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The Persistence of *Brucella melitensis* in Experimentally Infected Ewes Through Three Reproductive Cycles

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With 4 figures and 4 tables

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Summary

The authors studied the persistence of infection in 46 ewes experimentally infected with *Brucella melitensis* biovar 3 and monitored through three subsequent reproductive cycles. The entire experimental period lasted for 151 weeks. Infection of ewes and elimination of *Brucella* in milk, or its presence in vaginal discharges, persisted throughout the duration of the trial, as demonstrated by recurrent elimination of *Brucella* in milk and vaginal discharges. *Brucella melitensis* was recovered from the tissues of one ewe killed at the end of the trial. The strain was recovered from vaginal swabs and milk following parturition in the third reproductive cycle from an ewe that had aborted in the first cycle but was not pregnant in the second cycle. From a public health point of view, the periodical recovery of *Brucella* from the milk during the entire trial period illustrated that brucellosis in sheep remains a continuous occupational risk and a significant public health problem for consumers of fresh milk and milk products. That risk may persist for at least 3 years following the initial infection of the flock. Lamb antibody titres became negative in all lambs within 5 months after birth. This suggested that serological tests on lambs may have no practical diagnostic significance if performed during the first 5 months of life. Nevertheless, the birth of three infected lambs suggested that the phenomenon of latent carrier state may represent another way for *B. melitensis* to persist in a flock.

Introduction

Long-term *Brucella melitensis* infections of sheep flocks were investigated in France during the 1940s and 1950s. Lafenêtre (1947) suggested that it was possible to clear an infected flock following 6 months of enforced sexual abstention. Paltrinieri et al. (1956), in Italy, studied the possibility of spontaneous clearance of brucellosis infection in 63 ewes experimentally infected and killed at progressive time intervals. However, they were able to isolate *B. melitensis* even after 6 months of sexual abstention in some of the animals that had been pregnant at the time of infection, and also from three non-pregnant ewes between 184 and 199 days post-infection (p.i.). Their findings indicated that spontaneous clearance of brucellosis infection does not occur in all infected animals. None of these studies, however, was extended to consider subsequent reproductive cycles nor was the possible transmission of infection to offspring considered. Thus, it remains unclear as to whether, or not, spontaneous clearance of infection of ewes actually occurs following prolonged sexual abstention. For these reasons the present study was conducted to evaluate, in experimentally infected ewes, and during three subsequent reproductive cycles, the following:

1. the long-term evolution of infection and the likelihood of spontaneous clearance of brucellosis infection after prolonged sexual abstention;
2. the evolution of the serological responses in lambs born to infected dams;
3. the possible transmission of the infection from ewes to their offspring and the possible development of latent carrier state as regards *Brucella* infection.

Materials and Methods

Experimental design

Animals

Forty-six Fabrianese and cross-bred sheep, aged between 1 and 5 years, from an officially *B. melitensis*-free flock were naturally inseminated after oestrus synchronization. Twenty-five of the 46 sheep became pregnant of which 24 aborted between 4 and 6 weeks p.i.; only one sheep had normal parturition (first reproductive cycle), and three died within one to 5 months p.i. The remaining 43 sheep were naturally inseminated, after oestrus synchronization, 8 months p.i. Twenty-six sheep became pregnant and all had normal parturition between weeks 56 and 58 p.i. (second reproductive cycle). Of 39 lambs born, 26 survived for longer than 3 weeks and were included in this second stage of the study. Seven sheep died within 3 months following parturition. One sheep died after 8 months. Twenty-two months p.i., the remaining 35 sheep were naturally inseminated after oestrus synchronization. Twenty-two sheep became pregnant and all had normal parturition between weeks 120 and 126 p.i. (third reproductive cycle). Sixteen of the 23 lambs born survived for longer than 3 weeks and were included in this final stage of the study. The entire experimental period lasted for 151 weeks. All ewes under experiment were subjected periodically to bacteriological examination of samples available from the living animals (milk, vaginal swabs, and blood) and collected from ewes dead during the experiment or killed at the end of the trial. Moreover, the study included the serological examination of 34 ewes that had had parturition during the second and third reproductive cycles and their offspring. All pregnancies were con-

firmed by ultrasound scanning; no early fetal loss occurred. All the animals were housed in shelters with adequate space and fed with hay and nutritional supplements for the entire duration of the experiment; all stages were conducted with consideration for their welfare.

