Experimental infection of pregnant sheep with attenuated *Salmonella typhimurium*

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ABSTRACT

Salmonellosis occurs in many animal species including sheep, and may cause pathology of the gut and possible abortion. If attenuated strains were to be used for future control, their establishment in the host and shedding pattern needs to be determined. Fifty-one pregnant ewes were obtained from the Lincoln University Research Farm and assigned randomly to five treatment groups. At approximately 1 month before lambing, the groups were given attenuated *S. typhimurium* (*cya*$^-$, *crp*$^-$) by different routes and doses as follows: 10^{10} colony-forming units (cfu) intranasally (i.n.), 10^9 cfu i.n., 10^{10} cfu subcutaneously (s.c.), 10^9 cfu s.c., and control (saline i.n. and s.c.). Selected animals were slaughtered at days 4 and 14 post infection for bacteriological culture from the mesenteric and retropharyngeal lymph nodes, intestinal contents and lamb stomach contents. The experimental organisms were isolated using selective cultures and identified by latex agglutination test and fermentation reactions. Following infection there were no changes in rectal temperature or significant clinical signs except two cases of abortion in the 10^{10} cfu s.c. group. The number of animals shedding organisms in their faeces decreased with time from 12 ewes at day 1 to one ewe at day 4 and day 7. Most of them were from the 10^{10} i.n. group. Subcutaneous injection of 10^{10} cfu induced abortion in two of 11 ewes, as organisms were detected in their faeces and the aborted lambs. However, no organisms were detected in faeces, intestinal contents or the lamb stomach contents of the ewes that were killed at days 4 or 14. In the two ewes that had received 10^{10} cfu i.n. and were killed at day 4, organisms were detected in the mesenteric and retropharyngeal lymph nodes. In summary, the administration of the attenuated *S. typhimurium* (*cya*$^-$, *crp*$^-$) at 10^{10} cfu caused asymptomatic short-term faecal shedding and abortion when given i.n. and s.c., respectively.

Keywords: attenuated; *Salmonella typhimurium*; pregnant sheep.

INTRODUCTION

Infection of sheep with *Salmonella* serovars has been recorded all over the world and has been associated with gastrointestinal symptoms and possible abortion in pregnant sheep. The more common serovars associated with ovine salmonellosis are *S. typhimurium*, *S. abortusovis*, *S. arizonae*, *S. derby*, *S. dublin* and *S. montevideo* (Wray and Linklater, 2000). In New Zealand, the most commonly reported serovars are *S. hindmarsh* and *S. typhimurium*, which are generally associated with diarrhoea and death in adult sheep during the summer and autumn periods. *Salmonella Brandenburg* has been identified recently in many areas in New Zealand and diagnosed as the causative organism of ovine and bovine abortion (Boxall *et al.*, 1999). With the exception of *S. abortusovis* which is host-specific, there are many sources of infection, e.g. food, water, other animals, wild birds and human, and infection is most probably by the oral route. Experimental *Salmonella abortusovis* infection of pregnant ewes has been found to induce abortion when given by different routes (Pardon *et al.*, 1980; Linklater 1985; Sanchis *et al.*, 1991). When pregnant ewes were infected intra-conjunctivally with *S. abortusovis*, eight of 18 aborted and another four gave birth to stillborn lambs (Sanchis *et al.*, 1991). Pardon *et al.* (1980) found that the subcutaneous route gave more consistent results than intra-gastric infections with *S. abortusovis*. With subcutaneous doses varying between $2.5x10^7$ and 10^{10} colony-forming units (cfu), they produced abortion in 7 of 11 pregnant ewes infected between the 64th and 84th days of pregnancy. Linklater (1985) found that an oral dose of 10^9 cfu of *S. typhimurium* produced an acute systemic disease, characterised by fever, anorexia, dullness and profuse diarrhoea, similar to that described in a field outbreak (Hunter *et al.*, 1976). Infection was more severe in pregnant ewes than in non-pregnant ones, and abortion was produced 5-9 days later by doses of organisms that were not lethal to non-pregnant animals (Linklater, 1985). In addition to imposing an economic loss on the farmer, *S. typhimurium* belongs to the group of zoonotic serotypes of *Salmonella* associated with human food poisoning (WHO, 1988). This serotype is the most frequent cause of human salmonellosis in New Zealand (ESR Lablink report, 2000). Also, *S. Brandenburg* was associated with human septicaemia and urogenital infection in the human female (Baquar *et al.*, 1994; Boxall *et al.*, 1999) and in New Zealand, it has been an increasing cause of gastroenteritis in humans (ESR Briefing, 1999).

