 mg)	12
6	$1{\cdot}1\times10^9$	4	Oxytetracycline	3 × 71	6 × IM (10 mg)	12
7	$1{\cdot}1\times10^8$	4	Oxytetracycline	3 × 71	1 × IM (20 mg)*	—
1	$0{\cdot}7\times10^9$	12	Oxytetracycline	3 × 71	—	—

IM, Intramuscular injections. IV, intravenous injections. * Long-acting oxytetracycline

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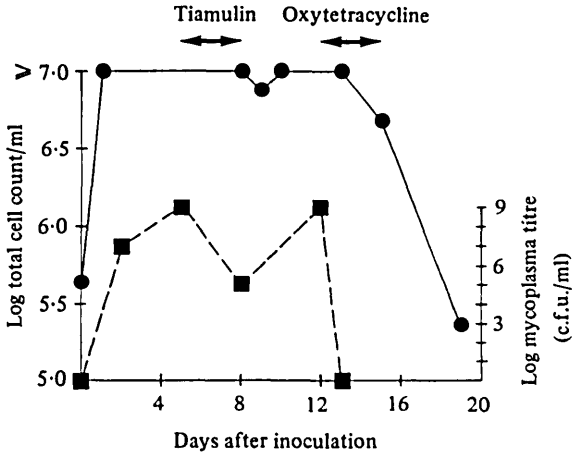

Fig. 3. Number of cells and *Mycoplasma californicum* titre in milk of ewe following inoculation with strain 136 at day 0, and treatment first with tiamulin, and then with oxytetracycline. ●—●, Cells in infected gland; ■—■, mycoplasma; ◄—►, duration of antibiotic treatments.

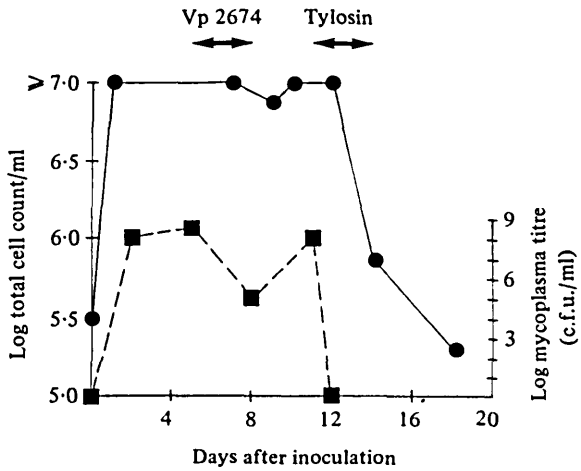

Fig. 4. Number of cells and *Mycoplasma californicum* titre in milk of ewe following inoculation with strain 136 at day 0, and treatment first with Vp2674, and then with tylosin. ●—●, Cells in infected gland; ■—■, mycoplasma; ◄—►, duration of antibiotic treatments.

Combined intramammary and intramuscular tiamulin (Fig. 3) caused only a slight temporary drop in both mycoplasma titre and milk cell count. A similar slight and temporary effect was observed following intramammary treatment with Bay Vp2674 (Fig. 4). When intramammary treatment of this drug was combined with intramuscular injection (10 mg/kg body weight) the infection was resolved, but the milk cell count remained high, dropping to between 10^6–$10^{6\cdot5}$ cells/ml by 12 days after treatment and staying at this level for at least an additional 14 days.

Both the regimes used for tylosin and oxytetracycline were effective in eliminating the infection; tylosin when the intramammary treatment was accompanied by intramuscular inoculation every 12 hours or every 24 hours; and oxytetracycline whether it was accompanied by parenteral treatment or not. The

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Table 3. *Persistence of antibiotic levels in milk and serum during and after treatment*

Ewe no.	Antibiotic	Antibiotic concentration greater than (μg/ml)	Period (days from start of treatment) for which antibiotic was present in: milk from infected gland	milk from control gland	serum
1	Tiamulin	0·4	3	4	0
2	Bay Vp2674*	0·5	0	0	0
3	Bay Vp2674	0·5	3	3	0
2	Tylosin	0·5	4	4	0
4	Tylosin	0·5	7	5	0
5	Oxytetracycline	0·4	$\geqslant$4	$\geqslant$4	6
6	Oxytetracycline	0·4	$\geqslant$6	5	$\geqslant$5
7	Oxytetracycline	0·4	4	3	4
1	Oxytetracycline*	0·4	4	0	0

* Treatment did not include parenteral injection.

milk cell count from the treated glands rapidly returned to normal levels within 4–5 days after the end of the treatment (Figs. 3 and 4).

