Q FEVER IN BRITAIN: ISOLATION OF *RICKETTSIA BURNETI* FROM PLACENTA AND WOOL OF SHEEP IN AN ENDEMIC AREA*

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INTRODUCTION

Soon after Q fever was identified in man in this country, infection was also found in dairy cows (Marmion & Stoker, 1950), and subsequent surveys of these animals (Marmion, Stoker, McCoy, Malloch & Moore, 1953; Slavin, 1952) revealed infection to be widespread. Even so the epidemiological findings did not indicate that cattle were the only source of infection, and recently some evidence has been obtained in the Romney Marsh area of Kent that sheep are involved in the epidemiology of Q fever in that area.

In the Romney Marsh five flocks of sheep were found to have complement-fixing antibody to *Rickettsia burneti* in their sera and this finding, together with a certain seasonal and occupational pattern of Q fever in the local inhabitants, led to the supposition that not only were the sheep infected, but that they could act as a source of Q fever in man (Marmion, Stewart, Richmond, Barber & Stoker, 1954).

Although the evidence of infection in sheep in the Romney Marsh, and also in sheep from a wider area of Kent, sampled at various Kentish slaughterhouses, depends on a well tried serological method of diagnosis, it nevertheless seemed essential to isolate the rickettsia from these animals as final proof of the presence of *R. burneti* in English sheep.

Investigations in other countries have shown that *R. burneti* can be isolated from the milk of infected sheep (Caminopetros, 1948; Jellison, Welsh, Elson & Huebner, 1950), and, more constantly, from the placenta and birth fluids of the ewes at lambing (Welsh, Lennette, Abinanti & Winn, 1951; Abinanti, Lennette, Winn & Welsh, 1953) and, thereafter, from their faeces for a variable, and generally short period (Winn, Lennette, Welsh & Abinanti, 1953). Attempts have been made, therefore, to isolate the organism from such materials collected from Romney Marsh sheep.

While our investigation was being planned we were informed by Dr Lennette (personal communication) that *R. burneti* had been isolated from the wool of naturally infected sheep in Northern California (see Abinanti, Welsh, Winn &

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Lennette, 1955). As a result of receiving this information, and because wool is easy to collect, we also attempted to isolate the organism from this material as well.

At the start of the investigation into the epidemiology of Q fever in the Romney Marsh in the early months of 1953, only two cases of Q fever were known to have occurred among farm workers in contact with sheep. In the lambing season of that year 197 placentas and fifty-nine milk samples were collected from the flock with which these workers had been in contact (flock 1 of Marmion *et al.* 1954), but these samples proved to be negative on examination, as did 304 milk samples from four additional flocks. By the spring of 1954, however, several more human cases of Q fever and other flocks of sheep with serological evidence of infection had been found. Two of these (flocks 3 and 5), with evidence of the highest rates of infection, were chosen for further investigation. This paper describes the isolation of *R. burneti* from the placenta and wool of sheep in one of these flocks.

METHODS

(1) *Collection of specimens*

Specimens were collected from both flocks during the entire lambing season during late March, April and May 1954. As our intention was to isolate the organism rather than estimate the frequency of its excretion from the ewes, no special precautions were taken to collect placentas in individual sterile containers or to use a separate set of sterilized instruments to harvest specimens from each placenta.

Placentas were collected from the ground as soon as practicable, and not more than 10 hr. after they had been dropped by the ewe, and were taken in a bucket to a shepherd's hut or barn adjoining the lambing fields. Here they were placed, one at a time, on a metal tray, and the outer membranous portion of the placenta was cut open with scissors and forceps to reveal the cotyledons. Portions of cotyledon were then taken from several different areas of the placenta, so as to ensure a comprehensive sample of the whole. The samples of cotyledon were placed in screw-capped bottles. The remainder of the placenta was then discarded, and the tray washed clean and wiped over with cotton-wool soaked in methylated spirit before a fresh placenta was placed upon it. Forceps and scissors were cleansed of gross contamination by wiping with cotton-wool and spirit, then dipped in spirit and heated in the flame of a spirit burner.

Wool tags, a term applied here to clippings of the matted wool from the perineal region, were obtained from the ewes within 48 hr. of lambing and mostly consisted of a mixture of wool, faeces and dried birth fluids. On the occasions when ewes had lambed but the placenta was lost, faeces and milk as well as wool tags, were collected from the ewe concerned within 24 hr. of lambing.

A random sample of faeces from the whole of each flock was also made by collecting droppings, after the majority of ewes had lambed, from twenty-five evenly distributed areas in each lambing field.

Post-mortem examinations were performed on any dead lambs and also on one ewe which died during pregnancy. Samples of liver and spleen and occasionally lung were removed for attempted isolation of *R. burneti*.