Infection of ewes

The animals were experimentally infected intraconjunctivally with a field strain of *B. melitensis* biovar 3 during the third month of the first reproductive cycle. This isolated field strain was first inoculated into two guinea pigs and then re-isolated from their spleens 21 days later in 10 ml of Farrell's medium (Farrell, 1974). After 48 h of growth in the medium at 37°C, the re-isolated *Brucella* was titrated. A dose of 5×10^8 colony-forming units in 100 μl was inoculated into the conjunctiva of both eyes (50 μl each) of the ewes, according to the provisions of the European Pharmacopoeia (Conseil de l'Europe, 2000).

Sampling procedures

Samples from ewes

Sera and blood All animals tested negative for antibodies to *Brucella* spp. prior to experimental infection. From pregnant ewes 3 ml of serum was collected by jugular venipuncture on the day of parturition in their second and third reproductive cycles. Using an ethylenediaminetetraacetic acid (EDTA) system 3 ml of whole blood was collected in the first week p.i., then weekly for 2 months p.i., and then periodically until 134 weeks p.i. A total of 784 blood samples were collected.

Milk Milk was collected weekly, commencing after the first abortion (or parturitions) onwards and in all reproductive cycles. A total of 139 samples of milk were collected.

Vaginal swabs Vaginal swabs were collected twice in the 14th and 15th week p.i. during the first reproductive cycle. During the second reproductive cycle, vaginal swabs were collected in the first week following parturition and then monthly for the next 4 months. During the third reproductive cycle, vaginal swabs were collected in the first week following parturition and then every second week for 1 month only. A total of 362 vaginal swabs were collected.

Tissues Spleen, udder and lymph nodes (retropharyngeal, submandibular, iliac and supramammary) were taken from all ewes, those that died during the experiment and those killed at the end of the trial. A total of 276 samples were collected.

Samples from lambs

Sera and blood From each animal, 3 ml of serum was collected by jugular venipuncture. For lambs born in the second reproductive cycle serum samples were taken weekly for 2 months and then periodically until 26 weeks following birth. For lambs born in the third reproductive cycle serum samples were taken weekly for 2 months and then periodically until 23 weeks following birth. A total of 358 sera were collected. Using an EDTA system 3 ml of whole blood was collected from each lamb; the blood was collected at birth and then periodically until the end of the trial. A total of 236 blood samples were collected.

Tissues Liver, brain, lung and spleen samples were taken from dead and killed lambs. Eight lambs born in the second reproductive cycle survived until the end of the experiment; they were between 23 and 24 months old at the time of bacteriological examination. Six lambs born in the third reproductive cycle survived until the end of the experiment; they were between 6 and 8 months old at the time of bacteriological examination. A total of 204 tissue samples were collected.

Aborted fetuses

Liver, abomasum fluid and brain samples were taken from 24 aborted fetuses.

Bacteriological tests

The culturing of *Brucella* from blood, milk, vaginal swab and tissues was performed using *Brucella* broth (401274 Biolife, Sarasota, FL, USA) and/or *Brucella* medium base (CM169 Oxoid Ltd, Basingstoke, UK) with the addition of antibacterial supplement (modified *Brucella* selective supplement, SR 209E Oxoid Ltd) and equine serum 5% (v/v). The tests were conducted according to the OIE Manual of Diagnostic Tests and Vaccines for terrestrial animals (Office International des Epizooties, 2004) and Alton et al. (1988). *Brucella* cultures were identified on the basis of colonial morphology and slide agglutination tests, with pure cultures submitted for *Brucella* typing tests. Identification and typing were conducted according to the OIE Manual (Office International des Epizooties, 2004), Corbel and Thomas (1983) and Alton et al. (1988).

