Inoculation of lactating ewes by the intramammary route with Mycoplasma agalactiae: comparative pathogenicity of six field strains

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Inoculation of lactating ewes by the intramammary route with *Mycoplasma agalactiae*: comparative pathogenicity of six field strains

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Abstract – Contagious agalactia affects goats and sheep. In most infected sheep, the causal agent, *Mycoplasma agalactiae*, induces mastitis and/or agalactia, keratoconjunctivitis and arthritis. However, a few strains of *M. agalactiae* were isolated from tank milk from flocks without any clinical signs. The present study was undertaken to compare these apparently "asymptomatic" strains to classical virulent strains in order to assess the pathogenicity of four "asymptomatic" strains. Six groups of lactating ewes were inoculated by the intramammary route with 10^8 viable mycoplasmas of each strain. The clinical signs were regularly evaluated; the excretion of bacteria in milk and the serological response were measured. Ewes were necropsied 7 weeks after inoculation and the level of infection in retromammary lymph nodes was determined. Among the 4 apparently "asymptomatic" strains, 2 were fully virulent as were the strains isolated from diseased animals, and the other 2 induced somewhat less severe clinical symptoms. The other parameters, in particular the level of excretion in milk and the level of infection of regional lymph nodes following necropsy were similar for all strains. Mean antibody response was also comparable between the apparently "asymptomatic" and virulent strains, in spite of great individual variability. This observation shows that flocks without any clinical sign from which *M. agalactiae* is isolated in bulk milk, must be kept under strict control since mycoplasmas may induce severe outbreaks later with changing conditions of breeding.

contagious agalactia / *Mycoplasma agalactiae* / virulence / experimental infection / sheep disease

Résumé – **Inoculation de *Mycoplasma agalactiae* par voie intramammaire chez des brebis en lactation : virulence comparée de 6 souches de terrain.** L'agalactie contagieuse affecte les chèvres et les moutons. Chez la plupart des moutons infectés, l'agent causal, *Mycoplasma agalactiae*, induit des mammites et/ou de l'agalactie, des kératoconjonctivites et des arthrites. Cependant, quelques

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souches de *M. agalactiae* ont été isolées de lait de tank provenant de troupeaux ne présentant aucun signe clinique d'agalactie. L'étude présente a été entreprise afin de comparer 4 souches dites « asymptomatiques » à 2 souches virulentes et de mesurer la pathogénicité de ces souches « asymptomatiques ». Six groupes de 4 ou 5 brebis en lactation ont été inoculés par voie intramammaire avec 10^8 bactéries viables de chaque souche. Les signes cliniques ont été régulièrement évalués ainsi que l'excrétion des mycoplasmes dans le lait et la réponse sérologique. Les brebis ont été autopsiées 7 semaines après l'inoculation et le niveau d'infection des nœuds lymphatiques rétromammaires a été mesuré. Parmi les 4 souches apparemment asymptomatiques, 2 étaient aussi virulentes que les souches isolées de brebis malades, tandis que les 2 autres ont induit des signes cliniques moins sévères. Les autres paramètres, en particulier le niveau d'excrétion dans le lait et le niveau d'infection des nœuds lymphatiques rétromammaires après autopsie, étaient comparables. La réponse anticorps moyenne était elle aussi comparable, malgré une grande variabilité individuelle. Cette observation a pour conséquence que les troupeaux sans signe clinique desquels des mycoplasmes sont isolés à partir du lait de tank doivent rester sous contrôle strict car ces mycoplasmes peuvent induire plus tard de l'agalactie contagieuse à la faveur de conditions d'élevage changeantes.

agalactie contagieuse / *Mycoplasma agalactiae* / virulence / infection expérimentale / maladie du mouton

1. INTRODUCTION

Contagious agalactia, a worldwide disease of sheep and goats, is a syndrome which principally affects the mammary glands, joints and eyes [2, 10]. The main causal agents are *Mycoplasma agalactiae* in sheep and goats and *M. mycoides* subsp. *mycoides* large colony type, *Mycoplasma capricolum* subsp. *capricolum* or *Mycoplasma putrefaciens* in goats [3, 7]. In some French regions where contagious agalactia of ewes due to *M. agalactiae* is enzootic, the prevention of the disease is based on a strict control of infected flocks. The protocol includes culling of sheep with clinical signs, a bacteriological survey of bulk milk and a restriction of movements of flocks based on serology, using a quantitative ELISA leading to the classification of flocks with a serological index [9, 12]. Most flocks in the infected areas of France are at present free of contagious agalactia using this sanitary control but a few remain infected at low levels [11, 14]. In combination with the serological testing, the use of bacteriological methods, such as MF-dot for identifying mycoplasmas following isolation of mycoplasmas in bulk milk, has rapidly lowered the percentage of infected flocks. In some cases, however the sensi-