This experiment was conducted to determine the safety and shedding pattern of an attenuated strain of *S. typhimurium* (*cya*$^-$ *crp*$^-$) when given to pregnant ewes by different routes and doses, with the hope of using it as a vaccine. The main advantage of using such live attenuated *Salmonellae* as vaccines is that they can stimulate secretory, humoral and cellular immune responses following oral administration, while dead vaccines tend to stimulate strong antibody production only (Collins, 1974).

MATERIALS AND METHODS

Animals

The experiment was conducted on 51 Coopworth ewes obtained from the Lincoln University Research Farm. The trial was started approximately one month prior to the start of lambing and extended from September to December 2000. All procedures were approved by the Lincoln University Animal Ethics Committee.

Infecting organisms

The attenuated strain of *S. typhimurium* was prepared

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by deleting the genes encoding adenyl cyclase (*cya*) and cyclic AMP receptor protein (*crp*) (Curtiss & Kelly, 1987). The organisms (*cya⁻ crp⁻*) were lyophilised and at 10^{11} cfu per vial were supplied by Pacificvet Ltd, New Zealand. The organisms were reconstituted and diluted to the desired doses using sterile pyrogen-free water.

Experimental infection

Animals were assigned randomly to five treatment groups, which were mixed as one flock and grazed on autumn-saved pasture. They were given 10^{10} cfu intranasally (i.n.) (n=12), 10^{10} cfu subcutaneously (s.c.) (n=11), 10^{9} cfu i.n. (n=12), 10^{9} cfu s.c. (n=8) or phosphate-buffered saline solution (PBS) i.n. and s.c. (n=8). For intranasal infection, 0.5ml of the organism suspension was bilaterally sprayed approximately 50mm into the nostrils using flexible PVC tubing. For subcutaneous infection, organisms were injected subcutaneously into the dorsum of the neck. Daily clinical observations were made and rectal temperatures taken at each time of sample collection. Selected animals infected with 10^{10} cfu i.n. (n=4), 10^{10} cfu s.c. (n=4) or 10^{9} cfu i.n. (n=4) were killed at day 4 or 14 to allow bacterial cultures from mesenteric lymph node (MLN), retropharyngeal lymph node (RLN), intestinal contents (SI), rib bone marrow (rib) and foetal lamb stomach contents (LSC). To monitor the survival of organisms on the grazed pasture, autoclaved cotton "stocking" swabs were impressed on many random spots of the paddock 31 and 48 days after the start of the infection, 2 weeks after the ewes had been removed from the paddocks.

Bacteriological examinations

Faeces and nasal secretions were collected for bacteriological examination to monitor shedding of organisms. Faecal samples were collected from the rectum of each animal at days 0, 1, 4, 7, 14, 21 and 28 post infection as well as at the time of lambing or abortion. Nasal secretions were collected using cotton swabs at days 0, 1, 4, 7 and 14 only, as there had been only one isolation (day 1) prior to that time. Faecal, nasal or tissue samples were keep moist in buffered peptone water (Merck, Germany), and then subcultured into each of tetrathionate broth and RVS broth (Fort Richard Ltd, NZ) for 24h, followed by plating onto each of xylose lysine desoxycholate and brilliant green agars for 24 - 48 h. Suspected colonies were inoculated into triple sugar iron and lysine iron agar slopes to distinguish between the experimental attenuated *S. typhimurium* organisms (*cya⁻ crp⁻*), field-type *Salmonellae* and other *Enterobacteriaceae*. Slide agglutination (Serobact, Medvet, Australia) and fermentation reactions (API, bioMerieux, France) were conducted to confirm positive results.

Statistical analysis

Time between infection and lambing was presented as mean ± SEM. Shedding of organisms in faeces and the number of dead or aborted ewes were presented as a proportion of positive cases to the total number of tested samples and total number of ewes respectively. Effect of dose and route of infection on rectal temperature and time of lambing post infection was measured using analysis of variance (Systat INC, USA). Effect of treatments on faecal shedding of organisms, incidence of death or abortion and survival of lambs was determined by Pearson's Chi square test (Genstat, USA).

RESULTS

Cultural examination of faeces and nasal secretions from ewes for the presence of field-type *Salmonella* or the attenuated *S. typhimurium* strain (*cya⁻ crp⁻*) before the start of the experiment at day 0 was negative.