Blood culture

From each blood sample 0.1 ml was sown onto *Brucella* agar and incubated for 7 days at 37 ± 1°C in an atmosphere containing 5–10% CO_2. The plates were examined after 72 h and then each following day to observe the development of the bacterial colonies. Another 0.1 ml of each blood sample was transferred into a screw-top aseptic tube containing 10 ml of *Brucella* broth. The tubes were incubated at 37 ± 1°C in an atmosphere containing 5–10% CO_2 for up to 6 weeks, with weekly subcultures transferred onto solid selective medium. The plates were examined after 72 h and then each following day to observe the development of the bacterial colonies.

Milk

The milk samples were centrifuged at 2000 ***g*** for 15 min. The skimmed milk was discarded, while the cream and the deposit were mixed and 0.1 ml of this mixture spread onto two *Brucella* agar plates. One plate was incubated at 37 ± 1°C, the other also at 37 ± 1°C, but in an atmosphere containing 5–10% CO_2. The plates were examined after 72 h and then each following day to observe the development of the bacterial colonies. One millilitre of each milk sample was transferred onto *Brucella* broth and the tubes were incubated at 37 ± 1°C in an atmosphere containing 5–10% CO_2 for up to 6 weeks. Weekly, subcultures from each broth were made onto selective solid medium and incubated at 37 ± 1°C in an atmosphere containing 5–10% CO_2. The plates were examined after 72 h and then each following day to observe the development of the bacterial colonies.

Vaginal swabs

The swabs were transferred into screw-top tubes containing 10 ml of *Brucella* broth. Plates of *Brucella* agar were streaked with the saturated swabs after 2 h of incubation at 37 ± 1°C, while the tubes containing broth were left at 37 ± 1°C in an atmosphere containing 5–10% CO_2 for up to 6 weeks. Weekly, subcultures from each broth were made onto selective solid medium and incubated at 37 ± 1°C in an atmosphere containing 5–10% CO_2. The plates were examined after 72 h and then each following day to observe the development of the bacterial colonies.

Tissues and aborted fetuses

Small pieces (about 2 cm^3 in size) were cut from all sampled tissues and prepared for culture by maceration in a Stomacher bag (Seward, Thetford, UK), containing 10 ml of sterile phosphate buffer saline (PBS), pH 6.8. The bag was processed for about 1 min; 0.1 ml of tissue suspension was spread onto two plates of *Brucella* agar. Of these, one plate was incubated at 37 ± 1°C and the other at 37 ± 1°C in an atmosphere containing 5–10% CO_2. The plates were examined after 72 h and then each following day to observe the development of bacterial colonies. From the same suspension 1 ml was transferred into tubes containing *Brucella* broth and incubated at 37 ± 1°C in an atmosphere containing 5–10% CO_2 for up to 6 weeks. Weekly, subcultures from each broth were placed onto selective solid medium and incubated in an atmosphere containing 5–10% CO_2. The plates were examined after 72 h and then each following day to observe the development of bacterial colonies.

Serological tests

All sera were tested for the presence of antibodies against *Brucella* spp. using the complement fixation test (CFT). The test was performed using the *Brucella abortus* biovar 1 strain S99 as antigen (VLA, Weybridge, UK); it was prepared according to the methods described in the fifth edition of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Office International des Epizooties, 2004).

