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## INTERNATIONAL PhD COURSE IN IMMUNOPHARMACOLOGY

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**Study of ovine immune response against *Mycoplasma agalactiae* during natural and experimental infection and comparison of different vaccine formulations.**

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## **Introduction: Mycoplasmas of animals**

### **Classification and phylogeny**

The mycoplasmas (class Mollicutes) represent a wide spectrum of phylogenetically related parasitic bacteria. The Mollicutes group includes the smallest known self-replicating organisms carrying the smallest number of genes that are characterized by the lacking of a cell wall. There is no wonder, therefore, that mycoplasmas have a special appeal to those interested in the definition of the minimal set of genes essential for life considering this as an important step on the way of reaching the goal of defining in molecular terms the entire machinery of a self-replicating cell<sup>1</sup>. Recently the attention of researchers was also focused on the study of the pathogenic mechanism of *Mycoplasma* and on its ability to evade the host immune response by antigenic variation of surface proteins<sup>2</sup>. Mollicutes phylogeny has been controversial for a long time. Thanks to the 16S rRNA sequence data Woese and co-workers<sup>3</sup> were able to define the classification that is currently in use. A simplified list of the most common genera is listed in Fig. 1.

Classification	Current no. of recognized species	Genome size (kb)	Mol% G+C of genome	Cholesterol requirement	Distinctive properties	Habitat
Order I: <i>Mycoplasmatales</i>						
Family I: <i>Mycoplasmataceae</i>						
Genus I: <i>Mycoplasma</i>	102	580–1,350	23–40	Yes	Optimum growth at 37°C	Humans, animals
Genus II: <i>Ureaplasma</i>	6	760–1,170	27–30	Yes	Urea hydrolysis	Humans, animals
Order II: <i>Entomoplasmatales</i>						
Family I:						
<i>Entomoplasmataceae</i>						
Genus I: <i>Entomoplasma</i>	5	790–1,140	27–29	Yes	Optimum growth at 30°C	Insects, plants
Genus II: <i>Mesoplasma</i>	12	870–1,100	27–30	No	Optimum growth at 30°C; 0.04% Tween 80 required in serum-free medium	Insects, plants
Family II:						
<i>Spiroplasmataceae</i>						
Genus I: <i>Spiroplasma</i>	33	780–2,220	24–31	Yes	Helical motile filaments; optimum growth at 30–37°C	Insects, plants
Order III: <i>Acholeplasmatales</i>						
Family I:						
<i>Acholeplasmataceae</i>						
Genus: <i>Acholeplasma</i>	13	1,500–1,650	26–36	No	Optimum growth at 30–37°C	Animals, some plants, insects
Order IV: <i>Anaeroplasmatales</i>						
Family: <i>Anaeroplasmataceae</i>						
Genus I: <i>Anaeroplasma</i>	4	1,500–1,600	29–34	Yes	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Genus II: <i>Asteroleplasma</i>	1	1,500	40	No	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Undefined taxonomic status						
Phytoplasma	ND <sup>b</sup>	640–1,185	23–29	Not known	Uncultured in vitro	Insects, plants

**Figure 1: Taxonomy and characteristics of the genera of the class Mollicutes. Modified from Razin S *et al.*, (2002). Molecular biology and pathogenicity of mycoplasmas<sup>4</sup>, p. 1096.**

The ancestral mycoplasma arose from the *Streptococcus* phylogenetic branch about 600 million years ago. The mycoplasma phylogenetic tree split later into two major branches: one branch (the “AAA” branch) led to the *Asteroleplasma*, *Anaeroplasma*, and *Acholeplasma* branches, and the other (the “SEM” branch) led to the *Spiroplasma*, *Entomoplasma*, and *Mycoplasma* branches<sup>4</sup>.

The members of the class Mollicutes are characterized by their small genome size (0.58 – 2.2 Mbp), a low G+C content (23 – 40 mol%) of the genome and a permanent lack of a cell wall, but a deeper insight into their classification reveals a discrepancy between taxonomy and phylogeny, due to the fact that the genus *Mycoplasma* is distributed in four of the five phylogenetic groups and three of these groups also contain mollicutes of other genera. However is widely accepted that phylogeny is a naturally dynamic subject, because of the numerous parameters that influence its results.

The aim of this project was the evaluation of the host T cell immune response elicited against *M. agalactiae* infection and vaccination, but little is known about the immune dialogue between *M. agalactiae* and its host, particularly the T cells reaction to this pathogen; hereby is depicted a panorama of the immune interaction with the host immune system of other better-known Mycoplasmas.

## **Pathogenesis and immunomodulation by *Mycoplasma* and its components**

### **Invasion of nonphagocytic cells**

Among *Mycoplasma* species, many are classified as commensal organisms. They may inhabit mucosal tissues of animals without causing a disease. Also pathogenic mycoplasmas are often able to interact with host cells without causing acute cytopathic effects, therefore they can be defined as the “ideal parasites”<sup>1</sup>, being able to establish with the host an harmonic equilibrium.

Pathogenic mycoplasmas have a pronounced affinity for mucosal tissues and consequently show a predilection for the respiratory system, mammary gland, serous membranes and the urogenital tract. Although it is believed that mycoplasmas remain attached to the surface of epithelial cells, some mycoplasmas have evolved mechanisms for invading host cells that are not naturally phagocytic. Invasion of nonphagocytic host cells, even if for a short period of time, may provide mycoplasmas the ability to cross mucosal barriers and gain access to internal tissues. The ability to invade and live within host cells has been intensively studied in the case of *Mycoplasma penetrans*<sup>5</sup>. This microorganism adheres to cell surfaces, deeply penetrates into the cell using a tip-like structure and extensive invasion of the mycoplasma into the cytoplasm may kill the cells.

Other mycoplasmas, known to be surface parasites, such as *M. fermentans*<sup>6</sup>, *M. pneumoniae*<sup>7</sup>, *M. genitalium*<sup>8</sup>, *M. gallisepticum*<sup>9</sup> and *M. synoviae*<sup>10</sup> were also proved, under certain conditions, to reside within non-phagocytic cells. The mechanism of invasion seems to be dependent on the polymerization/depolymerization of microtubules or microfilaments<sup>1</sup>. For example, *M. penetrans* invasion of HeLa cells depends on the capacity of the cells to assemble actin microfilaments<sup>11</sup>, and the entry of *M. gallisepticum* into the chicken embryo fibroblast is inhibited by the microtubule inhibitor nocodazole<sup>9</sup>. The information about the ability of *Mycoplasma* to multiply within the host cells still needs to be investigated.

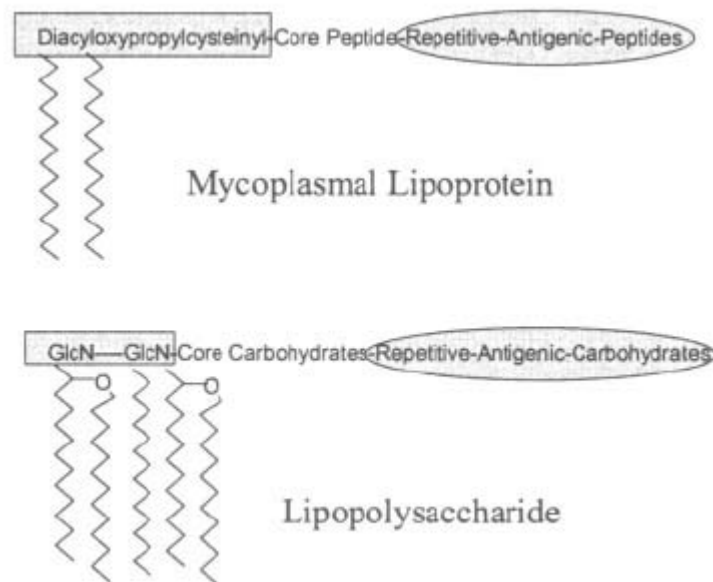
Many studies have shown that mycoplasma lipoproteins stimulate monocytes and induce the secretion of pro-inflammatory cytokines [ie. tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 6 (IL-6) and IL-1]<sup>4,12</sup>. Interestingly it happens by a mechanism distinct from that of bacterial lipopolysaccharide. Northern (RNA) blot analysis of cytokine expression in these cells showed that the induction of IL-1 $\beta$  by mycoplasmas involves, unlike that by LPS, posttranscriptional events<sup>12</sup>.

Another pathogenic mechanism related to mycoplasma infection is the high-frequency variation of surface proteins. This aspect will be discussed in the chapter related to *M. agalactiae*.

### **Macrophages activation**

Several mycoplasma-derived factors have been reported that act on immune cells in various ways, but it was only in the early '90 that it was described for the first time a "high-molecular-weight material" (MDHM) found in *M. fermentans* able to activate macrophages and leading to an indirect formation of cytotoxic T-cells (CTL)<sup>13</sup>. This macrophage activator was demonstrated to be a lipopeptide with only two fatty acids

and a free amino terminus and a molecular weight of about 2 kDa. The substance was therefore called MALP-2 for Macrophage Activating Lipopeptide of 2 kDa molecular weight. The comparison with a similar molecule found in *M. hyorinis*<sup>14</sup> allowed establishing that it is the lipid portion which imparts the macrophage stimulatory activity to these molecules. This activity is very similar to that caused by Gram-negative LPS, and the structural similarities between the two classes of molecules is surprising (Fig 2). Under certain circumstances, MDHM alone does not suffice to trigger cytokine release from THP-1 cells and that additional lipid membrane components could be required<sup>12</sup>, but at least in vitro MALP-2 and LPS display the same activities when added to macrophage/monocyte cultures, and both substances act at similarly low concentrations.



**Figure 2: Structural similarities between Mycoplasmal lipoprotein and bacterial lipopolysaccharide. From Razin S, Herrmann R (2002), Molecular biology and pathogenicity of mycoplasmas, p. 457.**

As previously mentioned, the interaction of monocytes and macrophages with several mycoplasmas, including *M. fermentans*, induces the production of several proinflammatory cytokines and mediators, as TNF $\alpha$ , IL-1, IL-6, nitric oxide and

oxygen radicals<sup>15</sup>. Thus proinflammatory cytokines, chemokines and prostaglandins<sup>16,17</sup> are released in response to MALP-2 and other mycoplasmal lipoproteins and lipopeptides. Using a mouse mutant strain (C3H/HeJ) that exhibit a very low susceptibility to endotoxin, it was possible to demonstrate that MALP-2 and LPS utilize different receptors<sup>18,19</sup>

### **Alternative mechanisms of immunomodulation**

Stuart *et al.* have reported that heat-inactivated mycoplasmas from some species of mycoplasmas can stimulate the expression of MHC class I and class II antigens in the mouse myelomonocytic cell line WEHI-3 and that it is due to increase in the transcriptional activity of MHC genes<sup>20</sup>. In a follow up study it was shown that the immunomodulatory effect was exerted also in other murine and human cell lines and that neither IL-4 nor GM-CSF were required<sup>21</sup>. Other Authors reported that not only the stimulation but also the suppression of expression of MHC class II antigen on peritoneal macrophages can be caused by MALP-2 containing preparations<sup>22</sup>. Recently, the over expression of MHC class II molecules was also related to catarrhal bronchointerstitial pneumonia caused by *M. bovis* in cattle<sup>23</sup>.

### ***Mycoplasma agalactiae* and contagious agalactia**

*Mycoplasma agalactiae* is the main etiological agent of Contagious agalactia (CA), a severe mastitis occurring in small ruminants that represents a relevant constraint for milk and cheese production.

Contagious agalactia is an important disease in the Mediterranean countries of Europe, Asia, and North Africa, in the former Soviet Union, in India and Pakistan, and in countries of the Middle East. It has also been reported from Australia, South Africa, and South America. Although three isolations of *M. agalactiae* have been



reported from the United States, it appears that North American strains are non pathogenic and do not cause classical contagious agalactia syndrome.

Young ruminants become infected directly at suckling, while the adults are contaminated during the milking procedures, both by milkers' hands and by milking machinery. The incubation period often varies considerably and can last several weeks or months. The main reservoir of the mycoplasmas is the infected animal, in which the organisms can persist for over a year after clinical recovery. Infected animals which do not show any clinical signs of disease play an important role as carriers and are often the main obstacle in the control and eradication of mycoplasmoses<sup>24</sup>.

In the first stage the disease is visible as mild unilateral or bilateral mastitis with warm, swollen, painful parenchyma, later followed by sclerosis and typical reduction in size of one or both the organs. Mammary lymph-node is often involved showing from mild to marked enlargement in size. The affected animal can also suffer for arthritis, which usually involves carpal and tarsal joints, and, in smaller percentage, conjunctivitis. Joint fluid is often a rich source of specific antibody, often at a higher titre than in the serum, and of the causative mycoplasmas themselves<sup>25</sup>. *M. agalactiae* can be isolated from milk when mastitis is present; the milk may become discolored and granular. Lambs born from affected females can show a wide range of symptoms, from null to severe keratoconjunctivitis and polyarthritis (Loria G.R., personal communication). New born animals have been also reported to be ataxic, and this may be related to septicaemia and/or the presence of the mycoplasma in the brain causing a non-purulent encephalomyelitis<sup>26</sup>.

### **Pathogenic mechanisms of *M. agalactiae***

*M. agalactiae* genome has been entirely sequenced in 2007, when it became easier to investigate into the hypothesis that an horizontal gene transfer phenomenon could had been be the basis for the exchange of genetic material among mycoplasmas<sup>27</sup>. *M. agalactiae* strain PG2 genome possesses a typical mollicute genome, with a small size (877,438 bp), a low GC content (29.7 moles %), a high gene compaction (88% of coding sequence), and UGA preferentially used as a tryptophan codon over UGG. It contains 751 coding DNA sequences, half of which have unknown functions or encode hypothetical proteins, 66 of which are lipoproteins<sup>27</sup>.

As previously reported, the immune response against *M. agalactiae* and its pathogenic mechanisms have never been investigated in the whole. Nevertheless some aspects have been evaluated thanks to the information available about other mycoplasmas. Different mechanisms are thought to be responsible for *M. agalactiae* pathogenic effects:

- phase variable related surface proteins (Vpma)

*M. agalactiae* possesses a family of lipoproteins named Vpma (Variable Protein of *Mycoplasma agalactiae*) encoded by the correspondent genes<sup>27,2</sup>. They are represented as orthologues in other *Mycoplasma* species and it was hypothesized that they are related in adhesion to host cells<sup>28</sup>, but the correlation between expression of variable surface proteins and adherence rates still seems controversial<sup>29</sup>. Nevertheless, the antigenic variation of *M. agalactiae* surface antigens could reasonably be one of the causes of the loss of efficacy of host immune response.

- P40: a protein involved in host adhesion

Nucleotide sequence-derived amino acid sequence comparisons revealed a similarity of P40 to the adhesin P50 of *Mycoplasma hominis* and to protein P89 of *Spiroplasma citri*, which is expected to be involved in adhesion<sup>30</sup>. A direct adhesion analysis and inhibition experiments of the purified recombinant protein P40 revealed that it is involved in adhesion of *M. agalactiae* to lamb synovial membrane cells, this protein is thus part of the pathogenic machinery mounted by *M. agalactiae*.

- P48: a probable macrophage stimulatory protein

As previously mentioned, one of the pathogenic mechanism adopted by Mycoplasmas is explicated through macrophages activation. P48, one of the surface antigens of *M. agalactiae* was demonstrated to be an homologous to a MALP product of *Mycoplasma fermentans*<sup>31</sup>, and thus is implied in the production of several proinflammatory cytokines and mediators.

- Biofilm formation, antimicrobial production and adaptation to specific environments.

McAuliffe *et al.* have demonstrated that *M. agalactiae* is able to produce a biofilm layer<sup>32</sup>. Biofilms consist in an extracellular polysaccharide matrix that exhibit different phenotypes and organizations. The presence of biofilm increases *Mycoplasma* resistance to heat, desiccation, antimicrobials and, probably, host defences.

### **Immune response against *Mycoplasma agalactiae*: state of art**

The immune response against *M. agalactiae* infection has been initially studied in terms of immunoglobulin production. The amount and the timing of antibody production was investigated<sup>33,34</sup>, but results vary depending on the test used and the initial amount of live strain used for the infection. According to experimental studies

in sheep with *M. agalactiae* carried out by Buonavoglia *et al*<sup>35</sup>, and confirmed in our experience<sup>36</sup>, the presence of antibodies is not detectable by serology until 25-30 days. In 2007 de la Fe and *et al*<sup>37</sup> analyzed the immunogenicity of two polyvalent vaccines against *M. agalactiae* and *Mycoplasma mycoides* subsp. *Mycoides* in terms of antibody production, and also in this case there was a slight increase of antibody production around day 25 after vaccination, a peak around the second month and a persistence of a humoral immunity till day 210 after vaccination. In 2009 and 2010 Castro Alonzo *et al*<sup>38,39</sup> correlated the clinical course of mycoplasma mastitis with its immune response inoculating mammary glands of lactating goats with a huge amount of live *M. agalactiae*. Authors demonstrate an early involvement of the innate immune response, and in particular of neutrophils and macrophages, but unable to control mycoplasmal invasion. Post-mortem immunohistochemical analyses were then performed to establish the cellular subsets contribution to *Mycoplasma* immunity. Their results confirmed expansion of the CD8 compartment at the expense of the CD4 subset leading to a reduced CD4/CD8 ratio 45 days post infection.

Our analyses deepened those performed by Castro Alonzo *et al* by the using of a irradiated antigen of *M. agalactiae* as stimulus, in order to detect only the cellular subsets involved in the defense against *Mycoplasma* infection.

### **Vaccines against *M. agalactiae***

Immunity to infection depends on a combination of innate mechanisms and antigen specific adaptive responses. The immune system regulates which specific responses predominate (humoral vs. cell-mediated) based on the body infected compartment (intracellular vs. extracellular) and on cytokine signals present at initial antigen contact (Th1 vs. Th2 responses). Disease-causing microbes have virulence

mechanisms that resist or evade innate and/or specific immune effector functions. Recovery from natural infection or artificial immunization promotes specific long-term immunity to re-infection (immunological memory). The protective immunity against intracellular pathogens mainly involves cellular immunity. A key role in immunological memory is played by T lymphocytes. It is well known that naïve T cells are those lymphocytes that have never encountered the antigen they are specific for. This cell line can be characterized by the presence of the cell surface antigen CD45RA. Upon stimulation by specific antigen, naïve T cells lose CD45RA antigen, acquire CD45R0 antigen and are finally recruited into the peripheral pool of memory T cells<sup>40,41</sup>. In the study of the immune response elicited against *M. agalactiae* antigen, the attention was focused both in the memory cell populations (CD45R0<sup>+</sup> – T lymphocytes), whose expansion was considered a consequence of the vaccination, and in the naïve cell populations (CD45RA<sup>+</sup> – T lymphocytes) that could be considered the reservoir of memory cells<sup>42</sup>.

The first efforts aimed to study the immune response elicited against *M. agalactiae* vaccines have been done many years ago, but were mainly addressed to test the best vaccine formulation in terms of inactivation and administration procedures<sup>43,44,45,46,47</sup>.

Different vaccines have been produced in Italy in the last decades, most of them are prepared starting from a monovalent inactivated broth cultures added with different kinds of adjuvants which have been gradually improved in immunogenic performance. Inactivation is normally performed by addition of small percentage of formalin, phenol or more recently saponine-purified products<sup>48</sup>. Farm vaccines made by Istituti Zooprofilattici Sperimentali are veterinary products prepared from wild strains directly isolated from the outbreak. These normally are utilized under

veterinary prescription to immunize the remaining healthy heads kept in the farm. Normally farm vaccines are formalin inactivated and with aluminium hydroxide as adjuvant. In Sicily “Mirri vaccine” or “milk inactivated vaccine” was the first vaccine ever utilised in Italy<sup>49</sup>, when selective *Mycoplasma media* and strain culture were not yet available. This vaccine, which still is requested by farmers, was made from diluted milk from clinical cases with addition of 4% formalin and aluminium hydroxide.

In the last decade immunogenic proteins of MA have been identified as suitable for vaccines against the disease<sup>48</sup>, but a practical development of a vaccine has been actually improbable. Many authors don't recommend single proteins as potential protective vaccine, also due to antigenic variation of the pathogen<sup>50,51</sup>. Nevertheless the real efficacy of currently circulating products (inactivated monovalent vaccines) is still doubtful in field conditions<sup>37</sup>. In Italy the current regulation obliges the farmer confining the whole herd until one month from the last clinical case of contagious agalactia. Unfortunately, with the exception of few drugs as tylosin and new fluoroquinolones that were demonstrated to have a good efficacy *in vitro* (Minimal Inhibitory Concentration<sup>52</sup>), antibiotics are poorly effective against *M. agalactiae*, and often their action is merely bacteriostatic, rather than bactericidal. A more economical alternative is vaccination, which could massively reduce the cost of treatment when used as preventive tool. Nowadays, farmers use vaccines also in course of outbreak, reporting sometimes a clinical recover also in diseased animals.

The efficacy of vaccine against CA have been reported for some inactivated products<sup>53,46,48</sup>, showing data on clinical efficacy (udder changes, milk changes, fever, general body conditions, arthritis, conjunctivitis, etc). No data have been reported on effectiveness of vaccines in terms of reduction of excretion of the

pathogen in the environment. Analogously no information has been collected on environmental impact following the use of the vaccine, especially when, in order to protect the remaining healthy milking sheep, farmers treat the whole group.

### **Aim of the project**

Little is known about the cell-mediated immune response against *Mycoplasma agalactiae*. Empirically it was noted that, under natural conditions, the animal infected by this pathogen becomes again susceptible after less than one year. Since in Europe the administration of live vaccine is not allowed, the attention was traditionally focused on killed microorganisms. The vaccine formulations including *Mycoplasma* inactivated by saponin or ethanol seem to be more effective than others<sup>54</sup>. Nevertheless, a live vaccine has been used in Turkey since the '30s, obtaining very good results.

The aim of this project has been the study of the host immune response to *M. agalactiae*, and the comparison of different vaccine formulations, commercially available, in order to define which of them is able to confer the best protection. The purpose of this work was thus to find a correlate of protection, both during a natural infection or after vaccination, and to provide information for veterinary management in course of infection about the more effective vaccine. The expansion of T-lymphocyte subsets has been investigated after *in vitro* stimulation - with irradiated *M. agalactiae* as a source of antigen - of sheep PBMCs, and intracellular IFN- $\gamma$  presence has been considered as a marker of the activation state.

### **Project design**

The project has been divided in two phases:

Phase 1- Immune response against *M. agalactiae*: the immune response against a wild strain of *M. agalactiae* has been evaluated after the experimental infection of four lactating sheep. The results were compared to those obtained from an uninfected control group.

Phase 2- Vaccine formulations evaluation: twenty-five lactating sheep were selected and divided in five group of five ewes each. Four groups were inoculated with four different vaccine formulations. The fifth group did not receive any vaccine. Two extra animals were intramammary infected with a wild strain of *M. agalactiae* and used as source of infection for the herd. Clinical symptoms, microbiological data, serological data and T-lymphocyte expansion data were analyzed in order to compare the efficacy of the different vaccines.

#### **- Immune response against *M. agalactiae***

In the first phase of the project, the percentage of antigen-specific lymphocytes positive for intracellular IFN- $\gamma$  during the infection of sheep with *Mycoplasma agalactiae* was analyzed, culturing peripheral blood mononuclear cells of infected or uninfected animals with an irradiated strain of *M. agalactiae* as *in vitro* stimulus<sup>36</sup>. Eight healthy ewes, 3–5 year old, belonging to the Comisana dairy breed originating from a flock with no history of CA, were selected for the experiment. Four ewes were inoculated at day 0 with 1 ml PBS containing  $1 \times 10^6$  CFU/ml of MA by intracanalicular route in both teats. The remaining four sheep were injected with PBS alone. During the following 2 months, all animals were monitored weekly for clinical changes, specific antibody production, cellular response and pathogen excretion in the environment. Blood samples from all the infected and healthy ewes were collected at days 0, 15, 30, 45, 60 after infection. After the experiment all the animals were clinically monitored until a full remission.



### **- Vaccine formulations evaluation**

Twenty-five animals were divided in five groups of 5 ewes each, while 2 extra sheep were chosen (but excluded from data analysis) as reservoir of *M. agalactiae* for “in contact” later challenge<sup>37,48</sup>. Four groups were inoculated with four different vaccines, following manufacturer instructions. The fifth group was not vaccinated and was considered as control. The two previously selected animals were used as source of infection by inoculation via intracanalicular route of live *M. agalactiae* wild strain. Once vaccination protocols were completed, these “challenge” sheep were infected and joint to the flock. All ewes were farmed and milked together as one flock, in order to get the same chance to be exposed to the pathogen, resembling natural infection. All the animals were monitored once a week for 26 weeks. Whole blood samples were collected at days 0, 7, 15, 30, 90 after infection. Milk and swabs samples were collected weekly from all ewes; all the data were then statistically analysed. Infected animals have been left in the flock until the 4th week of the study, than pharmacologically treated to induce drying off and complete recovery. Four different vaccines were utilized (Tab 1), all of them were initially compared, evaluating those that in our opinion are good indicators, together with immune parameters, of vaccine effectiveness: clinical symptoms and pathogen excretion. The attention was then focused on three of them, in order to investigate the immune response they were able to induce in the host. Pendik vaccine was not included in the immune data analysis, because of the restriction dictated by EU in the matter of live attenuated vaccine.

