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

M. Tittarelli¹,², M. Di Ventura¹, F. De Massis¹, M. Scacchia¹, A. Giovannini¹, D. Nannini¹ and V. Caporale¹

Addresses of authors: ¹Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’, Campo Boario, Teramo, Italy; ²Corresponding author: E-mail: m.tittarelli@izs.it

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, 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 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 x 10⁶ colony-forming units in 100 ml 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% CO₂. 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% CO₂ 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% CO₂. 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% CO₂ 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% CO₂. 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% CO₂ 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% CO₂. The plates were examined after 72 h and then each following day to observe the development of bacterial colonies.

Tissues and aborted fetuses

Small pieces (about 2 cm³ 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% CO₂. 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% CO₂ for up to 6 weeks. Weekly, subcultures from each broth were placed onto selective solid medium and incubated in an atmosphere containing 5–10% CO₂. 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 Brucella abortus biovar 1 strain S99 was used 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).

Persistence of B. melitensis in Infected Ewes

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

Mean OD of tested sample

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 statistical analysis was performed on the CFT results of each lamb born in the third reproductive cycle.

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.

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

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Milk</th>
<th>Blood</th>
<th>Vaginal swabs</th>
<th>Tissues at slaughter</th>
<th>Lamb (n = 1)</th>
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</thead>
<tbody>
<tr>
<td>Tested</td>
<td>9</td>
<td>46</td>
<td>46</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>24</td>
<td>2</td>
<td>2</td>
<td>24</td>
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<tr>
<td>% Positive</td>
<td>77.8</td>
<td>52.2</td>
<td>4.3</td>
<td>66.7</td>
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Table 2. Bacteriological results of second reproductive cycle: animals positive on tested, according to sample collected

<table>
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<tr>
<th>Sample Type</th>
<th>Milk</th>
<th>Blood</th>
<th>Vaginal swabs</th>
<th>Tissues at slaughter</th>
<th>Lambs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested</td>
<td>22</td>
<td>43</td>
<td>43</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>% Positive</td>
<td>63.6</td>
<td>0.0</td>
<td>0.0</td>
<td>14.3</td>
<td>18.8</td>
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</table>

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

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Milk</th>
<th>Blood</th>
<th>Vaginal swabs</th>
<th>Tissues at slaughter</th>
<th>Lambs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested</td>
<td>16</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>14</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>% Positive</td>
<td>37.5</td>
<td>0.0</td>
<td>2.9</td>
<td>2.9</td>
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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
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.

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.

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

<table>
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<tr>
<th>Ewe number</th>
<th>First isolation (WPI)</th>
<th>Last isolation (WPI)</th>
<th>Median value (WPI)</th>
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</table>

WPI, weeks post-infection; n.i. = *Brucella* never isolated from the ewe.
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 $\rho = 0.228; P > 0.05$).

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 agglutin-
ating antibody levels had high and low levels of agglutin-
atlng 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 con-
firmed 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 serolog-
ically 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
persistance in a flock. Actually, this phenomenon has been
studied in cattle; such animals are usually termed ‘latent
carrier’, 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 ‘symptom-
less’ (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. There-
fore, 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 depopu-
lation of chronically infected brucellosis flocks is often
warranted.

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