Storage. All samples were placed in a local refrigerator at $-5°$ to $-10°$ C. for up to 3 weeks before removal to Cambridge, where they were stored without preservative at $-20°$ C. until examined.

(2) *Preparation of specimens*

(*a*) *Placentas and organs collected at post-mortem*

The placental samples were thawed, smears were made for microscopy and 0·5 g. of tissue were removed from not less than five cotyledons. Material collected in this way from five placentas (i.e. weighing 2·5 g.), was pooled and ground in a microblender with 10 ml. of 10 % horse serum broth. The resulting 20 % suspension was then centrifuged at 1000 r.p.m. for 15 min. 500 or 1000 units of penicillin per ml. were added to the supernatant, which was then allowed to stand at 4° C. for 24–48 hr. 20% suspensions were also made in the same way from organs collected at post-mortem.

(*b*) *Wool tags*

These were grouped in pools of tags from five ewes, so that the same animals contributed to each pool of tags as had contributed to each pool of placentas. To each pool of tags sufficient Triton X–100, a non-ionic surface active agent, (0·4 % in distilled water) was added to make a 20 % suspension and the wool tag and fluid were shaken for 15 min. at room temperature in a mechanical shaker. The fluid was then decanted from the wool, centrifuged at 1000 r.p.m. for 15 min., and the resulting supernatant was treated with 500 units of penicillin per ml. for 24 hr. at 4° C. (see Abinanti *et al.* 1955).

Tags from a few individual sheep were prepared separately as 10 % suspensions.

(*c*) *Faeces*

Samples collected from individual ewes which had just lambed were ground with horse serum broth to make a 20 % suspension, then centrifuged at 1000 r.p.m. for 15 min. The supernatant was treated with 1000 units of penicillin per ml. for 24 hr.

Each of the samples of faecal droppings collected at random from different areas of the fields, weighing about 6 g. was allowed to soak in 50 ml. of horse serum broth overnight at 4° C. It was then emulsified by shaking with glass beads in an automatic shaker for 1 hr. A pool composed of equal quantities of the suspension from each of the five samples was prepared, centrifuged at 1500 r.p.m. for 5 min., and the supernatant treated with 1000 units of penicillin per ml. for 24 hr at 4° C.

(*d*) *Milks* were mixed with 500 units of penicillin and allowed to stand for 24 hr. at 4° C., but were otherwise untreated.

(3) *Inoculation of guinea-pigs*

Pairs of adult guinea-pigs, weighing from 300 to 500 g., were inoculated intraperitoneally with 2 ml. quantities of each suspension, whether made from an individual specimen, or from a pool of specimens. Uninoculated guinea-pigs were occasionally placed in the animal house, but on no occasion was there evidence of

cross-infection. All the animals were bled 6 weeks after inoculation, and the sera examined for Q fever complement-fixing antibodies. If antibodies were present at 1:10 or above, the material inoculated was retested in guinea-pigs which were bled both before and 6 weeks after inoculation.

In some instances both the inoculated guinea-pigs died before the 6-week period had elapsed; the sample was then retested in two or more guinea-pigs. If only one of the original guinea-pigs died, however, the test was not repeated, save in exceptional circumstances.

RESULTS

Flock 3

Flock 3, composed of 125 Romney Marsh ewes, was kept on permanent pasture and largely maintained from lambs born on the farm. In June 1952, however, some 40 ewe tegs (i.e. 1- to 2-year-old ewes, about to lamb for the first time) had been introduced into the flock from elsewhere. In September 1953, 33·7 % of the flock had Q fever antibodies (Table 2).

In 1954 lambing began on 9 March and was not completed until 25 May, but the great majority of ewes lambed in the month of April. Seven were barren and placentas were collected from ninety-six of the remaining 118 ewes. Nineteen were ewe tegs and the remaining 77 were in their second, third or fourth lambing season. The placentas were examined for *R. burneti* by inoculation into guinea-pigs in twenty pools of five or less specimens, as already described. One pool representing placentas from five sheep gave rise to Q fever antibodies in both inoculated guinea-pigs, and the remaining nineteen pools were negative. 20 % suspensions made from individual placentas in the positive pool were then tested separately by inoculation into groups of two previously bled guinea-pigs. Suspensions from one of these placentas gave rise to fever in both guinea-pigs after 10 days, and another gave rise to fever in both guinea-pigs after 11 days. There was a subsequent appearance of Q fever antibody at a dilution of at least 1 in 80 in the animals inoculated with these two placentas but not in those inoculated with the other three placentas, which had been part of the original positive pool.