In animals that were infected intranasally, there was a close relationship between the infective dose and excretion in the faeces ($P < 0.01$). Eleven out of 12 ewes that were infected intranasally with the higher dose of 10^{10} cfu, excreted the organisms in faeces at day 1, compared with one of 12 ewes given the lower dose 10^{9} cfu by the same route (Table 1). Furthermore, two of the 12 ewes given the higher dose (10^{10} cfu i.n.) excreted field-type *S. typhimurium* in faeces, in addition to the attenuated strain (*cya⁻ crp⁻*). The number of ewes shedding the organisms in faeces decreased dramatically with time (Table 1), from 12 ewes at day 1 to one ewe at days 4, 7 and 21 - a different individual animal at each time - and none at days 14 or 28. Intermittent recovery was recorded in one ewe given 10^{10} cfu i.n., as organisms were detected in faeces at days 1 and 7, but not on day 4. Organisms were detected in the nasal secretion of one ewe at day 1 when given 10^{10} cfu i.n.

TABLE 1: Shedding pattern of the attenuated organisms (*cya⁻ crp⁻*) in faeces collected from ewes infected by different doses and routes.

Infection (cfu)	Day 0	Day 1	Day 4	Day 7	Day 14	Day 21	Day 28
10^{10} i.n.	0/12[a]	11/12	1/12	1/10	0/10	0/8	0/8
10^{10} s.c.	0/11	0/10[1]	0/10	0/8	0/8	0/6	0/6
10^{9} i.n.	0/12	1/12	0/12	0/10	0/10	1/8	0/8
10^{9} s.c.	0/8	0/8	0/8	0/8	0/8	0/8	0/8
PBS	0/8	0/8	0/8[2]	0/7	1/7[3]	0/6	0/6

[a] Results indicate number of positive samples/total number of tested samples.
[1] One ewe died at day 1 following 10^{10} cfu s.c. infection and *S. typhimurium* (*cya⁻ crp⁻*) was detected in MLN and LSC. Faecal sample was not collected.
[2] One ewe excreted a field type *Salmonella* in faeces at day 4 and died at day 5.
[3] One ewe died at day 15 and *S. typhimurium* (*cya⁻ crp⁻*) was detected in faeces but not in MLN or LSC.

No significant changes ($P > 0.05$) were detected over time with the rectal temperatures of the infected or control groups, nor were there clinical signs attributable to the experimental infection. In the control group, one ewe was treated for hypocalcaemia at day 1, excreted field-type *Salmonella typhimurium* (phage type 160) in faeces at day 4 and died on day 5. Another ewe died on day 15 and the experimental organism was detected in faeces, but not in MLN or the lamb stomach contents, and was not believed to be causal.

In animals that were infected subcutaneously, there was a relationship between the infective dose and lambing success $P < 0.05$ (Table 2). The experimental organism was recovered from the faeces of two ewes, and the stomach contents of their respective lambs, that had been given 10^{10} cfu s.c., and had aborted at days 9 and 15 post infection. It was also recovered from the MLN and lamb stomach contents of a ewe (10^{10} cfu s.c.) that died of other (metabolic) causes. Shedding of *Salmonella typhimurium*

TABLE 2. Effect of dose and route of infection of *S. typhimurium* (*cya⁻ crp⁻*) on survival of ewes and lambs, shedding from ewes at lambing, and time of lambing.

Infection (CFU)	Total ewes	Faecal shedding[a]	Ewes died	Ewes aborted[b]	Time of lambing[c]	Lambs died[d]
10^{10} i.n.	12	0/8	0/12	0/8	32 ± 2.63	2/11
10^{10} s.c.	11	2/6[1]	1/11[3]	2/6[1]	32 ± 4.37	0/5
10^{9} i.n.	12	1/8	0/12	0/8	23 ± 2.9	0/8
10^{9} s.c.	8	1/7[2]	1/8[4]	0/7	27.25 ± 4.15	1/8
PBS	8	0/6	2/8[5]	0/6	24.25 ± 0.5	0/7

