

***Salmonella* Abortusovis experimental infection induced by the conjunctival route: clinical, serological and bacteriological study of the dose effect in female lambs**

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Summary — The aim of this work was to explore the first stages of infection with *Salmonella* Abortusovis after a mucosal inoculation, and to establish an experimental model limiting the bacteriological investigations to the inoculation site. Four groups of 6 ewe-lambs were infected by the conjunctival route with decreasing doses of *Salmonella* Abortusovis. The clinical and serological survey was completed by a daily bacteriological examination of nasal swabs and faeces. Enumeration of viable bacteria was performed on liver, spleen, lungs and lymph nodes harvested 1, 8, and 22 d after inoculation. A rapid colonization of the cephalic lymph nodes was observed, associated with a transient spreading to prescapular and subiliac lymph nodes without dissemination to the liver and spleen. An irregular and low level faecal excretion was associated with the colonization of mesenteric lymph nodes. The infection by the conjunctival route may either systemically propagated or be locally restricted by the lymphoid system in relation to doses administered. This model uses low infective doses similar to those occurring in field conditions and offers the possibility of limiting the bacteriological control to the regional lymph nodes. It confirms that live attenuated *Salmonella* strains may be used as vaccinal vectors by the mucosal route.

***Salmonella* Abortusovis / experimental infection / conjunctival route / ovines**

Résumé — Infection expérimentale par voie conjonctivale avec *Salmonella* Abortusovis : étude clinique, sérologique et bactériologique de l'effet dose chez des agnelles. Ce travail se propose d'étudier les premiers stades de l'infection par *Salmonella* Abortusovis après inoculation par voie muqueuse, et d'établir un modèle expérimental en limitant les contrôles bactériologiques à la sphère d'inoculation. Quatre lots de 6 agnelles ont été inoculés par voie conjonctivale avec des doses décrois-

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santes de *Salmonella Abortusovis*. Le suivi clinique et sérologique a été complété par l'examen bactériologique quotidien d'écouvillons nasaux et de fèces. Des numérations bactériennes ont été effectuées à partir du foie, de la rate, du poumon, et de ganglions lymphatiques prélevés 1, 8, et 22 j après l'inoculation. On a observé une colonisation précoce des ganglions céphaliques, une extension irrégulière aux ganglions préscapulaires et précruraux sans mise en évidence d'une contamination de la rate et du foie. Une excrétion fécale intermittente était associée à la colonisation des ganglions mésentériques. L'infection par la voie conjonctivale a conduit soit à la dissémination, soit au contrôle de l'infection par voie lymphatique en fonction des doses inoculées. Ce modèle, utilisant de faibles doses infectantes proches de celles rencontrées en conditions naturelles, permet de limiter le contrôle bactériologique aux ganglions régionaux. Il confirme la possibilité d'utiliser la voie muqueuse avec des souches de *Salmonella* à virulence atténuée comme vecteurs vaccinaux.

Salmonella Abortusovis / infection expérimentale / voie conjonctivale / Ovins

INTRODUCTION

Salmonella enterica subsp *enterica* ser *Abortusovis* (hereafter *Salmonella Abortusovis*), a sheep-adapted serotype, is one of the main causes of abortion in ewes and has been isolated in France and in several other countries (Pardon *et al*, 1988). Colonization of the foetoplacental unit leads to a massive peripartum vaginal excretion following either abortion, or at-term lambing of infected live lambs.

Different routes of experimental infection with *Salmonella Abortusovis* have been explored (Pardon *et al*, 1983). They did not regularly reproduce an infection leading to abortion except when the subcutaneous route was used during the third month of gestation (Pardon *et al*, 1983; Sanchis and Pardon, 1984). This model was used to evaluate diagnostic tests and vaccination (Pardon *et al*, 1980, 1983; Sanchis and Pardon, 1981) and leads to bacteriemia and foetoplacental colonization, but short-circuits the early steps of infection occurring at the mucosal level.

The conjunctival route used in experimental brucellosis (Plommet and Plommet, 1975, 1976) was also used with *Salmonella Abortusovis* (Sanchis and Pardon, 1986; Sanchis *et al*, 1991). In these studies, with an infective dose of 1×10^{10} cfu, clinical and bacteriological effects corresponded to

those seen in natural conditions, including abortion and lambings of infected lambs. The high inoculation dose used by this mucosal route was chosen to make sure abortion took place, but the early events of infection were again not investigated.