The first placenta to contain *R. burneti* was collected on 9 April from a ewe in its third lambing season, and the other was taken on 10 April from a ewe in its second lambing season. The strain of *R. burneti* from the first placenta was passaged twice in guinea-pigs, giving rise to a typical febrile response and to Q fever antibody. The strain was also grown in the yolk sacs of fertile hens' eggs. It showed the morphology typical of rickettsiae, and purified suspensions of organisms from the yolk sacs fixed complement with serum from guinea-pigs inoculated with the Nine Mile strain of *R. burneti*. Late guinea-pig serum samples, taken 90 days after inoculation, were used because of the failure of some strains to react with early samples of serum (Smadel, Snyder & Robbins, 1948).

No rickettsiae could be seen in direct Macchiavello stained smears prepared from the surface of the cotyledons of the two positive placentas or of the remaining placentas which had proved to be negative on guinea-pig inoculation.

From twenty-two ewes which lambed the placenta was not obtained, but faeces

and wool tags were collected from seven, and from five of these milk was obtained as well, all within 24 hr. of lambing. From two ewes, wool tags were the only specimens obtained. None of the guinea-pigs inoculated with these materials developed Q fever antibodies. No specimens were tested from thirteen ewes. Thus *R. burneti* was isolated from two of the total 105 ewes from which specimens were tested, or of 118 ewes which lambed.

Wool tags were collected within 24 hr. of lambing from ninety-three ewes. These were tested after grouping in pools of tags from five sheep (see Methods), but tags were tested separately from each of the five sheep which had contributed to the positive pool of placentas (see above). One of these tags, which was tested by itself, gave rise to Q fever antibody in both the inoculated guinea-pigs. This tag was collected on 9 April from the ewe which had voided an infected placenta a few hours previously. None of the other wool tags, including that from the other ewe from which an infected placenta was obtained on 10 April, gave rise to Q fever antibodies in the guinea-pigs.

Random sample of faeces

On 1 May, after most of the ewes had lambed, twenty-five samples of faeces were collected from the ground in evenly distributed areas of the lambing field in order to determine the extent of faecal excretion by the whole flock. They were tested in pools of five samples but *R. burneti* was not isolated.

Post-mortem material

R. burneti was not isolated from the liver or spleen of seven lambs, or of one ewe which died of pregnancy toxaemia while the investigation was in progress.

Flock 5

Flock 5, which was 4 miles from flock 3, consisted of about 203 Border-Leicester Cheviot ewes. In April 1953 a new shepherd had developed Q fever during the first lambing season he worked with this flock (see Marmion *et al.* 1954). Flock 5 was maintained on pastures which were ploughed up in rotation and used for crops. None of the lambs born in this flock were used as replacements for the breeding stock, which was maintained with lambs purchased in Scotland. In the spring of 1954 about a quarter of the flock were ewe tegs in their first lambing season. Q fever complement-fixing antibodies had been found in 7·7 % of ewes on 26 June 1953 (Table 2).

Lambing began on 28 March 1954. Of the 203 ewes, 199 lambed before 7 May, and the remaining four ewes were barren. Placentas were collected from 194 ewes and tested by guinea-pig inoculation as described, in forty pools of five or less. One of two guinea-pigs inoculated with a pool of placentas collected on 4 April was found to have an antibody titre of 1 in 10 when bled 6 weeks later. When individual placentas in this pool were tested separately, however, no guinea-pigs developed antibodies. No isolations were made from any of the remaining placentas, or from faeces taken from four of the five remaining ewes from which the placentas were lost.

On 19 April samples of faeces were collected from the ground in twenty-five evenly distributed areas, but no *R. burneti* was isolated from them.

Samples of livers and spleens were taken from twenty-nine dead lambs and samples of liver from three dead ewes, which had not yet been bred, but none of these contained *R. burneti*.

INFECTIVITY TITRATIONS OF POSITIVE MATERIAL

In order to determine the degree of infectivity of the two positive placentas and the wool tag from flock 3, ascending tenfold dilutions of the suspensions prepared from those specimens were inoculated into groups of two or three guinea-pigs which were tested for development of Q fever antibodies 6 weeks later.

Table 1. *Results of infectivity titrations on suspensions from placentas and wool tags*

(Numerator denotes number of guinea-pigs developing Q fever antibody at 1 in 40 or greater. Denominator denotes number inoculated.)

	Dilution of inoculum					
Inoculum	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Placenta from sheep 2617	3/3	2/2	3/3	2/2	0/2	0/3
Wool tag from sheep 2617	2/2	2/3	2/3	1/3	0/3	—
Placenta from sheep 2618	1/2	1/2	0/2	0/2	—	—
Wool tag from sheep 2618	0/2	—	—	—	—	—

The results given in Table 1 show that the first placenta (sheep 2617) had a moderately high infectivity titre ($ID_{50} = 10^{-4 \cdot 3}$) and also that the suspension from the wool tag of this sheep was almost as infective ($ID_{50} = 10^{-3 \cdot 25}$). The second placental suspension (sheep 2618) was infective only in low dilution, however, and it was not surprising that no organisms were found on the wool tag.