Milk-ELISA test

The milk-enzyme-linked immunosorbent assay (ELISA) was performed according to a method described previously (Biancifiori et al., 1996), but with some modifications. A smooth lipopolysaccharide antigen from *B. abortus* biovar 1 strain S99 for the ELISA was prepared according to the technique described by Hendry et al. (1985). The milk samples and the controls were distributed in duplicate into medium capacity binding microplates (Polisorp, Nunc, Denmark). The antigen was diluted to the optimal working concentration in carbonate–bicarbonate buffer 0.05 M (pH 9.6). The diluent/washing buffer was 0.01 M PBS (pH 7.2) plus 0.05% Tween 20 (PBST). A commercial donkey anti-sheep IgG (whole molecule) peroxidase conjugated (Sigma, St. Louis, MO, USA) was used. The chromogen was a 0.16 M solution of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) in citrate buffer (pH 4.5). Sodium fluoride was used to stop the enzymatic reaction. The optical density (OD) values were read in a Jupiter (Asys Hitech, Eugendorf, Austria) microplate reader ($\lambda = 405$ nm). Strong positive (considered as 100% positivity) and negative standards were used. The results were expressed as percent positivity (PP) of the sample tested in relation to the strong positive control using the following formula:

$$PP = \frac{\text{Mean OD of tested sample}}{\text{Mean OD of positive control}} \times 100.$$

The test cut-off value was set at 10% PP.

Statistical analysis

Correlation between antibody titres in ewes and their lambs was evaluated using Spearman's correlation coefficient. A least-square linear regression of the natural logarithm of antibody titre and a standardized residuals analysis was performed on the CFT results of each lamb born in the third reproductive cycle.

Results

Clinical results

All ewes (except one) aborted in the first reproductive cycle; no abortions occurred during the second and third reproductive cycles.

Bacteriological results

Ewes

Bacteriological results, in terms of animals positive on testing according to the type of sample tested and relative reproductive cycles, are summarized in Tables 1–3. First isolation on

Table 1. Bacteriological results of first reproductive cycle: animals positive on tested, according to sample collected

	Milk	Blood	Vaginal swabs	Tissues at slaughter	Lamb ($n = 1$) and fetuses ($n = 24$)
Tested	9	46	46	3	25
Positive	7	24	2	2	24
% Positive	77.8	52.2	4.3	66.7	96.0

Table 2. Bacteriological results of second reproductive cycle: animals positive on tested, according to sample collected

	Milk	Blood	Vaginal swabs	Tissues at slaughter	Lambs
Tested	22	43	43	7	16
Positive	14	0	0	1	3
% Positive	63.6	0.0	0.0	14.3	18.8

Table 3. Bacteriological results of third reproductive cycle: animals positive on tested, according to sample collected

	Milk	Blood	Vaginal swabs	Tissues at slaughter	Lambs
Tested	16	35	35	35	14
Positive	6	0	1	1	1
% Positive	37.5	0.0	2.9	2.9	7.1

Table 4. First isolation on the living animals, last isolation on the living animals and median value

Ewe number	First isolation (WPI)	Last isolation (WPI)	Median value (WPI)
101	61	61	61
102	5	126	91
103	5	6	5
104	3	3	5
105	n.i.	n.i.	n.i.
106	5	122	64
107	5	61	15
108	56	56	56
109	2	126	122
110	3	8	5
111	n.i.	n.i.	n.i.
112	56	56	56
113	4	8	5
114	3	57	5
115	5	8	6
116	n.i.	n.i.	n.i.
117	3	15	9
118	n.i.	n.i.	n.i.
119	56	56	56
120	n.i.	n.i.	n.i.
121	2	3	3
122	2	5	3
123	5	61	56
124	n.i.	n.i.	n.i.
125	4	8	6
126	n.i.	n.i.	n.i.
127	3	6	5
128	6	7	7
129	56	61	59
130	4	126	59
131	3	8	5
132	56	57	57
133	5	126	57
134	3	124	33
135	4	61	56
136	n.i.	n.i.	n.i.
137	3	3	3
138	2	5	4
139	3	6	5
140	3	6	5
141	5	5	5
142	3	5	4
143	5	5	5
144	n.i.	n.i.	n.i.
145	5	8	6
146	5	5	5

WPI, weeks post-infection; n.i. = *Brucella* never isolated from the ewe.

the living animals, last isolation on the living animals and the median value are reported in Table 4.