Group name	Vaccine	Number of animals	Producer	Inactivation procedure	Adjuvant
Pendik	Live attenuated	5	Pendik Institute Turkey	100 repeated passage in eggs	---
Saponin	Saponin vaccine	5	Pharmaceutical company 1	2 mg/ml saponin	---
IZSSi	Farm vaccine	5	IZS Sicilia, Italy	0.4% formalin	200µl/ml Quil A
Commercial	Commercial	5	Pharmaceutical company 2	0.2% formalin	Aluminium hydroxide

**Tab 1: Overview of the vaccine formulations used in this experiment**

## Results and conclusions

### **Paper 1: Expansion of intracellular IFN- $\gamma$ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep**

The expansion of antigen-specific IFN- $\gamma$  positive lymphocytes was evaluated. It was initially sustained by CD4<sup>+</sup> T cells, that play a role around 15 days after infection. In a later phase of infection, CD8/IFN- $\gamma$  double positive cells increase.  $\gamma\delta$  T-cells were not expanded at any analyzed time point. IFN $\gamma$ <sup>+</sup> T cells disappear 60 days after infection, suggesting that antigen specific IFN $\gamma$ <sup>+</sup> T cells, mainly detected in the early phase of the disease, could be useful to understand the role of cell-mediated immunity during *M. agalactiae* infection.

### **Paper 2: Pathogen excretion and clinical symptoms as indicators for the comparative analysis of vaccines efficacy during *Mycoplasma agalactiae* infection**

The data regarding the pathogen excretion in conjunctiva, nasal mucosa and milk were analyzed in order to determine the role of the vaccines in conferring protection

both to the vaccinated animal (direct protection) and to the herd (indirect protection)<sup>55</sup>. The differences of pathogen excretion through the milk resulted to be statistically significant among groups, and each group showed different ability to spread the pathogen in the environment. The sheep belonging to the Pendik and Saponin vaccinated groups eliminated significantly less bacteria in the environment in comparison with commercial product treated group thus reducing the incidence of infection in the herd. This phenomenon has a huge impact in the improving of economic management related to the milk products. Similar results were obtained considering the incidence of clinical symptoms: live-attenuated vaccine was able to confer to the entire group a good clinical protection, and none of them showed typical contagious agalactia symptoms. Saponin and IZSSi vaccines conferred a similar degree of protection, and only one animal per group showed clinical symptoms. Eighty per cent of animals belonging to commercial-vaccine group showed severe clinical symptoms.

### **Paper 3: Timing of activation of CD4 memory cells as a marker of protection for contagious agalactia in the sheep**

Data obtained from this preliminary trial demonstrated that two out of the three vaccines used in this study protected sheep from the disease<sup>56</sup>; memory CD4<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells increased in the groups protected by these vaccines ( $p < 0.05$  when compared to unprotected groups). On the contrary, memory CD8<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells increased in non-protected animals ( $p < 0.05$ ).  $\gamma\delta$ <sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells reached peaks of expansion in infected and in two vaccinated groups indicating that these cells aren't preferentially involved in protection or pathology ( $p < 0.05$ ). Memory CD4<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> lymphocytes could induce protection from contagious agalactiae also activating other effector immune mechanisms such as antibody response and/or

antibody dependent cell cytotoxicity. CD8<sup>+</sup>CD45RO<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> lymphocytes could induce pathogenic effects killing the protective lymphocyte subset not only by themselves, but also cross-talking with other cytotoxic cells such as Natural Killer and/or  $\gamma\delta$ <sup>+</sup> T cells. Taken together, our results suggest that antigen-specific Interferon- $\gamma$ <sup>+</sup> T cells provide subset correlates of protection and infection with *M. agalactiae*.

## **Overall conclusions**

From the data obtained during this project, the cell-mediated immune response against *M. agalactiae* and *M. agalactiae* vaccines have been elucidated as follows:

- The natural infection with a live *M. agalactiae* strain causes an early expansion of specific CD4<sup>+</sup>-IFN- $\gamma$ <sup>+</sup> T-cells, followed by a later expansion of CD8<sup>+</sup>-IFN- $\gamma$ <sup>+</sup> T-cells, 30 days post infection.
- The immune monitoring of vaccinated and infected sheep suggests that timing of activation of CD4<sup>+</sup>-IFN- $\gamma$ <sup>+</sup> memory cells is a key step for the successful protection against *M. agalactiae*. Hereby we propose that CD4<sup>+</sup>-IFN- $\gamma$ <sup>+</sup>-CD45RO<sup>+</sup> T-cells could be used to determine vaccine efficacy
- The pathogen excretion in the environment was used as parameter, together with clinical symptoms, to evaluate vaccine efficacy, and live attenuated vaccine, in spite of the limitation dictated by EU, was the most protective vaccine, while among inactivated vaccine, the one treated with saponin conferred the best protection after the experimental challenge.

This was the first work aimed to analyze the T cell response occurring in experimental contagious agalactia and to determine the role of antigen-specific naïve

or memory IFN- $\gamma$  positive T cell subsets activated by different vaccine formulations for the treatment of contagious agalactia.

## **Future perspectives**

Since this work can't exclude the involvement of other T-cell subsets producing other cytokines in the immunity against *M. agalactiae*, and in monitoring the efficiency of *M. agalactiae* vaccines, we would like to insight this aspect, analyzing the role of other cytokines and their cellular sources eventually involved in the protective or pathogenic process.

Preliminary reports show that antibodies directed against some proteins are, more than others, represented in protected sheep rather than infected (data not shown). We plan to delve into the role of these proteins or their epitopes, studying their direct effects as vaccines in the natural host of contagious agalactia. We also plan to delete the open reading frame responsible of the protein production from a live *Mycoplasma* strain and demonstrate the consequent variation of clinical symptoms.

We are going to develop an anti-Mycoplasma IgM ELISA for an early detection of the infection in the herd. Since there is a long time span during which IgG are not detectable, the early identification of the pathogen in the herd would protect the other animals from the infection.

Finally, to confirm which cells are responsible for protection, it would be possible to use a immunocompromized mouse that accept sheep bone marrow and develops sheep immune system. This mouse could be infected with *M. agalactiae* and its immune response could be studied without the limits dictated by the lacking of commercial reagents directed to sheep antigens.



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## Expansion of intracellular IFN- $\gamma$ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep

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## ABSTRACT

A method to assess the expansion of antigen-specific intracellular IFN- $\gamma$  positive T cell subsets during the infection will be helpful for a better understanding of mycoplasmal infections physiopathology in the sheep. We analysed the percentage of antigen-specific lymphocytes positive for intracellular IFN- $\gamma$  during the infection of sheep with *Mycoplasma agalactiae* by culturing peripheral blood mononuclear cells of infected or uninfected animals with irradiated *M. agalactiae*. The expansion of antigen-specific IFN- $\gamma$  positive lymphocytes in infected sheep was initially sustained by CD4<sup>+</sup> T cells at day 15 after infection, when antigen specific IgG start to be detectable, followed by CD8/IFN- $\gamma$  double positive cells.  $\gamma\delta$  T-cells were not expanded at any time point analysed. IFN- $\gamma$ <sup>+</sup> T cells disappear 60 days after infection, suggesting that antigen specific IFN- $\gamma$ <sup>+</sup> T cells, mainly detected in the early phase of the disease, could be useful to understand the role of cell-mediated immunity during *M. agalactiae* infection.

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*Mycoplasma agalactiae* (MA) is a major pathogen of sheep in many areas in the world but particularly in Mediterranean countries. It causes contagious agalactia (CA), an infectious disease primarily affecting the mammary gland (Da Massa et al., 1992; Bergonier and Poumarat, 1996). The largely chronic nature of CA is usually explained by the capacity of mycoplasma to evade the host immune system by changing surface structure and expressing lipoproteins that induce cytokines modulating the immune system (Razin et al., 1998). Changes in the inflammatory cell subsets due by MA infection have previously been reported during the mammary infection (Castro-Alonso et al., 2010). Based on the recent knowledge about CA, we have proposed that the detection of intracytoplasmic inflammatory cytokines may be useful in shedding light on the functions of peripheral blood lymphocytes as markers of the infection phases. In this report, we describe a method to analyse the expansion of antigen-specific IFN- $\gamma$ <sup>+</sup> T lymphocytes during the infection with MA.

Eight healthy ewes, 3–5 year old, belonging to the Comisana dairy breed originating from a flock with no history of CA, were selected for our experiment. Four ewes were inoculated after daily

milking at day 0 with 1 ml PBS containing  $1 \times 10^6$  CFU/ml of MA (cloned strain from a recent, confirmed outbreak of MA) by intracanalicular route in both teats. This dose produce signs of CA in our previous experiments. The procedure was performed using a syringe linked to small size catheter (1.67 mm size French sterile urinary catheter) in order to reach the mammary gland sinus. The remaining four sheep were injected with PBS alone. During the following 2 months, all animals were monitored weekly for clinical changes, specific antibodies production, cellular response and pathogen excretion in the environment. Blood samples from all the infected and healthy ewes were collected at days 0–15–30–45–60 after infection and sera and PBMC were analysed. After the experiment all the animals were clinically monitored until a full remission.

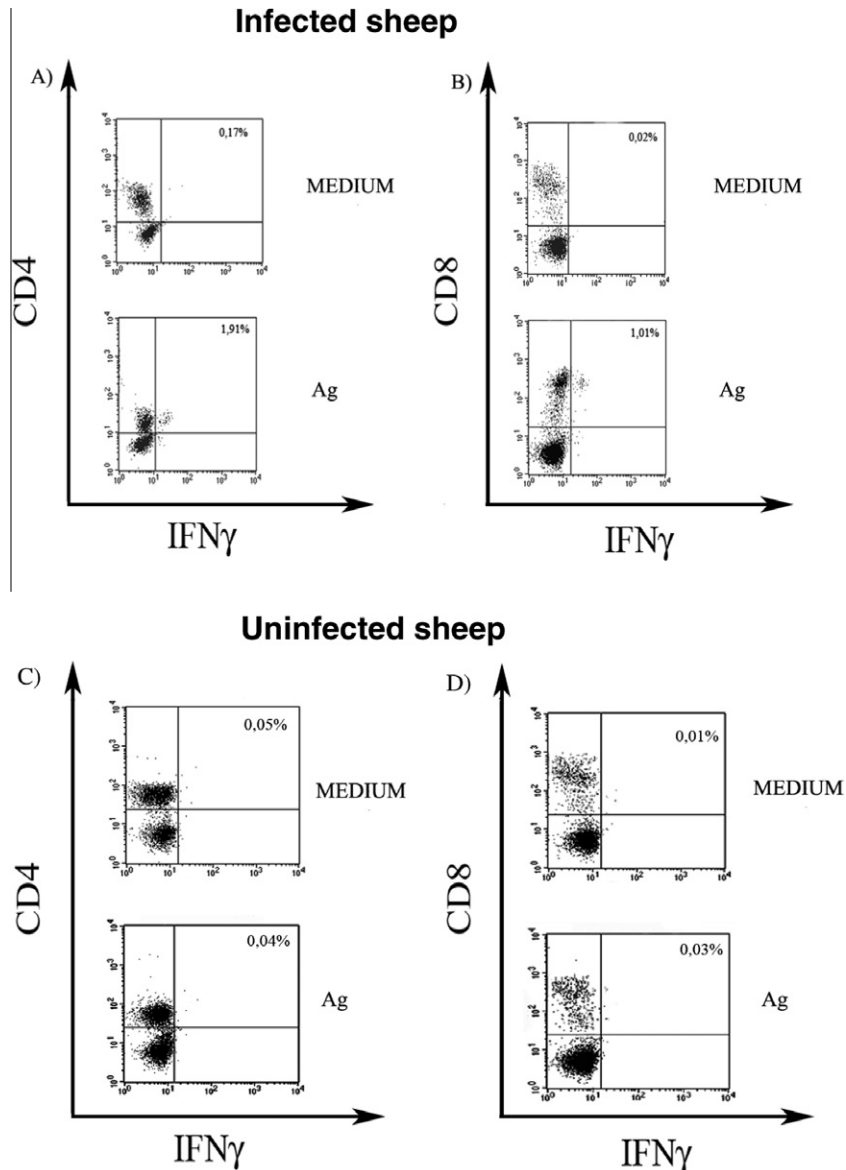
The rate of expansion of CD4<sup>+</sup>, CD8<sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> T-lymphocytes positive for intracellular IFN- $\gamma$  after *in vitro* exposure to irradiated MA was analysed on peripheral blood mononuclear cells (PBMC) from infected and healthy ewes. PBMC were separated by centrifugation of sheep blood on Lympholyte<sup>®</sup> solution (Cedarlane labs, Canada) and quantified by Trypan blue dye exclusion test. Cells were resuspended ( $1 \times 10^6$ /ml) in RPMI 1640 medium plus FCS 10%, glutamine 1%, streptomycin, penicillin, gentamycin and Hepes (EuroClone, Italy).

Ionomycin (10 ng/ml) and Phorbol Myristate Acetate (PMA) (5 ng/ml) (Sigma–Aldrich, Italy), as positive control for intracellular IFN- $\gamma$  staining, medium alone, as negative control, and

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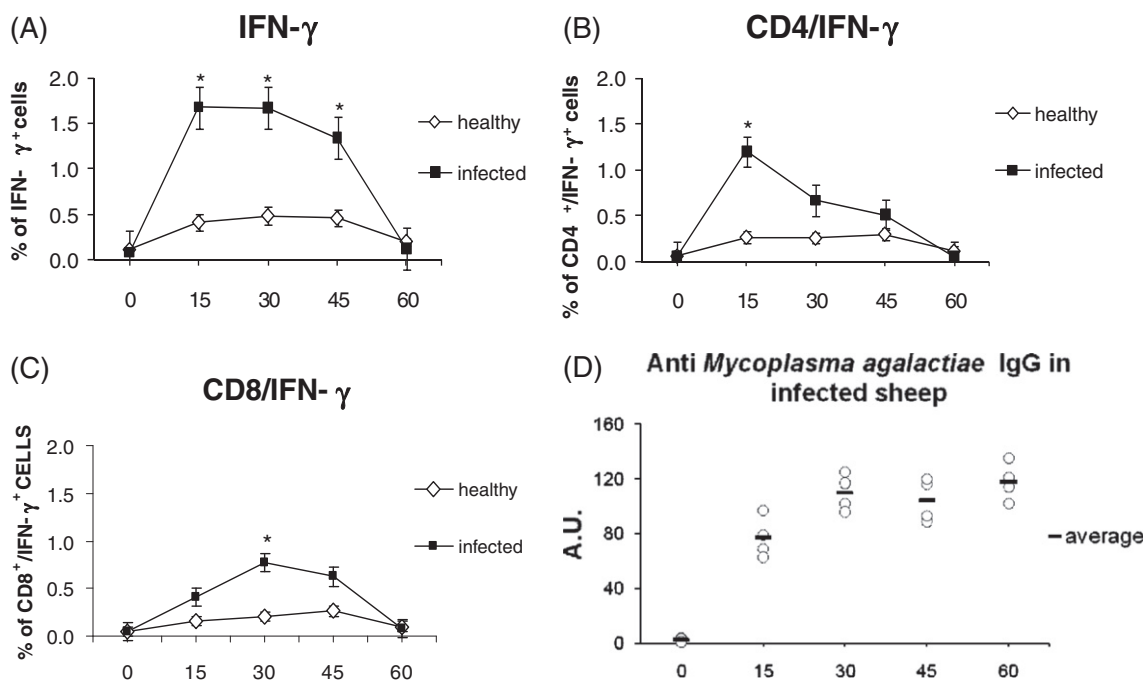
**Fig. 1.** Panels showed in the figure were obtained acquiring to the FACS-CAN (Becton Dickinson) 10000 events after gating lymphocytes. In infected sheep, panel of CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> were obtained 15 days after infection, CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> 30 days after the infection.

$1 \times 10^6$  CFU/ml of irradiated (60 Gray) MA, as *stimulus* for antigen-specific cells, were added to cell cultures. After an overnight *in vitro* antigen exposure, 3  $\mu$ M Monensin (Sigma–Aldrich, Italy) was added to each well to avoid IFN- $\gamma$  release. PBMC were collected and pooled after 48 h of culture at 37 °C 5% CO<sub>2</sub> and flow-cytometric analyses were assessed. These assays were performed staining the cells with anti surface-FITC-labelled mAbs and anti-IFN- $\gamma$  PE-labelled mAb at the concentration suggested by the suppliers in 100  $\mu$ l PBS/0.1% sodium azide. Surface immunofluorescence was detected by anti-CD8 (FITC-labelled, clone CC63, mouse IgG2a, Serotec, UK), anti- $\gamma\delta$  (FITC-labelled WC1, clone 19.19, mouse IgG1, Serotec, UK) and anti-CD4 (FITC-labelled, clone 44.38, mouse IgG2a, Serotec, UK). After three washes, cells were fixed by 1% formalin buffer and permeabilized by 0.01% saponin/PBS for 10 min, washed three times and incubated with anti-IFN- $\gamma$  mAb PE-labelled (clone CC302, mouse IgG1, Serotec, U.K.) for 15 min at 4 °C. After three washes, cells were collected using a FACS-CAN cytometer (Becton Dickinson, USA) and analysed by CELL-QUEST PRO program (Becton Dickinson, USA). We tested the presence of

anti MA-IgG in sera of infected sheep by an indirect ELISA test (Institut Pourquier, Montpellier, France) in each infected sheep. The corrected OD 450 nm value of the sample divided by mean corrected OD 450 nm value of the positive control  $\times$  100 (S/P%), samples with a S/P% equal to or lower than 50% are considered to be negative, samples with a S/P% between 50% and 60% are considered to be doubtful, samples with a S/P% equal to or greater than 60% are considered to be from a sheep which has been in contact with *M. agalactiae* (Poumarat et al., 2009).

The Mann–Whitney *U* test was used to compare statistical significance of data from infected and healthy sheep, and the value of  $P < 0.05$  was chosen to be significant comparing the data obtained from infected and control group.

All MA inoculated sheep started to show swelling, rise in temperature and pain of both the udders followed by a severe decrease in milk production in 4–5 days after infection and reduction in size of the organs. In one case left carpal joint was affected by arthritis: joint were swollen, warm and painful. No ocular lesions were recorded in this trial.



**Fig. 2.** PBMC from healthy (◇) and infected (■) ewes were obtained at the time points showed in the figure. The percentages of double positive cells were obtained analysing 10000 events on lymphocytes gate (Mann-Whitney  $U$  test \*  $< 0.05$ ). IgG from sera of infected ewes (○) were obtained at the points showed in the figure and analysed by commercial indirect ELISA assay anti-*M. agalactiae* IgG. We calculated for each infected sheep the corrected OD ratio and we show the mean for each time points.

Analysis of PBMC cultured with irradiated MA antigen showed the expansion of antigen-specific IFN- $\gamma$ + T cell subsets at day 15, 30 and 45 after MA infection. Fig. 1 shows representative panels of CD4 and CD8 positive cells from infected sheep *in vitro* re-exposed to MA antigen and stained with intracellular anti-IFN- $\gamma$ .

As shown in Fig. 1A, CD4+ IFN- $\gamma$ + cells increase, in infected sheep, from 0.17% to 1.91% at day 15 post infection while CD8+ IFN- $\gamma$ + increase from 0.02% to 1.01% at day 30 post infection (Fig. 1B). No expansion of double positive cells was detected in uninfected animals (Fig. 1C and D).

Flow-cytometric analysis of PBMC from infected sheep showed an expansion of IFN- $\gamma$ + cells reaching a plateau from day 15 to day 30, compared to the non-infected ones, followed by a relevant decrease at day 60 (Fig. 2A). At day 15 a peak of antigen-specific CD4+ IFN- $\gamma$ + T lymphocytes expansion was detected (Fig. 2B) while CD8+ IFN- $\gamma$ + T cells had a two fold increase 30 days after infection (Fig. 2C). From day 30, TCR $\gamma\delta$ + IFN- $\gamma$ + T lymphocytes showed no significant expansion (data not shown).

CD4+ cells seem to be responsible for an early cell-mediated immune response, since the maximum value of CD4+ IFN- $\gamma$  producing cells was reached at day 15 of infection, together with, or maybe few days before, the appearance of specific IgG. In fact, indirect ELISA assays of infected sheep sera, showed the presence of specific IgG after 15 days from the infection, with a plateau reached 30 days after the infection (Fig. 2D).

The peak of CD4, overlapping with the increase of serum IgG, has been described and related to an activation of the cellular and humoral immunity (Rodríguez et al., 2000, 2001; Byrne et al., 2005).

The role of cell-mediated immunity in mycoplasmal infections has not been well established (Tajima et al., 1984; Razin et al., 1998; Rodríguez et al., 2000; Sarradell et al., 2003; Byrne et al., 2005). Differently from previous papers (Razin et al., 1998; Rodríguez et al., 2000, 2001; Byrne et al., 2005), we describe an expansion of MA antigen-specific CD4+ IFN- $\gamma$ + T cells 15 days after infection and an antigen-specific CD8+ IFN- $\gamma$ + T cells increase at day 30, when CD4+ IFN- $\gamma$ + cells decrease. Accordingly to our data,

Castro-Alonso et al. (2010) reported that, lately, at 45 days post infection in the breast of infected goats, the inflammatory process was mainly supported by CD8+ cells. The different timing of CD8+ IFN- $\gamma$ + T cells increase, reported by us in the blood of the sheep at day 30 post infection and by Castro-Alonso in the mammary gland of infected sheep at day 45, could be explained because, differently from us, Castro-Alonso did not detect MA-specific CD8+ IFN- $\gamma$ + T cells. Probably, in the blood the increase of CD8+ IFN- $\gamma$ + T cells occurs before than in mammary glands. Our findings support the hypothesis that, after an early activation (2 weeks after infection) of CD4/IFN- $\gamma$  double positive cells and a later activation (4 weeks after infection) of CD8/IFN- $\gamma$  double positive cells, the anti-MA immune response is mainly sustained by the immunoglobulin secretion. As the total percentages of IFN- $\gamma$ + cells almost coincided with the CD4+ IFN- $\gamma$ + 15 days after infection or with CD8+ IFN- $\gamma$ + 30 days after infection, NK cells seem to be a minor component of MA-specific IFN- $\gamma$ + cells.

We cannot exclude the possibility that IL-4 or other intracytoplasmic cytokines positivity could be involved in T cell activation during CA.

The contribute of antigen-specific MA IFN- $\gamma$ + T cells to protective immunity to CA could be a direct involvement in killing of infected cells and/or MA itself, and/or these cells could play a supporting role IFN- $\gamma$  mediated in the activation of B lymphocytes and subsequent secretion of MA specific antibodies (Snapper et al., 1988; Mohr et al., 2010). Further analyses of the immunological features characterizing sheep infection, are needed to deeply understand the role of IFN- $\gamma$ + cells expansion in the protective immune response to MA.

The assessment of our antigen-specific intracellular IFN- $\gamma$ + T cells analysis could represent a new approach in monitoring immune response of sheep infected with MA.

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Title: Pathogen excretion and clinical symptoms as indicators for the comparative analysis of vaccines efficacy during *Mycoplasma agalactiae* infection

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Abstract: In the scenario of the available vaccines against Contagious Agalactia, little is known about the protection they confer and their efficacy. This paper approaches the comparison of four different vaccine formulations, in terms of pathogen spreading control and containment of clinical signs. In particular, a vaccine that reduces the load of *Mycoplasma agalactiae* in milk, nasal and ocular secretions could be very important to minimize the spreading of this pathogen in the herd. Here we show that some vaccines used protect with different efficacy the sheep both in terms of clinical manifestations and in terms of pathogen excretion. In spite of the limited number of animals, results show how the live attenuated vaccine, not allowed in European Union, conferred the best clinical protection, when compared to a control group, followed by saponin-treated vaccine.



1 **Pathogen excretion and clinical symptoms as indicators for the comparative analysis of**  
2 **vaccines efficacy during *Mycoplasma agalactiae* infection.**

3

4

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21

22 **Abstract**

23 In the scenario of the available vaccines against Contagious Agalactia, little is known about the  
24 protection they confer and their efficacy. This paper approaches the comparison of four different  
25 vaccine formulations, in terms of pathogen spreading control and containment of clinical signs. In  
26 particular, a vaccine that reduces the load of *Mycoplasma agalactiae* in milk, nasal and ocular  
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28 show that some vaccines used protect with different efficacy the sheep both in terms of clinical  
29 manifestations and in terms of pathogen excretion. In spite of the limited number of animals, results  
30 show how the live attenuated vaccine, not allowed in European Union, conferred the best clinical  
31 protection, when compared to a control group, followed by saponin-treated vaccine.

32 *Keywords:* Vaccines comparison, Contagious Agalactia, *Mycoplasma agalactiae*

33

## 34 **Introduction**

35 Contagious agalactia (CA) is a major veterinary problem of many countries involved in small  
36 ruminants farming. This disease affects small ruminants livestock causing a severe mastitis, arthritis  
37 and keratoconjunctivitis. *Mycoplasma agalactiae* is the most common causative agent, a wall-less  
38 bacterium belonging to Mollicutes, characterized by high virulence and contagiousness.

39

40 Different vaccines have been produced in Italy (Tola et al., 1999) they are made by Istituti  
41 Zooprofilattici Sperimentali and are veterinary products prepared from wild strains directly isolated  
42 from the outbreak. These are usually utilized under veterinary prescription to immunize the  
43 remaining healthy heads kept in the farm.