tivity of the technique has enabled mycoplasmas to be detected in bulk milk from a few flocks without any clinical signs. We investigated whether the "asymptomatic" strains of *M. agalactiae* isolated in these situations differ from the virulent strains isolated in true clinical circumstances. For this purpose, lactating ewes were experimentally infected with strains from various origins and clinical, serological, bacteriological and necropsy findings were recorded.

According to the inoculation routes, different models of infection of small ruminants with mycoplasmas were used in order to evaluate diagnostic tests, treatments or vaccines [5, 8, 21, 22]. The inoculation of lambs by the conjunctival route, which was previously used [19], led to the constant colonisation of the regional lymph nodes (LNs) and allowed the first steps of the infection to be analysed. When applied to lactating ewes, this mucosal route confirmed the colonisation of the retromammary LNs and the excretion of *M. agalactiae* in the milk of some animals (Sanchis et al., unpublished results). In the present study, the intramammary route was preferred in order to assess the pathogenicity of various field strains of *M. agalactiae* based on comparative analyses of clinical signs.

2. MATERIALS AND METHODS

2.1. Animals and protocol

Twenty-nine lactating ewes, free of contagious agalactia, of the local breed, "Manech", from the Pyrénées Atlantiques region (France) were identified and randomly distributed into 6 groups, 4 to 6 weeks after lambing. Each group of 5 (or 4 for 1 group) ewes was housed in separate experimental pens one week before inoculation. Ewes were inoculated by the intramammary route with 10^8 viable bacteria (CFU) of 6 different strains of *M. agalactiae*. The two virulent strains, including the strain P89 already described in a previous work [19], were isolated from ewes of two flocks with severe clinical signs including mastitis and keratoconjunctivitis. The 4 "asymptomatic" strains were isolated from bulk milk from 4 different flocks without any clinical sign of agalactia at the time of sampling. Infected animals were submitted to regular clinical and serological examinations. Excretion of bacteria in milk was controlled by quantitative bacteriological examination of the milk. All infected ewes were slaughtered at the end of the experiment (7 weeks post-inoculation [pi]) to quantify *M. agalactiae* in the retromammary LNs.

2.2. Bacterial strains and experimental infection

The list of the strains of *M. agalactiae*, with their virulent or "asymptomatic" status used in this study is presented in Table I.

For the preparation of inocula, the strains were cultured as previously described [19]. Briefly, 500 mL flasks with Mycoplasma Broth enriched Medium (MBM; Axcell, St Genis l'Argentière, France) were inoculated with each strain. After 3 days at 37°C, the culture was harvested by centrifugation at 10 000 *g* for 30 min and the pellet was suspended in a hundred-fold dilution in phosphate buffer saline pH 7.2 (PBS). It was then distributed in 1 mL sterile tubes and frozen at –70 °C. Before inoculation, a frozen specimen was thawed and examined in serial dilutions in MBM for enumeration of viable bacteria on sterile 96-well microplates. On the inoculation day, another tube of the same batch was thawed and adjusted to the required concentrations by dilution in PBS.

In each group, the animals received 10^8 CFU of each strain of *M. agalactiae* diluted in 5 mL of PBS and instilled via the intramammary route through the right teat.

2.3. Clinical examination and sample collection

Behaviour of the infected animals and clinical signs were observed every day after inoculation. To quantify the clinical signs and to estimate the time-course of the clinical development, an average clinical index was determined each week including the appearance of the milk (from 0 for a normal milk to 4 for agalactia) and the clinical status of the udder (from 0 for a normal udder to 4 for severe mastitis). Four additional points were added when signs of keratoconjuntivitis were detected.

Venous blood was harvested each week for serological examination. Serum samples were stored at -20 °C before testing all sera together at the end of the experiment.

Milk from right and left teats of each ewe was separately harvested once a week and examined the same day to quantify viable *M. agalactiae*.

Necropsy of all infected ewes was performed at day 47 pi. Retromammary LNs were collected from each ewe, placed individually in sterile vials and stored at –70 °C before quantitative bacteriological examination.

