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An oil-emulsion vaccine induces full-protection against *Mycoplasma agalactiae* infection in sheep

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SUMMARY

The immunogenicity and efficacy of three inactivated vaccines (A, B, C) prepared with *Mycoplasma agalactiae* (*M. agalactiae*) and with different oil-emulsion adjuvants were evaluated in sheep. Twenty-eight animals were used, divided into four groups (a, b, c, d) of seven animals each. Three groups were immunized with the same vaccine, but using different adjuvants, while one group (d) was used as an unvaccinated control group. All the vaccine formulations were able to induce clinical protection of animals after challenge with *M. agalactiae*, but only vaccine C, emulsioned with Montanide ISA-563, Marcol-52 and Montane-80 (ratio: 30%, 63%, 7% respectively), was able to induce full protection in challenged animals, preventing both the onset of clinical signs and infection by *M. agalactiae.*

KEY WORDS: Mycoplasma agalactiae, Sheep, Oil-adjuvant vaccine, Immunogenicity

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INTRODUCTION

Contagious agalactia (CA) is an infectious disease of small ruminants caused mainly by *Mycoplasma agalactiae (M. agalactiae)* and characterised by mastitis, agalaxia, arthritis and keratoconjunctivitis. CA-like clinical signs either alone or in conjunction with respiratory symptoms may also be caused by M*ycoplasma capricolum* subsp. *capricolum*, *Mycoplasma mycoides* subsp. *mycoides* Large Colony, *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma putrefaciens* (DaMassa *et al.,* 1992; Nicholas, 2002). In the areas where CA is endemic, economic losses are reported due to decreased milk production and to reduction of productive life of the infected animals, but prophylaxis measures are not always applied due to the lack of complete knowledge of the pathways of infection and to the limited availability of highly effective vaccines.

Early in the study of CA, tools for immuno-prophylaxis against CA were developed that were based on milk or organ-derived preparations from infected animals.

These vaccines were poorly effective and were able to transmit other pathogens such as the scrapie agent (Caramelli *et al.,* 2001). Accordingly, in recent years several studies have been done to develop novel safe and effective vaccines against *M. agalactiae* (Leon Vizcaino *et al.,* 1995; Buonavoglia *et al.,* 1998; Tola *et al.,* 1999; Greco *et al.,* 2002). Experimental vaccines combined with aluminium hydroxide ($Al(OH)_3$) or mineral-oil adjuvants (Buonavoglia *et al.,* 1998; Tola *et al.,* 1999; Greco *et al.,* 2002) proved to be effective, but additional data on the safety and immunogenicity of the vaccines are needed. Although the aluminium hydroxide-adjuvated vaccine proved to be safe, it elicited low antibody titers that persisted for short periods. Conversely,

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the mineral-oil-adjuvated vaccine proved to be highly immunogenic with high antibody levels persisting for long periods, but it induced granulomatous reactions around the point of inoculation (Buonavoglia *et al.,* 1998; Greco *et al.*, 2002). The present study reports the results of vaccine trials done to evaluate the safety and efficacy of three vaccines against CA, prepared with different mineral-oil adjuvants.

MATERIALS AND METHODS

Animals

Twenty-eight sheep of comisana breed at the 2nd lactation were selected from a flock free from CA. The experimental study was performed at the isolation unit of the Animal Hospital, Faculty of Veterinary Medicine of Bari, according to the European and Italian animal health and well-being regulations. The animals were clinically examined to exclude any pathologies involving the mammary gland; additionally, milk samples, nasal and auricular swabs and sera from each animal were analyzed to exclude any previous contact with *M. agalactiae*. Milk samples, auricular and nasal swabs were examined by polymerase chain reaction (PCR) (Tola *et al.,* 1996; 1997), whereas sera were screened for antibodies against *M. agalactiae* by ELISA test according to Romano *et al.* (1995) with minor modifications as described below. The animals were divided into four groups (a, b, c, d) of seven animals each. The groups (a, b and c) were vaccinated while group d was used as a control (unvaccinated animals).