The aim of this work was to study the first stages of infection after conjunctival inoculation, and to establish a model in non-pregnant animals. The experiment was conducted in female lambs with varying doses of *Salmonella Abortusovis* to study the relationship between doses and subsequent spreading of infection to the lymph nodes. This model used lower infective doses than those previously used and offers the possibility of establishing a persistent mucosal infection which limits the bacteriological control to the regional lymph nodes.

MATERIALS AND METHODS

Animals and protocol

Twenty-four 5–6 month-old Lacaune female lambs were purchased from a salmonellosis-free flock with no history of abortion during the 3 previous years. Serological tests for salmonellosis and for chlamydiosis, Q fever, toxoplasmosis and Border disease were performed on the whole flock from which the animals came, and twice on the experimental group during the month before inoculation. Animals were ran-

domly distributed in 4 groups of 6 lambs, and housed into 4 different isolated pens. Two randomly selected lambs were slaughtered and necropsied in each group on days 1, 8 and 22 after inoculation.

Bacterial strain and inoculation

The *Salmonella* Abortusovis strain 15/5 SR is a mutant resistant to streptomycin (500 µg/ml). It was obtained from the parental strain 15/5 and possesses the same bacteriological and virulent characteristics as those of the parental strain (Pardon and Marly, 1979). For the inoculum preparation, cultures on tryptic soy agar (TSA; Sanofi, Diagnostics Pasteur, Marnes-la-Coquette, France) with 500 µg/ml streptomycin added (TSA/S; Spécia, Paris, France) were harvested in phosphate buffer saline (PBS) pH 7.2, standardized turbidimetrically and adjusted to the required concentration by dilution in PBS. Viable bacteria were enumerated before and after inoculation by plating serial 10-fold dilutions in TSA/S.

Each animal received two 50 µl drops of the suspension in the right eye conjunctival sac. For the 4 groups, the doses were respectively: 5×10^9 , 5×10^7 , 5×10^5 and 5×10^3 colony forming units (CFU) per sheep.

Clinical examination and sample collection

Behavioural and clinical examinations of the animals were performed daily. Rectal temperatures were recorded on each lamb each morning before sampling venous blood for serological examination. Individual faeces were taken daily from rectum with disposable latex gloves and stored in sterile vials. Mucus was also daily sampled with sterile swabs in right and left nostrils for bacteriological examination.

At necropsy, left and right mandibular, retropharyngeal, parotid, prescapular and subiliac lymph nodes (LNs), jejunal, caecal and retro-mammary LNs were collected and placed individually in sterile vials. Pieces of liver, spleen and lungs were also removed and placed in sterile dishes. All samples were stored at 4°C before being processed.

Serology

Serum agglutinating antibodies were measured by a microagglutination test (MAT) using formalin-killed *Salmonella* Abortusovis stained with triphenyl-2,3,5-tetrazolium chloride (Prolabo, Paris, France) as previously described (Sanchis *et al*, 1985). Serum was tested in 2-fold dilutions between 1/20 and 1/40 960, the threshold dilution being 1/320.

Bacteriological examinations

Nasal swabs were smeared on Petri dishes with TSA/S. Each faeces sample was weighed, diluted 1:10 (wt/vol) in PBS, homogenized with a blender (Stomacher-Colworth, Cofralab, Bordeaux, France) in sterile plastic bags, and 0.5 ml of suitable dilutions were plated on *Salmonella-Shigella* agar (SS; Sanofi, Diagnostics Pasteur, Marnes-la-Coquette, France) with 500 µg/ml streptomycin added.

After removing the fat, the LN and organ samples were weighed, singed, fragmented with sterile blades, diluted in PBS (1:5 wt/vol) and homogenized with the blender in sterile bags. Ten-fold dilutions were plated on TSA/S, and 1 ml was cultivated for enrichment in 9 ml of tryptic soy broth (TSB; Sanofi, Diagnostics Pasteur, Marnes-la-Coquette, France) added with streptomycin.