The glands of the five ewes from which the infection was successfully eliminated following treatment during the acute stage, did not differ noticeably from the control glands within one week after the end of the treatment. Atrophy and difference in milk content was minimal. The glands of the two ewes which did not respond to the first antibiotic, and which were infected for a longer period before successful treatment, showed similar gland shrinkage and lower milk content to the ewes where the infection was allowed to resolve naturally.

Antibiotic assay

In most cases, the relevant antibiotics were detectable in the milk samples for at least 1 day after the completion of treatment. The persistence of antibiotics in the milk of the infected and control glands and in the serum at levels greater than the minimal inhibitory levels in the *in vitro* tests (0·4 or 0·5 μg/ml) are listed in Table 3. Tiamulin, Bay Vp2674 and tylosin were not detected in the serum whereas oxytetracycline persisted in the serum for as long as it was in the milk, with the exception of ewe 1, which was not injected parenterally when treated in the chronic stage of infection. Bay Vp2674 did not stay at levels greater than 0·5 μg/ml in milk when administered by intramammary inoculation only, but appeared to concentrate in the milk and maintain higher levels there when given parenterally as well as intramammarily. In the ewe inoculated intramammarily without parenteral treatment, oxytetracycline was not detectable in the control gland or the serum. In this ewe, the antibiotic persisted in the infected gland for as long as in the ewe that was treated with one parenteral injection of long acting oxytetracycline, but not for as long as in the two ewes treated parenterally with six intravenous or six intramuscular injections. Similarly, tylosin persisted for longer in the milk of the ewe injected intramuscularly six times than in the one injected three times over the same period.

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DISCUSSION

M. californicum does infect the mammary gland of the ewe and cause mastitis. The duration of infection in ewes as shown in the present study, and confirmed by other unpublished experimental work, is not as long as in the cow, but the clinical effect appears to be the same. The milder infection observed in one ewe, with low milk cell count and low mycoplasma titre, is indicative of host variation as has been seen with experimental ureaplasma mastitis (Ball & Mackie, 1984/85) and has been observed in cows in field cases (Mackie, Ball & Logan, 1986). The difference in severity of infection produced by the two *M. californicum* strains in the present study also indicates a mycoplasma strain variation.

The high milk cell count maintained in the uninfected control gland of the first ewe infected with strain 136 (Fig. 1), and observed also in the ewes infected for treatment studies, has not been seen in ewes experimentally infected at this laboratory with ureaplasmas (Ball & Mackie 1984/85, 1985), *M. canadense* (Ball & Mackie, 1986) or *M. bovigenitalium* (unpublished results). Neither has this effect been seen in the control quarters of cows infected with *M. californicum* (Mackie, Ball & Logan, 1982). Whether it is due to a diffusable toxic factor is open to speculation.

The 3-day period of treatment was selected because of the previous lack of success of a 2-day treatment of a cow (Mackie, Ball & Logan, 1982) and ewes (unpublished results), and because of the *in vitro* demonstration that the effects of the antibiotics were static rather than cidal. The aim of the treatment was the maintenance of a static effect to enable the natural defences to work. The elimination of the antibiotics from the body determines the persistence of inhibitory levels following the administration period. Although prolonged inhibitory levels in the milk appear to be important, whether these could be replaced by higher antibiotic levels over a shorter period needs to be investigated.

The glands of the ewes that were successfully treated during the acute stage of infection did not become noticeably indurated and a drop in milk yield was not detected, unlike the glands that were infected for a longer period. It appears that treatment early in infection may limit the extent of damage to the gland.

The ovine mammary gland appears to be susceptible to infection with some of the mycoplasmas which cause bovine mastitis. As well as *M. californicum*, these include bovine ureaplasma (Ball & Mackie, 1985), *M. canadense* (Ball & Mackie, 1986) and *M. bovigenitalium* (unpublished results). There are economic advantages to using the ewe as opposed to the cow as an experimental model. In the present study, these included the need for lower amounts of antibiotics. Whether the results demonstrated using the ewe can be extended to the cow remains to be established.

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