2.4. Bacteriological examination

– *Milk.* After collection, each fresh sample of milk was immediately refrigerated at +4 °C and divided in two parts. The first part was kept at –70 °C and the second was immediately examined in serial ten-fold dilutions on sterile microplates in MBM with amoxicillin added (MBMA) for the enumeration of viable *M. agalactiae*. After incubation for 7 days at 37 °C, the titres were expressed as viable bacteria per mL of milk. If this first trial was negative, the remaining frozen specimen was thawed, diluted 1:5, 1:10 and 1:100 in 2 mL MBMA and incubated at 37 °C. If it was negative again after 3 days, sub-cultures were realised in 2 mL MBMA. If no turbidity occurred after 7 days at 37 °C, the culture was considered as negative. Positive cultures were streaked onto mycoplasmal agar medium (Axcell, St Genis l'Argentière, France) with added amoxicillin and incubated for 3 days at 37 °C. Plates were examined, and colonies were transferred into separate tubes and used as inoculum for identification tests achieved by classical procedures [10, 16].

– *Lymph nodes.* LNs were thawed, cut into pieces with sterile blades, diluted 1:5 (weight per volume) in MBMA and homogenised with a blender (Stomacher LB80, SewardMedical, UK) in sterile bags. Specimens were treated for enumeration as previously described for the milk, and the remaining specimen suspensions were kept at –70 °C. If these primary cultures were negative, the frozen specimens were thawed and subcultured as above.

2.5. Serological examination

The serological examination was performed by an enzyme-linked immunosorbent assay (ELISA) according to the procedure already described [9]. The antigen used in this ELISA was prepared from the reference strain P89. The results were expressed in units according to the curve of a titrated standard serum included in each microtitration plate [12].

2.6. Statistical analysis

Data are expressed as mean ± SEM. Differences between the groups of ewes (or between strains) were analysed by ANOVA or the Chi2 test. A probability value of less than 0.05 was considered significant.

3. RESULTS

3.1. Clinical observations

For all experimentally infected ewes, the sign observed during the first week after inoculation was an alteration of the quality of the milk collected from the right inoculated side. This alteration rapidly extended to the left udder-half and, in some animals, led to a transitional or a true clinical mastitis. The most severe clinical signs appeared between 10 and 25 days pi.

With the virulent strains P89 and 9512, clinical mastitis appeared in 4 of the 5 ewes: 3 and 4 of them, respectively, developed agalactia associated with keratoconjunctivitis from the second week after inoculation to the week before necropsy. The clinical course was similar with the "asymptomatic" strains 9523 and 8751. The 2 other "asymptomatic" strains, 7783 and 7784, induced a decrease in milk production and slight clinical signs of mastitis in the right udder-half in 2 and 3 ewes from day 7 to day 28 pi (Tab. I; Chi2 test: $p = 0.02$). Agalactia occurred in the right udder at day 25 pi in one ewe inoculated with strain 7783. The change in the average clinical index of each group (Fig. 1) illustrated the differences in the expression of clinical signs of these two strains, 7783 and 7784, in comparison with the constant increase in clinical signs with the four other strains (ANOVA: $p < 0.02$).

Table I. Clinical signs observed in lactating ewes experimentally infected by the intramammary route with 10^8 viable bacteria of 6 different strains of *M. agalactiae.*

| Strains | Apparent status of pathogenicity | No of ewes per group | No of positive ewes | | | |
			Mastitis	Agalactia	Kerato-conjunctivitis	Total[a]
9512	virulent	5	4	4	2	10
P89	virulent	5	4	3	2	9
9523	asymptomatic	5	4	3	3	10
8751	asymptomatic	5	5	2	1	8
7783	asymptomatic	5	3	1	0	4
7784	asymptomatic	4	2	0	0	2

[a] Sum of positive findings.
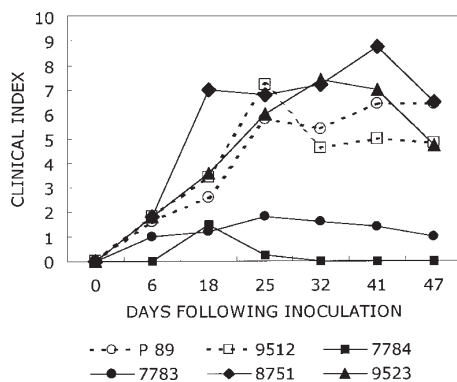
Figure 1. Mean clinical index as a function of time in 6 groups of ewes inoculated by the intramammary route with different strains of *Mycoplasma agalactiae* (10^8 viable bacteria per ewe). Standard errors of the mean are not represented and do not exceed ± 1.