Cultures were incubated at 37°C and examined daily for 4 d. On solid medium, colonies were enumerated, and identification of *Salmonella* Abortusovis was confirmed by slide agglutination with specific antisera of 'O' and 'H' phases (Sanofi, Diagnostics Pasteur, Marnes-la-Coquette, France). Enrichment cultures in TSB/S were plated on TSA/S after 48 h incubation, and the colonies identified as above.

RESULTS

Clinical observations

Animal behaviour was normal throughout the experiment, without any prostration, spoiled appetite, or faecal softness. The right eye and the palpebral mucosa did not show any visible adverse reaction.

In the group receiving the 2 higher doses the thermic acme exceeded 40°C between d 3 and d 7 post-inoculation (PI), and a second thermic phase was observed between the d 14 and d 17 PI (table I). In the other 2 groups rectal temperatures reached 40–41°C from only d 13 to d 18 PI for the 3 animals still alive at this date.

Serology

At the beginning of the experiment, all the animals had serum titers lower than 1:160. In the last group receiving 5×10^3 cfu, no serological response was detectable with MAT. In the other 3 groups the overall change of antibody titers appeared to be directly related to the doses (fig 1); the plateau was reached on d 9, d 14 and d 18 PI for the 5×10^9 , 5×10^7 and 5×10^5 groups, respectively. Moreover, the plateau was lower in the group receiving 5×10^5 CFU than in the other 2 groups.

Bacteriological results

Nasal swabs

The technique used for these samples did not allow enumeration of viable bacteria, but it was possible to assess positive culture frequency and intensity. These parameters showed an evolution in relation to the inoculation dose. The results shown in table II indicate that *Salmonella Abortusovis* was

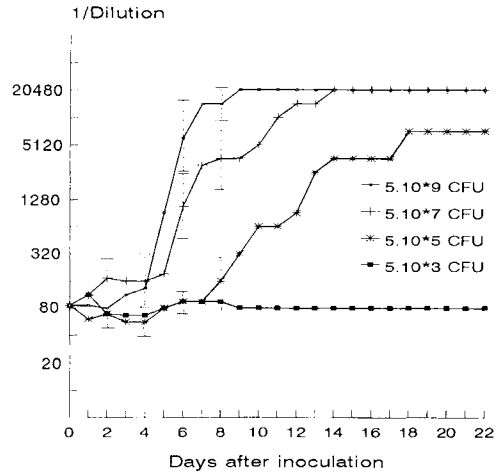


Fig 1. Kinetics of agglutinating antibodies after inoculation by the conjunctival route with 4 different doses of *Salmonella Abortusovis* (strain 15/5 SR). Each point represents the geometric mean of serum titers per group (6 lambs on d 0 to d 2, 4 lambs on d 3 to d 8, 2 lambs on d 9 to d 22).

never isolated from nasal mucus from the lambs in the 5×10^3 group and only during the last period from the 2 remaining lambs in the 5×10^5 group at the end of the observation period. In contrast, it was regularly isolated from groups 5×10^7 and 5×10^9 , but frequency and number declined with time. While the colonies were confluent in the first 5 d for group 5×10^9 , they were less numerous thereafter. Samples from the left nostril were usually negative, with 2 excep-

Table I. Number of lambs with rectal temperature >40°C after inoculation by the conjunctival route with *Salmonella Abortusovis* (strain 15/5 SR).

Days post inoculation	No of animals	Dose (cfu)			
		5×10^9	5×10^7	5×10^5	5×10^3
1–8	4	4	3	0	0
9–22	2	0	1	2	1

Table II. Number of positive cultures from swabs of the right nostril following inoculation with *Salmonella* Abortusovis (strain 15/5 SR) by the conjunctival route ^a.

Days post inoculation	No of animals	No of samples	Doses group (cfu)			
			5 x 10 ⁹	5 x 10 ⁷	5 x 10 ⁵	5 x 10 ³
1	6	6	5	1	0	0
2-8	4	28	22	14	0	0
9-22	2	28	11	12	7	0

^a Samples from left nostril were positive in only 4 samples: 2 in group 5 x 10⁹ (d 1 and 5) and 2 in group 5 x 10⁵ (d 15 and 16).

tions, indicating that the nasal infection does not usually propagate from the right to the left nasal cavity.