44

45 In the last decade immunogenic proteins of *M. agalactiae* have been identified as suitable for  
46 vaccines against the disease (Tola et al., 1999) but their practical development to become a vaccine  
47 has been actually improbable. Many authors don't recommend single proteins as potential  
48 protective vaccine, also due to antigenic variation of the pathogen (Bergonier et al., 1996; de la Fe  
49 et al., 2006). Nevertheless the real efficacy of currently circulating products (inactivated  
50 monovalent vaccines) is still doubtful in field conditions (de la Fe et al., 2007).

51

52 Nowadays, control of CA is mainly based on the application of veterinary regulations. In Europe  
53 some different inactivated vaccines are available, but live attenuated vaccines are not acceptable, in  
54 spite of positive clinical feedback from countries where it is currently utilised (Turkey) (OIE  
55 manual, 2008). Unfortunately, with the exception of few drugs as tylosin and new fluoroquinolones  
56 that were demonstrated to have a good *in vitro* Minimal Inhibitory Concentration (Loria et al.,

57 2003), antibiotics are poorly effective against *M. agalactiae*. Vaccination could massively reduce  
58 the cost of treatment when used as preventive tool.

59

60 The efficacy of vaccine against CA have been reported for some inactivated product (Tola et al.,  
61 1999; Buonavoglia et al., 2008; Consenti et al., 1999), showing data on clinical efficacy (udder  
62 changes, milk changes, fever, general body conditions, arthritis, conjunctivitis, etc.) among different  
63 products. No data have been reported on effectiveness of vaccines in terms of reduction of excretion  
64 of the pathogen in the environment. Analogously no information has been collected on  
65 environmental impact following the use of the vaccine, especially when, in order to protect the  
66 remaining healthy milking sheep, farmers treat the whole group.

67

68 The aim of the study is to investigate current vaccines available against the disease, including  
69 either inactivated vaccine or attenuated products. Inactivated vaccines allowed in EU, concern  
70 commercial products and farm vaccines. They are based on similar procedures of antigen  
71 production and inactivation, but the commercial ones can utilize only an officially registered strain,  
72 whereas farm vaccine are prepared with the wild strain isolated in the outbreak.

73

74 This is the first report aimed to determine the efficacy of different vaccine formulations in the  
75 treatment of CA. The purpose of this work is to provide evidence of the effectiveness of vaccines  
76 and the protection that they are able to confer, giving information for veterinary management in  
77 course of infection. A comparison of different vaccines formulations was thus performed, evaluating  
78 those that in our opinion are good indicators, together with immune parameters that we're  
79 analysing, of vaccine effectiveness: clinical symptoms and pathogen excretion.

80

## 81 **Materials and methods**

### 82 *Experimental design*

83 Twenty-seven adult lactating sheep were selected from a dairy herd microbiologically and  
84 serologically negative for *M. agalactiae*, other mycoplasmas or other mastitis etiological agents. All  
85 ewes belonged to the same breed (Comisana) and were aged between 2 and 4 years. All animals  
86 were subjected to synchronisation of their estrum. After parturition, a proper weakening interval of  
87 time was additionally considered in order to feed new-born lambs with colostrum. After this period,  
88 27 sheep were ready and synchronized for milking season. Twenty-five of them were divided in five  
89 groups of 5 ewes each, while 2 were chosen (but excluded from data analysis) as inoculated “in  
90 contact” animals for later challenge (Tola et al., 1999; de la Fe et al., 2007). Four groups were  
91 inoculated with a first dose of vaccine, following manufacturer instructions. The fifth group was not  
92 vaccinated and was considered as control. Four weeks after the first vaccination, all the groups  
93 treated with inactivated products were boosted with an identical dose of vaccine whereas attenuated  
94 vaccine was administered once only. The two remaining animals were used as source of infection  
95 by inoculation via intracanalicular route of live *Mycoplasma agalactiae* wild strain. These  
96 “challenge” sheep were infected and joint to the flock few days post booster vaccination. Once  
97 vaccination protocols were completed, all ewes were thus farmed and milked together as one flock,  
98 in order to get the same chance to be exposed to the pathogen, resembling natural infection. All the  
99 animals were monitored once a week for 26 weeks. Milk, blood and swabs samples were collected  
100 weekly from all ewes; all the data were then statistically analysed. Infected animals were than  
101 pharmacologically treated to induce drying off and complete recovery.

102

### 103 *Vaccines*

104 Performances of four different vaccines (Tab. 1) have been studied. The farm vaccine used was  
105 formalin inactivated with Quil A as adjuvant (hereafter “farm” vaccine). Another vaccine,  
106 commercially available, is formol-treated and contains Aluminium hydroxide as adjuvant. It was  
107 bought from a local veterinary pharmacy (hereafter “commercial” vaccine). Alive vaccine was  
108 kindly supplied by a Turkish Institute (hereafter “live” vaccine) and a novel saponin-inactivated  
109 vaccine, in which saponin acts as inactivant and adjuvant in the same time (hereafter “saponin”  
110 vaccine) was also provided by the producing pharmaceutical company and used.

111

## 112 *Challenge*

113 The field strain used for inoculum preparation was isolated from a Sicilian outbreak of CA, and  
114 then confirmed by biochemical and PCR analysis. After isolation the strain was cultured in  
115 modified Hayflick broth (500 ml) (Nicholas et al., 2009), after 72 hours incubation at 37°C, the  
116 culture was harvested at 10.000g for 30 min and re-suspended in the same volume of phosphate  
117 buffered saline (PBS) solution, pH 7.2. *M. agalactiae* antigen suspension was further stored in  
118 1.5ml criovials and frozen at -80°C, when necessary. Viable *M. Agalactiae* antigen was quantified  
119 before inoculation by the method described by Postgate (Postgate, 1969).

120

121 A 1.5 ml-inoculum containing  $10^5$  CFU of *M. agalactiae* was injected intracisternally into both  
122 halves of the mammary glands of 2 sheep belonging the group of non-vaccinated ewes. Animals  
123 were kept together and milked daily two times per day (7.00 AM and 4.00 PM) before and after  
124 grazing, as traditionally performed in Sicily. After milking the infected ewes, the operator would dip  
125 his hands in the infected milk and milk the vaccinated and control sheep, in order to resemble  
126 natural infection (Tola et al., 1999; de la Fe et al., 2007).

127

128 *Ethics*

129 The experiment was carried out in accordance with the Code of Practice for Housing and Care of  
130 Animal used in Scientific Procedures (EU Directive 2010/63/EU). The work was reliable using the  
131 animal facility of Istituto Zootecnico Sperimentale per la Sicilia (Palermo), where lactating sheep  
132 were managed according to traditional production management. The experiments were carried out  
133 with the authorization of the Italian Ministry of health (Decreto Ministeriale N° 101/2006 –A).

134

135 *Clinical assessment*

136 All sheep were clinically monitored in order to determine pathologic changes in the udder.  
137 Udders and milk were evaluated once a week, during milking procedure with a simple score (Yes-  
138 No) enrolling those symptomatic as mastitic sheep. Clinical data (presence of mastitis, arthritis,  
139 conjunctivitis) were recorded till the complete recovery of the sheep that was correspondent to week  
140 26 after challenge.

141

142 *Mycoplasma cultures and PCR*

143 Milk samples (300µl) or nasal and ocular swabs, were cultured both in solid agar plates and  
144 liquid medium. Purification of the organism by further cloning sub-cultures was always essential  
145 before identification (Nicholas and Baker, 1998). The samples were additionally cultivated on 5%  
146 sheep blood agar (Merck, Darmstadt, Germany) at 37°C under both aerobic and anaerobic  
147 conditions, as well as on Tryptic Soy Agar (DIFCO Laboratories, Detroit, Michigan, USA) in order  
148 to monitor eventual other bacterial infections. Isolates were then analysed using standard  
149 biochemical tests (Poveda et al., 1998) and molecular biology method (16S rRNA gene PCR) as  
150 described previously (McAuliffe et al., 2003; McAuliffe et al., 2005).

151

152 The surface drop method of Postgate (Postgate, 1969) for the determination of viable cells in  
153 milk samples was adopted. The 95% confidence limit of the number of the colonies counted ( $x$ )  
154 was calculated as approximately  $x \pm 1.96\sqrt{x}$  (Meynell and Meynell, 1970).

155

#### 156 *Statistical analysis*

157 The data of *M. Agalactiae* excretion in milk, ocular and nasal swabs were analysed considering  
158 the mean of CFU  $\pm$  SD obtained by the determination of viable cells from the collected samples.  
159 The data were analysed with a statistical model of analysis of variance ANOVA. A risk analysis was  
160 also calculated separately analysing the probability for the animal to suffer of clinical symptoms,  
161 and the spreading of the pathogen in the environment (ocular and nasal swabs). These values were  
162 obtained using the Relative Risk and their I.C. 95%. Antibodies optical density were analysed with  
163 a statistical model of analysis of variance ANOVA for repeated measures. The confidence value was  
164 fixed at 0.05. The analyses were performed using Statistica 5.0 (StatSoft, Inc., 2010) and Openepi  
165 (Dean et al., 2011) software.

166

## 167 **Results**

### 168 *Live and saponin vaccine conferred the best protection against clinical symptoms*

169 Clinical data were recorded weekly, till the week 26 after challenge, period when complete clinical  
170 recovery was observed in all vaccinated ewes still enrolled at the end of the study. The two  
171 intramammary inoculated animals, utilised for the challenge, showed mastitis few days post  
172 infection, but they were excluded from data collection. In the Fig.1 is shown the mean percentage of  
173 symptomatic animals during the whole observation period. Control group showed a typical  
174 behaviour of natural infection, characterised by high morbidity and sub-acute course of the disease



175 (Stazzi and Mirri, 1986). The groups of “in contact” vaccinated sheep showed clinical signs of CA  
176 after the second week post challenge with highest incidence of mastitis 4 wpi (weeks post  
177 infection). At this time point a total of 10 sheep showed CA symptoms and 9 sheep were severely  
178 affected: 4 belonging to control group, 4 to the commercial vaccine group and 1 to the farm vaccine  
179 group (Tab 2). In the first stage the disease was visible as mild unilateral or bilateral mastitis with  
180 warm, swollen, painful parenchyma, later followed by sclerosis and typical reduction in size of one  
181 or both the organs. Mammary lymph-node was always involved showing from mild to marked  
182 enlargement in size. The presence in the udder of one of the above-mentioned changes was  
183 considered as “mastitis”. Two ewes only (8% of the total number) showed arthritis (1 ewe  
184 belonging to saponin inactivated vaccine group) and monolateral conjunctivitis (1 sheep belonging  
185 to control group). Arthritis involved all limbs, particularly visible in carpal and tarsal joints.

186

187 Vaccinated groups were then analysed in terms of relative risk to be affected by symptoms of  
188 CA, giving the value of control group equal to 1, which represents the certainty to suffer of mastitis,  
189 arthritis and/or conjunctivitis. The relative risk to be affected by mastitis, conjunctivitis and/or  
190 arthritis was thus calculated, considering more effective a vaccine able to protect by clinical  
191 symptoms. From this point of view the range of efficacy of vaccines resulted to be:

192  $LIVE (0,12) > SAPONIN (0,24) > FARM (0,4) > COMMERCIAL (0,96)$

193 The limited number of units per group (5) is not statistically significant; nevertheless the  
194 statistical analysis gave an interesting model about the different behaviour of the groups treated  
195 with the different vaccine formulations. The value of 0.12 obtained by the analysis of Live vaccine  
196 group means a protection of about 88% to develop clinical signs of CA. Among inactivated  
197 vaccines the best results in terms of clinical protection was recorded in saponin-vaccinated group,  
198 followed by farm vaccine. On the contrary, a value of protection near to zero was unexpectedly

199 found in the commercial vaccine. For humanitarian reasons, between the 12<sup>nd</sup> and the 13<sup>th</sup> wpi,  
200 three sheep from control group and other 4 from the commercial vaccine group, were  
201 pharmacologically treated and excluded from the experiment, because of severity of symptoms. At  
202 26 wpi (experiment conclusion), complete clinical recovery was observed in all the ewes.

203

#### 204 *Microorganism excretion as a parameter of vaccine efficiency*

205 The behaviour of the different vaccines in terms of reduction of pathogen excretion in milk was  
206 also monitored: all the groups secreted the bacteria through the milk since the first wpi, but each  
207 group showed a different behaviour regarding the excretion of the pathogen in the milk. The highest  
208 number of *Mycoplasma*-secreting sheep was recorded in the first week, when the most of animals  
209 were asymptomatic. However, the peak of the microorganism excretion in terms of CFU/ml values  
210 was reached around the 4<sup>th</sup> wpi. The peak of *Mycoplasma* excretion in each group showed different  
211 trends: at week 2 in the live vaccine-treated group, at week 3 in the control group, at week 4 for  
212 saponin vaccine and at week 5 for the commercial vaccine and farm groups. In spite of the  
213 encouraging clinical trend, residual excretion (one head per group) was still recorded in Control and  
214 farm groups at the end of the study (26<sup>th</sup> week). Live and saponin inactivated treated groups showed  
215 complete clearance of the pathogen after 22 and 19 weeks respectively. The pathogen excretion in  
216 the milk, measured by CFU, was also analysed with the ANOVA test in order to know if the  
217 differences among groups were statistically significant. It was rejected the null hypothesis since  $p$   
218 was calculated to be 0.023, assuming a significant value equal to 0.05 (data not shown). The  
219 variation of pathogen excretion with respect to the vaccine used, and the relative standard  
220 deviations, during the whole period of observation, is shown in Fig. 2. Commercial vaccine cause  
221 an increase of 94.57% of excretion of the pathogen through the milk, while farm vaccine allows a  
222 contraction of the pathogen spreading of 29.75%, together with saponin and live vaccines, which  
223 allow a reduction of *M. agalactiae* excretion of 75.02% and 74.65%, respectively. It can be noted

224 how the vaccines able to protect from clinical symptoms are also able to limit the spreading of  
225 bacteria in the environment.

226

227 By the comparison at week four (period considered as “at maximum risk” of exposure) of all  
228 vaccinated sheep with control animals, the Fisher exact test applied on milk, ocular and nasal swab  
229 cultures, demonstrated that the risk ratio between non vaccinated and vaccinated animals resulted in  
230 most cases  $<1$ . The values are showed in Table 3. These data underline the importance of the  
231 vaccines in conferring protection not only to the single animal, but also to the whole herd. Fig. 3  
232 summarizes clinical performances and excretion data in milk for all groups. The reported trends  
233 underline, at selected weeks throughout the experiment, the difference between clinical behaviour  
234 and antigen elimination in the flock. In control as well as in animals treated with Commercial  
235 vaccine there are higher incidence of clinical symptoms at 4 and 8 wpi, when compared to the  
236 incidence values detected in groups treated with farm, saponin and live vaccines. Saponin and live  
237 vaccines are able to decrease from the 4<sup>th</sup> wpi on the amount of *M. agalactiae* secreting sheep. No  
238 reduction of *M. agalactiae* secreting sheep was detected at 4 wpi in farm vaccine treated animals  
239 while after 8 wpi a drastic reduction of this value was observed in this group.

240

## 241 **Discussion**

242 The present study, for the first time, compared some vaccines currently available against CA  
243 suggesting more correct veterinary choice in case of outbreak. The prolonged monitoring of antigen  
244 excretion in the environment in control and vaccinated animals is one of the tools to determine the  
245 risk of spreading infection and, as a consequence, the effectiveness of a vaccine. The data regarding  
246 the pathogen excretion in conjunctiva, nasal mucosa and milk were thus analysed in order to  
247 determine the role of the vaccines in conferring protection to the vaccinated animal. The differences

248 of pathogen excretion through the milk resulted to be statistically significant among groups, and  
249 each group showed different ability to spread the pathogen in the environment.

250

251 Vaccinated animals showed a significant reduction of the pathogen elimination in the  
252 environment, when compared to the non-vaccinated, strengthening the importance of vaccines in  
253 controlling the disease either in the farm or in the area. The results showed a significant decrease in  
254 relative risk of elimination of the bacteria through the milk and nasal mucosa. The variation of the  
255 excretion of the pathogen by ocular fluids between the two groups was not significant, but in our  
256 experience the role of the transmission of the disease by this route should be considered less  
257 relevant.

258

259 The sheep belonging to the live and saponin vaccinated groups eliminated significantly less  
260 bacteria in the environment in comparison with commercial product treated group, thus reducing the  
261 incidence of infection in the herd. This phenomenon has a huge impact in the improving of  
262 economic management related to the milk products.

263

264 Similar results were obtained considering the incidence of clinical symptoms: live-attenuated  
265 vaccine was able to confer to the entire group a good clinical protection, and none of them showed  
266 typical CA symptoms. Saponin and farm vaccines conferred a similar degree of protection, and only  
267 one animal per group showed clinical symptoms. Eighty per cent of animals belonging to  
268 commercial-vaccine group showed severe clinical symptoms.

269

270 The whole data analysis suggests that the live attenuated vaccine was the most effective one.  
271 Among the inactivated vaccines, the saponin inactivated formulation resulted to be the most  
272 effective, followed by the farm vaccine that had a weaker protective effect in terms of pathogen  
273 excretion in the milk as well as of number of *M. agalactiae* secreting sheep. Basing on the data  
274 collected by this study, a more relevant role should be ascribed to vaccination against CA utilizing  
275 the vaccines that resulted protective by our analysis (live, saponin and farm). These vaccinated  
276 groups show better health conditions, milk production and protection against the clinical symptoms  
277 when compared with control and commercial vaccine groups. Among inactivated vaccine  
278 formulations, hereby tested, the vaccine inactivated with saponin resulted to be the most effective,  
279 but live attenuated vaccine conferred an excellent protection in all the animals tested.

280

281 It can be hypothesized that the different protective effects of vaccine formulations depends on  
282 their intrinsic features. Commercial product is less protective than other vaccines probably because  
283 of the strain used. The strain, in fact, has to be registered and it is possible a gradual loss of  
284 immunogenic activity due to the antigenic variability of *M. agalactiae* strains. In other words the *M.*  
285 *agalactiae* strain used to prepare commercial vaccine could display many antigenic differences with  
286 *M. agalactiae* strain used to infect the sheep in this work. As above mentioned, live vaccine is an  
287 attenuated strain cultured in chicken eggs. As live vaccine, it can take advantage of the natural  
288 amplification of antigenic load consequent to bacteria proliferation. To the best of our knowledge,  
289 the saponin used in both farm and Saponin vaccine, should act through the same mechanism for the  
290 stimulation of the host immune response. Unfortunately, the exact composition of Saponin vaccine  
291 is not known (because of commercial rules), thus the reason why farm vaccine is less effective than  
292 Saponin vaccine cannot be completely defined. The difference could be due to the antigen damage  
293 caused by formalin, whereas saponin inactivation effect is mainly based on disruption of  
294 mycoplasma membranes without denaturing immunogenic proteins (Bangham et al., 1962).

295

296 This is the first evidence of the different efficacy of vaccine formulations in the control of CA in  
297 terms of pathogen excretion and its potential environmental impact. Furthermore vaccine utilization  
298 may reduce the risk of residues of antibiotics in dairy products for human consumption.

299

### 300 **Conflict of interest statement**

301 None of the authors has any financial or personal relationships that could inappropriately  
302 influence or bias the content of the paper.

303

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308

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376 **Table 1**

377 **Overview of the vaccine formulations used in this experiment**

<b>Group name</b>	<b>Vaccine</b>	<b>Number of animals</b>	<b>Producer</b>	<b>Inactivation procedure</b>	<b>Adjuvants</b>
Live	Live attenuated	5	Turkish Veterinary Institute	100 repeated passages in eggs	---
Saponin	Saponin vaccine	5	Pharmaceutical company 1	2 mg/ml saponin	---
Farm	Farm vaccine	5	Italian Veterinary Institute	0.4% formalin	200µl/ml Quil A
Commercial	Commercial	5	Pharmaceutical company 2	0.2% formalin	Aluminium hydroxide
Control	None	5	---	---	---

378

379 Animals were vaccinated with a proper dose of vaccines, following manufacturer instructions, and

380 boosted after 30 days. Control group was not subjected to any vaccine injection.

381

382 **Table 2**

383 **Percentages of sick animals in each group 4 weeks after infection**

<b>Group</b>	<b>Number of Animals per group</b>	<b>% of sick animals</b>
Live	5	0
Saponin	5	20
Farm	5	20
Control	5	80
Commercial	5	80

384

385 In control and commercial vaccines groups 4 sheep out of five presented classical CA symptoms  
386 (mainly mastitis, only one ewe in control group showed mastitis and arthritis). In saponin vaccine  
387 group one sheep presented joints constraint, in Farm vaccine group a ewe was mastitic. In Live  
388 vaccine group, no ewes showed CA symptoms

389

390

391 **Table 3**

392 **Risk values in vaccinated and non-vaccinated sheep**

	<b>Risk value in non-vaccinated group (%)</b>	<b>Risk value in vaccinated groups (mean) (%)</b>	<b>Ratio</b>
Nasal swab	<i>23.53</i>	<i>50</i>	<i>0.47</i>
Ocular swab	<i>8.33</i>	<i>7.14</i>	<i>1.167</i>
Milk excretion	<i>29.41</i>	<i>83.33</i>	<i>0.35</i>

393

394 The table compares the risk values to take contact with the pathogen between vaccinated and non-  
395 vaccinated animals. The Fisher exact test demonstrates how important is the vaccination to limit the  
396 risk to be affected by CA by pathogen excreted through nasal mucosa and milk.

397

398 **Figure legends**

399

400 Fig. 1. Incidence of clinical symptoms. The percentage of CA symptoms as an average of clinical  
401 manifestations during the whole monitoring period is shown. All the classical clinical symptoms  
402 were considered: mastitis, conjunctivitis and arthritis. The animals out of experiment were still  
403 considered symptomatic.

404

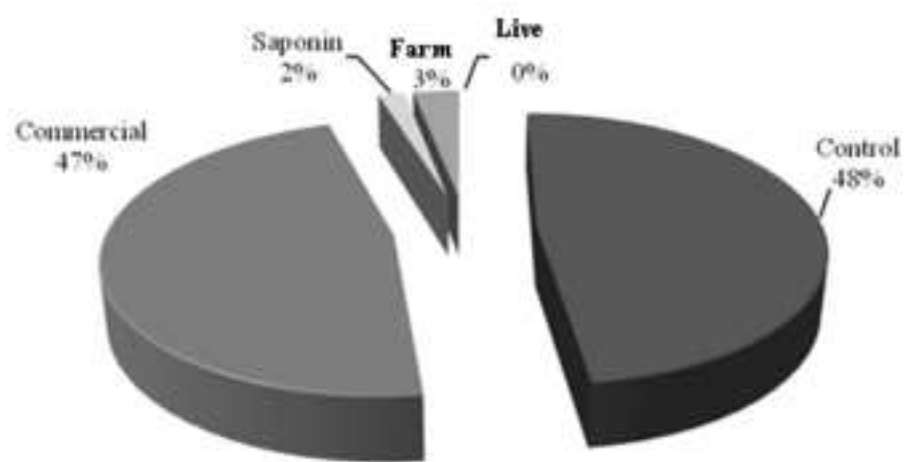
405 Fig. 2. Modification in excretion of *M. agalactiae* in the milk in vaccinated animals compared to  
406 Control group. The figure shows the mean values of pathogen excretion during the whole period of  
407 observation per each group compared to control group. The value of CFU/ml in the milk sample of  
408 control group was considered as “zero” and the percentages with respect to this group together with  
409 the relative standard deviations, were calculated. Positive values represent an increase of excretion  
410 of the pathogen while negative values mean a contraction of the pathogen spreading.

411

412 Fig. 3. Comparison of trends of clinical symptoms and milk excretion among groups at different  
413 time points. Data were analysed in order to compare the clinical behaviour and the pathogen  
414 spreading of the different groups 1, 4 and 8 wpi. In the first week after infection, all animals were  
415 still asymptomatic, but they were already secreting *Mycoplasma* by milk. Four wpi, it was recorded  
416 the higher number of symptomatic animals when compared to the other time points, mainly due to  
417 the contribution of control and commercial vaccine-treated animals. At the same time, there was the  
418 higher amount, in terms of CFU/ml of *Mycoplasma* excretion in the milk (see text). After 8 weeks  
419 post challenge, some ewe stopped to produce milk because of mastitis, and they were no longer  
420 considered «secreting» but still symptomatic (data not shown).