Blood samples Blood culturing showed 24 of 46 experimentally infected ewes to be *B. melitensis* positive. The first positives were detected in four animals at 15 days p.i. and the last in another four animals at 57 days p.i. Positivity was recorded only during the first reproductive cycle; no bacteraemia was detected subsequently (Tables 1–3).

Milk samples During the first reproductive cycle, the inoculated strain was recovered from seven of the nine ewes that produced milk; during the second and third reproductive cycles, it was recovered from 14 of 22 lactating ewes and six of 16 lactating ewes respectively (Tables 1–3). Considering all the milk samples collected during the experiment, the strain was recovered from the milk of 21 of 31 ewes that produced milk in at least one of their reproductive cycles.

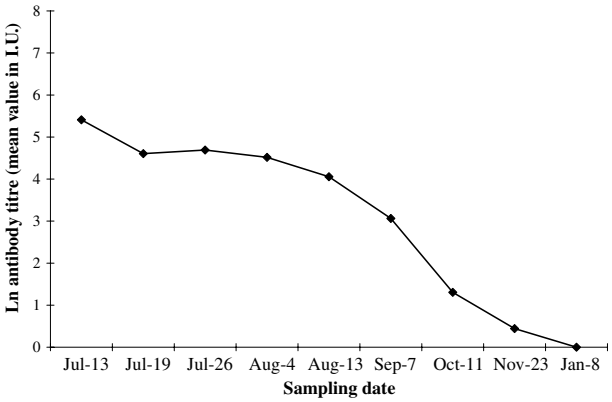

Fig. 1. The complement fixation test antibody kinetics of lambs born in the second reproductive cycle.

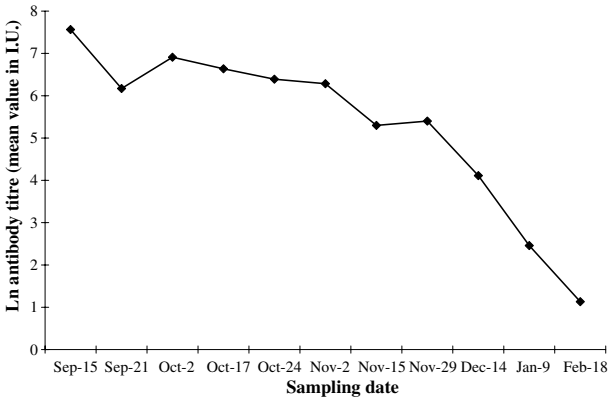

Fig. 2. The complement fixation test antibody kinetics of lambs born in the third reproductive cycle.

Vaginal swabs The inoculated strain was recovered from two ewes up to 68 days post-abortion in the first reproductive cycle and up to 44 days post-partum in another ewe that had normal parturition in the third reproductive cycle. During the second reproductive cycle all vaginal swabs were negative (Tables 1–3).

Tissues The inoculated strain was recovered in the tissues of four of 46 experimentally infected ewes (Tables 1–3). Three of these ewes died respectively five, 18 and 54 weeks p.i.; the fourth ewe was killed at the end of the trial.

Considering all bacteriological tests performed on ewes during the experiment, *B. melitensis* was isolated at least once in 37 living ewes out of the 46 ewes experimentally infected (Table 4). The infection of one more ewe was detected only by bacteriological examination of tissues when killed.

Lambs

Blood samples Blood culturing gave negative results for all lambs except one born in the third reproductive cycle and which was found positive 1 month following birth.

Tissues *Brucella melitensis* biovar 3 was recovered from three lambs born in the second reproductive cycle; they died between 1 and 54 days following birth.

Fetuses

The inoculated strain was recovered from all 24 aborted fetuses.

Serological results

Lambs

The CFT antibody kinetics of lambs born in the second and third reproductive cycles is shown in Figs 1 and 2 respectively. All regressions are significant for all but one of the lambs born in the third reproductive cycle; for this single exception, the low number of observations made could explain the absence of significance in the regression. For all lambs, the standardized residuals of the last observation were included between the expected value and ± 2 standard deviation.

