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From the State Veterinary Research Station for Small Ruminants, Høyland, Sandnes, Norway.

LISTERIOSIS IN SHEEP

LISTERIA MONOCYTOGENES EXCRETION AND IMMUNOLOGICAL STATE IN HEALTHY SHEEP*

By

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GRØNSTØL, H.: *Listeriosis in sheep. Listeria monocytogenes excretion and immunological state in healthy sheep.* Acta vet. scand. 1979, *20*, 168—179. — The excretion of Listeria monocytogenes (Lm) in the faeces and milk, and humoral and cell mediated immunity against Lm, were examined in a sheep flock where no cases of listeriosis had occurred during the last 3 years. The investigation was carried out during the indoor season. During the first part of the season 2 of the 10 pregnant, 8 months old lambs excreted Lm in the faeces, but none of the 106 ewes, 2—10 years old. At lambing the organism was isolated from the faeces of 6 of the 10 1 year old lambs and from 64 % of the ewes, and from the milk of 1 of the lambs and 41 % of the ewes. Nearly all the isolates (98.5 %) belonged to serotype 1.

Antibody titres against Lm were found in sera and whey by an indirect haemagglutination method. The titres were higher for the ewes than for the hoggs and seemed to be influenced by the number of foetuses the animals carried.

Cell mediated immunity was determined by a skin test where delayed hypersensitivity against an antigen prepared from Lm, was measured. Animals fed grass silage had a stronger reaction than animals fed hay, and a stronger reaction was found in animals with $\geq$ 3 foetuses than in the remainder.

The investigation indicates that even in a healthy sheep flock all the animals may be exposed to Lm, and the majority may be latent carriers and excrete this organism in the faeces and milk during periods of stress.

Listeria monocytogenes; sheep; bacteria in faeces and milk; immunity.

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Listeria monocytogenes (Lm) can be isolated from the faeces, nasal secretions and milk of healthy sheep. This has been shown in connection with spontaneous outbreaks of listeriosis and in experiments (*Ivanov et al.* 1964, *Lehnert* 1964, *Killinger & Mansfield* 1970).

Since no extensive investigations of healthy flocks have been reported, the present work was undertaken to study the excretion pattern of Lm during and after pregnancy in a flock where clinical listeriosis had not occurred for 3 years, and to examine whether sera and whey from sheep in that flock contained antibodies against Lm.

Cell mediated immunity (CMI) seems to be the main defence system against listeric infections (*Mackaness* 1962). A skin test which measures delayed hypersensitivity (DHS) against Lm and is a good indicator of CMI (*Grønstøl & Larsen,* unpublished) was used to examine whether healthy animals fed hay and grass silage developed CMI against Lm. In addition, the influence of age, and number of foetuses on excretion of Lm, and on humoral immunity (HI) and CMI against Lm was recorded.

MATERIALS AND METHODS

Flock

The investigation was carried out in the experimental flock belonging to this Research Station. Only 3 cases of clinical listeriosis had been diagnosed in the flock during the preceding 14 years, and none of them during the latest 3 years. The material comprised 106 ewes, from 2 to 10 years old, and 10 hoggs, about 8 months old, of the Dala and Rygja breeds. All the sheep were pregnant and due to lamb in April/May.

During the summer the animals had been kept on mountain pastures, and from the middle of September the sheep had grazed on farm leys at the Research Station. They were moved indoors on 5th December and housed during the gestation and lambing period, and let out about 3 weeks after lambing. They were kept in pens with slatted floors, each pen containing 12—13 sheep. The animals were fed hay and grass silage ad libitum, and each sheep was given an additional daily ration of 0.3 kg concentrates with a crude protein content of 12.5 %. From lambing this ration was increased to 1 kg. Grass silage, hay and concentrates were not fed after the animals were moved onto pasture.

The grass silage was examined for content of digestible protein, total nitrogen and dry matter at the Institute of Animal Nutrition, Agricultural College of Norway.

All the sheep were shorn on the 4th—6th of March, i.e. 4—8 weeks before lambing. The first week after shearing an electric fan heater was used in the sheep house to prevent cold stress.

The sampling dates are recorded in Table 1.

Table 1. The sampling dates for bacteriological and serological examination of 106 ewes, 10 hoggs and silage. All the sheep were pregnant and lambed in April/May.

Date	Sampling material			
Dec. 5*	Faeces	Sera		
Dec. 27				Silage***
Jan. 10				Silage
Feb. 12	Faeces	Sera		
Apr. 5				Silage
At lambing	Faeces	Sera	Milk	
June 2**	Faeces			

* Before start of the grass silage feeding.
** The sheep had been on pasture for 3 weeks.
*** Four samples were examined at each sampling date.