421

Agnone et al, Fig 1



Agnone et al, Fig 2

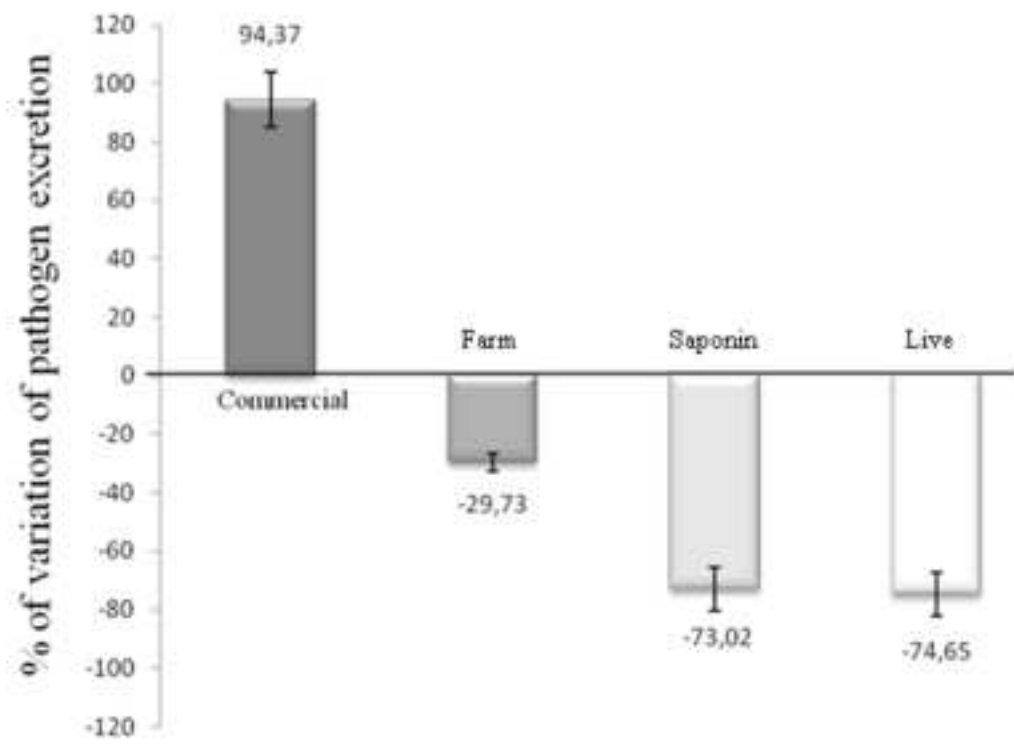
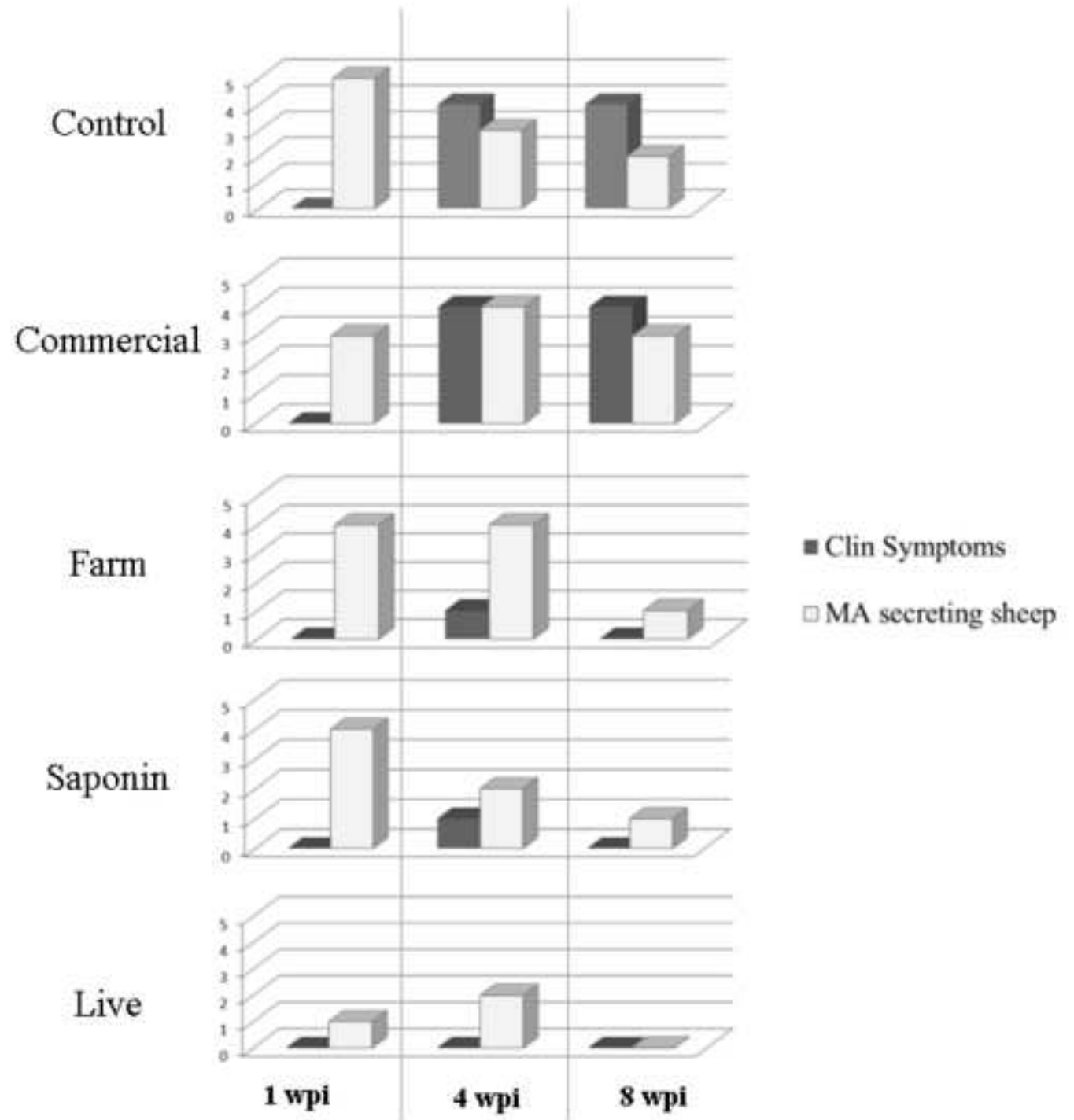


Figure 3  
[Click here to download high resolution image](#)

Agnone et al, Fig 3



**To the editorial board of  
The Veterinary Journal**

Dear Editors,

We are sending you a manuscript entitled “Pathogen excretion and clinical symptoms as indicators for the comparative analysis of vaccines efficacy during *Mycoplasma agalactiae* infection”. This study introduces a novel approach in evaluate control tools against Contagious agalactia, a great concern of sheep and goat livestock. It regards effectiveness of vaccines available to control the infection and moreover data on environmental impact of vaccines in reducing the excretion of the pathogen in the flock/farm. The study could be of great interest for the veterinarian management, since it illustrates with simplicity and reliability the effects caused by the different vaccines, and underlines the difference between vaccinated and not vaccinated animals. In our opinion, considering the relevance of our study not only among academics, but also for their application in the field, The Veterinary Journal is an appropriate platform where our data can be showed.

We hope you will retain our paper suitable for publication in The Veterinary Journal.

Sincerely,

Guido R. Loria



# Timing of activation of CD4<sup>+</sup> memory cells as a marker of protection for Contagious Agalactia in sheep

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# Abstract

## Background

*Mycoplasma agalactiae* is a major pathogen of sheep in many areas of the world and particularly in Mediterranean countries. It causes Contagious Agalactia, an infectious disease primarily affecting mammary glands. Many vaccines are currently under revising with the aim to protect the animals from the disease. The aim of the study is to investigate the involvement of T cell-mediated immunity in protection and pathogenic mechanism of Contagious Agalactia. A comparison of the antigen-specific Interferon gamma positive T cell memory and naïve subsets was performed between vaccinated and non vaccinated sheep to identify cellular correlates of protection and/or disease.

## Results

Data reported in this manuscript demonstrated that two out of the three vaccines used in this study protected sheep from the disease; in the protected groups memory CD4<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells underwent an early expansion ( $p < 0.05$  when compared to unprotected groups), whilst memory CD8<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells have increased in non-protected animals since 7 days post infection ( $p < 0.05$ ).  $\gamma\delta$ <sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells reached peaks of expansion in infected and in two vaccinated groups indicating that these cells aren't preferentially involved in protection or pathology ( $p < 0.05$ ).

## Conclusions

Hereby we propose that the early activation of memory CD4<sup>+</sup> Interferon- $\gamma$ <sup>+</sup> T cells, rather than CD8<sup>+</sup> or  $\gamma\delta$ <sup>+</sup> Interferon- $\gamma$ <sup>+</sup> cells should be considered as a marker of protection from the disease as well as a tool to establish vaccine efficacy.

**Keywords:** *Mycoplasma agalactiae*, cellular immunity, IFN- $\gamma$ <sup>+</sup> cells

## Background

*Mycoplasma agalactiae* is the major pathogen cause of Contagious Agalactia (CA), a severe syndrome of small ruminants characterized by mastitis, arthritis and keratoconjunctivitis [1]. The disease is a real priority in Mediterranean livestock and it is included in the list of notifiable diseases issued by the OIE (World Organization for Animal Health) because of its economic impact and widespread distribution [2].

Little is known about the immunity acquired during infection and/or vaccination against *M. agalactiae* [3-5], and the expansion of T cells subsets under specific stimulation *in vitro* has been never investigated [6].

In Italy available vaccines are commercial *M. agalactiae* inactivated products (formalin or saponin inactivated) and autologous products (so called “farm vaccine”) supplied by Italian Experimental Zooprophyllactic Institutes (IZSSs) limited in their use where outbreak is officially notified in the infected farm. Some vaccine formulations (saponin or ethanol inactivated vaccines) seem to be more effective than others [2]. However, a live attenuated vaccine, not yet allowed in EU, has been used in Turkey since the ‘30s, with good clinical results. As a consequence, no standard vaccine against CA has been universally utilized and, in spite of its severe economic losses [7, 8], control of CA in endemic areas is still a controversial issue [6].

The antigenic variation of *M. agalactiae* surface antigens [9-11] could reasonably be one of the causes of the loss of efficacy of host immune response, but there is still lack of information regarding the immune response itself elicited by *M. agalactiae* and related lymphocyte subsets and cytokines involved [3-5].

The aim of this study is to investigate the involvement of T cell subsets in sheep treated with different vaccine formulations followed by an experimental challenge with *M. agalactiae*, in order

to find a correlate of protection suitable to be used as a marker of the efficacy of the host immune response and/or of the protection conferred by the vaccine.

## Results

### **Two vaccines protect sheep from contagious agalactia**

The previously selected ewes were vaccinated, infected and monitored as described in Fig. 1A. The time points to analyse were chosen in order to investigate memory cells involvement useful to define a protection induced by a vaccine. Control group wasn't inoculated with any vaccine.

Intracanalicular infected ewes showed classical symptoms of CA five days post infection. The presence of *M. agalactiae* infection was confirmed microbiologically from milk and swabs collected from these animals. *M. agalactiae* was isolated from each inoculated sheep. These animals act as a good source of infection for the other ewes, and the presence of *M. agalactiae* in all secreting sheep was confirmed both by specific PCR and by cultural techniques.

The percentages of symptomatic sheep in infected and/or vaccinated groups during the period of observation are showed in Fig 1B. The clinical and immunological data were collected from day 0 to day 90 after infection. Clinical signs of CA in the naturally infected and vaccinated animals arose only after the second week post challenge.

The highest value of prevalence of mastitis was recorded four weeks after challenge. A total of 9 sheep were affected by mastitis: 4 sheep belonging to group 1 (infected control group), 4 belonging to group 4 (sheep treated with Commercial vaccine), 1 belonging to group 2 (farm vaccine) and none belonging to group 3 (saponin vaccine). Only two ewes of the total amount of treated sheep (8%) showed other CA symptoms: one was affected by arthritis (group 3) and one by monolateral conjunctivitis (control group) that overlapped with classical mastitis.

From the data recorded, it was possible to conclude that the vaccine better involved in protection from clinical symptoms is the saponin vaccine, followed by farm vaccine. Commercial vaccine was not able to confer protection in the 80% of the group.

### **Immunoglobulin presence doesn't relate to protection**

The specific anti-*Mycoplasma agalactiae* antibodies trend was evaluated in order to determine if there was a significant difference among groups in the time-span of 26 weeks. The presence of immunoglobulin was thus monitored with a qualitative ELISA since one week before vaccination. The day before the infection, after the two doses of vaccines, about 75% of vaccinated sheep resulted positive for anti-*Mycoplasma* IgG. In the control group, anti-*Mycoplasma* specific IgG have not been detectable until 4 weeks after challenge. Time of detection of antigen-specific IgG in our model is consistent with those found in other reports [17, 18]. The statistical analysis confirmed as significant the variation of OD values in the groups in the time course, but not relevant the differences among groups (data not shown).

### **CD4<sup>+</sup>- CD45R0<sup>+</sup>- IFN- $\gamma$ <sup>+</sup> T-lymphocytes increased in the early phase of immune response in protected animals**

The T cell subsets monitoring was assessed comparing the vaccinated groups with natural-like infected group, in terms of *in vitro* expansion of cellular subsets specifically activated by an irradiated *M. agalactiae*, and IFN- $\gamma$  was considered as a marker of cellular activation, as previously published [3, 19]. The data related to the cellular subsets of CD4<sup>+</sup>CD45R0<sup>+</sup>- CD4<sup>+</sup>CD45RA<sup>+</sup>- CD8<sup>+</sup>CD45R0<sup>+</sup>- and CD8<sup>+</sup>CD45RA<sup>+</sup>- and  $\gamma\delta$ <sup>+</sup> T-lymphocytes were obtained collecting PBMC at days 0, 7, 15, 30 and 90 after infection and culturing the cells for 48 hours with the specific irradiated antigen. The cellular trend was analysed indicating the percentage of antigen-specific IFN- $\gamma$ <sup>+</sup> cells, distinguishing between CD4<sup>+</sup> (CD45R0<sup>+</sup> or CD45RA<sup>+</sup>) and CD8<sup>+</sup> (CD45R0<sup>+</sup> or CD45RA<sup>+</sup>) and  $\gamma\delta$ <sup>+</sup> cells.

Data showed in figure 2A describe the relative percentages of CD45R0/CD45RA in gated CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells in each group at different time points. Animals treated with vaccines used in the group 3 and 2 show a mild cellular expansion at day 7, followed by a more relevant expansion of CD4<sup>+</sup>CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> at day 30 in the group 3, and 15 dpi in the group 2, maintaining high percentages of memory cells at day 90 after infection in both groups.

In the group 4, this subset shows a mild expansion not earlier than day 15, reaching and maintaining a plateau until the day 90 after the infection.

In the control group there is a slight increase of the value of antigen-specific CD4<sup>+</sup>- CD45R0<sup>+</sup>- IFN- $\gamma$ <sup>+</sup> T-lymphocytes, reaching a plateau from day 30 on, even if the percentages reached a value significantly lower than those of the same time points of clinically protective vaccine groups.

The level of CD4<sup>+</sup>- CD45RA<sup>+</sup>- IFN- $\gamma$ <sup>+</sup> T-lymphocytes does not show a significant increase in the group 2 but until day 15 high percentages of naïve CD4 T cells are detected. On the contrary, control and Commercial vaccine treated groups show higher percentages of naïve CD4 cells 7 dpi, when compared to the same time points of protected groups.

Figure 2B describe the absolute percentages of CD4 memory cells in the time points analysed to show the levels of CD4<sup>+</sup>CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells during the time of observation. Data obtained from animals of groups 1 and 4 don't reveal any significant modifications of the absolute percentage of the memory subset in all time points studied. The expansion of CD4 memory IFN- $\gamma$ <sup>+</sup> in the group 2 becomes significant only from day 30 post infection ( $p < 0.05$ ). Maximal absolute percentages of CD4<sup>+</sup>CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells were observed 30 and 90 dpi in the group 3 ( $p < 0.05$ ).

### **CD8<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T-lymphocytes increase in sick animals**

Figure 3A shows the relative percentages of CD45R0/CD45RA in gated CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells. In the group 1 high percentages of CD8 memory cells from day 15 to day 90 have been detected. CD8<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T-lymphocytes increase from day 0 to day 30 in the group 4. In the group 3, two peaks of CD8<sup>+</sup> memory IFN- $\gamma$ <sup>+</sup> cells were observed at days 15 and 90. The higher percentages of CD8<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells were observed in the group 2 in two time points of immunological monitoring: one occurs at day 7 and the other reach a plateau from day 30 to day 90.

Figure 3B shows the absolute percentages of CD8 memory cells during the observation period.

Cells from animals of Group 1 increase significantly, when compared to protected group 3, from day 15 to day 90. In the unprotected group 4 CD8<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells increase only at day 30 in absolute percentages.

### **$\gamma\delta^+$ IFN- $\gamma^+$ T lymphocytes quickly expand in a vaccinated and unprotected group**

In the group 4 no significant expansion of  $\gamma\delta^+$  IFN- $\gamma^+$  T lymphocytes was detected during all the time points of observation (Fig 4). The trend of  $\gamma\delta^+$  IFN- $\gamma^+$  T lymphocytes shows an early statistically significant expansion at day 7 in the control group ( $p<0.05$ ), followed by a new expansion 30 dpi when compared to the same time points of group 3. They reach again lower levels at day 90.  $\gamma\delta^+$  IFN- $\gamma^+$  T lymphocytes raised in the group 2 30 dpi ( $p<0.05$ ). A relevant peak of this cellular subset was also detected in the group 3 at day 15 after infection.

## **Discussion**

The study of the antigen-specific cellular subsets activated in response to *M. agalactiae*, was aimed to the deepening in the protective mechanisms conferred by the different vaccine formulations to the natural host of CA. Even if the data reported concern a limited number of animals, they represent a step ahead in the knowledge of small ruminants *Mycoplasma* infections.

The attention was focused both in the memory cell populations (CD45R0<sup>+</sup> – T lymphocytes), whose timing of expansion was considered a consequence of the efficiency of vaccination, and in the naïve cell populations (CD45RA<sup>+</sup> – T lymphocytes) that could be considered the reservoir of memory cells [20]. It is well known that upon stimulation by specific antigen, naïve T cells lose CD45RA, acquire CD45R0 antigen and are finally recruited into the peripheral pool of memory T cells. [21, 22].

Even if the cytokine IFN- $\gamma$  was used as a marker of the activation state in other *Mycoplasma* models of infection [19, 23], it should not be considered exhaustive of the cytokines scenario that indeed could be involved under the stimulus of the *M. agalactiae* infection. However, antigen-specific IFN- $\gamma^+$  T cell subsets could be useful to monitor the T-cell mediated immune response during the infection. The T cell response of IFN- $\gamma^+$  lymphocytes to *M. agalactiae* infection detected in the control group during this experiment confirmed our previous results describing, in infected sheep, a first increase of CD4<sup>+</sup> IFN- $\gamma^+$  cells followed by an expansion of CD8<sup>+</sup> IFN- $\gamma^+$  lymphocytes after short *in vitro* re-exposure to irradiated *M. agalactiae* [3] (data not shown). The same method,

improved with the detection of CD45R0 or CD45RA, could give an idea of the instauration of immunological memory. Since in other systems memory cells are involved in an effective immune response induced by a vaccination [17], the contribution of this population could be considered a goal for a good vaccine.

The results have emphasized how in the group treated with the effective vaccines (saponin and farm), the most represented T cell subset is CD4<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup>, able to increase since day 7 after infection to day 90. The early increase of CD4<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> is not traceable in the control group and in the group treated with the commercial vaccine. We thought that the timing of expansion of this subset could be considered as a correlate of protection. In control group and in the group 4, its activation undergoes a consistent delay, which can be the cause, together with the expansion of CD8<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T-lymphocytes, of the bad clinical conditions of animals. It would be interesting to perform a deeper analyses in order to classify memory cells in effector memory, central memory or Terminal Effector Memory RA and understand their different timing of activation, as showed in other systems [24], but unfortunately such reagents are not commercially available for small ruminants. Another explanation for the different time point when CD4<sup>+</sup> memory T cells reach the maximum of expansion could be a delay in the antigen presentation in Commercial vaccine when compared to saponin vaccine. In all cases, CD4<sup>+</sup> CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells are a well-known correlate of protection, as suggested by other models of infective diseases in humans [25] and in bovine. In order to assess an effective subunit vaccine against *Mycoplasma mycoides* subsp. *mycoides*, researchers are studying molecules able to activate CD4 central and effector memory cells, since it seems they are the most suitable peptides to be included in such vaccines [26, 27]. In the group that is not protected by vaccine, the infection causes an increase of the CD8<sup>+</sup>CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T-lymphocytes at day 30 after infection. This group suffered of a sort of delay in cellular response, due to the absence of any previous contact with the pathogen (both natural or by vaccination). The unavailability of data around the day 60 after infection does not allow confirming or excluding the expansion, also in this group, of a memory subset effective in the protection



against *Mycoplasma*. An analogous trend could have been happened in the group treated with the Commercial vaccine, where until the day 30 after infection any effective protection against the pathology is detected. In this case, the strain wouldn't be able to stimulate any cellular immune response, with respect to the wild type strains that are circulating nowadays.

It would be interesting the study of the whole cytokine pattern production in the context of the natural infection, in order to determine if, together with the presence of CD4<sup>+</sup>CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T-lymphocytes, the absence of clinical symptoms can be related with other cytokine patterns (Th-2, Th-17, Th-9, Th-22 subsets etc.) rather than Th-1.

Other *Mycoplasma* infection models in bovine suggested that the microorganism could support the expansion of  $\gamma\delta$  IFN- $\gamma$ <sup>+</sup> T cells releasing mevalonate metabolites [28]. In control animals (group 1) two peaks of  $\gamma\delta$  IFN- $\gamma$ <sup>+</sup> T cells were recorded, 7 dpi and 30 dpi. As  $\gamma\delta$  T cells are responsible of early innate immune response by recognition of non peptidic ligands, it could be argued that in control group, during the first days of infection, *M. agalactiae* could release non peptidic ligands that stimulate T cells with  $\gamma\delta$  T cell receptor. On the contrary, 30 dpi these metabolites could be also available in the environment as a consequence of the cytotoxic activity on infected cells probably due to the cytokines released by activated CD8<sup>+</sup> cells. The peak of  $\gamma\delta$  IFN- $\gamma$ <sup>+</sup> T cells in the group treated with the commercial vaccine 30 dpi could be again explained by an increase of cytotoxic T cells that, through the killing of *Mycoplasma*-infected cells, could release ligands for the expansion of  $\gamma\delta$  cells. The high amount of  $\gamma\delta$  IFN- $\gamma$ <sup>+</sup> T cells 15 dpi in the group 3 (saponin vaccine) could be due to the cytokine milieu produced by CD4 memory cells expanded 7 dpi that could support  $\gamma\delta$  IFN- $\gamma$ <sup>+</sup> T cells increase.

## Conclusions

The detection of T lymphocyte subsets responsible of the protection against CA, represents an important tool to evaluate the efficacy of available and experimental vaccines, and could be also useful to select the more immunogenic antigens for the inclusion in a subunit vaccine. On the

contrary the identification of a subset correlate to the infection could be useful as a biomarker of disease.

## Methods

### Experimental design

Twenty-two ewes, belonging to Comisana breed, aged from 2 to 4 years, were microbiologically and serologically confirmed as negative for *Mycoplasma spp* and any other mastitis etiological agent. All animals were subjected to synchronisation of their estrum. Forty days after parturition – to allow new-borns sheep to receive natural colostrum from their mothers – the herd was divided into 4 groups of 5 animals. The two remaining sheep were chosen to be later experimentally inoculated with live *Mycoplasma*, as natural source of pathogens, but not considered in the data analysis. Three groups were treated with the proper dose of the same number of vaccines (as recommended by manufacturer instruction). The fourth was considered as control group. Vaccines tested in this experiment were supplied by different sources (Tab. 1): farm vaccine (inactivated with formalin and combined with Quil A as adjuvant) from internal Vaccine laboratory of Istituto Zooprofilattico Sperimentale della Sicilia (hereafter “farm” vaccine); commercial vaccine (inactivated with formalin and combined with Aluminium hydroxide as adjuvant) that was bought from a local veterinary pharmacy (hereafter “Commercial” vaccine); and a novel saponin-inactivated vaccine, in which saponin acts as inactivant and adjuvant in the same time (hereafter “Saponin” vaccine).

Three weeks after the first inoculation, all the animals were boosted with an identical dose of the same vaccine. Immunoglobulin levels were weekly monitored to determine the presence of an acquired immunity against *M. agalactiae*. In the meantime, in a separate paddock, the two selected sheep were intracisternally inoculated with a live strain of *M. agalactiae*. Microbiological and biomolecular analyses demonstrated that the CA induced in these sheep was exclusively due to *M. agalactiae*. At day 0 of the experiment, the infected ewes were put in contact with the other animals, thus allowing the herd to stay permanently in contact with sick animals, imitating natural

infection [12]. Immunological data were collected at days 0, 7, 15, 30 and 90 after challenge (Fig. 1A).

### **Challenge**

For inoculum preparation a field strain recently isolated from a Sicilian outbreak of CA, confirmed by biochemical and PCR analysis, was utilized. After isolation the strain was cultured in modified Hayflick broth (500 ml) [13] after 72 hours incubation at 37°C, the culture was harvested at 10.000g for 30 min and re-suspended in the same volume of phosphate buffered saline (PBS) solution, pH 7.2. *M. agalactiae* antigen suspension was further stored in 1.5ml criovials and frozen at -80°C. Viable *M. agalactiae* antigen was quantified before inoculation by the method described elsewhere [13].

The challenge was designed in order to realize a system as more similar as possible to the natural infection. A 1.5 ml-inoculum containing  $10^5$  CFU of *M. agalactiae* was injected intracanalicularly into both halves of the mammary glands of 2 sheep. Milk samples collected from these ewes were tested in order to confirm the presence, the excretion of *M. agalactiae* and the absence of any other mastitis agents [13]. As previously reported by Tola and coll. [12, 14], all other remaining animals didn't receive any direct inoculum and they were simply left in contact with others and milked together. The *Mycoplasma* CFU was monitored both in infected and in-contact animals (data not shown). Twice a day, after milking the infected ewes, the operator would dip his hands in the infected milk and milk the vaccinated and control sheep [12]. The experiment was carried out in accordance with the Code of Practice for Housing and Care of Animal used in Scientific Procedures (EU Directive 2010/63/EU). The experiments were carried out with the authorization of the Italian Ministry of health (Decreto Ministeriale N° 101/2006 –A). The work was reliable using the animal facility of Istituto Zootecnico Sperimentale per la Sicilia (Palermo).