3.2. Bacteriological results

– *Milk*. From Day 4 pi, *M. agalactiae* was recovered from the two udder-halves in all ewes, whatever the group. However, the excretion was always higher in the milk collected from the inoculated udder-halves (10^8 to 10^{12} viable bacteria per mL) than in the milk from the left ones (10^2 to 10^6 viable bacteria per mL). The excretion progres-

sively decreased and, in spite of some individual variability, excretion persisted until the end of the experiment (mean 10^2 to 10^3 CFU · mL^{-1} of milk) without significant differences between the different strains (Fig. 2).

– *Lymph nodes*. At necropsy, the right retromammary LNs were always larger than the left ones in all groups. There was no significant difference for the mean weight of the LNs in the 6 groups. No other signs were observed except a weak congestion of some LNs in all inoculated groups. In the strain 7784 group, all retromammary LNs were infected ranging from 10^2 to 10^7 CFU · g^{-1} of organ. In the strain P 89 and strain 8751 groups, the left LNs were not infected and the level of infection of the right LNs was lower (10^2 to 10^3 CFU · g^{-1}) (Tab. II). In the 3 other groups, which were infected by the other virulent strain (9512) or by two "asymptomatic" strains (9523, 7783), only a few left LNs remained uninfected and the level of infection of infected LNs was from 10^2 to 10^3 CFU · g^{-1} (Tab. II). Regarding the number of infected LNs or the level of infection, the differences between the virulent strains (P89 and 9512) and the 4 "asymptomatic" strains (7783, 7784, 8751, 9523) were not significant.

Table II. Level of infection in retromammary lymph nodes (LNs) after necropsy of lactating ewes experimentally infected by the intramammary route with 10^8 viable bacteria of 6 different strains of *M. agalactiae.*

Strain	Status	No of positive LNs / No. of LNs	No of viable bacteria in LNs (range)
9512	virulent	8/10	10^2-10^3
P89	virulent	4/10	10^2-10^3
9523	asymptomatic	6/10	10^2-10^3
8751	asymptomatic	4/10	10^2-10^3
7783	asymptomatic	7/10	10^2-10^3
7784	asymptomatic	8/8[a]	10^2-10^7

[a] 4 ewes per group.
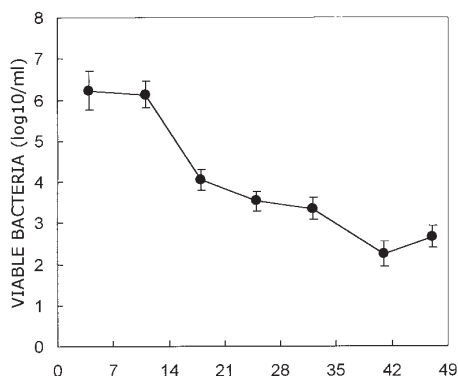
Figure 2. Time-course of the milk excretion of *Mycoplasma agalactiae* in ewes experimentally infected by the intramammary route. Mean excretion was calculated from data of 29 ewes inoculated with 6 different strains (10^8 viable bacteria per ewe).
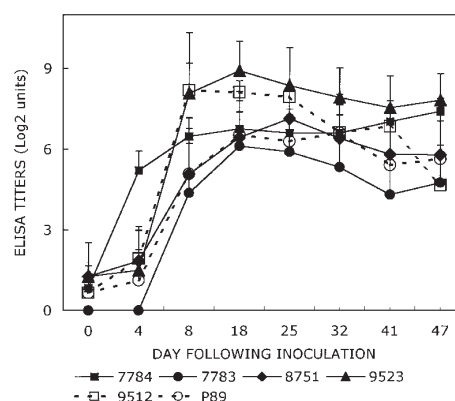
Figure 3. Time-course of antibody response after experimental infection of 6 groups of 5 ewes (4 ewes for strain 7784; ± SEM) inoculated by the intramammary route with 6 different strains of *Mycoplasma agalactiae* (10^8 viable bacteria per ewe).

3.3. Antibody response (Fig. 3)

Significant titres (>150 units) were obtained from the first week following inoculation; however, a few ewes (at least 1 animal per group) remained negative until the end of the experiment. The lowest ELISA titres were obtained in the group inoculated with the 7783 strain, and the highest titres (>1000 units) were obtained in the group infected with the 9523 strain. Because of the small number of animals per group and the great individual variability observed in all groups, no significant relationship could be observed between the "asymptomatic" and virulent strains.