Strain and growth conditions

The field strain *M. agalactiae* Ba/2 was used in the study for vaccine preparation and challenge trials. The strain, isolated from a milk sample of a CA affected sheep, was cloned according to Tully (1983), identified on the basis of its cultural and biochemical characteristics (Clyde, 1983; DaMassa, 1996) and finally confirmed as *M. agalactiae* by specific PCR (Tola *et al.,* 1996). The strain was used at the 3rd passage level. The genotype and phenotype of the Ba/2 strain had been previously analysed and compared to the other field strains isolated in Southern Italy, by using restriction fragment length polymorphism (RFLP) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Greco, 1999) showing a 100% similarity.

Vaccines

The *M. agalactiae* Ba/2 strain at the 3rd passage level was used to prepare the experimental vaccines. The strain was cultivated at 37°C in 500 ml of *Modified Hayflick Broth* (MHB) supplemented with equine serum (10% v/v). After 3 days the titer of the culture was evaluated according to Taylor's method (Meynell and Meynell, 1973). After centrifugation at 10,000 x *g* for 30 min at 4°C to remove the culture medium, the pellet was washed twice with saline solution (0.8% of NaCl) and finally suspended in the original volume (500 ml) with the same solution; then, the washed culture was inactivated with beta-propiolactone 0.3% (v/v) for 2 h at 37°C.

In order to control against residual infectivity and sterility, the suspension was tested according to the guidelines of the Italian pharmacopeia for the quality control of vaccines for veterinary use (Ciuchini *et al.,* 1998).

Before making the oil-emulsion the titre of the washing culture was evaluated by real-time PCR (Lorusso *et al.,* 2007) and adjusted with saline solution to a concentration of $2x10^9$ DNA copies of *M. agalactiae* per ml of suspension. The suspension of strain Ba/2 was initially emulsified with tween-80 (4,1% v/v) and subsequently emulsified in a 1:1 ratio with each of the mineral-oil adjuvants:

a) mineral oil Montanide ISA 563 (Seppic Inc., Paris, France) in a 1:1 ratio (Vaccine A);
b) a mixture of "Montanide ISA 563, Marcol 52 (Esso Italiana S.r.l., Rome, Italy) and Montane 80 (Seppic Inc., Paris, France)" oils in a 50:45:5 ratio (Vaccine B);
c) a mixture of "Montanide ISA 563, Marcol 52 and Montane 80" oils in a 30:63:7 ratio (Vaccine C). Each dose of vaccine (2 ml) contained $2x10^9$ DNA copies of *M. agalactiae*.

Experimental design

Animals of group a were inoculated with vaccine A; animals of group b were inoculated with vaccine B and animals of group c were administered vaccine C. Each animal received 2 doses of vaccine at a 30 day interval (T^0 and T^1) by intra-tail inoculation.

Challenge
Experimental infection was carried out by using the *M. agalactiae* Ba/2 strain at 3rd passage level. The challenge strain was propagated as for the vaccine preparation and the mycoplasma titre was adjusted with saline solution to a concentration of 10^6 CCU/ml. Twenty-one days after administration of the 2nd dose of vaccine (T^3), the vaccinated (group a, b, c) and unvaccinated animals (group d) were inoculated by the nasal route using 0.5ml of the culture per nostril.