### **Data and samples collection**

Blood, serum, milk, ocular and nasal swabs samples and clinical data about the presence or absence of mastitis, arthritis and keratoconjunctivitis were recorded weekly till the week 26 after challenge,

while immunological data were collected at days 0, 15, 30 and 90 after infection as previously shown in Fig. 1A.

### **Serological and microbiological tests**

Screening for anti-*M. agalactiae* antibodies was performed utilizing a commercial ELISA kit (Institut Pourquier, France) following manufacturer instructions. The value of antibodies optical density were analysed with a statistical model of analysis of variance (ANOVA) for repeated measures. The confidence value was fixed at 0.05. The presence of *M. agalactiae* from infected and in-contact animals was confirmed by specific PCR and culturing milk and swabs samples as described elsewhere [15, 16]. Microbiological tests also included screening for conventional mastitis agents (data not shown).

### **Antigen preparation and flow cytometric analysis**

A selected *M. agalactiae* strain was isolated from cultures in semi-solid medium (Mycoplasma Experience, UK; Mycoplasma broth and agar, Oxoid, USA) and radiated to be used as a source of antigenic *in vitro* stimulus for lymphocytes [3]. Peripheral Blood Mononuclear Cells (PBMC) were collected from whole blood and cultured as described elsewhere [3]. Cells were thus collected after 48h of *in vitro* antigen exposure and flow-cytometric analyses were assessed. These assays were performed staining the cells with anti-surface-FITC-labelled mAbs and anti-IFN- $\gamma$  PE-labelled mAbs at the concentration suggested by the suppliers in 100  $\mu$ l PBS/0.1% Sodium Azide. Surface immunofluorescence was detected by anti-CD8 (FITC-labelled, clone CC63, mouse IgG2a, Serotec, U.K.), anti-CD4 (FITC-labelled, clone 44.38, mouse IgG2a, Serotec, U.K.), anti-WC1 (FITC-labelled, clone 19.19, mouse IgG1, Serotec, U.K.); anti-CD45R0 (purified, clone GC42A1 mouse IgG1) and anti-CD45RA (purified, clone 73B1 mouse IgG1) stainings were assessed by indirect labelling using a rat anti mouse IgG1 (PerCP-labelled, cat.n. 340272, rat IgG). After three washes, cells were fixed and permeabilized by Leucoperm<sup>®</sup> reagent set (Serotec, U.K.) and then incubated with anti-IFN- $\gamma$  mAb PE-labelled (clone CC302, mouse IgG1, Serotec, U.K.) for 15 minutes at 4°C. After three washes, cells were collected using a FACS-CAN cytometer (Becton Dickinson, U.S.A.)

and analysed by CELL-QUEST PRO program (Becton Dickinson, U.S.A.). The data related to the cellular subsets of CD4<sup>+</sup>CD45R0<sup>+</sup>- CD4<sup>+</sup>CD45RA<sup>+</sup>- CD8<sup>+</sup>CD45R0<sup>+</sup> and CD8<sup>+</sup>CD45RA<sup>+</sup>- and  $\gamma\delta^+$  T-lymphocytes were analysed and the values of the fluorescence were determined subtracting those of the corresponding negative controls that is the fluorescence of PBMC cultured with media alone and treated with the same reagents for staining. Each analysis was performed collecting 10000 events of live lymphocytes previously physically gated.

### **Statistics**

The Mann-Whitney test was used to analyse the significance between values of different groups; the confidence value was fixed at 0.05.

### **List of abbreviation**

CA: Contagious Agalactia; MA: *Mycoplasma agalactiae*; dpi: days post infection, PBMC:

Peripheral Blood Mononuclear Cells

### **Competing interests**

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

### **Authors' contributions**

AA: participated in study design and coordination, assessed the experimental procedures described in the paper and drafted the manuscript.

MPLM: participated in study design and coordination, assessed the experimental procedures described in the paper and helped to draft the manuscript.

GRL: conceived the study, participated in its design and coordination and helped to draft the manuscript.

RP: participated in study design and helped in data and sample collection.

SV: assessed the experimental procedures described in the paper and helped in data collection.

RAN: conceived the study and participated in its design.

GG: helped to draft the manuscript and performed statistical analysis.

GS: conceived the study, participated in its design and coordination and helped to draft the manuscript.

All authors read and approved the final manuscript.

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The authors would like to thank Anna Tamburello and Giovanni Cassata for their precious help, as well as Valeria Chiaracane and M. Todaro for their technical support. These experiments were supported by funds provided by Italian Ministry of Health (Ricerca Corrente 2008).

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## Figures

### Figure 1 - Clinical trend of vaccinated and infected groups of sheep

The figure 1A represents the scheme of the experimental design, indicating the time points before and after the infection (day 0). Fig 1B shows the incidence of clinical symptoms in the four groups, considered as presence of symptoms during the monitoring period of 90 days. During this period some animals recovered from the symptom, thus allowing the decrease of the incidence. All the main symptoms of CA were considered (mastitis, arthritis, conjunctivitis).

### Figure 2 - CD4 memory and naïve IFN- $\gamma$ <sup>+</sup> T cells during the observation period

The figure 2A shows the relative percentages of CD4<sup>+</sup>CD45R0<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD4<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T-lymphocytes in CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells. In panel B the absolute percentages of CD4<sup>+</sup>CD45R0<sup>+</sup>IFN- $\gamma$ <sup>+</sup> are shown. It can be noted that the comparison of the percentage of activated CD4<sup>+</sup>CD45R0<sup>+</sup> T-lymphocyte subset shows a significant difference between groups 3 and 2 and control group ( $p < 0.05$ ) at days 30 and 90 post-infection.

### Figure 3 - CD8 memory cells increase in sick animals

The figure 3A shows the relative percentages of CD8<sup>+</sup>CD45R0<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T-lymphocytes gated on CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells. The comparison of the absolute percentages of CD8<sup>+</sup>CD45R0<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T-lymphocyte subsets among groups, illustrated in panel B, shows the significant difference between non-protected groups (groups 4 and 1) and protected groups (2 and 3,  $p < 0.05$ ) at days 7 and 30 post-infection.

### Figure 4 - IFN- $\gamma$ positive $\gamma\delta$ T-cells trend in vaccinated and control groups

The mean percentages of IFN- $\gamma$  positive  $\gamma\delta$  T-cells in the time course of the experiment in the different groups of sheep is showed.

## Tables

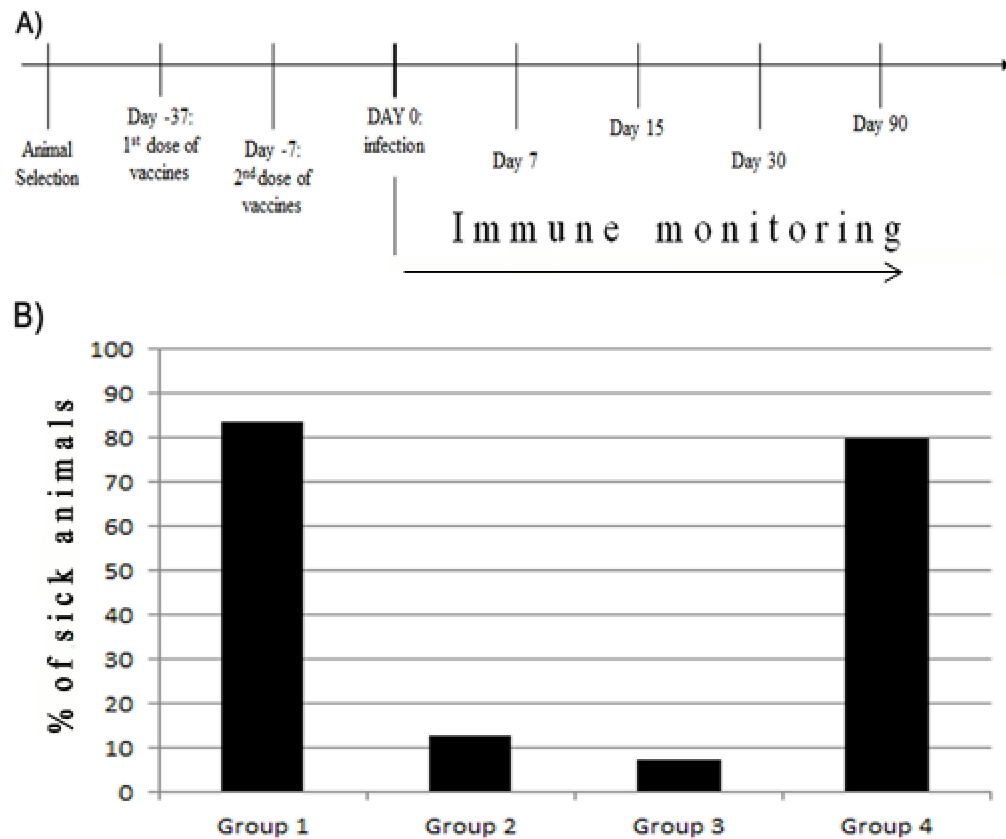
### Table 1 - Overview of the vaccines formulations used in this experiment

The group 1 was treated with PBS only in place of the vaccine injections. Groups 2, 3 and 4 were treated with a proper dose of vaccine, and boosted following the manufacturer instruction.

Agnone et al., Table 1

<b>Group #</b>	<b>Vaccine</b>	<b>Producer</b>	<b>Inactivation procedure</b>	<b>Adjuvants</b>
<b>1</b>	None	---	---	---
<b>2</b>	Farm vaccine	IZS Sicilia, Italy	0.4% formalin	200µl/ml Quil A
<b>3</b>	Saponin vaccine	IZO	2 mg/ml saponin	---
<b>4</b>	Commercial	Aglovax	0.2% formalin	Aluminium hydroxide

Agnone et al, Fig 1



Agnone et al, Fig 2

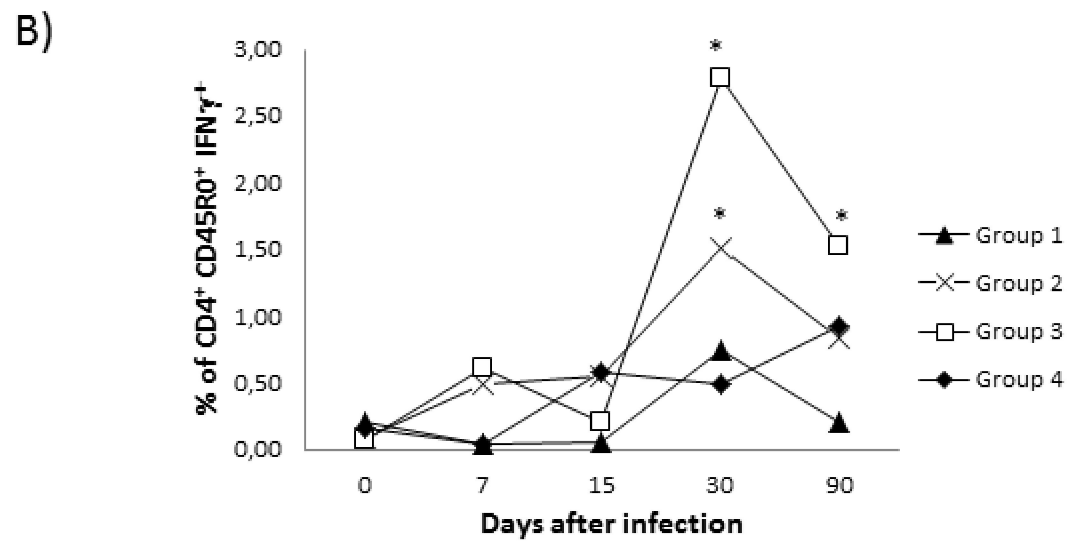
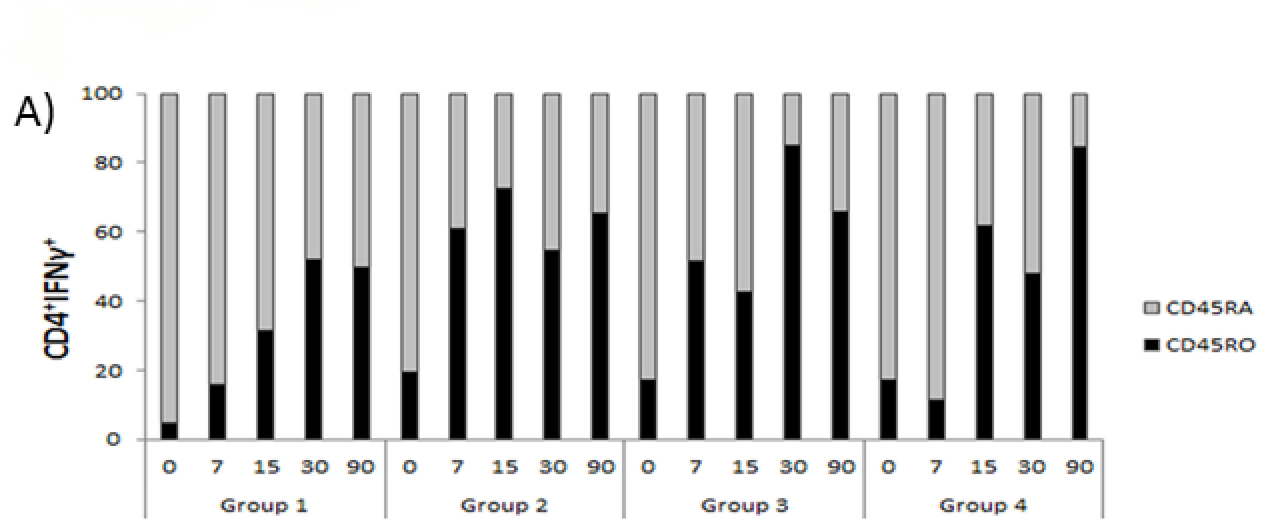


Figure 2

Agnone et al, Fig 3

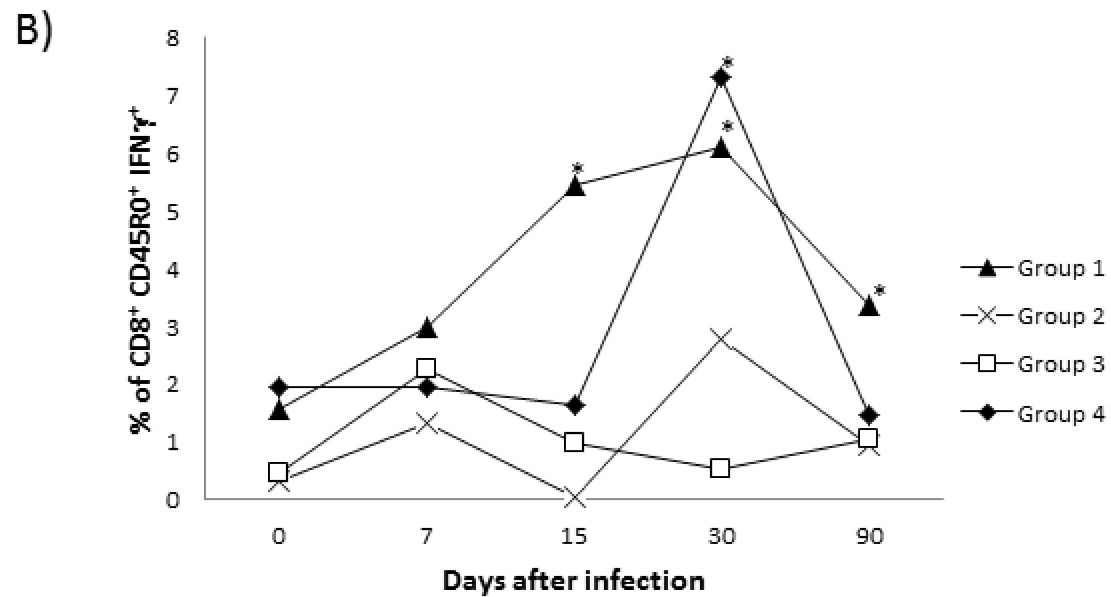
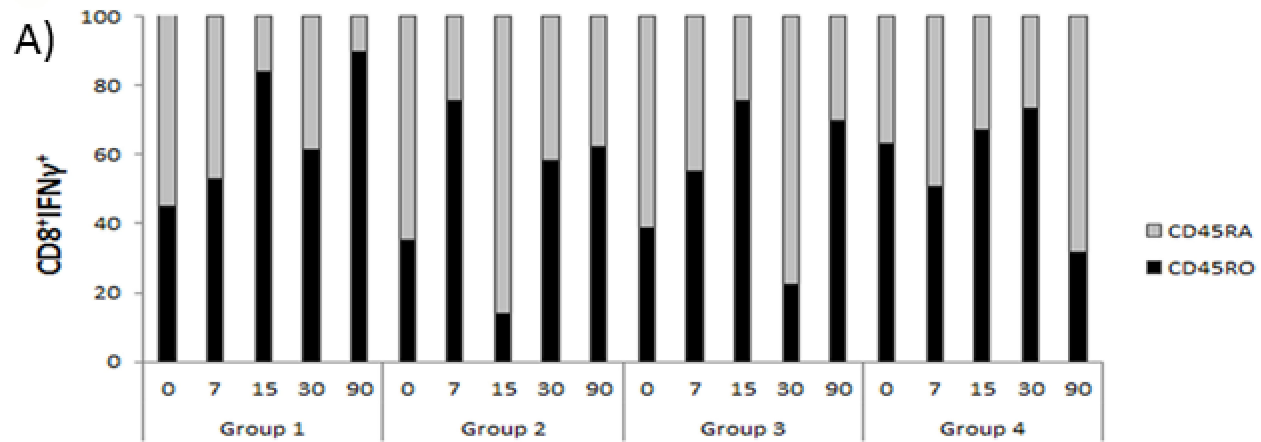
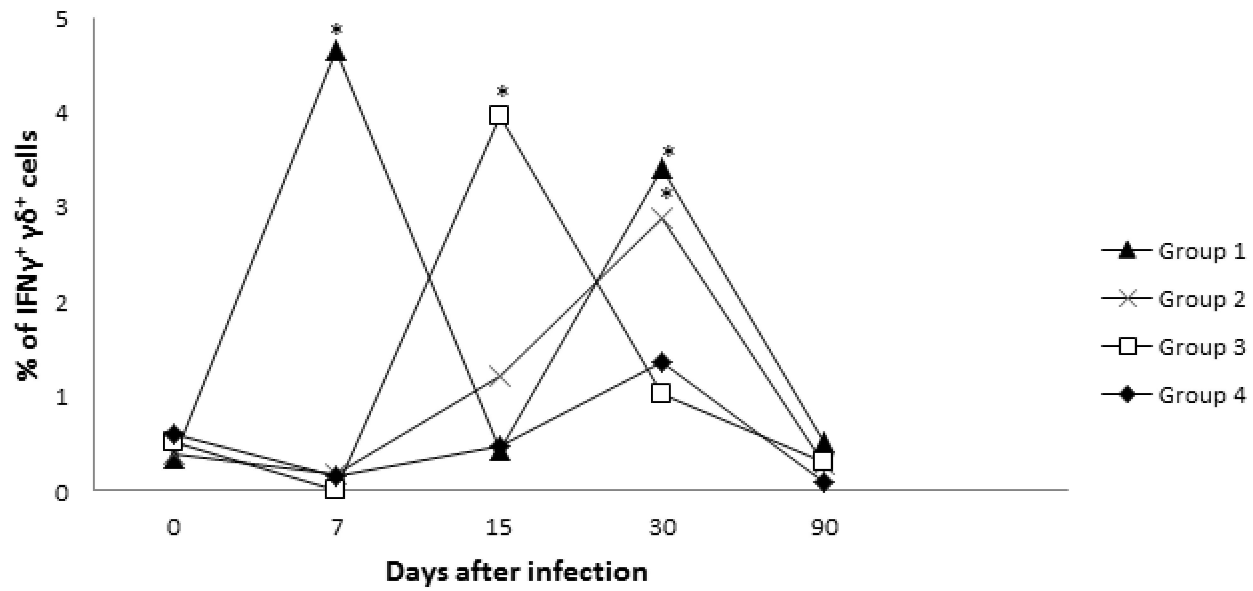


Figure 3



## Review Article

# Antigen-Specific T Cells and Cytokines Detection as Useful Tool for Understanding Immunity against Zoonotic Infections

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Zoonoses include a broad range of diseases, that are becoming of great interest, due to the climate changing, that cause the adaptation of vectors to new niches and environments. Host immune responses play a crucial role in determining the outcome of infections, as documented by expansion of antigen-specific T cells during several zoonotic infections. Thus, understanding of the contribution of antigen-specific T-cell subsets in the host immune response is a powerful tool to evaluate the different immunological mechanisms involved in zoonotic infections and for the development of effective vaccines. In this paper we discuss the role of T cells in some eukaryotic and prokaryotic infectious models.

## 1. Introduction

Zoonotic diseases are a significant burden on global economies and public health [1] and are due to the unaware role of wild and domestic animals, which act as reservoir or hosts of the etiological agents. More than 60% of emerging infectious diseases are constituted by zoonoses and the majority of these are increasing significantly over time [2]. In 2009 the World Organization for Animal Health (OIE) has commissioned Civic Consulting to conduct a study on the Cost of National Prevention Systems for Animal Diseases and Zoonoses, estimating that in developing and transition countries substantial differences in the public expenditure for the National Prevention System for Animal Diseases and Zoonoses exist, reaching from 10 million international dollars to 167 million international dollars [3]. The impair they cause should be attributed not only to human and animal suffering but also to the hampering agricultural production, the decreasing of food availability, and the creation of barriers to international trade [1], as well as the veterinary management, the maintenance of surveillance

plans, and the capillary control in the food industry chain of production.

Many zoonotic agents are transmitted by vectors, others by contaminated water or food, and others by direct transmission. A broad range of pathogens can be responsible for zoonoses, ranging from virus to prokaryotic to eukaryotic (unicellular or multicellular), and the great difference in the antigenic input for the immune system of the hosts implies that many different branches of immunity could be involved in protection or pathogenesis.

T cells play a pivotal role in immune functions since they are able to act not only differentiating in different subsets (including  $\gamma\delta$  T-lymphocytes and Cytotoxic T-Lymphocytes) but also inducing the production of antibodies that inhibit the pathogen spreading, both directly and with the help of other branch of the immune system.

Homeostatic cytokines are those factors able to regulate multiplication and differentiation of many cell types; T cells are dependent on contact with IL-2, IL-7, and IL-15, for their survival and intermittent homeostatic proliferation [4]. T-helper cell differentiation is instructed by distinct



environmental cytokines, that upregulate the expression of lineage-specific transcription factors and inhibit the alternate differentiation pathways [5]. The contact between the naïve T cell and the antigen induces the expression of IL-2 and IL-2 receptor leading to the entry of the T cell into several rounds of proliferation and to the differentiation in Th1, Th2, Th17, and induced regulatory T (iTreg) cells. The process consist of an intriguing cytokines puzzle, where IL-4 plays a major positive feedback role in Th2 differentiation, and IFN- $\gamma$ , together with IL12, determines Th1 induction [6]. IL6 and IL1 are necessary for Th17 production, while the role of TGF $\beta$  needs still to be deeper investigated [7, 8]. Finally, activated naïve CD4 T cells stimulated by TGF- $\beta$  in the absence of proinflammatory cytokines develop into iTreg cells [9].

The complex network of cytokines function is resolved in a balance from different T-cell activation pathways (Th1/Th2, Th1/Treg, Th2/Treg, Th1/NK, and/or  $\gamma\delta$  T cells). Although T-cell-mediated immune response during zoonotic infections is poorly studied, the facilities in the setting-up experimental conditions make it good system for a deeper investigation on the specific activation of T-lymphocytes.

It is well known that protozoan, helminthic parasites, and intracellular bacteria are able to survive within the host, in spite of the activation of both innate and adaptive immune response [10]. Zoonotic infections caused by eukaryotic organisms are intriguing systems where the antigen-specific T-cell expansion can be studied [11].

Helminthes have the ability to drive the differentiation of naïve CD4 T cells to the Th-2 subset of effector cells which are able to eliminate the pathogens by the actions of antibodies induced by Th2 cytokines. During a protozoarian infection, protozoa are usually phagocytosed into macrophages, previously activated by Th1 lymphocytes, and are able to survive evading host immune response. As it happens in the case of intracellular bacteria, infected cells loose the ability to kill the pathogen, and Cytotoxic T-Lymphocyte- (CTL-) mediated immune response is needed for the elimination of microorganisms into macrophages [12] (Figure 1). The naïve T cells encounter the antigen in the peripheral lymph node, develop toward effector cells, and migrate to the site of infection for the killing of infected cells. This process is finely tuned by cytokines cross-talk and microbial ability to evade host immune response.

B cells and humoral response play the main role in the clearance of extracellular bacteria. Nevertheless, a certain enrolment of T-cells has been demonstrated [13]. In this paper, we draw attention on different mechanisms of T-cell-mediated immunity, in order to compare the mechanisms of immune modulation induced by various zoonotic agents.

