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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...
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), 10^6 cfu i.n. (n=12), 10^6 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 approximatley 55mm 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 10^6 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 kept moist in buffered peptone water (Merck, Germany), and then subcultured into each of tetraionate 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 Salmonella 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>
<thead>
<tr>
<th>Infection (cfu)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^6 i.n.</td>
<td>0/12</td>
<td>1/12</td>
<td>1/12</td>
<td>1/10</td>
<td>0/10</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>10^6 s.c.</td>
<td>0/12</td>
<td>0/10</td>
<td>0/10</td>
<td>0/8</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>10^9 i.n.</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/10</td>
<td>1/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>10^9 s.c.</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>PBS</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/7</td>
<td>1/17</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

1 Results indicate number of positive samples/total number of tested samples.
2 One ewe died at day 1 following 10^6 cfu s.c. infection and S. typhimurium (cya crp) was detected in MLN and LSC. Faecal sample was not collected.
3 One ewe excreted a field type Salmonella in faeces at day 4 and died at day 5.
4 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.

<table>
<thead>
<tr>
<th>Infection (CFU)</th>
<th>Total ewes</th>
<th>Faecal shedding*</th>
<th>Ewes died</th>
<th>Ewes aborted*</th>
<th>Time of lambing</th>
<th>Lambs died‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁴ i.n.</td>
<td>12</td>
<td>0/8</td>
<td>0/12</td>
<td>0/8</td>
<td>32 ± 2.63</td>
<td>2/11</td>
</tr>
<tr>
<td>10⁴ s.c.</td>
<td>11</td>
<td>2/6</td>
<td>1/11</td>
<td>2/6</td>
<td>32 ± 4.37</td>
<td>0/5</td>
</tr>
<tr>
<td>10³ i.n.</td>
<td>12</td>
<td>1/8</td>
<td>0/12</td>
<td>0/8</td>
<td>23 ± 2.9</td>
<td>0/8</td>
</tr>
<tr>
<td>10³ s.c.</td>
<td>8</td>
<td>1/7</td>
<td>1/8</td>
<td>0/7</td>
<td>27.25 ± 4.15</td>
<td>1/8</td>
</tr>
<tr>
<td>PBS</td>
<td>8</td>
<td>0/6</td>
<td>2/8</td>
<td>0/6</td>
<td>24.25 ± 0.5</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* 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.
* Number of ewes aborted / total number of ewes at the time of lambing.
‡ Number of lambs died shortly after birth / total number of lambs.

(cya *crp*) was detected at the time of lambing in two ewes given 10⁷ cfu i.n. and 10⁸ cfu s.c.; organisms were recovered shortly before and after lambing respectively, but not at (cya *crp*) - was non-pathogenic compared to *S. abortusovis* when given at a dose as high as 10⁹ 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 10⁹ cfu i.n. or s.c. and survived until the end of pregnancy gave birth to healthy lambs, as did the 10⁹ 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 10⁹ 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 10⁹ 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 10⁹ 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*

**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 10¹⁰ cfu i.n. excrated organisms in faeces at day 1 compared with only one ewe given 10⁹ 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 10⁹ cfu i.n. or s.c. and survived until the end of pregnancy gave birth to healthy lambs, as did the 10⁹ 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 10⁹ 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 10⁹ 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 10⁹ 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*
varies between $2.5 \times 10^7$ 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 $10^5$ cfu i.n. and $10^6$ 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 (phase 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 affenter 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 of 14 days or more. Intermittent excretion of organisms in faeces was recorded from intermittent excretion, especially around parturition (Linklater, 1985), which means a survival time on pasture. The origin of these organisms could have been communally grazing contaminated water.

REFERENCES