4. DISCUSSION

The present study compared the pathogenicity of six strains of *M. agalactiae*. The objective of our previous work using lambs inoculated by the conjunctival route was to develop an experimental model in order to quantify the infection, allowing

investigation of the first stages of the contamination and measurement of the persistence of the pathogen in non-milking ewes [18, 19]. In milking ewes inoculated by the conjunctival route with 10^7 viable bacteria of two different strains of *M. agalactiae,* the infection disseminated to the retromammary LNs and led to an established infection in the udder without clinical signs during the two months of observation, in spite of a sporadic excretion of mycoplasmas in milk (Sanchis et al., unpublished results). These preliminary results supposed that, by this mucosal route, the peak of the infection could happen later; longer observation times or higher doses were needed in order to observe clinical signs, as described in another report [4]. So, since such conditions are not fully compatible with experimental requirements, a more drastic inoculation by the intramammary route with 10^8 CFU was chosen in order to obtain clinical signs so that the pathogenicity of 6 field strains of *M. agalactiae* could be compared.

Using this route of inoculation, the infection was rapidly established in the right udder-half and became bilateral with the 6 strains. The high level of excretion in milk (10^2 to 10^{12} CFU $\cdot$ mL^{-1}) during the first two weeks suggests a multiplication of mycoplasmas in the udder-halves of all ewes; the infection remained at a high level until the end of the experiment (Fig. 2). The only difference between the strains was in the clinical index which discriminated 2 "asymptomatic" strains from the 4 other strains (Fig. 1). These 2 strains (7783 and 7784) induced only mild and transitional disgalactiae and mastitis while the 2 other "asymptomatic" strains (8751, 9523) induced clinical signs as severe as the 2 virulent strains. However, since the level of infection and excretion remained comparable within all strains, the difference of clinical symptoms caused by these two strains indicates that, in our experimental conditions, the clinical signs are independent of the level of infection. These differences suggest that eco-epidemiological or physiolog-

ical conditions, such as the route of inoculation in natural infections [8], probably play an important role in the expression of the pathogenicity. Given the short time of observation required by our experimental conditions (7 weeks), it remains possible that, as for the dose effect studied with other pathogens [15, 17], the differences between strains could be related to a difference in the time-course of the infection induced by the different strains. These differences in induction of clinical symptoms may also be influenced by phenotypic surface antigen variation of *M. agalactiae* strains, which might play a key role in the establishment and persistence of mycoplasma infections [6].

Regarding the serological responses of ewes following inoculation with various strains of *M. agalactiae*, high levels of antibodies have been previously reported to be closely associated with induction of clinical signs and high levels of infection, as in other *Mycoplasma* infections such as *M. ovipneumoniae* in adult sheep [13] or *M. agalactiae* in lambs [19]. In the present study, in spite of comparable levels of infection, great individual variations were observed and some ewes remained seronegative while others developed very high titres; thus the highest titres (>1000 units) were obtained with the supposed "asymptomatic" strain 9523, while 2 ewes remained negative with the reference virulent strain P89. No correlation could be established between the individual ELISA titres and the severity of clinical signs. These variations in the antibody response detected by ELISA may be due to the well-established antigenic variability of mycoplasmas [6]*,* including *M. agalactiae* [1, 20]. The infection of the udder seemed to be a prerequisite for the development of a serological response but did not appear to be sufficient. These results confirmed the observations made in naturally-infected flocks in which the strains studied in this report were isolated: high ELISA titres were observed in animals with active or latent infections while a few other

infected animals remained seronegative. These observations confirmed that while ELISA is an excellent tool for establishing a flock infection, it is of little value in detecting all infected animals.

In conclusion, in our experimental conditions, the 6 strains studied in this report induced a constant infection reflected by a comparable level of infection in the retromammary LNs and the level of excretion of strains in milk. The 6 strains also induced clinical signs of agalactia, but for 2 of them these signs remained low during the 47 days of observation. Among the four "asymptomatic" strains, the results show that there is a variability in the pathogenicity of strains of *M. agalactiae*; these differences cannot be interpreted as a true attenuation of the virulence for the strains 7783 and 7784. These strains rather represent fully virulent *M. agalactiae* isolated from healthy or apparently healthy carrier animals [4]. This highlights the need to adopt a strict control of these asymptomatic flocks with excretion of mycoplasmas in milk.

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