CFT correlations between lambs and their mothers

In the second reproductive cycle the correlations between the CFT titres of week-old lambs and those of their mothers (Fig. 3) were not statistically significant (Spearman's correlation coefficient $\rho = 0.228$; $P > 0.05$).

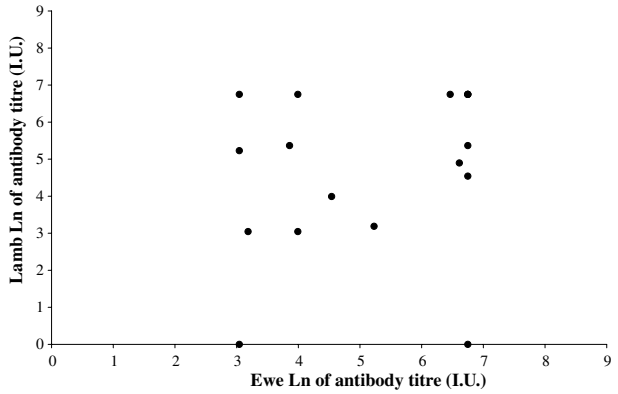

Fig. 3. Correlation of complement fixation test titres between lambs and their dams 1 week after birth in the second reproductive cycle.

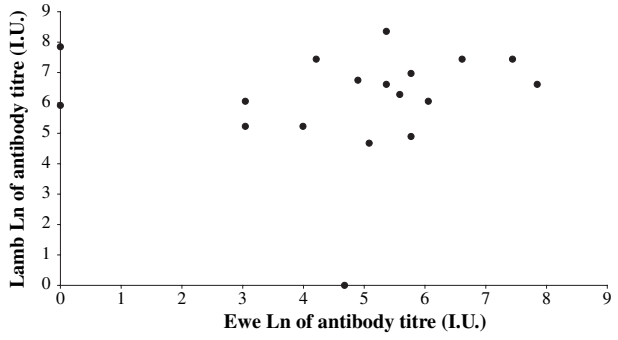

Fig. 4. Correlation of complement fixation test titres between lambs and their dams 1 week after birth in the third reproductive cycle.

In the third reproductive cycle the correlations between the CFT titres of week-old lambs and those of their mothers (Fig. 4) were also not statistically significant (Spearman's correlation coefficient $\rho = 0.218$; $P > 0.05$).

Milk-ELISA results

During the first reproductive cycle, the milk was not submitted to milk-ELISA test. During the second reproductive cycle, the test resulted positive for at least one sampling on 21 out of the 22 lactating ewes. During the third reproductive cycle, the test resulted positive for at least one sampling on 16 out of the 16 lactating ewes.

Discussion

As demonstrated by the number of ewes that were culture positive (38 of 46) and those serologically positive (46 of 46, as described in a previous study by Tittarelli et al., 2004), *B. melitensis* biovar 3 had infected all ewes in the experiment. From the clinical point of view, abortions in the flock did not persist in the second and third reproductive cycles; however, from the bacteriological point of view infection of ewes and elimination of *Brucella* in milk, or its presence in vaginal discharges, persisted throughout the duration of the trial. Four ewes eliminated *Brucella* in their milk during all three lactation periods (at least until 125 weeks p.i.). Previous studies demonstrated excretion in milk for up to 140 days p.i. (Itabashi et al., 1938) and even up to 180 days p.i. (Biggi, 1956) following abortion. Alton (1990) stated that in other trials excretion of *B. melitensis* was not detected in the second lactation. In our study, *B. melitensis* was recovered from the tissues of one ewe killed at the end of the trial. In a previous study (Alton, 1990) using 54 experimentally and 35 naturally infected sheep, all animals were bacteriologically negative between 4 and 13 months after infection, while Taylor et al. (1938) reported the persistence of *B. melitensis*, in exceptional cases, for up to 17 months. In our study, the strain was recovered from vaginal swabs and from milk following parturition in the third reproductive cycle from an ewe that had aborted in the first cycle but was not pregnant in the second cycle. Previous studies (Tazima et al., 1940) revealed that sheep, after a first abortion, continued to excrete *B. melitensis* via the vaginal route during a subsequent pregnancy, and following a normal birth, but occurred only in rare instances. There are no previous reports on the vaginal route of excretion following a second pregnancy and over 2 years (790 days) following a first abortion. Our study demonstrated that this is indeed possible.