Bacteriological examination

Faeces. Three g of faeces was suspended in 10 ml of phosphate-buffered saline (PBS), pH 7.4, and stored at 4°C for up to 1 year. Monthly an alginate swab was dipped into the suspension and then transferred to Stuarts transport medium (Difco) and incubated at 22°C for 2 weeks. The swab was then suspended in 10 ml of Ringer-Calgon solution (Oxoid) and held at 22°C for 48 h (*Kampelmacher & Janssen* 1969), after which 1 loop-ful was spread on trypaflavine-nalidixic acid-serum-agar (TNSA, *Ralovich et al.* 1971, as modified by *Bockemühl et al.* 1974) and on propolis-agar (PA, *Grønstøl & Aspøy* 1977). The plates were incubated at 37°C for 48 h after which Lm colonies were identified presumptively by the oblique light technique (*Gray et al.* 1948). Their identity was confirmed by slide agglutination using bacto anti-1 and anti-4 sera (Difco), by Gram-staining and by their motility in semi-solid agar (nutrient broth (Oxoid) with addition of 0.3 % agar) incubated at 22°C for 1 week.

M i l k. Ten ml of milk was centrifuged, the sediment suspended in 10 ml of PBS, and held at 4°C for up to 1 year and examined as described for faeces.

S i l a g e. Two hundred g of grass silage was added to 0.5 l PBS, stored at 4°C for 6 days, filtered through a double layer of sterile gauze and the filtrate centrifuged. The sediment was suspended in 10 ml of PBS, kept at 4°C for up to 1 year and examined by the methods described above.

Serological examination

Sera were tested for antibodies against Lm by an indirect haemagglutination method (IHA, *Herbert* 1973), having first been inactivated at 56°C for 30 min.

Inactivated sheep serum was used as stabilizer for the coated blood cells. A protein-containing fraction was prepared from Lm serotypes 1 and 4 as described by *Patocha & Mara* (1973) and used as antigen. The tannin-treated sheep red blood cells were coated with antigen and diluted to a concentration of 1.5 %. The titration was carried out in wells of plastic agglutination plates* by adding 0.2 ml of the 1.5 % cell suspension to a doubling dilution series of the test sera in 0.2 ml of PBS. The plates were held at room temperature and read after 18 h. Milk samples were treated according to *Kohler et al.* (1968), and the whey was titrated as described for serum.

Skin test

The protein-containing fraction (*Patocha & Mara*) was standardized to 1 mg/ml, and 0.3 ml of this antigen solution was injected intradermally on a shaven area of the neck. The same volume of PBS was injected as a control on another spot. After 24 h the thickness of the 2 skin folds was measured using a cutimeter designed for tuberculin testing, and the difference was recorded. Seven ewes fed hay and concentrates were also tested in the same way. The test was performed on the 25th of March, i.e. shortly before the lambing started.

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RESULTS

Bacteriological examination

Faeces and milk. The number of samples which were found to contain Lm on the various sampling dates can be seen from Table 2. Lm was not isolated from the faeces of any ewe on the first 2 sampling dates, but from 64 % of the samples collected at lambing. Lm was isolated from the faeces of 2 of the 10 hoggs on the first sampling date and from 6 hoggs at lambing. At lambing, 41 % of the milk samples from the ewes and 1 of the 10 samples from the hoggs contained Lm, with 98.5 % of the isolates during the investigation period belonging to serotype 1. The excretion of Lm in the faeces and milk was not related to age or number of foetuses.

Table 2. Number of animals with faeces and milk containing Listeria monocytogenes (Lm) on the various sampling dates, in a flock consisting of 106 pregnant ewes and 10 pregnant hoggs.

	Faeces		Milk	
Date	ewes	hoggs	ewes	hoggs
Dec. 5	0	2		
Feb. 12	0	0		
At lambing	68	6	43	1

Grass silage. The grass silage was of good quality according to chemical analysis (*Nedkvitne,* personal communication 1975). Lm was isolated from 1 of the 12 samples examined.

Serological examination

Both sera and whey contained antibodies against Lm. The reciprocal geometrical mean titres (GMT) in sera from the whole flock and for the ewes and hoggs are recorded in Table 3. GMT

Table 3. Reciprocal geometrical mean titres (GMT) for the whole flock, the ewes and the hoggs on the various sampling dates.

	Sera			Milk		
Date	whole flock	ewes	hoggs	whole flock	ewes	hoggs
Dec. 5	28	31	11			
Feb. 12	17	18	<10			
At lambing	12	12	14	13	14	<10

in the ewes was at its lowest at the time of lambing, while the trend was opposite for the hoggs. GMT for the different age groups followed the pattern for the ewes.