Faeces

Salmonellae were isolated from the faeces of 3 animals in group 5 x 10⁹ (10-100 cfu/g), either on d 1 and d 2 PI for 2 animals, or on d 8 and d 10 PI for the third. In group 5 x 10⁷, the culture was positive from only 1 lamb on d 1 PI. All other cultures remained

negative from the faeces collected in the other groups.

Lymph nodes

At slaughter, *Salmonella* Abortusovis was isolated from different LNs as indicated in table III, but it was not isolated from retro-mammary LNs, liver, spleen, and lungs. The bacteriological examination showed a dose effect on kinetics, spreading and infection level.

Table III. Number of positive cultures from lymph nodes in 6 animals necropsied at different times after inoculation with *Salmonella* Abortusovis (strain 15/5 SR).

Days post inoculation	Lymph node ^a	Dose groups (cfu)				Total
		5 x 10 ⁹	5 x 10 ⁷	5 x 10 ⁵	5 x 10 ³	
1	RC	6	1	0	0	7
	I	1	0	0	0	1
8	RC	6	5	3	1	15
	I	4	2	0	0	6
22	RC	1	2	2	0	5
	I	0	1	0	0	1

^a RC: right cephalic = mandibular, parotid and retropharyngeal LNs (n = 6). I: intestinal = caecal and jejunal LNs (n = 4).

On d 1 PI, positive cultures from the cephalic LNs of the group receiving 5×10^9 cfu were obtained after enrichment, and corresponded to about 5 cfu/g of collected LNs. On d 8 PI, the right cephalic LNs of the groups receiving 5×10^9 , 5×10^7 and 5×10^5 were enlarged and the infection level varied from 800 to 10^5 cfu/g according to the doses. The colonization was bilateral in groups 5×10^9 and 5×10^7 , but the left LNs, were always less infected than the right homologous ones. At this time, the infection spread to the intestinal LNs and the prescapular and subiliac LNs of 1 animal in these 2 groups. In the 5×10^3 group, a weak infection (10 cfu/g) was limited to the right parotid LN in only one lamb. On d 22, the level of infection decreased (5–2 300 cfu/g) and was limited at the right cephalic LNs in the groups receiving 5×10^9 , 5×10^7 and 5×10^5 cfu.

The parotid LNs were always the most frequently and most heavily infected of the LNS. The other most affected LNs, according to the frequency and infection level, were the retropharyngeal and mandibular LNs.

DISCUSSION

In previous papers, a high dose of 10^{10} by subcutaneous route was chosen to obtain a reproducible model of abortion with a limited number of animals (Pardon *et al*, 1983; Sanchis *et al*, 1991). In the present report, for an experimental model *via* the conjunctiva, variable doses were chosen to limit the colonization to the draining LNs, and to approach the range of doses of contamination occurring in husbandry conditions. Regarding the clinical signs following the inoculations there was no modification of the behaviour of the animals, even with the higher doses used by this route, except for a transient increase of rectal temperatures. As in previous studies (Sanchis and Pardon, 1984, 1986), this confirms that abortion is the main and sometimes the only clin-

ical sign in *Salmonella* Abortusovis infection in sheep.

In spite of the limitations due to the low number of animals in each group, the results strongly suggest changes in relation to the dose (table I) and the time following inoculation. A similar dose effect varying with time was also observed in early and later isolations on swabs (table II). No bacteriological examination of the inoculation site was done to avoid any lesion of the conjunctival mucosa. The infection probably reaches the nasal cavity by lachrymal route, and the positive nasal swabs provide an indication of the contamination level in this area. The technique used for sampling in this study did not allow bacterial enumeration in nasal mucus, and so it is difficult to conclude that a multiplication occurs at this site. Nevertheless, the positive cultures in the group receiving 5×10^5 at d 10 PI suggest such an increase in bacterial population. The sensitivity of the detection technique of the bacteria could effectively be too low to detect a weak contamination resulting from the low inoculated dose, but sufficient to detect a further local multiplication of bacteria overcoming the local immune system. At this time, the infection also became bilateral, showing an invasion of the left nasal cavity explained either by nostril leak or by a retrograde colonization from the nasopharynx. The dose effect observed with these parameters, including agglutinating antibodies, which reached a plateau between d 9 and d 18 PI according to the dose (fig 1), reflects the kinetics of infection of the draining LNs as previously described for brucellosis (Fensterbank, 1987). The isolation of more than 10^5 cfu per parotid LN on d 22 in the group receiving an inoculation dose of 5×10^5 cfu is sufficiently late and close to the inoculated dose to involve the overcoming of the local lymphatic system, and a probable multiplication of *Salmonella* Abortusovis.