Bacteriological examinations and PCR
Starting from 1st day post challenge (dpc) to day 60, milk samples and nasal swabs were collected daily from each animal. Each sample was inoculated in MHB and maintained at 37°C for one week and then inoculated in Hayflick agar. All the colonies resembling mycoplasmas were cloned and identified by biochemical and molecular assays.
The PCR was carried out by using two *primers* specific for a 375 bp-long sequence of *M. agalactiae* (Tola *et al.,* 1996; 1997). DNA extraction from the samples was made with QIAmp tissue kit (Qiagen). The PCR assays were performed using the DNA Thermal Cycler Gene AMP 9600. Reactions were made in a 25-µl mixture containing 5µl of DNA, 12.5µl of AccuPrime SuperMixII mix (Invitrogen) (40mM Tris.HCl pH 8.4, 3 mM $MgCl_2$, 100 mM KCl, 400 M of each dNTP, AccuPrime *Taq* DNA Polymerase), 200pM of each primers and DNase-free H_2O. The PCR amplicons were analyzed by electrophoresis on 2% Tris-Acetate-EDTA agarose gels after staining with ethidium bromide.

Serology
To evaluate the antibody response to *M. agalactiae*, blood samples were taken from each animal at the first vaccination (T_0), second vaccination (T_1), 10 days after the second vaccination (T_2), at challenge (21 days after the second vaccination, T_3) and subsequently at 10, 20, 30 and 60 dpc (T_4, T_5, T_6 and T_7, respectively). For evaluation of the antibodies, an ELISA test was used according to Romano *et al.,* (1995) with minor modifications. Briefly, the sheep sera, diluted 1:100 in PBS Tween-20 (PBS-T), were tested on flat bottom polystyrene plates (Polysorp NUNC) coated with 100 µl of Tween-20 extracted *M. agalactiae* Ba/2 strain membrane-protein. Then, anti-sheep IgG horseradish peroxidase-conjugated and substrate 2,2'-Azino-bis Diammonium (SIGMA-Aldrich) diluted in citrate-phosphate buffer (pH 5) with 25 µl of 30% hydrogen peroxide were added. Optical density (O.D.) was read at 405 nm. The cut-off was determined at 0.050 O.D., as calculated by analysis of 30 sheep sera collected from a CA-free flock and 30 sera whose 20 were field sera belonging to CA affected animals and 10 of them belonged to experimentally infected animals. All 60 sera were analyzed for the presence of the antibodies against *M. agalactiae* by evaluating the ability to inhibition the growth of both reference strain Fg/1 and field strain Ba/2 of *M. agalactiae*, according to the procedure of Cottew (1983).

Clinical examination
Starting from day 7 after the first vaccination, the areas of vaccine inoculation were inspected weekly to monitor the onset of adverse reactions (by using a calipers). Starting from the time of challenge, the animals were examined clinically every day, with particular regard to the body temperature and to the clinical status of mammary lymph nodes, udder, joints, conjunctives and corneas.

RESULTS

Clinical examination
After vaccination no adverse systemic effects were observed, while granulomatous inflammation was observed in all the vaccinated animals at the site of vaccine injection (Table 1). Starting from the 2nd week, in the animals inoculated with vaccine A, a local increase in skin thickness was observed with a median value (mv) of 7 mm. Skin thickness reached an mv of 9 mm by the 5th week and of 8 mm by the 7th week to the end of the study period. In the animals inoculated with vaccine B, starting from the 2nd week, the skin thickness increased to an mv of 12 mm, reaching an mv of 17 mm by the 5th week through the end of the study. Local adverse reactions were also observed in the animals inoculated with vaccine C. Starting from the 2nd week, skin thickness increased to mv of 12 mm, reaching mv of 16 mm by the 3rd week, 22 mm by the 5th week and 25 mm by the 6th week through the end of the study. After challenge, no specific clinical signs (hyperthermia, anorexia, arthritis, keratoconjunctivitis

TABLE 1 - *Increases in derma thickness in the side where the vaccines were inoculated.*

Group	Vaccine	Time of observation (week)								
		0	1	2	3	4	5	6	7	8
a	A	4[a]	4	7	7	7	9	9	8	8
b	B	5	5	12	12	12	17	17	17	17
c	C	4	4	12	16	16	22	25	25	25

[a]Mean values expressed in mm.