Table 4 shows GMT for the ewes according to the number of foetuses they carried. Ewes with 3 foetuses or more had a lower GMT at lambing than ewes with 1 or 2 foetuses, but the difference was not statistically significant (Students t-test).

Table 4. Reciprocal geometrical mean titres (GMT) for the ewes according to the number of foetuses they carried, on the various sampling dates.

	3 foetuses		2 foetuses		1 foetus	
Date	sera	milk	sera	milk	sera	milk
Dec. 5	22		34		30	
Feb. 12	18		19		13	
At lambing	<10	12	12	12	14	15

No specific pattern was found for GMT in whey from the different age groups or from ewe groups composed according to number of foetuses.

Skin test

The results of the skin test are recorded in Table 5. The response to the test was much greater for the ewes fed silage than for the ewes fed hay, but the variances were too different to allow statistical evaluation by Students t-test. The response was also greater for ewes with ≥ 3 foetuses than for the remainder ($P < 0.05$).

Table 5. Average increase in skin thickness (mm) in the skin test.

Number of animals	Feeding	Number of foetuses	Increase (mm)
7	Hay	1—4	1.00
106	Hay + silage	1—4	1.90
16	— „ —	≥ 3	2.25
70	— „ —	2	1.90
20	— „ —	1	1.65

DISCUSSION

Although only 3 cases of clinical listeriosis had occurred in this flock during the last 14 years, the investigation indicates that most sheep, may be all, were latent carriers of Lm. The bacteriological examination also showed that Lm was present in the silage that otherwise was of good quality.

The excretion pattern of Lm in the hoggs was different from that found in the ewes. On the first examination, 5th December, Lm was isolated from the faeces of 2 of the 10 hoggs but from none of the ewes. This difference may reflect duration and intensity of exposure to Lm in earlier periods. However, the number of hoggs was too small to allow any conclusions.

The present investigation indicates that the hoggs had been exposed to Lm from birth through infected milk and from teats soiled with infected faeces. In addition, the hoggs had been kept together with the ewes on farm leys before the investigation started. These fields had been grazed by sheep for many years and had been fertilized routinely with sheep dung in late winter or early spring. The present findings, i.e. the high frequency of Lm excretors, indicate that the soil and the vegetation on the farm leys had been contaminated with Lm. This is also supported by the findings of *Weis* (1975) who isolated Lm from a high proportion of soil and vegetation samples.

Grazing on these fields had probably represented an additional exposure to Lm for the hoggs, sufficiently strong to develop antibody titres against Lm. The ewes had probably been exposed to Lm in a way which induced systemic immunity, in earlier periods. The skin test had not been performed prior to this investigation and, hence, no comparison with these data is possible.

The ewes were also used to handling, and the stress when the animals were gathered, weighed and moved into pens might have been less for the ewes than for the hoggs.

On the next sampling date the animals had been kept indoors for more than 2 months, and the management and feeding regime had been fairly constant. None of the animals were excreting Lm, probably reflecting the low number of Lm ingested and the relative absence of stress. In addition, as GMT in the hoggs were not elevated, the reduction of excretors might have been associated with development of local immunity in the gut.

At lambing the picture had changed completely. A large proportion of the animals excreted Lm in the faeces and milk. Several factors were probably responsible for this increase in excretors. Although measures had been taken to prevent cold stress after shearing, the shearing process itself and the inevitable changes in protection against low temperature may have constituted stress factors (*Kilgour & Langen* 1970) which reduced the immunity against Lm. The animals were most likely latent carriers of Lm. As Lm was not isolated from the grass silage at that time, an increased supply of Lm through the feed was therefore unlikely. *Grønstøl et al.* (1974) working on salmonella in calves, showed that stress could activate excretion in latent carriers.

The changes associated with late pregnancy were probably important. The increase of excretors during this period seemed to follow the same pattern as the periparturient rise in faecal egg count from gastrointestinal nematodes, and the mechanisms behind the increase might be similar. Hormonal changes, such as an increase in progesterone (*Cox* 1975) may be of importance.

The animals which excreted Lm in the milk had no clinical symptoms of mastitis. The presence of Lm in the milk from sheep has been described by *Garcia* (1974) in association with abortion and by *Ivanov et al.* (1964) in cases of spontaneous listeriosis and in experimentally infected sheep. *Ivanov et al.* found the highest number of Lm in the milk during the septicaemic phase. However, as no clinical illness was seen in the animals in the present investigation, it is unlikely that those excreting Lm were in a septicaemic state, although one cannot exclude the possibility of a transient septicaemia without clinical symptoms in the period shortly before lambing. As an intracellular bacterium, Lm may be present in the udder of healthy animals, and it may be activated whenever the animal is subjected to stress.