## 2. T Cells and Cytokines Induced by Eukaryotic Zoonotic Agents

The nematode parasites *Toxocara (T.) canis* and *T. cati* choose dogs and cats as definitive hosts, respectively. Sometimes, when embryonated eggs are accidentally ingested

by humans, larvae hatch in the small intestine, penetrate the intestinal wall, and cause the larva migrans syndrome [14]. Toxocariasis symptoms are classified according to the organs affected in visceral larva migrans (VLMs) and ocular larva migrans (OLMs). In the latter toxocariasis pathological effects on the host are restricted to the eye and optical nerve [15], while in the case of VLM, symptoms can persist for more than one year and include abdominal pain, coughing, headache, and normal or mildly elevated eosinophilia [16]. A recent survey [17] emphasizes that the seroprevalence value among humans is considerably high, thus demonstrating the relevance of this pathology. *T. canis* is able to control host immune response, through the modulation of cytokines produced by immune cells. The immunomodulatory effect has been demonstrated in mice, where the stimulation of normal macrophages with *T. canis* antigen *in vitro* induced IL-1 $\alpha$ , IL-6, IL-10, and TGF- $\beta$ , but not IL-12 and TNF- $\alpha$  [18]. Prototypical immune responses are characterized by increased lymphoproliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, increased production of IL-4 and IL-5, eosinophilia, and augmented production of IgE, as previously described in humans and mice [13–15]. As regards the immune response in dogs, it has been demonstrated that *T. canis* is able to induce antigen-specific IFN- $\gamma$  production in pregnant dogs and in their puppies [19]. Blood mononuclear cells (BMCs) were isolated from pregnant dogs and their puppies and were cultured in the presence of ESAg (Excretory/Secretory Antigen of *T. canis*). Cytokine levels were tested in cultures' supernatants by ELISA, and it was noted that IL-10 concentration increases during pregnancy in infected animals while IFN- $\gamma$  production decreases. On the contrary IL-10 concentration decreases with the age of infected puppies while IFN- $\gamma$  amount increases. It appears clear that immune cells of infected dogs undergo *T. canis*-induced modifications. These modified pattern of cytokines detected in *T. canis* could be due to a synergistic effects of physiological changes of immunity during pregnancy and in the first month of life, and/or direct effects mediated by parasite interaction with host immunity. The finding that IL-10 and IFN- $\gamma$  levels were significantly modified in infected pregnant dogs and their puppies provides new perspectives for immunotherapeutic interventions based on switch of Th2 to Th1 cytokine pattern in females before pregnancy.

Another system to understand the role of T cells in eukaryotic zoonotic infections is echinococcosis. Alveolar echinococcosis is caused by the metacestode stage of *Echinococcus multilocularis*. The definitive hosts are the foxes, which release Echinococcus eggs in the foecal matter, spreading them in the environment. Little rodents acquire the infection by ingesting eggs and carry the infection in their liver. Humans are aberrant intermediate hosts [20]. In humans, metacestode stage of the worm affects the liver, where an abdominal mass develops; other symptoms may arise like abdominal pain, jaundice, and liver failure [21]. The severity of the disease is dependent on the genetic background of the host and on the balance between the Th1-related immune response, associated with protection, and the induction of the immune tolerance by the parasite itself [22]. In experimentally infected C57BL/6J mice the

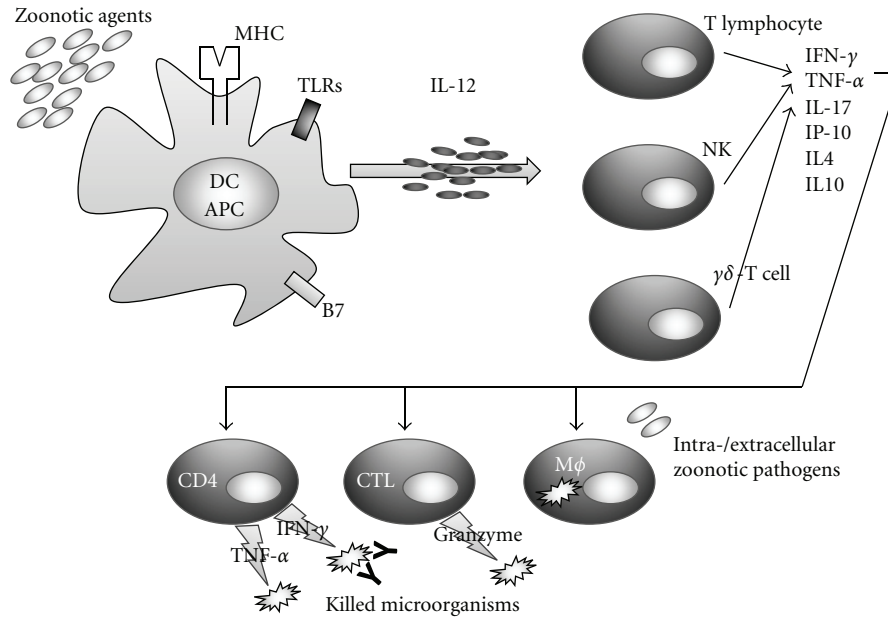


FIGURE 1: Schematic network of cells and molecules in response to zoonotic agents. An “oversimplified” scenario constituted by various cells and molecules involved both in binding of epitopes derived from pathogens and in the effector mechanisms hereby represented. APCs bind zoonotic derived epitopes and present them to various types of lymphocytes, in the context of MHC molecules and/or Toll-Like Receptors (TLRs). These subsets, producing different cytokines, could activate effector “protective” mechanisms involving macrophage killing, cytotoxic activity by CTL and/or CD4, and release of various cytokines, thus leading to the damaging of zoonotic pathogens. The killing by CTL, that could be not only CD8 but also NK cells, could be also due to an ADCC phenomenon with the contribution of antizoonotic epitopes,-specific antibodies.

promotion of the disease seems to be associated with the expansion of different T-cell subsets: spleen cells harvested at different time points after infection were stimulated *in vitro* with a crude parasite extract. A strong CD4<sup>+</sup> proliferative T-cell response was observed at the early stage of infection, and IFN- $\gamma$ , IL-2, and IL-5 were produced within the first weeks after infection whereas the detection of IL-10 was slightly delayed [23]. Cystic echinococcosis is caused by *E. granulosus*. The main domestic cycle is maintained between dogs and sheep, with man as accidental intermediate host. The disease is acquired by ingesting eggs, originating from the faeces of definitive hosts (dogs, wolves, and other carnivores) [24], and it typically affect, the liver. It is often asymptomatic, but in case of rupture of the cyst, secondary infection and anaphylactic reaction can occur. The most frequent complications are pain, obstructive jaundice, cholangitis and sometimes shock [25]. It has been demonstrated that a restimulation of PBMC from affected patients with the crude antigen induces an upregulation of IL-5 and IL-10 [26] as well as a downregulation of IL-1 and TNF- $\alpha$  mRNAs [27].

The opportunistic parasite *Toxoplasma gondii* belongs to the phylum apicomplexa. Feline acts as definitive hosts in its life cycle, while mammals, including humans, are intermediate hosts. Human toxoplasmosis is usually asymptomatic or paucisymptomatic, but the parasite is able to cross the intestinal barrier and disseminate through the body, reaching muscle, central nervous tissues, eyes, and

placenta [28]. Congenital toxoplasmosis may hesitate in retinochoroiditis and/or mental abnormalities [29].

The infection by *T. gondii* induces a strong cellular response essential for the host resistance [30]. In particular, it has been noted since 1990 that upon an *in vitro* stimulation with *T. gondii* antigen, a strong CD8<sup>+</sup> T-cells response, sustained also by CD4<sup>+</sup> cells expansion, is mounted [31]. The role of CD4<sup>+</sup> in the activation of CD8<sup>+</sup> has been demonstrated in mice [32], where the generation of optimal numbers of antigen specific CD8<sup>+</sup> effector T cells was found to require CD4<sup>+</sup> T-cells help. The parasite is also able to induce a strong natural killer (NK) cells activation and macrophages production of IL-12, both ending in a massive IFN- $\gamma$  production. The IFN- $\gamma$  production is sustained by  $\gamma\delta$ -T lymphocytes [33] that help CD4<sup>+</sup> and CD8<sup>+</sup> T cells to restrict parasite growth until the emerging of the complete adaptive response. It has been recently demonstrated that the CD8<sup>+</sup> T-cells response is sustained both by “homeostatic cytokines” IL-15 and IL-7 and that the absence of IL-15 or IL-7 alone does not affect CD8<sup>+</sup> T cell activation during acute toxoplasmosis [34], thus suggesting that these cytokines could act in synergy. Immune response of congenitally infected newborns to *T. gondii* undergoes to a process that leads to anergy [35, 36], probably due to a developing immune system of the infant. In this case, both  $\alpha\beta$ - and  $\gamma\delta$ -T cells become unresponsive when stimulated with *T. gondii*-specific antigen. Nevertheless, V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells are able to

lose tolerance before  $\alpha\beta$ -T-cells, and to confer protection against the chronic phase of infection in congenitally infected children [37]. Indeed,  $\gamma\delta$  T cells are considered to undergo peripheral tolerance, thus persisting in blood longer than  $\alpha\beta$  T lymphocytes which are deleted in the thymus during *T. gondii* infection [37].

A useful model to better understand immune response to eukaryotic zoonotic agents is constituted by Leishmaniasis and its related immunity. Leishmaniasis is a vector-borne disease caused by obligate intramacrophage protozoan parasite of the genus *Leishmania* and its incidence is increasing in nonendemic areas due to changing patterns of international travel and to population migration [38]. Visceral leishmaniasis (VL) or kala-azar is one of several diseases caused by more than 20 species of the protozoan parasite *Leishmania*. The infection tends to affect mainly children, but immunosuppression and HIV increase the possibility to contract the illness. The common symptoms are fever, malaise, shivering or chills, weight loss, anorexia, and discomfort in the left hypochondrium [39]. In experimental *L. major* infections genetically resistant mice develop a T-cell response dominated by a CD4<sup>+</sup> (Th1) phenotype characterized by IFN- $\gamma$  secretion while in susceptible mice the dominant response is a CD4<sup>+</sup> (Th2) phenotype characterized by interleukin IL-4, IL-5, and IL-13 secretion [40]. These observations of *L. major* in mice led to the emergence of the Th1/Th2 paradigm as opposing cytokine responses in the control of infections [41, 42]. The balance of Th1 to Th2 responses determines the outcome to infection. In the natural disease both Th1 and Th2 cellular subtypes are activated. Resistance to infection depends on production of cytokines such as IFN- $\gamma$ , TNF, IL-2, and IL-12. These cytokines stimulate cell-mediated immunity which eliminates the infection activating leishmanicidal activity of macrophages [41, 42]. The infection in dogs shows different clinical presentations, from subclinical/asymptomatic to a fully developed disease, depending on the host's immune responses. The Th1/Th2 dichotomy is not clear in the different forms of canine leishmaniasis, because it depends on physiological status of the infected subject. The production of IL-4, IL-5, IL-6, and IL-10, which in turn promote B-cell proliferation and antibody production, is the cause of susceptibility of dogs, which become not able to control the infection [43–45]. Our experience is focused to evaluate cytokine expression level with a quantitative real-time PCR assay to measure expression levels of cytokines relative to either Th1 or Th2 patterns in the blood of naturally infected asymptomatic dogs. High expression levels of IL-2 and IFN- $\gamma$  were detected at the first observation, which decreased over time. Opposite cytokine-based effects were detected in infected dogs. In those that had a clinically evident outcome, IL-2 and IFN- $\gamma$  were initially not expressed, but their levels suddenly increased with the appearance of clinical signs [43]. Furthermore from our study it was confirmed that IL-12 represents a marker of active disease, while IL-18 cannot be involved in the progression from asymptomatic to active disease. These data suggest that response to *Leishmania* in the dog does not fit into a specific cytokine profile.

### 3. Antigen-Specific T Cells and Derived Cytokines Detection in Prokaryotic Infections

Among prokaryotic microorganisms able to cause zoonotic disease, *Leptospira*, *Brucella*, and *Mycobacteria* offer suitable models to analyze the role of immune response against these pathogen since the related immunity could involve different antigen-specific T cell subsets. *Leptospira interrogans* is one of the main causative agents of leptospirosis. The pathogen is able to persist in the kidneys of infected (wild and domestic) animals and is spread in the environment through their urine. It is transmitted to humans through skin abrasions and causes haemorrhage, diarrhoea, renal impairment, and aseptic meningitis [46]. Phagocytosis is the main process that allows the clearance of the pathogen, and it has been recently demonstrated that the bacteria undergo a complex transcriptional regulation in order to evade host immune response [47]. In particular they downregulate the major OMPs (Outer Membrane Proteins) through the action of a hypothetical transcriptional factor. It is well accepted that humoral immunity has an important role for the elimination of extracellular bacteria, but sometimes antibodies alone could not be sufficient, especially in the case of *L. borgpetersenii* serovar Hardjo [48]. In this and other cases, IFN- $\gamma$  plays an important role for the activation of macrophages and the production of IgG2 class of immunoglobulins [49, 50]. The involvement of a cellular immune response has been recently demonstrated: a strong Th1 response was recorded by the observation of the IFN- $\gamma$  production following the *in vitro* stimulation of vaccinated bovine PBMC with the specific antigen [51]. The results from vaccinated animals indicated that approximately two-thirds of IFN- $\gamma$ <sup>+</sup> cells were within the CD4<sup>+</sup> T-cell population while the remaining one-third were  $\gamma\delta$  T cells [51]. Furthermore, Guo et al. have recently reported the existence of specific cytotoxic CD8<sup>+</sup> T cells in patients with leptospirosis and have detected a potential epitope of the leptospiral protein LigA, able to elicit specific cytotoxic T-lymphocyte (CTL) responses [13]. Naiman and Guo suggest that Th1 response to *Leptospira* requires the cooperation between two or more T cell subsets like  $\gamma\delta$ , CD8<sup>+</sup>, CD4<sup>+</sup>, and so forth. In *Leptospira*-infected hamsters a new soluble factor was shown to be important for the protection: IP-10 [52]. This evidence points to T cell-derived chemokines in zoonosis. These proteins are able to induce cell migration from lymphoid organs to affected tissues and they are also considered markers of T cell maturation [53]. Indeed, future approaches for a deeper analysis of T cell response in zoonoses could be comprehensive of the characterization of the released chemokines and their receptors.

A very hot field in veterinary immunology is represented by T cell responses against intracellular bacteria. Tuberculosis and Brucellosis remain major worldwide health emergencies among zoonotic bacterial infections, and a better understanding of the host immunological reactions to these pathogens is fundamental for improving both therapies and vaccines strategy, as well as to prevent dissemination of the

infectious agents in the herds. Tuberculosis causes in host mild fever and a wide range of symptoms depending on the localization of the Mycobacterium (pneumonia, kidney failure, meningitis especially in children, etc.) [54].

Animal tuberculosis is mainly observed in cattle (less frequently also in horses, swine, dogs, cats, sheep, and goats), caused by *Mycobacterium (M.) bovis*, and in birds, due to *M. avium*. Human tuberculosis is mainly caused by *M. tuberculosis*, but around 10% of total infections are due to *M. bovis*, typically as professional disease, while *M. avium* can cause disease in immunodeficient patients [55]. Dogs and parrots are highly susceptible to *M. tuberculosis* by the contact with infected humans. T-lymphocytes play a central role in the control of *M. tuberculosis* replication, as this infection evokes a strong cell-mediated immune response. Protective immunity against *M. tuberculosis* is due to adaptive cellular immune responses, and protective immunity correlates to the induction of T cell cytokines following antigen specific stimulation. CD4<sup>+</sup> and CD8<sup>+</sup> T cells are key components of anti-mycobacterial immunity [56, 57]. Both IFN- $\gamma$  production and cytotoxic activity against infected target cells contribute to bacteria killing with lysis of infected cells [58, 59].

T cells response after *in vitro* stimulation of human PBMCs with *M. tuberculosis*-specific antigens (e.g., Purified Protein Derivative, or PPD) can be assessed by measuring intra- and extracellular IFN- $\gamma$  [60]. The severity of *M. tuberculosis* infection may be detected by measuring CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as their numbers markedly decrease in patients with severe tuberculosis, which can be a sign of suppressed cellular immunity in these patients [60]. Particularly, patients with active TB have a lower number of both CD4<sup>+</sup> T cells and their naïve, effector, and late differentiated memory subsets [61], with a drop in all the three phenotypic populations. Similarly, CD8<sup>+</sup> T cells counts were also significantly different between infected and negative patients. At least partially, these disturbances seem to be restored to baseline after successful therapies [61].

In our experience with cattle [62] it has been showed that cocktails of epitopes from ESAT-6 (the 6kDa early secretory antigenic target of *Mycobacterium tuberculosis*) are recognized with high frequency by CD8<sup>+</sup> T lymphocytes of naturally infected cattle, thus confirming a role of ESAT-6 specific CD8<sup>+</sup> T cells in the response to *M. bovis*. Nevertheless, the number of IFN- $\gamma$ -positive CD8-negative cells was larger than that of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells, indicating that IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells are not the dominant subset responding to stimulation with ESAT-6-derived peptides. Nevertheless, ESAT-6-specific T-cell expansion could be useful to detect the early phase of the disease thus limiting the dissemination of *M. bovis*.

Other cytokines such as TNF- $\alpha$ , IL-2 [63], MCP-2 [64], and IP10 [65] were shown to be involved in the anti-mycobacterial immune responses in humans; Th1- and other cytokines interacting with macrophages are commonly considered as mediators of anti-mycobacterial biological agents. When reagents for the detection of these cytokines in vertebrates will be available, it could be intriguing to

understand the role of these cytokines in mycobacterial immune response also in veterinary infections.

Brucellosis is a multisystemic disease with a broad range of symptoms, usually beginning with acute febrile illness, headache, malaise, and myalgia. Gastrointestinal signs as vomiting, anorexia, and nausea may also occur [66]. Humans are susceptible to *Brucella (B.) suis*, *B. Abortus*, and *B. canis*, and, more frequently, to *B. melitensis*. The disease can be transmitted by both direct and indirect contact with infected animals or secretions, or by eating contaminated food (especially unpasteurized milk and fresh cheeses). Interhuman transmission is extremely rare [67].

*Brucella* invades and proliferates within monocytes. In addition to the central role of monocytes/macrophages, other cells of the innate immune response are recruited and influence the interaction between bacteria and host. For instance, human V $\gamma$ 9V $\delta$ 2 T cells play an important role in the early response to infection [67], and their number dramatically increases in the peripheral blood of patients with acute brucellosis [68], reaching 30% of the total T lymphocytes. V $\gamma$ 9V $\delta$ 2 T cells are specifically stimulated by *Brucella* to secrete TNF- $\alpha$ , important for the autocrine activation of macrophage functions, IFN- $\gamma$ , and other cytokines [69]. *In vitro*, V $\gamma$ 9V $\delta$ 2 T cells exhibit a strong cytotoxicity against *Brucella*-infected cells. V $\gamma$ 9V $\delta$ 2 T cells decrease the development of intracellular *Brucella* releasing lytic granules and/or acting through Fas-mediated signals to lyse infected macrophages. It was also shown that the recruitment of NKG2D by its ligands is sufficient to induce cytokine production and the release of lytic granules thus increasing the TCR-triggered responses of V $\gamma$ 9V $\delta$ 2 T cells. The interaction between NKG2D and its main ligand expressed on *Brucella*-infected macrophages, UL16-binding protein 1 (ULBP1), is involved in the inhibition of bacterium development [69]. As demonstrated in the case of V $\gamma$ 9V $\delta$ 2 T cells, it was shown that also NKT cells are able to exert an anti-*Brucella in vitro* activity, either secreting cytokines or killing infected macrophages [70]. NKT and V $\gamma$ 9V $\delta$ 2 are considered as quite unrestricted T cells as they do not recognize MHC and peptides, but they expand following stimulation with nonpolymorphic MHC-like molecules CD1 and/or with nonpeptidic and glycolipid ligands. A cross-talk between V $\gamma$ 9V $\delta$ 2 and NKT, due to cytokines released in the milieu, could be responsible for the activation of NKT in synergy with a possible upregulating role of CD1 molecules expression exerted by *Brucella* antigens. The previously described subsets activated during *Brucella* infection could exert a protective role during *Brucella* infection through their potent cytotoxic activity.

#### 4. Concluding Remarks

Each microorganism hereby evaluated elicits a particular type of immune response. A “classical” Th1-mediated protective immune response was detected during zoonotic infections like leishmaniasis or tuberculosis. Toxoplasma-, *Brucella*- and *Leptospira*-induced immune response involves a wide range of T cells including  $\gamma\delta$  and NKT cells. The *in vitro* and *ex vivo* detection of T cells upon stimulation with

the specific antigen allows going insight in the host/pathogen interaction. The equilibrium established after such dialogue is critical for the further ongoing of the infection. A complex network of T cells, cytokines, and chemokines could be studied to better understand the interactions between zoonotic agents and receptors of innate and adaptive immunity. This tool could be useful to develop vaccines and immunotherapies in the next future.

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**Development and validation of two PCR tests for the detection and differentiation  
between *Anaplasma ovis* and *Anaplasma marginale***

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Running title: *Anaplasma ovis* and *Anaplasma marginale* specific PCR

## **Abstract**

*Anaplasma ovis* and *Anaplasma marginale* are tick-transmitted bacteria that cause anaplasmosis in domestic and wild animals. Recent results show that some domestic and wild animals and ticks are susceptible to both *A. ovis* and *A. marginale*, thus supporting the need to differentiate between these species in hosts and ticks diagnosed with *Anaplasma* infection. However, although anaplasmosis is one of the most common diseases of grazing animals worldwide, rapid and effective tests are not available for the detection and discrimination between these two *Anaplasma* species. The objective of this research was to develop an easy and reliable method to identify and discriminate between the closely related pathogens, *A. ovis* and *A. marginale*. *A. ovis* and *A. marginale* major surface protein 4 (*msp4*) gene sequences were retrieved from different geographic strains and aligned to design two set of primers in a region with significant differences between the two species, but completely conserved among strains. PCR reactions using these primers were 100% species-specific and detected all strains from each pathogen previously identified with other methods. The two set of primers designed for the specific PCR amplification of *A. ovis* and *A. marginale* allow to easily detect and discriminate between the two pathogens, thus avoiding the time-consuming sequencing or multi-gene amplification procedures. This PCR provides a tool for the detection of *A. ovis* and *A. marginale* in ticks and in wildlife and domestic hosts.

**Keywords:** *Anaplasma*, *Diagnostics*, *major surface protein 4*, *Polymerase Chain Reaction*

## 1. Introduction

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes tick-borne pathogens that impact human and animal health (Dumler et al., 2001; Kokan et al., 2004). The genus *Anaplasma* includes the species *A. marginale*, the type species that infects ruminants and *A. ovis*, which infects sheep, goat and deer. Also included in this genus are other species such as *A. centrale*, *A. bovis*, *A. platys* and *A. phagocytophilum*. *A. marginale* and *A. ovis* infection could result in anaplasmosis, a mild to severe intraerythrocytic disease that produces considerable economic losses in some regions (Kokan et al., 2004).

It has been generally accepted that *A. marginale* infects cattle while only sheep and goats are susceptible to *A. ovis* (Kokan et al., 2010). However, it has been shown that deer species are susceptible to both *A. marginale* and *A. ovis* (de la Fuente et al., 2005a, 2006, 2007, 2008; Kuttler, 1984), and recent reports provide evidence for possible *A. ovis* infection in cattle (Hornok et al., 2010, 2011). Additionally, some tick species are also vectors of both of these pathogens (de la Fuente et al., 2005a, Torina et al., 2008). These results support the need to differentiate between *A. marginale* and *A. ovis* in domestic animals, wild animals and ticks diagnosed with *Anaplasma* infection.

Major surface proteins (MSPs) play a crucial role in the interaction of *Anaplasma* spp. with host cells and they are subjected to selective pressures exerted by host immune systems (Brayton et al., 2006; de la Fuente et al., 2005c; Dunning Hotopp et al., 2006; Kocan et al., 2004).

MSP4 is an immunodominant outer membrane protein with orthologs in all *Anaplasma* spp. examined so far (de la Fuente et al., 2005c). The *mSP4* gene has been used for phylogenetic studies providing information about the biogeography and evolution of *Anaplasma* spp (de la Fuente 2005b, 2005c, 2007).

MSPs PCR has been used for the diagnosis of *Anaplasma* infection (de la Fuente 2005a, 2005b, 2005c; Hornok 2010, 2011, Torina et al., 2008). Unfortunately, although very sensitive and easy to perform, this method is not able to discriminate between *A. ovis* and *A. marginale*. In a recent OIE publication (OIE Manual, 2008), they report that laboratories running this PCR assay should recognise problems associated with the inability to discriminate between some *Anaplasma* spp., thus requiring an additional step such as restriction enzyme analysis, Southern blot hybridisation or sequencing that can confirm the species *msp* amplified by PCR. However, these methods are expensive and time-consuming, thus requiring the development of reliable methods to identify and discriminate between the closely related *Anaplasma* spp.

## **2. Materials and methods**

### **2.1. Blood samples and DNA extraction**

Eight cattle, sheep and goat field samples were selected to validate our procedure. Samples were collected in different farms to increase the probability of finding different *Anaplasma* spp. strains. Genomic DNA was purified from blood samples using a DNA extraction kit (Invitrogen PureLink Genomic Mini kit). DNA extracted from uninfected animal blood samples were used as negative controls. All the DNA samples were examined both using a spectrophotometer method (Nanodrop™ Thermo Fisher Scientific, Wilmington, USA) and by 16S rDNA PCR (Stuen et al., 2003), to test their quantity and quality. The *Anaplasma* spp. *msp4* gene was amplified by PCR as reported previously (de la Fuente et al., 2003), using primers that amplify both *A. marginale* and *A. ovis msp4* (Table1). The amplicons were sequenced in order to identify the species.