or reduction of milk production) were observed in any of the vaccinated animals within the 3 groups. Starting from dpc 8 (group A) and 13 (group B) the animals displayed moderate enlargement of the mammary lymphoglands, while the animals of group C did not.
At dpc 7, all the control animals (group d) showed hyperthermia (39.5-39.7°C) (Table 2). At dpc 12, the temperature of 3/7 control animals was normal (38.3°C), while at dpc 15 the temperature was normal in all the animals. At dpc 25, 26 and 27 two animal exhibited peaks of hyperthermia, reaching values of 39.5°C.
Starting from dpc 4, all the animals of group d showed enlargement of the supramammary lymph nodes reaching 3±0.5 cm at dpc 9. Starting from dpc 9 pathological changes in the mammary gland and decrease in milk production were also observed. From dpc 18 to 20, watery secretion and fibrinous exudate was observed from the udder. This secretion steadily decreased and ended at dpc 33. In the animals of group d no signs of ocular or joint sickness were observed.

Bacteriological trials and PCR assay
The results of bacteriological and PCR analysis on the milk samples and nasal swabs are reported in Table 3. The samples were collected from all 28 animals, starting on dpc 1 through dpc 60. By bacteriological and molecular analysis, all the animals of group c tested negative and the results were not included in the table.
None of the animals of group a showed clinical signs and displayed uninterrupted shedding of *M. agalactiae* from dpc 6 to 18 in milk and from dpc 12 to 18 by the nasal route. All the animals of group b irregularly shed *M. agalactiae* and only for a few days. *M. agalactiae* was detected in milk samples at dpc 7 and 8 with bacteriological examinations and from dpc 7 to 16 by PCR. *M.*

TABLE 2 - *Clinical signs in the animals of group d (not vaccinated) after challenge with M. agalactiae.*

Animal	Clinical signs		
	Fever	Hypogalaxia	Mastitis
d1	6-14[a]	9b	18[b]
d2	6-11	11	19
d3	7-14	11	20
d4	6-11	10	18
d5	7-14; 25-27	12	20
d6	7-14; 25-27	9	20
d7	6-11	10	20

[a]Day of onset - last day with symptom; [b]Day of onset symptom.

TABLE 3 - *Bacteriological and PCR results on the milk samples and nasal swabs collected from animals of groups a and b (vaccinated) and group d (not-vaccinated) after challenge with M. agalactie.*

			Shedding of M. agalactiae			
			Bacteriological results		*PCR*	
Group	*Vaccine*	*Animals*	*Milk*	*Nose*	*Milk*	*Nose*
a	A	a1	7-14[a] (6)[b]	12-15 (3)	6-15 (8)	12-19 (4)
		a2	6-18 (6)	15-18 (2)	6-18 (6)	13-18 (6)
		a3	7-15 (6)	13-16 (3)	7-16 (8)	13-17 (3)
		a4	6-15 (7)	12-14 (2)	6-16 (8)	12-16 (5)
		a5	8-18 (3)	15-16 (2)	7-18 (6)	14-17 (4)
		a6	9-18 (7)	12-18 (4)	8-18 (8)	12-18 (4)
		a7	6-17 (6)	14-16 (3)	6-17 (8)	13-17 (4)
b	B	b1	7-8 (2)	17-18 (2)	7-11 (3)	14-18 (3)
		b2	8 (1)	17 (1)	8-16 (4)	16-17 (2)
		b3	7 (1)	18 (1)	7-14 (3)	15-17 (3)
		b4	7-8 (2)	18 (1)	7-9 (3)	16-18 (3)
		b5	7-8 (2)	17-18 (2)	7-15 (4)	14-18 (3)
		b6	8 (1)	18 (1)	8-13 (4)	15-18 (4)
		b7	7-8 (2)	17-18 (2)	7-11 (3)	17-18 (2)
d	D	d1	4-33 (20)	13-53 (19)	1-33 (29)	12-55 (23)
		d2	5-33 (22)	12-56 (28)	2-33 (30)	12-56 (29)
		d3	4-32 (20)	12-57 (23)	1-33 (30)	12-57 (24)
		d4	6-31 (21)	13-57 (20)	3-31 (27)	13-57 (23)
		d5	4-33 (20)	13-56 (20)	2-33 (28)	12-56 (23)
		d6	5-33 (25)	13-56 (16)	2-33 (31)	13-56 (17)
		d7	4-33 (23)	12-55 (18)	1-33 (29)	12-55 (20)