[a] Shedding of attenuated *S. typhimurium* (*cya⁻ crp⁻*) in faeces collected at the time of lambing or abortion. Results indicate number of positive samples / total number of tested samples.
[b] Number of ewes aborted / total number of ewes at the time of lambing.
[c] Mean ± SEM time between infection and lambing in days.
[d] Number of lambs died shortly after birth / total number of lambs. Deaths were unrelated to the experimental infection.
[1] Two animals aborted at days 9 and 15 post infection and S. *typhimurium* (*cya⁻ crp⁻*) was detected in ewe faeces at the time of abortion, and the stomach contents of their lambs.
[2] one ewe excreted *S. typhimurium* (*cya⁻ crp⁻*) in faeces at lambing (7 days post infection) but gave birth to a healthy lamb.
[3] One ewe died at day 1 and *S. typhimurium* (*cya⁻ crp⁻*) was detected in MLN and lamb stomach contents.
[4] Both ewe and its lamb died at day 39 from dystocia.
[5] One ewe excreted a field-type *S. typhimurium* (phage type 160) in faeces at day 4 and died at day 5 of dystocia. Other ewe died at day 15 of unknown causes and *S. typhimurium* (*cya⁻ crp⁻*) were detected in faeces but not in MLN or LSC.

(*cya⁻ crp⁻*) was detected at the time of lambing in two ewes given 10^9 cfu i.n. and 10^9 cfu s.c.; organisms were recovered shortly before and after lambing respectively, but not at any other time. There was no significant effect ($P > 0.05$) of treatments on the survival of lambs after birth. Seven out of eight ewes given 10^{10} cfu i.n. produced nine healthy lambs. One ewe gave birth to lambs that died shortly after lambing due to climatic causes. Five lambs born from four ewes given 10^{10} cfu s.c. survived, the two other ewes aborted probably due to the experimental infective agent which was isolated from the foetal stomach contents. However, all ewes given 10^9 cfu, either i.n. or s.c., produced healthy lambs, with the exception of one ewe given 10^9 cfu s.c. that produced twins, one of which died due to climatic causes (Table 2). The average time between infection and lambing (Table 2) was longer in ewes infected with 10^{10} cfu i.n. (32.25 ± 2.63) compared with 10^9 cfu i.n. (23 ± 2.9) ($P < 0.05$).

Salmonella typhimurium (*cya⁻ crp⁻*) was detected in the MLN of one ewe and RLN of another, both of which had been given 10^{10} cfu i.n. and killed 4 days post infection (Table 3). However, no organisms were detected in the MLN, RLN, intestinal contents, rib bone marrow or the lamb stomach contents of ewes that were killed at day 14 post infection.

TABLE 3: Detection of attenuated *S. typhimurium* (*cya⁻ crp⁻*) in tissues of animals killed at days 4 and 14 post infection (combined).

Infection (cfu)	LSC	SI	MLN	RLN	Rib
10^{10} i.n.	0/4[a]	0/4	1/4[1]	1/4[1]	0/4
10^{10} s.c.	0/4	0/4	0/4	0/4	0/4
10^{9} i.n.	0/4	0/4	0/4	0/4	0/4

[a] Results indicate number of positive samples/total number of tested samples
[1] *S. typhimurium* (*cya⁻ crp⁻*) was detected (separately) in the MLN and RLN of two animals killed on day 4.
LSC = lamb stomach contents SI = small intestine contents
MLN = mesenteric lymph node RLN = retropharyngeal lymph node
Rib = rib bone marrow

The experimental organisms were identified in the pasture swab obtained 2 weeks after spelling the pasture, that was 31 days after the beginning of the experimental infection. But the organisms were absent after 31 days of spelling or 48 days after the beginning of the experimental infection.

DISCUSSION

The experimental infection was administered after a period of cold and wet weather. This, plus the yarding procedures imposed a substantial stress on the pregnant ewes, which resulted in hypocalcaemia (and possibly hypomagnesaemia) occurring in 4 animals, two of which subsequently died due to related complications, and another aborted. There was no evidence that the experimental infection was responsible for any abnormal clinical signs in the ewes.