Although the lambs of the experimental ewes might have ingested Lm, they were all in good condition. The number of Lm ingested was probably too low to cause clinical illness.

The bacteria isolated in this investigation belonged almost exclusively to serotype 1. This is somewhat surprising since Lm isolated from grass silage samples from this Research Station during a 2-year period were equally divided between serotypes 1 and 4 (*Grønstøl,* to be published). A possible explanation may

be that serotype 1 is more prone to give a carrier state than serotype 4, but results from other investigations by the author do not support this hypothesis.

Serological examination of the whey showed a low GMT of IHA-antibodies, but the IHA-test mainly records the IgM-fraction (*Herbert* 1973, *Grønstøl,* to be published) and may not reflect the true immunity against Lm. Antibodies in the milk belong mainly to the IgG-fraction (*Lascelles & McDowell* 1974). Even if the newborn lambs had ingested Lm through the milk, the bacteria might have been neutralized by antibodies against Lm and T-lymphocytes primed against Lm, which they also received through the colostrum and the milk (*Parmely & Beer* 1977). More work is needed to determine the exact amount of antibodies against Lm in the milk and to assess the importance of these antibodies and the maternally derived T-lymphocytes in the protection of the newborn lamb.

Sera have been examined for antibodies against Lm by several methods. *Schierz & Burger* (1966) found indirect haemagglutination to be a reasonably specific and sensitive test, provided that suitable antigen was used. The IHA-method described in this report has been used in experimental work, and the results confirm their findings (*Grønstøl,* to be published).

GMT for ewes with 3 foetuses or more decreased towards the end of the gestation period. A similar, but much weaker trend was found for ewes with 1 or 2 foetuses. These findings support the result from an investigation of listeric abortion in sheep which indicated that the number of foetuses the ewes carried influenced their humoral immunity against Lm (*Grønstøl,* to be published). The increased level of progesterone in ewes with many foetuses (*Emady et al.* 1974) may be of importance.

Ewes with ≥ 3 foetuses reacted more strongly to the skin test than the remainder. The increased stimulation of CMI in these animals might have been associated with the decrease in humoral immunity. A stronger reaction was also found in ewes fed grass silage than in ewes fed hay. This may reflect a higher number of Lm in the grass silage than in the hay. The grass silage may also contain substances which have a suppressive effect upon humoral immunity and may thus produce a stronger stimulus for the CMI. These questions have been further investigated and discussed by *Grønstøl* (to be published).

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SAMMENDRAG

Listeriose hos sau. Utskiljing av og immunitet mot Listeria monocytogenes hos friske sauer.

Utskiljing av Listeria monocytogenes (Lm) i faeces og mjølk og utvikling av humoral og cellebunden immunitet mot denne bakterien blei undersøkt i ein saueflokk. I denne flokken hadde det ikkje vore noko tilfelle av klinisk listeriose i dei siste 3 åra, og i dei siste 14 åra var berre 3 sauer blitt behandla for denne sjukdommen. Sauene blei undersøkte i innefóringsperioden, og i fyrste delen av denne perioden var det ingen av dei vaksne sauene som skilde ut bakterien. Derimot blei han funnen hos 2 av dei 10 lamma. Etter lamming skilde 64 % av dei 106 sauene og 6 av dei 10 lamma ut Lm i faeces, og 41 % av sauene og 1 av årslamma skilde ut Lm i mjølka. 98.5 % av dei isolerte bakteriene tilhøyrde serotype 1.

Ved bruk av ein indirekte haemagglutinasjonsmetode blei det funne antistoff mot Lm både i sera og mjølk. Antistoff-titeret var høgare hos sauene enn hos årslamma, og titera såg ut til å vera påverka av kor mange foster dyra hadde.

Cellebunden immunitet blei undersøkt ved ein hudtest der seinka hypersensitivitet mot eit antigen framstelt frå Lm, blei målt. Dyr fóra

med silofór hadde større utslag på denne testen enn dyr fóra med høy, og dyr med ≥ 3 foster hadde større utslag enn resten av dyra.

Dette syner at også i flokkar med sjeldne utbrot av klinisk listeriose kan dei fleste dyra bli utsette for smitte med Lm, og storparten ser ut til å bli latente bærarar av denne bakterien. Dei kan då skilja ut Lm under påverknad av stress.

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