[a]Day of onset shedding - last day of shedding; [b]Days of shedding *M. agalactiae*. Note: Group C is not indicated because the bacteriological and PCR results for *M. agalactiae* shedding were constantly negative.

agalactiae was detected in nasal swabs at dpc 17 and 18 by bacteriological examinations and from dpc 14 to 18 by PCR.

In the animals of the group d (control), *M. agalactiae* was detected in milk samples of all the animals from dpc 4 to 33 by bacteriological examinations and from dpc 1 to 33 by PCR. It was not possible to detect shedding of *M. agalactiae* after dpc 33 as all the animals developed complete agalaxia. *M. agalactiae* was detected in the nasal swabs of all the animals from dpc 12 to 57 by both bacteriological and PCR examinations.

Serology

All the experimental vaccines were able to elicit specific antibodies in all the vaccinated animals. (Figure 1). After the 1st vaccination (T_1), the animals of group a resulted seronegative to *M. agalactiae* while they developed specific antibodies after the 2nd vaccination (T_3) with mean values of optical density (OD means) of 0.138 (*S.E.* 0.002). The animals of groups b and c elicited antibodies to *M. agalactiae* after the 1st vaccination (T_1) with mean OD values 0.065 (*S.E.* 0.001) and 0.125 (*S.E.* 0,001) respectively, reaching values of 0.170 (*S.E.* 0.001) and 0.308 (*S.E.* 0.002) OD after the 2nd vaccination (T_3), respectively.

At dpc 60 (T_7) the antibody levels further increased in the animals of groups a (ODm 0.180 (*S.E.*0,002)) and b (ODm 0.210 (*S.E.* 0,001)), while the antibody levels of the group c animals did not (ODm 0.313 (*S.E.* 0,001)).

In the control group d animals, the antibody kinetics after challenge was characterized, as observed in previous studies (Buonavoglia *et al.*, 1999), by low antibody levels, with an ODm of 0.044 (*S.E.* 0.003) at dpc 20 (T_5). Subsequently, the antibody levels reached ODm value of 0.083 (*S.E.* 0.001) at dpc 30 (T_6), and 0.140 (*S.E.* 0.002) at dpc 60 (T_7).