From an animal health viewpoint, our data indicate that infected flocks risk spreading the infection to other flocks and that it may persist for 3 years following the initial infection of the flock. Non-vaccinated ewes introduced into such flocks would thus be at great and continuous risk of infection. From a public health viewpoint, the data suggest that brucellosis represents a continuous occupational risk for those who are in contact with infected flocks, because of continuous excretion of *B. melitensis* in milk. Furthermore, the recovery of *Brucella* periodically from the milk of at least some ewes during the entire trial period illustrates that brucellosis in sheep remains a

continuous public health problem for consumers of fresh milk and milk products.

Haemocultures were positive up to 57 days p.i., a figure that falls within the range given in the literature (Biggi, 1956; Shimi and Tabatabayi, 1981). No bacteraemia was detected in subsequent pregnancies. No correlation was found between the CFT titres of ewes and their offspring 1 week following their birth in the second and third reproductive cycles. In cattle brucellosis it has been reported that calves from dams with high and low serum agglutinating antibody levels had high and low levels of agglutinating antibody respectively (Peiris, 1972). The present study suggests the absence of correlation in sheep, as it did not find one. This could be because of the fact that maternal antibody transmission to lambs may follow a different pattern in sheep. However, further studies are needed to better investigate if the correlation reported for cattle could exist in sheep also. Nevertheless, the lamb serology kinetics suggest that, even if not related to the antibody titres of the dams (Figs 3 and 4), the *B. melitensis* positivity of the lambs can be considered to be of colostral origin. This hypothesis is supported by the positivity of ewes to milk-ELISA. This hypothesis is also supported by the analysis of lamb CFT antibody titres (Figs 1 and 2), which became negative in all surviving lambs within 4 months in the second reproductive cycle, whereas the three tissue culture positive lambs died immediately after birth or within 54 days. This was confirmed even in the third reproductive cycle although four lambs were still positive at the end of the trial; in all probability this positivity would be because of the presence of colostral antibodies in decreasing phase, as demonstrated by the significance of the least-square linear regression. The only lamb blood culture that was positive was also serologically positive when the lamb died at the age of 119 days. One practical implication is that, despite some authors (Alton, 1990) stating that maternal antibody remain for 2 months, serological tests may have no practical diagnostic significance if performed during the first 5 months of life. The possibility that a lamb (born to an infected dam) can be infected but does not actively produce antibody against *Brucella* could represent another way for the bacteria persistence in a flock. Actually, this phenomenon has been studied in cattle; such animals are usually termed 'latent carriers', and the herds in which they occur 'problem herds' (Nicoletti and Muraschi, 1966; Luchsinger et al., 1973). The terms 'chronic' (Morgan and Richards, 1974) or 'symptomless' (Robertson, 1971) carriers have also been used to describe such animals. This has not been fully investigated in sheep brucellosis, even if some authors (Alton, 1990) have hypothesized the occurrence of this phenomenon in sheep also. The bacteriological negativity of all lambs survived over 2 months of age, their positivity to CFT and the high antibody titres in the single lamb blood culture positive at 1 month of age, would suggest that the induction of latent carrier state would be unlikely. Nevertheless, giving the flock husbandry in natural conditions, as the lambs were not separated from their dams, antibodies of colostral origin could have concealed a latent carrier state in lambs, and bacteriology may have not revealed this condition. Therefore, further studies are needed to evaluate to what extent the phenomenon of latent carrier state in sheep brucellosis may occur. On the contrary, the birth of three infected lambs, even if CFT positive, suggests that the phenomenon of latent carrier state may represent another way for *B. melitensis* to persist in a flock. Therefore, total depopulation of chronically infected brucellosis flocks is often warranted.

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