In view of their relative infectivities and of the fact that these two placentas were collected on consecutive days, we cannot exclude the possibility that the second placenta was contaminated from the first during the process of collection or removal of samples of cotyledon.

SEROLOGICAL FINDINGS IN THE FLOCKS

On 8 July 1954, some time after the lambing season was over, specimens of serum were collected from the ewes of both flocks in order to compare the Q fever antibody levels with those found in the previous year. The results of complement-fixation tests with Henzerling strain antigen on the serum samples collected in 1953 and in 1954 are shown in Table 2.

It will be seen that in 1954, there was a very marked drop in the proportion of sheep with Q fever antibodies in both flocks. As the individual sheep were not identified, it is impossible to know if the two ewes with antibody in Flock 3 in 1954 were those with positive placentas.

The results shown in both 1953 and 1954 were obtained by complement-fixation tests made after storage of the serum samples at −20° C. for less than 1 month after their collection. In tests made after longer periods of storage at −20° C. there was an increase in the proportion of sera showing fixation at low titre, but this did not affect the obvious fall in the serological rate of infection between 1953 and 1954.

Table 2. *Results of complement-fixation tests for Q fever antibody on sera collected on different dates from ewes in flocks* 3 *and* 5

Flock	Date of collection	Total tested	Total less than 1:10	Total 1:10 or more	Percentage 1:10 or more	Distribution of titres				
						10	20	40	80	160
3	3. ix. 53	95	63	32	33·7	12	10	6	4	0
	8. vii. 54	115	113	2	1·7	2	0	0	0	0
5	26. vi. 53	143	132	11	7·7	5	4	1	1	0
	23. x. 53	219	212	7	2·7	4	1	0	1	1
	8. vii. 54	200	198	2	1·0	2	0	0	0	0

DISCUSSION

The isolation of *R. burneti* from placentas and wool of sheep in south-east England confirms the previous supposition of infection in these animals, based on serological findings alone.

The number of sheep excreting the organism appears at first sight to be surprisingly low in view of the high proportion of animals which showed serological evidence of infection in the previous year. We did not take special precautions to prevent cross-contamination between placentas (see Method), but *R. burneti* was only isolated from two of ninety-six placentas from ewes in flock 3, presumably indicating excretion from not more than two, and possibly from only one ewe. This is in marked contrast to the findings of Welsh *et al.* (1951) who found *R. burneti* in twenty-one of seventy-two placentas collected at random from flocks in Northern California which had previously shown serological evidence of infection. The actual proportion of sheep with antibodies in the flocks as a whole was not given in this paper, but fifteen out of forty-three serologically positive sheep (35%) excreted the organism. It appears from their experiments that hamsters were somewhat more sensitive than guinea-pigs for detection of *R. burneti*, and it is possible that by using hamsters we might have found a few more positive placentas in flocks 3 and 5.

The apparently small number of ewes excreting *R. burneti* in the 1954 lambing season is in keeping with the considerable fall in the proportion of sheep with antibodies in that year (see Table 2).

It seems likely that in a given flock the number of ewes which excrete varies considerable from year to year and may depend, for example, on the number and susceptibility of ewe tegs introduced in the previous year, and on climatic and other

environmental conditions which might affect the spread of infection to the new susceptibles in that year.

Nevertheless, placental excretion by very few animals can lead to considerable dispersal of *R. burneti* because of the heavy contamination of the fleece and the prolonged viability of the organisms. The infectivity of one of the placentas and the wool was moderately high, and Welsh *et al.* (1951) have shown that naturally infected placentas may contain even up to 10^9 guinea-pig or hamster infective doses per gram. This provides a possible source of infection for man, while other sheep of the same flock are even more likely to be exposed because they are in closer contact. Unfortunately, in south-east England at least, one cannot be certain that this is the only mode of spread amongst the sheep themselves. Flock 3 was heavily infested with the tick *Haemaphysalis punctata*, and the isolation of *R. burneti* from these ticks, which is the subject of another communication (Stoker & Marmion, 1955), suggests that an alternative mode of transmission might have been possible in this flock.

SUMMARY

1. In an endemic area of Q fever in south-east England, two flocks of sheep, with serological evidence of infection by *Rickettsia burneti*, were examined for excretion of the rickettsia at parturition.

2. *R. burneti* was isolated from two out of ninety-six placentas collected from 118 ewes which lambed in one flock. The organism was also isolated from 'wool tags' obtained from one of the ewes which voided an infected placenta.

3. Infectivity titrations showed that the two placentas contained respectively $10^{4.5}$ and $10^{1.5}$ guinea-pig infective doses per gram of cotyledon. The wool tag from the sheep which voided the more highly infected placenta contained $10^{3.25}$ infective doses per gram.

4. Except for one doubtful result, which could not be confirmed, no isolations were made from 194 placentas collected from 199 ewes in the other flock.

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