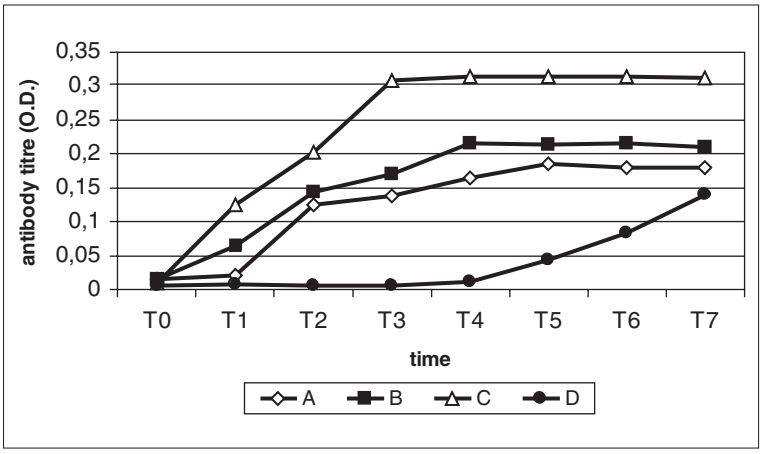

FIGURE 1 - *Antibody kinetics (Elisa test) in sheep immunized with some vaccines and challenged with M. agalactiae.*
T0: time of 1st vaccination
T1: time of 2nd vaccination
T2:10 days after the 2nd vaccination
T3: time of challenge
T4:10 days after challenge
T5: 20 days after challenge
T6: 30 days after challenge
T7: 60 days after challenge
A: vaccine A (Montanide ISA-563) 100%
B: vaccine B (Montanide ISA-563 + Marcol-52 + Montane-80) 50%, 45%, 5%
C: vaccine C (Montanide ISA-563 + Marcol-52 + Montane-80) 30%, 63%, 7%
D: not vaccinated animals (control group)

DISCUSSION

The three experimental vaccines caused weak local inflammatory reactions although different rates of tissue damage were observed. Administration of the three vaccines was not associated with systemic adverse reactions, unlike the observations of previous studies (Buonavoglia *et al.,* 1998) that reported the onset of ulcerations at the injection site and systemic clinical signs.
All the vaccines were able to elicit active immunization, as evidenced by the antibody response, although differences in the kinetics and magnitude of the humoral immune response were observed among the various vaccine formulations. Vaccine A elicited the lowest immune response with a low and delayed production of antibodies. Vaccine B elicited good antibody titers soon after the 1st vaccine administration. However, of the three experimental vaccines, the best results with regard to the antibody kinetics and magnitude were obtained with vaccine C.
All the experimental vaccines evaluated in this study were able to prevent the onset of CA-related clinical signs in the animals after challenge with *M. agalactiae,* although a weak swelling of the mammary lymphogland was found in the animals of groups a and b.
Interestingly, the animals inoculated with vaccine C were protected not only clinically but also from the infection. Shedding of *M. agalactiae* was not observed by the milk or the nasal route, whereas the animals of groups a and b shed *M. agalactiae* for a few days both at the udder and nasal level. By analyzing the antibody titers after challenge, different kinetics were observed among the various groups. In the animals of groups a and b the antibody levels appeared to increase after challenge, while the animals of group c did not. This may suggest that the animals immunized with vaccine C developed a strong immunity that was able to prevent replication of the challenge *M. agalactiae* and, consequently, a new stimulation of the immunity system after challenge did not occur.
Montanide ISA-563 mineral oil is characterized by a low viscosity. This makes the oil a good adjuvant as administration of the vaccine is easy and local adverse effects are kept at minimum. However, in our study the immunity elicited by this vaccine (A) was not able to prevent the infection.
Autogenous vaccines adjuvated with Marcol-52 mineral-oil, produced by the Regional Laboratories for animal prophylaxis (Istituto Zooprofilattico Sperimentale di Foggia) are widely used in the veterinary field. These vaccines promote protective and long-lasting immune responses, but are also associated with severe local adverse effects (personal and farm-veterinary remarks; unpublished data). Accordingly, farmers tend to prefer aluminium hydroxide adjuvated vaccines that appear to be less prone to induce local adverse reactions, but are less immunogenic (Buonavoglia *et al.*, 1998).
In the present study, an emulsion made up of a combination of 3 mineral oils (Montanide ISA-563 + Marcol-52 + Montane-80) with the ratio 30:63:7 proved to make the vaccine safer and more immunogenic.

The findings of this study warrant further investigations, with particular regard to evaluation of the duration of protective immunity elicited by vaccine C, adjuvated with a mix of 3 mineral oils. The burden of CA in countries where ovine and caprine livestock are the primary or sole economic source and the recognition that CA represents a major priority for animal health organizations have increased the efforts to investigate the pathogenetic pathways of the disease and to develop effective prophylactic tools. Accordingly, the study and development of safe, protective and low-cost vaccines is important to facilitate the adoption of prophylaxis measures for CA worldwide, notably in the developing countries.

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