The isolation of *Salmonella* Abortusovis from only 1 parotid LN of 1 lamb inoculated

with 5×10^3 cfu, without any serological sign, showed that the translocation through the conjunctival mucosa is still possible with this dose, but that such a dose is not sufficient to overcome the regional lymphatic system (Plommet and Plommet, 1976; Pardon and Marly, 1979; Collins and Campbell, 1982). It may also suggest that in unfavourable physiological or eco-epidemiological conditions, or during pregnancy when active immunity can be altered (Reynolds and Griffin, 1985), this weak dose might be sufficient to induce a mucosal translocation and a subsequent LN colonization leading to an established infection.

With other *Salmonella* serotypes (Collins *et al*, 1977), a few bacteria reach the blood stream after LN colonization, and colonize liver and spleen, which leads to septicemia. With *Salmonella* Abortusovis, the bacteriemic phase, which had previously been shown to be transient and require numerous samplings (Sanchis and Pardon, 1984), was not explored in this study. However, if no spleen or liver colonization was observed, even with the highest dose, subiliac LNs were positive in some cases, indicating that a weak and transient bacteriemia could occur after inoculation on the conjunctiva. The negative bacteriological examination of lambs inoculated with the highest dose and necropsied on d 22 PI seems to confirm the control of the infection by the lymphatic system (Sanchis and Pardon, 1984, 1986).

The caecal lymph nodes were colonized on d 1 PI, and this colonization was concomitant with positive cultures in faeces from lambs of the 5×10^9 group. This early transit from the inoculation site to the intestine probably follows the lachrymal apparatus, nasal cavities and nasopharynx. In spite of the presence of salmonella in faeces, no diarrhoea was observed. The transient and weak contamination of the caecal content (< 100 cfu/g) does not therefore suggest a multiplication of the bacteria in the intestine, but shows that a small number of

Salmonella Abortusovis reaching the intestinal content is sufficient to get a translocation through the intestinal mucosa to the intestinal lymph nodes.

These results confirm the possibility of a mucosal contamination by conjunctival, nasal or intestinal route, and its dissemination through the lymphatic system. This translocation appears to be possible with 5×10^5 bacteria, with an inoculum 10^5 times lower than those previously used (Sanchis and Pardon, 1986; Sanchis *et al*, 1991). The persistent contamination over 22 d on the nasal mucosa and in the draining LN with this low dose could explain why *Salmonella* Abortusovis infection could lead to abortion, excretion and enzootic salmonellosis in relation to particular environmental and physiological conditions including pregnancy (Sanchis and Pardon, 1984).

This model of infection does not require pregnant ewes, and bacteriological investigations can be limited to cephalic LNs and especially to the parotid ones which are the most heavily and constantly infected, as previously described for brucellosis by using the same route of infection (Plommet and Plommet, 1976). The conjunctival mucosa appears to be a suitable and practical route for infection or vaccination with attenuated strains of facultative intracellular pathogens (Fensterbank *et al*, 1981; Sanchis *et al*, 1991). The low doses that could penetrate the mucosa and colonize the lymphatic system beyond the first lymphatic barrier involve a stimulation of the cellular immunity leading to a strong protection with attenuated strains using this route (Plommet and Plommet, 1976; Pardon *et al*, 1980, 1984, 1990). The vaccinal dose might be lower than that using the subcutaneous route (Plommet and Plommet, 1975), and could induce a protective immunity associated with a low and short serological reaction, allowing a distinction between infected and vaccinated animals. Moreover, the results reinforce the idea that attenuated live *Salmonella* can be

used as vaccinal vectors both at the systemic and mucosal level (Chatfield *et al*, 1989; Cardenas and Clements, 1992).

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