As with previous work using *S. typhimurium* (Brown *et al*., 1976; Humphrey *et al*., 1991) there was a direct relationship between the dosage level and shedding pattern or severity of side effects (in this case abortion). Almost all animals that received 10^{10} cfu i.n. excreted organisms in faeces at day 1 compared with only one ewe given 10^9 cfu i.n. This presumably reflects passage of the experimental infection and transitory colonisation of the gastrointestinal tract (Wray & Linklater, 2000), rather than the upper respiratory tract or oral cavity, as no organisms were isolated from the nasal secretions with the exception of one ewe. All ewes that received 10^9 cfu i.n. or s.c. and survived until the end of pregnancy gave birth to healthy lambs, as did the 10^{10} cfu i.n. group. In this experiment, the attenuated *S. typhimurium* (*cya⁻ crp⁻*) was non-pathogenic compared to similar doses of a field-type *S. typhimurium* which induced acute systemic disease and abortion in sheep (Linklater, 1985). However, in ewes given 10^{10} cfu s.c., four out of six ewes gave birth to healthy lambs and two aborted. This was indicated by identification of the *Salmonella typhimurium* (*cya⁻ crp⁻*) in the aborted lamb stomach contents from these two ewes that aborted at days 9 and 15 post infection. The experimental organisms were identified in the mesenteric lymph node and lamb stomach contents of a ewe that died at day 1 post infection, due most probably to hypocalcaemia, and thus were probably incidental. It appears that the attenuated *Salmonella typhimurium* used in this experiment can induce abortifacient effects only when given at a dose as high as 10^{10} cfu, and by the subcutaneous route. Stage of pregnancy could influence the incidence of abortion and shedding of organisms. In the current experiment, infection was given approximately one month before the expected time of lambing. In a previous experiment using a wild type *S. abortusovis*, there was an apparent effect of stage of gestation on the response of pregnant sheep to *S. abortusovis*. Subcutaneous injection of 10^{10} organisms at 37 days before mating did not cause abortion in the subsequent pregnancy. However, subcutaneous injection of the same dose aborted one of nine ewes at 33 days after service and eight of ten at 89 days of gestation (Wray & Linklater, 2000). Also, Pardon *et al*. (1980) found that subcutaneous doses of *S. abortusovis*

varying between 2.5 x 10^7 and 10^{10} cfu produced abortion in seven of 11 pregnant ewes infected between the 64th and 84th days of pregnancy. Abortion epidemics caused by *Salmonella Brandenburg* in New Zealand have peaked typically 4-6 weeks prior to lambing (Smart, 1999) and were a major determinant on the timing of the current experiment where infection was given 1 month prior to predicted lambing dates (in actuality 23-32 days). The average time between infection and abortion was 19 days in ewes infected subcutaneously (Wray & Linklater, 2000), or 12-19 days in ewes infected orally (Linklater, 1985), which approximates our findings (9 and 15 days).

Physiological changes associated with lambing could stimulate the excretion of organisms, as shedding of the experimental organism was only detected at the time of lambing in two ewes, given 10^9 cfu i.n. and 10^9 cfu s.c., but not at any other time during the experiment, aside from the immediate post-infection period. In previous experiments, stress was found to aggravate *Salmonella* infection (Cooper, 1967; Linklater, 1985). The climatic stress (which is not unusual in Canterbury at this time of the year) imposed on the sheep during infection could account for the transient isolation of field-type *Salmonella typhimurium* (phage type 160) in the faeces of 3 sheep during the immediate post infection period. One was from the control group (PBS) and subsequently died. Detection of the experimental infection in the mesenteric lymph node of one ewe that died on day 1 and other one that was killed on day 4 shows the movement of organisms away from gut probably via the afferent lymph vessels and indicates the value of mesenteric lymph gland cultures in the diagnosis of *Salmonella* infection. Detection of *Salmonella typhimurium* (*cya*$^-$ *crp*$^-$) in the faeces of one ewe from the control group indicates that transmission of the attenuated strain between animals is possible when communally grazing contaminated pasture. The origin of these organisms could have been shedding at infection time, which means a survival time on pasture of 31 days. Or alternatively they could have arisen from intermittent excretion, especially around parturition, which would indicate a survival period of 14 days or more. Intermittent excretion of organisms in faeces was recorded in a ewe given 10^{10} cfu i.n. in this, and a previous, experiment (Brown *et al*., 1976). Under New Zealand conditions, field-type *S. typhimurium* have been reported to remain viable in faeces and soil for up to 12 weeks (Tannock & Smith, 1971).

The results showed that a high level of the attenuated *S. typhimurium* (*cya*$^-$ *crp*$^-$) given intra-nasally caused short-term faecal shedding of organisms. High levels given subcutaneously caused abortion, presumably following a bacteraemia. Colonisation of mesenteric lymph nodes by these attenuated organisms could be valuable for preventing the re-colonisation with virulent *Salmonella* organisms. Shedding of organisms via nasal secretions appears to be of less importance as organisms were detected in only one animal given 10^{10} cfu i.n. for one day.

It is hoped that, as a deliberate administration, this strain as a vaccine will provide protection to animals against subsequent infection with wild-type Group B Salmonellae, particularly *S. Brandenburg*, which is problematic as a cause of abortion in the New Zealand sheep flock. Efficacy studies are currently underway.

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