### **2.2 Design of PCR primers**

*A. marginale msp4* gene sequences were selected from strains identified in the following countries: USA (GenBank accession numbers AF428081, AF428088, AY010252, AY010254, (AY010253), France (AY010249), Mexico (AF428085, AF428089, AF428084, AF428083), Argentina (AF428086, AF428087), Brazil (AF428082), Nigeria (EU106082), and Hungary (EF190508). *A. ovis msp4* gene sequences were selected from strains identified in the following countries: Italy (EU436161, EU436160), USA (DQ674246, DQ674249, DQ674248, DQ674247), Hungary (EF190509, EF190510, EF190511, EF190512, EF190513), Iran (EU925811) and Spain (EF067341).

The *msp4* sequences were aligned using ClustalW2 (Larkin et al., 2007) and analyzed to identify an appropriate region to design the two set of primers. The designed primers were analyzed and adjusted so that 3' end hairpin  $\Delta G$  values were lower than -2 kcal/mol and the  $\Delta G$  values of internal hairpin were lower than -3 kcal/mol. Forward and reverse primers had similar melting temperatures.

### **2.3 PCR conditions optimization**

In order to set up the two PCR reactions, different melting temperatures, ranging from 58 to 64 °C, were tested, as well as different MgCl<sub>2</sub> concentrations (from 1.2 to 2 mM) to assure the best ratio between specificity and sensitivity. Many different commercial Taq polymerases were also tested, but the differences among them were not significant (data not shown). The reactions were always conducted in a final volume of 25  $\mu$ l. The obtained PCR products were visualized by UV lamp in a 1.5% agarose gel containing 0.1  $\mu$ g/ mL ethidium bromide.

### **2.4 Design of PCR primers**

The *msp4* gene sequences derived from 15 and 13 strains of *A. marginale* and *A. ovis*, respectively were aligned to design two set of primers in a region with significant difference between the two species, but completely conserved among strains. In particular we took advantage of a region of *A. marginale msp4* at nucleotide position 120, harbouring a deletion

of three nucleotide not traceable in *A. ovis msp4*. The primer forward were designed between nucleotide positions 113 and 131 of *A. marginale* and between nucleotides 114 and 134 of *A. ovis*. The primers reverse were designed between nucleotide positions 435 and 456 of *A. marginale* and between nucleotides 438 and 460 of *A. ovis*.

The primer set specific for *A. marginale* was composed by the forward primer AmargMSP4Fw (5'-CTGAAGGGGGAGTAATGGG-3') and the reverse primer AmargMSP4Rev (5'-GGTAATAGCTGCCAGAGATTCC-3'). The primer set specific for *A. ovis* included the forward primer AovisMSP4Fw (5'-TGAAGGGAGCGGGGTCATGGG-3') and the reverse primer AovisMSP4Rev (5'-GAGTAATTGCAGCCAGGGACTCT-3').

## **2.5 PCR amplification**

The optimized PCR conditions were established as follows: reaction buffer 1x, 0.4 µM of each primer, 1.5mM of MgCl<sub>2</sub>, 0.2 mM of dNTP, 1.25 U of Taq Polymerase (Invitrogen Taq DNA Polymerase). The thermal profiles of PCRs were: 10 sec at 95°C for denaturation, followed by 30 cycles with denaturation at 94°C for 30 sec, annealing at 62°C for 15 sec for *A. ovis* and 60°C for 15 sec for *A. marginale*, and extension at 72°C for 30 sec. The final extension step was 5 min at 72°C.

## **2.6 Cloning and sequencing**

Once optimized, PCRs were used to amplify *A. ovis* and *A. marginale msp4* region from all the selected samples. PCR products were randomly selected, purified by a PCR and gel purification kit (Promega Wizard SV Gel and PCR Clean-up System), cloned into a cloning vector (Invitrogen pCR2.1 TOPO TA cloning kit) and sent for sequencing (Macrogen, Inc., Seoul, South Korea) both strands to confirm the identity of the amplicons for both *A. ovis* and *A. marginale msp4*.

## **2.7 Specificity and sensitivity**

To characterize the specificity of the reactions, the two set of primers were used to analyze samples positive by PCR (Table 1) for *A. phagocytophilum*, *A. platys*, *Ehrlichia canis*, *Rickettsia conorii*, *Coxiella burnetii*, *Babesia ovis*, *Babesia bovis*, *Babesia bigemina*, *Babesia canis*, *Babesia gibsoni*, *Babesia caballi*, *Babesia microti*, *Theileria equi*, and *Theileria annulata*. Samples positive for *Anaplasma* spp. PCR but negative for *A. phagocytophilum*, *A. ovis* and *A. marginale* were also tested. Controls included reactions without DNA template and with DNA from *Anaplasma*-negative blood samples. Serial dilutions of a quantified plasmid DNA containing the *A. ovis* and *A. marginale msp4* inserts were done and the PCR sensitivity was calculated in terms of pg of DNA per  $\mu\text{l}$ .

### 3. Results

Selected field samples, 8 positive for *A. marginale* and 8 positive for *A. ovis*, were amplified using the specific set of primers AmargMSP4Fw and AmargMSP4Rev, as well as AovisMSP4Fw and AovisMSP4Rev with the optimized PCR conditions. All cattle samples gave an amplicon of the expected size (344 bp) when amplified with primers specific for *A. marginale* and resulted negative when amplified with primers specific for *A. ovis* (Fig. 1). All sheep and goat samples gave an amplicon of the expected size (347 bp) only when amplified using the set of primers specific for *A. ovis* (Fig. 2). Some PCR products were randomly selected, sequenced and confirmed the specificity of the reactions. In particular two PCR products, one from *A. ovis* and one from *A. marginale*, were cloned and their sequences showed a percentage of identity with the closest strain equal to 100% (GenBank #HM063433) and 98.8% (GenBank #AF428081), respectively. The sequences obtained were submitted to GenBank (Accession numbers JF714148 and JF714147).

All the samples positive for other related pathogens resulted negative using the newly designed primers. These results demonstrated that the new PCRs were specific and able to

differentiate between *A. marginale* and *A. ovis* with a sensitivity equivalent to 0,5 pg/μl for *A. marginale* and 0,005 pg/μl for *A. ovis*. (Figs. 3a and 3b).

#### **4. Discussion**

Anaplasmosis is one of the most spread tick-borne diseases not only in Mediterranean countries, but also in all tropical and sub-tropical regions of the world. The diagnostic PCRs used for *A. ovis* and *A. marginale* did not allow differentiation between these pathogens without the support of other techniques such as sequencing and multi-gene amplification. To resolve this deficiency in screening for these two pathogens, we developed a new sensitive PCR based on two different set of primers specific for the detection and differentiation between *A. ovis* and *A. marginale*.

*A. ovis* and *A. marginale* have been detected not only in their natural hosts, but also in ticks that become persistently infected (Kokan et al., 2010). Wild ruminants act as reservoirs for *A. ovis* and *A. marginale*. (de la Fuente et al., 2005a, 2006, 2008; Krier et al., 1963; Kuttler, 1984; Zaugg, 1987, 1988; Zaugg et al., 1996). Recently, a possible *A. ovis* infection in cattle has been reported (Hornok et al., 2010, 2011), supporting previous experiments conducted by Kuttler (Kuttler, 1981). Little is known about the role of wild ruminants in the epidemiology of *A. ovis* and *A. marginale* (de la Fuente et al., 2005a). However, these results suggest that some hosts can act as reservoirs of both *A. ovis* and *A. marginale*.

Recent works have focused on the presence of pathogens in ticks for epidemiological studies and to develop surveillance strategies. Ticks are the natural vectors of both *A. ovis* and *A. marginale* and some tick species can transmit both of these pathogens (de la Fuente et al., 2005a; Torina et al., 2008). Additionally, recent reports have shown evidence of simultaneous infection with two or more *Anaplasma* spp. in ticks, deer and cattle in different areas of the world (Aubry et al., 2011; de la Fuente et al., 2005a).



These results support the need to differentiate between *A. marginale* and *A. ovis* in domestic animals, wild animals and ticks diagnosed with *Anaplasma* infection.

In summary, the sensitive and species-specific PCRs developed here for the detection and differentiation between *A. ovis* and *A. marginale* have the potentiality to become an easy and fast tool for the diagnosis of *A. ovis* and/or *A. marginale* infection in ticks and vertebrate hosts.

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**Table 1.** PCRs used for pathogen detection

<b>Assay</b>	<b>Primers</b>	<b>Target</b>	<b>Amplicon size (bp)</b>
<i>A. ovis/A. marginale</i> <sup>7</sup>	MSP43 / MSP45	<i>msp4</i>	851
<i>Anaplasma</i> spp. <sup>30</sup>	16SANAF / 16SANAR	16S rRNA	467
<i>A. phagocytophilum</i> <sup>9</sup>	MSP4AP5 / MSP4AP3	<i>msp4</i>	849
<i>A. platys</i> <sup>20</sup>	EPLAT-5 / ERB2	16SrRNA	780
<i>Ehrlichia canis</i> <sup>17</sup>	ECC / ECB	16SrRNA	480
<i>Rickettsia conorii</i> <sup>33</sup>	Tz 15-19/Tz 16-20	17KDa	246
<i>Coxiella burnetii</i> <sup>31</sup>	Q3 / Q5	htpB	501
<i>Babesia ovis</i> <sup>1</sup>	Bbo-F / Bbo-R	ssu rRNA	549
<i>Babesia bovis</i> <sup>16</sup>	BOF/BOR	RAP 1	356
<i>Babesia bigemina</i> <sup>16</sup>	BiIA / BiIB	RAP 1	278
<i>Babesia canis</i> <sup>5</sup>	PIROA / PIROB	ssrRNA	400
<i>Babesia gibsoni</i> <sup>27</sup>	PIRO A1 / Piro B	18SrRNA	450
<i>Babesia caballi</i> <sup>3</sup>	Bc48R3 / Bc48F1	48KDa rhoptry protein	530
<i>Babesia microti</i> <sup>29</sup>	BAB 1 / BAB 4	ssrRNA	238
<i>Theileria equi</i> <sup>3</sup>	EMA5 / EMA6	merozoite antigen 1	268
<i>Theileria annulata</i> <sup>6</sup>	N516 / N517	30KDa	721

## Figures legends

**Figure 1.** AmargMSP4Fw/AmargMSP4Rev primers amplify a specific band of 344 bp only in samples positive to *A. marginale* (lanes from 9 to 16) but not in samples positive to *A. ovis* (lanes from 1 to 8). Lane 17: negative control, lane 18: 100-bp ladder, lane 19: positive control.

**Figure 2.** AovisMSP4Fw/AovisMSP4Rev primers amplify a specific band of 347 only in samples positive to *A. ovis* (lanes from 1 to 8) but not in samples positive to *A. marginale* (lanes from 9 to 16). Lane 17: negative control, lane 18: 100-bp ladder, lane 19: positive control.

**Figure 3.** Sensitivity of the PCR method. Ethidium bromide stained agarose gel electrophoresis of amplification products from 10-fold diluted samples (from 50ng/μl to  $5 \times 10^{-8}$  ng/μl – lanes 1 to 10). Lane N: negative control, Lane M: 100-bp ladder. **(a):** *A. marginale* sensitivity: the value resulted to be 0,5 pg/μl. **(b) :** *A. ovis* sensitivity: the value resulted to be 0,005 pg/μl.

**Figure 1**  
[Click here to download high resolution image](#)

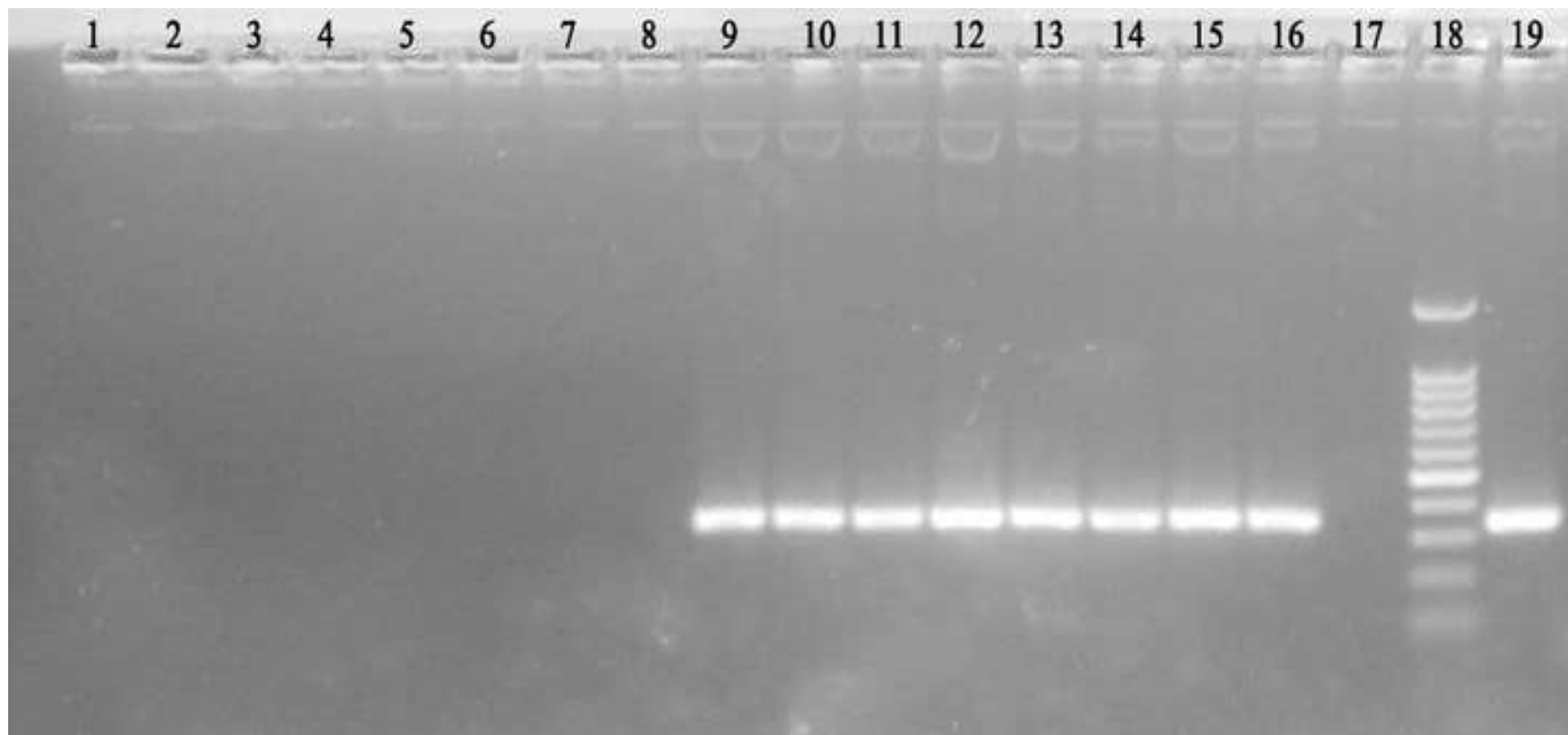


Figure 2  
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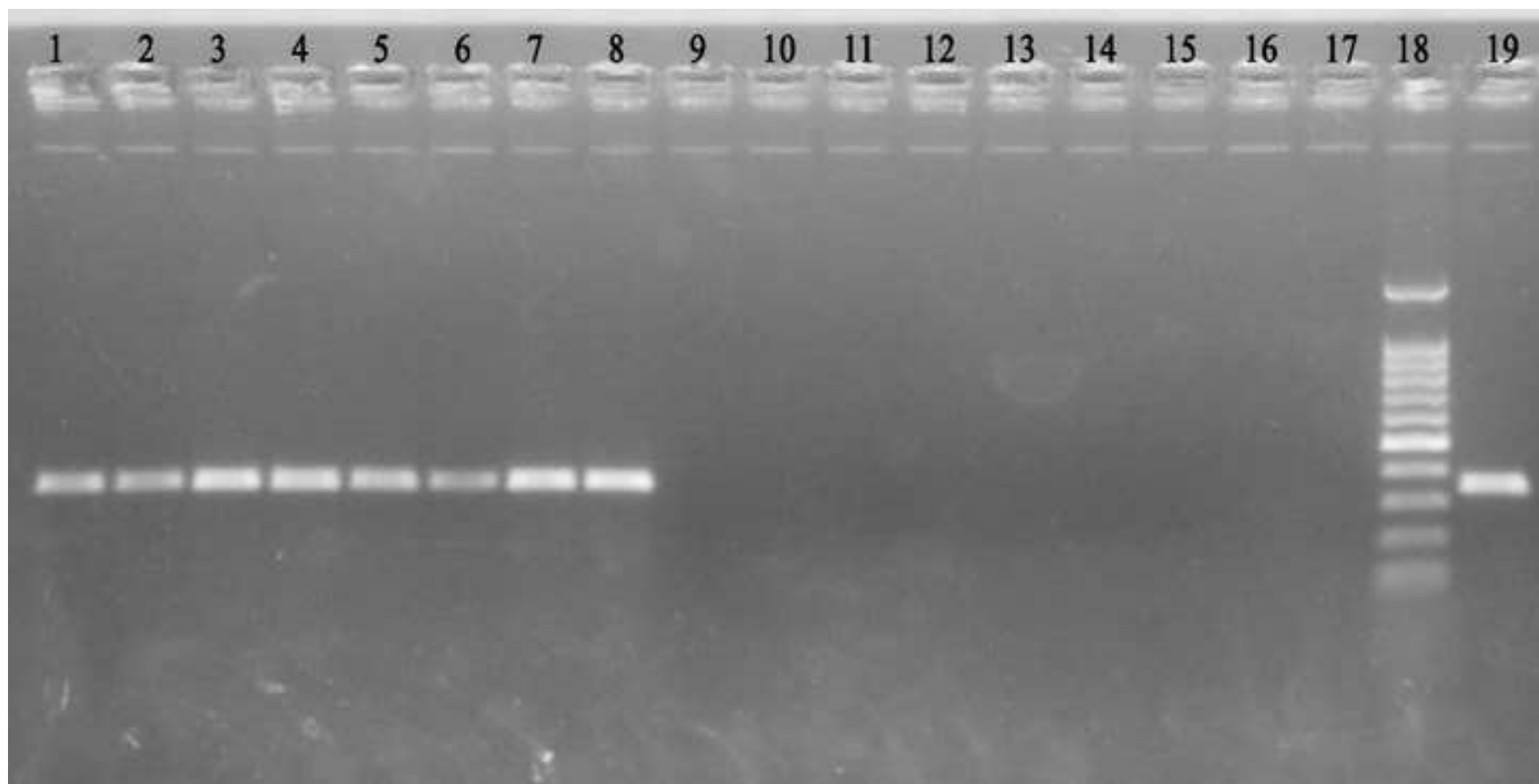
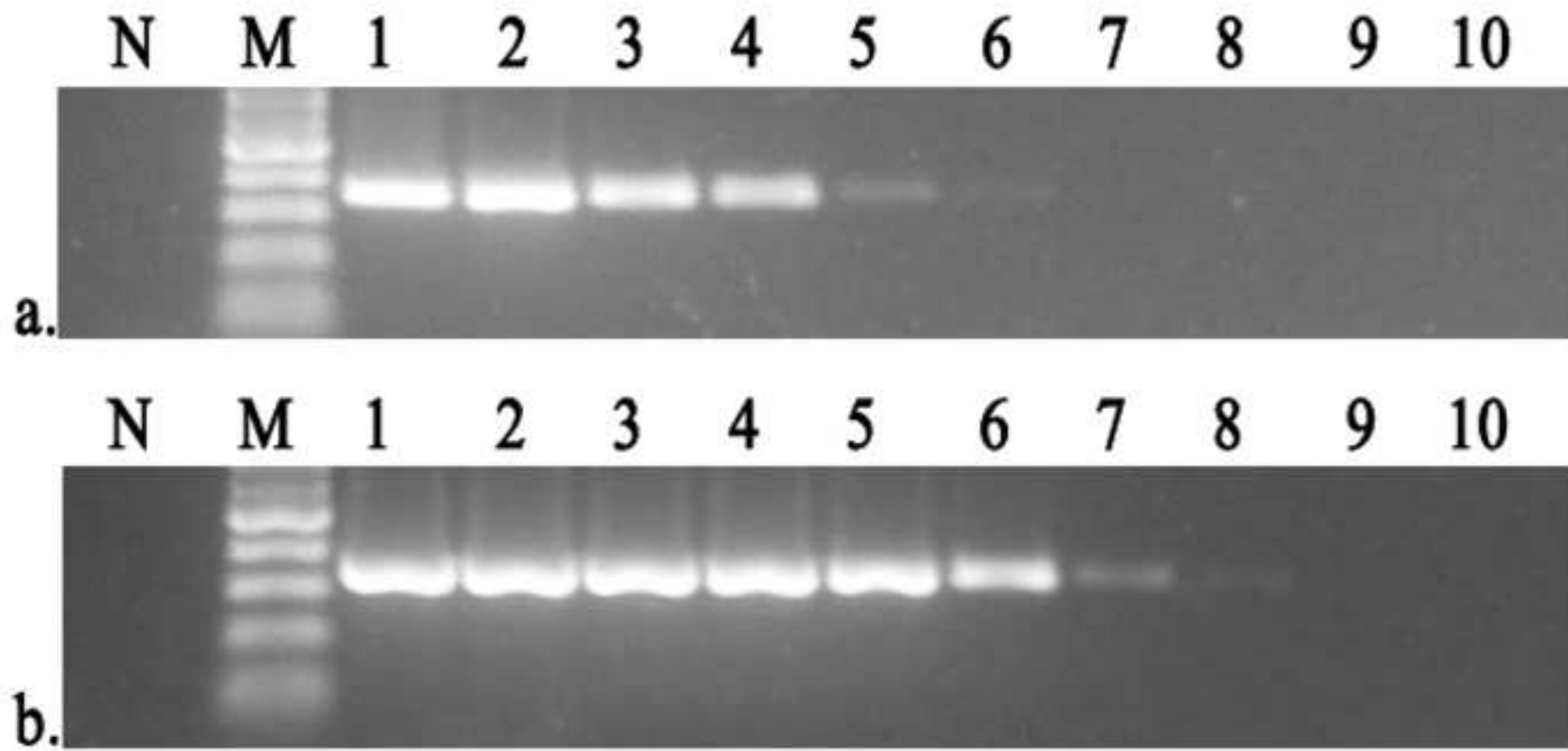


Figure 3  
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Manuscript Number:

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Article Type: Original Research Article

Keywords: Ixodidae, Anaplasma, Ehrlichia, Rickettsia , Coxiella, Italy

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**Abstract:** For the control of tick-borne diseases a deeper knowledge about the incidence and prevalence of the tick species involved in their transmission, as well as their role as pathogens vectors, would be helpful. Pathogens belonging to Anaplasma, Ehrlichia, Rickettsia and Coxiella cause important pathologies in animal and human. In our study we have analysed the ticks to detect the presence of these pathogens, in order to evaluate the tick species mainly involved in the transmission of disease. During the period 2004-2010, 5349 PCRs were performed in tick samples collected from infested animals or questing ticks collected in Sicily. Ticks were tested by specific PCRs for Anaplasma spp, A.marginale, A.phagocytophilum, Ehrlichia spp, Rickettsia spp and C.burnetii. Prevalence was calculated for each pathogen in different tick species. The pathogen Anaplasma spp was found in all the tick species tested; the highest prevalence was calculated in Hy. marginatum (17,7%) followed by Rh. bursa, Rh. turanicus, D. marginatus and I. ricinus (prevalence ranging from 10,2% to 8,2%), while Hy. lusitanicum and Rh. sanguineus had prevalence value less than 2,0%. A. marginale was isolated only from Rh. bursa (3,8%) and Rh. sanguineus (2,8%). Interestingly, A. phagocytophilum, was detected in Hy. marginatum (15,0%) Rh. turanicus (9,4%), Rh.bursa (6,1 %). I. ricinus had a prevalence of 5,7%. Ehrlichia spp showed a highest prevalence in D .marginatus (9,4%). As concerns Rickettsia spp prevalence, D. marginatus (34,4%), Rh. sanguineus (29,9%) and Hy. marginatum (20,8%) were the tick species with highest values. The Rickettsia prevalence for all the other species ranged from 10,3% to 1,6%. Finally, C. burnetii was mostly present in Hy. marginatum (8,9%) and Hae. punctata (6,3%). Taken together, our data support the idea that there's a significant difference in the ability of the tick vector to transmit different pathogens.

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Opposed Reviewers:



**Prevalence of *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Coxiella* in different tick species**

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Running title: *Prevalence of tick borne pathogens in Sicily*

**Abstract**



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For the control of tick-borne diseases a deeper knowledge about the incidence and prevalence of the tick species involved in their transmission, as well as their role as pathogens vectors, would be helpful. Pathogens belonging to *Anaplasma*, *Ehrlichia*, *Rickettsia* and *Coxiella* cause important pathologies in animal and human. In our study we have analysed the ticks to detect the presence of these pathogens, in order to evaluate the tick species mainly involved in the transmission of disease. During the period 2004-2010, 5349 PCRs were performed in tick samples collected from infested animals or questing ticks collected in Sicily. Ticks were tested by specific PCRs for *Anaplasma* spp, *A.marginale*, *A.phagocytophilum*, *Ehrlichia* spp, *Rickettsia* spp and *C.burnetii*. Prevalence was calculated for each pathogen in different tick species. The pathogen *Anaplasma* spp was found in all the tick species tested; the highest prevalence was calculated in *Hy. marginatum* (17,7%) followed by *Rh. bursa*, *Rh. turanicus*, *D. marginatus* and *I. ricinus* (prevalence ranging from 10,2% to 8,2%), while *Hy. lusitanicum* and *Rh. sanguineus* had prevalence value less than 2,0%. *A. marginale* was isolated only from *Rh. bursa* (3,8%) and *Rh. sanguineus* (2,8%). Interestingly, *A. phagocytophilum*, was detected in *Hy. marginatum* (15,0%) *Rh. turanicus* (9,4%), *Rh.bursa* (6,1 %). *I. ricinus* had a prevalence of 5,7%. *Ehrlichia* spp showed a highest prevalence in *D .marginatus* (9,4%). As concerns *Rickettsia* spp prevalence, *D. marginatus* (34,4%), *Rh. sanguineus* (29,9%) and *Hy. marginatum* (20,8%) were the tick species with highest values. The *Rickettsia* prevalence for all the other species ranged from 10,3% to 1,6%. Finally, *C. burneti* was mostly present in *Hy. marginatum* (8,9%) and *Hae. punctata* (6,3%). Taken together, our data support the idea that there's a significant difference in the ability of the tick vector to transmit different pathogens.

**Keywords:** Ixodidae, *Anaplasma*, *Ehrlichia*, *Rickettsia* , *Coxiella*, Italy

## Introduction

1  
2 Ticks (Acari: Ixodidae) are important parasites present in almost every geographic zone  
3  
4 in the world. Ticks are vectors of (viral, bacterial, and protozoal) pathogens that cause  
5  
6 disease in humans and animals worldwide (de la Fuente et al., 2008). The deep  
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8 knowledge of the incidence and prevalence of the tick species involved in the  
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10 transmission, as well as their geographical distribution, would be very important for the  
11  
12 control of tick-borne diseases (TBDs). Several species of ticks are indigenous in Italy  
13  
14 and have been reported to infect companion and production animals as well as humans.  
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18 A number of Spotted fever group Rickettsiae is associated with ticks and these  
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20 pathogens can be transmitted both in trans-ovarian and trans-stadial way. The main  
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22 vectors are ticks belonging to *Dermacentor*, *Rhipicephalus*, *Haemaphysalis* and *Ixodes*  
23  
24 genera.  
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28 Although Mediterranean spotted fever (MSF) due to *Rickettsia conorii* was thought for  
29  
30 a long time to be the only tick-borne rickettsial disease present in Europe, five more  
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32 spotted fever rickettsiae have been described as emerging pathogens in the last decade  
33  
34 (Parola et al., 2004). Sicily is the Italian region with a high number of TBDs cases,  
35  
36 probably due to particular climatic aspects. TBDs diagnosed in Sicily are mainly  
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38 theileriosis, anaplasmosis, babesiosis, and other rickettsioses, besides bacterial and virus  
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40 diseases (Caracappa et al., 1999). Every year, several outbreaks due to *Theileria*,  
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42 *Babesia* or *Anaplasma* infections in cattle are recorded. Imported breeds are the most  
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44 susceptible.  
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51 Sicily represents a typical Mediterranean ecosystem to study tick infestations and the  
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53 prevalence of endemic tick-borne pathogens (Torina et al., 2008a). Several studies have  
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55 characterized the prevalence of tick-borne pathogens in vertebrate hosts in Sicily (de la  
56  
57 Fuente et al., 2005a,b, 2006; Torina et al., 2007, 2008b). However, limited data are  
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1 available on the presence and prevalence of these pathogens in ticks (de la Fuente et al.,  
2 2005a; Torina et al., 2008b, 2010).  
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4 The aim of the study was to analyze tick species and pathogen interactions present in  
5 Sicily. In this study, we have analyzed ticks collected in Sicily in the last seven years.  
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## 10 **Materials and methods**

11 Ticks were collected in Sicily, during the period 2004-2010 by dragging vegetation or  
12 directly removed from hosts. A total number of 5349 PCR samples were performed in ticks  
13 samples collected from infested animals (3703) or questing ticks (1646). All specimens  
14 were identified by morphological characters using standard taxonomic keys for Italian  
15 Ixodidae (Manilla, 1998) and kept alive for at least one week. It was previously  
16 demonstrated that ticks are able to clean the organisms by day 4 after being removed  
17 from a highly parasitized host (de la Fuente et al., 2001), ticks were thus disinfected and  
18 stored in 70% ethanol until rinsed with distilled water, and cut in sterile tubes prior to  
19 the processing. DNA from ticks was extracted using TriReagent (Sigma, St. Louis, MO,  
20 USA) following manufacturers recommendations. The DNA was resuspended in sterile  
21 distilled water and stored at -20°C until used.  
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41 PCR with specific primer sets were used for detection of *Anaplasma* spp (Stuenkel S,  
42 2003), *A. marginale* (de la Fuente J, 2003), *A. phagocytophilum* (de la Fuente J, 2005c),  
43 *Rickettsia* spp. (Tzianabos T, 1989), *Ehrlichia* sp (Ulrike G, 1996) and *Coxiella burnetii*  
44 (To H, 1996) as previously described with 0.1–10 ng of DNA using 10 pmol of each  
45 primer and the Ready-To-Go PCR beads (Amersham, Piscataway, NJ, USA).  
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51 Reactions were performed in an automated DNA thermal cycler for 35 cycles. The PCR  
52 products were analyzed in 1% agarose gels to check the size of amplified fragments.  
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58 Control reactions were performed without the addition of DNA in the reaction to rule  
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1 out contaminations during PCR. Positive PCR products for *A.marginale* were sent to  
2 Macrogen Inc. (Seoul, Korea) to confirm by sequencing the specificity of the reactions.

3  
4 Prevalence was calculated for each pathogen in different tick species. Data reported  
5  
6 were referred to at least fifty ticks tested for each pathogen.  
7

## 8 9 **Results**

10 After the identification step, ticks were classified as follows: *Rhipicephalus (Boophilus)*  
11  
12 *annulatus*, *Dermacentor marginatus*, *Haemaphysalis sulcata*, *Hae. punctata*, *Hyalomma*  
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14 *lusitanicum*, *Hy. marginatum*, *Ixodes ricinus*, *Rhipicephalus turanicus*, *Rh. bursa*, *Rh.*  
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16 *sanguineus*. In Figure 1 are reported the tick species and pathogen interactions for  
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18 *Anaplasma*, *Ehrlichia*, and *Rickettsia* species and for *Coxiella burnetii*.  
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21  
22 The pathogen *Anaplasma* spp was found in all the ticks species tested, the highest  
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24 prevalence was calculated in *Hy. marginatum* (17,7%) followed by *Rh. bursa*, *Rh.*  
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26 *turanicus*, *D. marginatus* and *I. ricinus* (prevalence ranging from 10,2% to 8,2%), while  
27  
28 *Hy. lusitanicum* and *Rh. sanguineus* had prevalence value less than 2,0%. *A. marginale*  
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30 was isolated only from *Rh. bursa* (3,8%) and *Rh. sanguineus* (2,8%). Interestingly, *A.*  
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32 *phagocytophilum*, was detected in *Hy. marginatum* (15,0%) *Rh. turanicus* (9,4%), *Rh.*  
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34 *bursa* (6,1 %). *I. ricinus* had a prevalence of 5,7%. *Ehrlichia* spp showed a highest  
35  
36 prevalence in *D. marginatus* (9,4%). As concerns *Rickettsia* spp prevalence, *D.*  
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38 *marginatus* (34,4%), *Rh. sanguineus* (29,9%) and *Hy. marginatum* (20,8%) were the  
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40 tick species with highest values. The *Rickettsia* prevalence for all the other species  
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42 ranged from 10,3% to 1,6%. Finally, *C. burnetii* was mainly present in *Hy. marginatum*  
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44 (8,9%) and *Hae. punctata* (6,3%).  
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## Discussion

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2 During the last decade several studies on tick infesting animals have been carried out in  
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4 Italy (Garippa and Sanna 1990; Di Todaro et al. 1999; Genchi and Manfredi, 1999;  
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6 Georges et al. 2001; Cringoli et al. 2002; Beninati et al. 2004, 2005; Rinaldi et al.  
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8 2004). In most cases, the above mentioned studies are related to occasional tick  
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10 collections, i.e. 2–3 moth periods, and/or to one host only.  
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14 Our study was conducted on ticks collected in Sicily, especially in the area near  
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16 Palermo, where MSF, mainly due to *R. conorii*, has been endemic for long time.  
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19 In a previous study, the clinical data of 176 MSF patients in a period ranging from 1996  
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21 to 1999 were analysed (Picciotto et al., 2000). It was showed that an occupational risk  
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23 exists among small farmers, but that there were also environmental risks for subjects  
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25 living in rural areas due to contact with infected dogs or other animals. In Sicilian ticks  
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27 (n.150) collected in 2001 and 2002 on livestock (Beninati et al. 2004, Beninati et al.  
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29 2005) and analysed by polymerase chain reaction (PCR), was evidenced the presence of  
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31 *R. slovaca* in *D. marginatus* and in *Hae. punctata* and *R. aeschlimannii* and *R. africae*  
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33 in *Hy. marginatum*.  
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38 The pathogenic role of *R. slovaca* in humans has been shown in France (Raoult et al.,  
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40 2002; Cazorla et al., 2003). It could be thus argued that our tick investigation has been  
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42 an important stimulus to implement studies for better understanding human TBDs  
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44 transmission in Sicily.  
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48 The tick species identified in this study have been previously associated with pathogen  
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50 transmission. Recent surveys carried out in Sicily on haemoparasites transmitted by  
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52 ticks to cattle have shown a very high prevalence of mixed infections: *Babesia* spp.  
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54 and/or *Theileria* spp. and/or *Anaplasma* spp. (Loria et al. 1999; Greco et al. 2000;  
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56 Sparagano et al., 2000; Georges et al.. 2001) or *Anaplasma*, *Ehrlichia* and *Rickettsia*  
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1 spp. were detected in *H. marginatum* ticks collected from cattle (Torina et al., 2010).

2 Some species sampled can also transmit *Rickettsia* to humans and animals. In fact, *R.*  
3 *conorii*, causative agent in Europe of Mediterranean Spotted Fever (MSF), was detected  
4  
5 in *R. sanguineus*, but also in *R. turanicus* and in *D. marginatus* (Torina et al 2010). *R.*  
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7 *slovaca*, responsible for the human disease known as tick-borne lymphadenopathy is  
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9 found in *D. marginatus* and other spotted fever group rickettsiae are present in *H.*  
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11 *marginatum* (Beninati et al. 2005).  
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16 Ticks in Sicily play an important role in the transmission of vector-borne diseases for  
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18 humans and animals.  
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21 We confirmed that *Rickettsia* infection is common in ticks found in Sicily. In fact, the  
22  
23 presence of *Rickettsia* was detected in all the eight tick species found in the area, and  
24  
25 that *D. marginatus* could play a relevant role in *Rickettsia* transmission rather than *R.*  
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27 *sanguineus* that shows high prevalence but is a less anthropophilic tick.  
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31 The high prevalence in the ticks species tested for *Anaplasma* and *Rickettsia* pathogens  
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33 could be due to the high number of pathogens belonging to the two genera circulating in  
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35 Sicily while *Coxiella Burnetii* and *Ehrlichia* have lower prevalence.  
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39 The characterization of tick-borne pathogens in ticks suggested differences in the role  
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41 that different tick species play in the pathogen life cycle and transmission. This  
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43 information is important for epidemiological studies of tick-borne pathogens in Sicily  
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45 and to evaluate the risks associated with pathogen transmission to humans and animals.  
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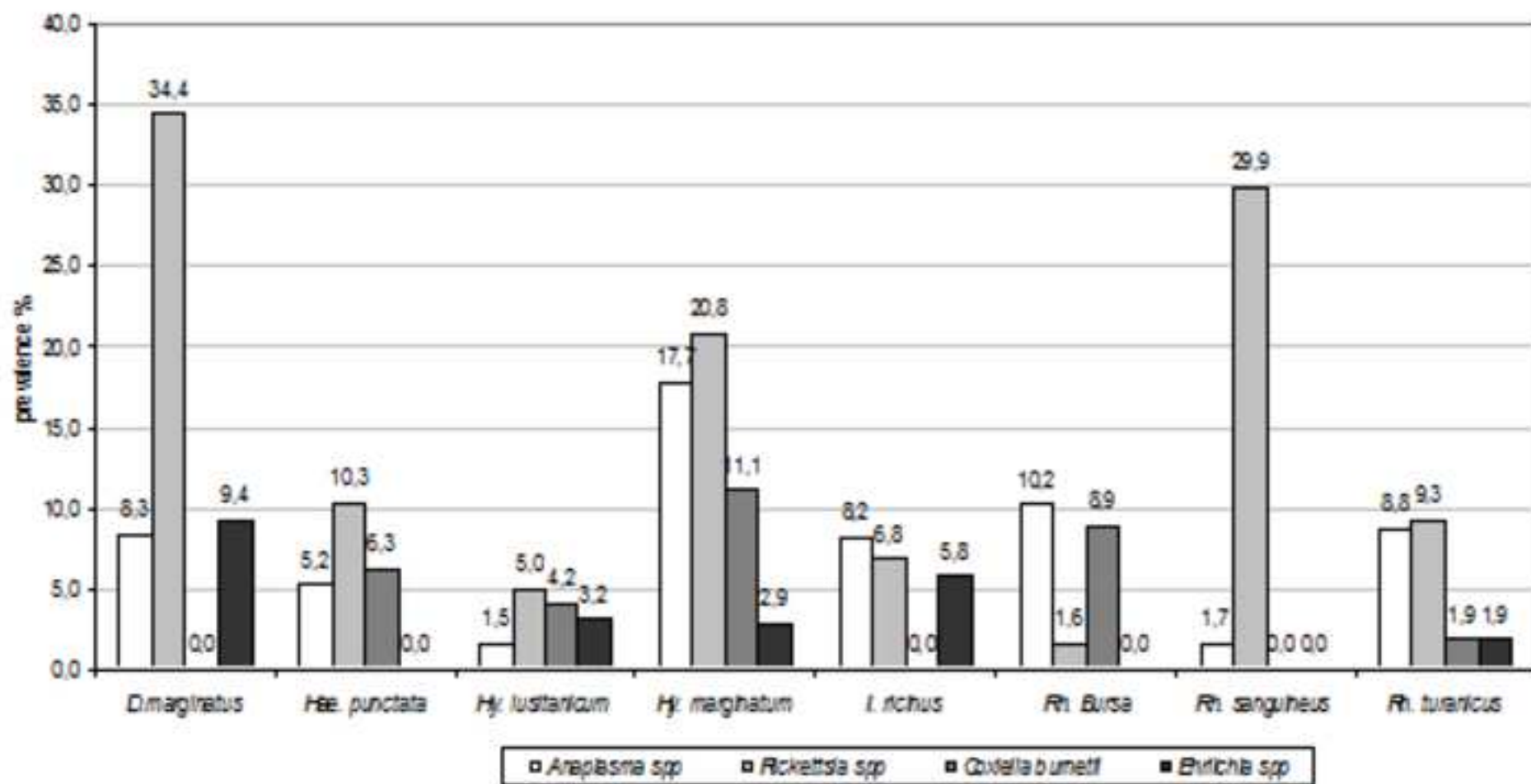
## Legend to Figures

**Figure 1.** Tick species identified in Sicily from year 2004 to 2010 and detection of *Anaplasma*, *Ehrlichia*, and *Rickettsia* species and *Coxiella burnetii* expressed in prevalence.

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Figure

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## ORIGINAL ARTICLE

## Characterization of the Apical Membrane Antigen-1 in Italian Strains of *Babesia bigemina*

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**Keywords:**

apical membrane antigen-1; *Babesia bigemina*; vaccine candidates

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**Summary**

*Babesia bigemina* is a parasite endemic in different parts of the world, including Europe and the Americas. One of the few genes characterized in this species codifies for the Apical Membrane Antigen 1 (AMA-1), a trans-membrane antigen recently identified. In this research, we characterized the *ama-1* gene from three Italian *B. bigemina* strains, two *B. bigemina* strains obtained from Ragusa, Sicily (ITA1 and ITA3) and a third one obtained from Benevento, Campania (ITA2). Italian sequences were compared with those of the Australian strain obtained from the Sanger Institute web site and to strains from different parts of the world. The results obtained confirmed that this newly described *ama-1* gene is highly conserved among Italian and foreign strains which has implications for vaccine development.

**Introduction**

Babesiosis is one of the most common infections of free-living animals worldwide. The disease is caused by intraerythrocytic parasites of the genus *Babesia*. *Babesia bigemina* is a cattle pathogen that is transmitted by the tick vector, *Rhipicephalus* (*Boophilus*) spp. Babesiosis is one of the major causes of economic losses in the cattle industry in the tropical and subtropical countries.

Although an attenuated strain of the parasite is still used as a vaccine in some countries of the world, this strain poses risk of reverting back to the wild type, and thus causing outbreaks of babesiosis. Therefore, this research is focused on molecular aspects of *B. bigemina* to identify and study the surface molecules that could be used to induce a protective immune response in cattle and could be incorporated into a subunit vaccine for control of babesiosis.

The design of anti-babesial vaccines has been hampered by extensive polymorphisms in some parasite proteins, particularly those expressed on the surface of the parasite. Many identified or suspected molecules may be involved in the erythrocyte invasion and may be targets for the development of vaccines, but few are characterized in *B. bigemina*. One of these recently identified invasion molecules, apical membrane antigen 1 (AMA-1), is a trans-membrane antigen.

The most characterized AMA-1 protein is the one codified by *Plasmodium falciparum* (PfAMA; Peterson et al., 1989). This protein has been extensively studied because, among the few anti-malarial vaccine candidates under development, it was one of the most promising erythrocyte stage candidates (Latitha et al., 2008). PfAMA-1 is a protein of 622 residues and a molecular weight of 83 kDa and three major domains defined by eight disulphide bonds (Escalante et al., 2001; Deans et al., 1988;

Hodder et al., 1996). In 2004, Eric de Vries et al. (reviewed by Gaffar et al., 2004) identified and characterized the *B. bovis* AMA-1 protein (BbAMA-1). They reported that the 62.2 kDa protein aligned with the full-length sequence of *P. falciparum*, *Plasmodium vivax* and *Toxoplasma gondii*, and had structural features similar to those of other AMA-1 proteins. As has been the case with several other Apicomplexa, AMA-1 is thought to play a crucial role in the invasion of erythrocytes by *Babesia bigemina* parasite (Yokoyama et al., 2006).

This study reports on features of *B. bigemina* AMA-1 gene from Italian strains of the parasite, and provides predictive information obtained using bioinformatics tools related to the protein.

## Materials and Methods

### Research on putative *B. bigemina* antigens from closely related microorganisms

The research is based on the data from the related microorganisms, *Plasmodium* and *B. bovis*, as well as on the valuable tool available in the Sanger Institute web site which provides published raw data derived from the *B. bigemina* whole-genome sequencing project. Through this tool, it was possible to compare genes of related organisms with the *B. bigemina* genome, thus allowing for identification of conserved sequences. In this manner, a region corresponding to AMA gene was identified in *B. bigemina*.

This region was submitted to two different software: ORF Finder (available at <http://www.ncbi.nlm.nih.gov/projects/gorf/>) and ORF Finder (available at [http://www.bioinformatics.org/sms/orf\\_find.html](http://www.bioinformatics.org/sms/orf_find.html)) to confirm that the sequence was a complete ORF without stop codons in frame. Once detected, the correct start and stop codons were determined and amplification was performed.

### Blood and spleen DNA extraction

Genomic DNA from *B. bigemina* naturally infected calves was extracted using the Sigma GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich Inc., St Louis, MO, USA), according to the manufacturer's instructions. Out of three samples, which resulted positive at the diagnostics PCR (Figuroa et al., 1993), two were obtained from whole blood, and one from a spleen-extracted *post-mortem* by a bovine died of Babesiosis.

### Amplification of the gene codifying for the surface antigen

The primers for the amplification of AMA were designed using the available sequence in the Sanger Institute web site (<http://www.sanger.ac.uk/cgi-bin/blast/submitblast/>

*b\_bigemina*). The primers were designed in such a way that the amplified region contained the entire ORF (Mosqueda et al., 2008).

### Cloning of the PCR products and sequence analysis

Purified PCR products were cloned in Invitrogen pCR2.1 TOPO TA cloning vector (Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's instructions.

Six positive colonies were chosen, from which plasmid DNA was extracted using the Promega Wizard SV Minipreps DNA purification system (Promega Inc., Madison, WI, USA). Plasmids obtained in such a way were restricted with *EcoRI*, quantitated and sent to Macrogen Inc. (Seoul, Korea) for sequencing. Sequencing was performed in the forward and reverse direction, using M13F and M13R universal primers. It was necessary to adopt the technique of primer walking because the fragment was too long to be sequenced.

The sequences were then aligned using the software ClustalW2.0.10 (Larkin et al., 2007) and Bioedit (Tom Hall Ibis Biosciences, Carlsbad, CA, USA).

The software, MEGA (Kumar et al., 2008; Tamura et al., 2007) and DAMBE (Xia and Xie, 2001; Xia, 2000), was used to calculate the percentage of similarity among each of the analyzed sequences. All results are based on the pairwise analysis of the sequences. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4. To obtain the main predictive features of the protein, the sequence was submitted to EXPASY proteomic server (Gasteiger et al., 2003).

Each aminoacidic sequence was analyzed to seek the sequence of possible signal peptides using the SignalP software (Center for Biological Sequence Analysis, University of Denmark, Copenhagen, Denmark), the presence of possible trans-membrane helices using TMHMM software (Center for Biological Sequence Analysis), putative B-cell epitopes (Predicting Antigenic Peptides, available at <http://bio.dfci.harvard.edu/Tools/antigenic.pl>) and hypothetical disulphide bonds (DiANNA 1.1 web server – Ferre and Clote, 2005a,b).

## Results

The whole coding region of the AMA-1 gene was amplified and sequenced from three Italian strains of *Babesia bigemina*. The sequences were submitted to GenBank with accession numbers GQ257738, GQ257739 and GQ257740.

The full-length AMA-1 gene of 1788 bp encodes a 595 amino acids product similar to the 622 aminoacids AMA-1 product of *P. falciparum* and to the 605 aminoacids AMA-1 product of *B. bovis*. The molecular weight of the Italian strain protein results 65.9 kDa (EXPASY).



Comparison of *Babesia bigemina* AMA-1 to the *B. bovis* and *P. falciparum* AMA-1 proteins

A consensus sequence was obtained by the comparison of the three Italian sequences that resulted in a high degree of identity. To verify the degree of conservation of AMA-1 protein among different Apicomplexa, the amino-acidic sequence of *B. bigemina* AMA-1 was compared with those of *B. bovis* and *P. falciparum*.

The percentage of similarity of *B. bigemina* AMA-1 with *P. falciparum* AMA-1 was low (28.12%; ClustalW2.0.10), but some of its features are typical of AMA-1 family members, among which the TM helix, the short-cytoplasmic tail, and a putative structural/functional conservation. The functional homology between PfAMA and *B. bigemina* AMA-1 protein was strongly supported by the results obtained using EPipe Server (Tress et al., 2007) and

P-Fam (Finn et al., 2008) that included the protein in AMA-1 family. The hydrophobic N-terminal domain of *B. bigemina* AMA-1 protein was predicted to form a signal peptide (SignalP) of 30 amino acids, that can be found also in PfAMA-1 (24 a.a.) and BbAMA-1 (39 a.a.).

A bioinformatic study was conducted using DiANNA 1.1 web server (Ferre and Clote, 2005a,b) to predict hypothetical disulphide bonds occurring in the Italian strain. The results showed 16 cysteine residues putatively involved in disulphide bridge formation. The multi-sequence alignment (ClustalW2.0.10) of *B. bigemina*, *B. bovis* and *P. falciparum* AMA-1 proteins (Fig. 1) demonstrated that most of the cysteine residues forming disulphide bonds in *P. falciparum* AMA-1 (13 of 16) are conserved in the three organisms which strongly supports the hypothesis of a structural and functional homology among AMA-1 proteins.



Fig. 1. Multiple-sequence alignment of AMA-1 proteins of *B. bigemina* (BbigAMA), *B. bovis* (BbovAMA) and *Plasmodium falciparum* (PfAMA). "\*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, ":" means that semi-conserved substitutions have been observed. Cysteine residues that form disulfide bonds in *P. falciparum* AMA-1 are marked and coupled by numbers.

### Comparison of Italian *B. bigemina* AMA-1 sequences with the Australian strain

The Italian strains sequences were compared with the corresponding Australian strain sequence. Because of the complete sequence genome, the Australian strain is presently considered as a reference strain. From the three sequences analyzed, the variation in the AMA-1 gene, at the nucleotide level, shows point mutation at 54 positions along the length of the gene. Out of these, 36 are synonymous mutations and 18 are non-synonymous changes. Fifteen mutations occur at the first base of codon, 4 at the second base and 35 at the third base of the codon.

Analysis of the amino acid substitutions in the AMA-1 protein revealed a total of 18 amino acid changes across the length of AMA-1 protein. The analysis of the amino-acidic sequence of the protein by means of TMHMM software, revealed the presence of a TM helix between amino acids 513 and 531 near the C-terminus. The helix divides the extracellular portion of the protein (amino acids 1–512) from the predicted short cytoplasmic C-terminal tail (532–595). Out of the 54 mutations, the most of them (51) occurs in the extracellular part of the protein, two occur in the cytoplasmic tail, while the trans-membrane region has only a synonymous mutation.

The average percentage of identity among the Italian and the Australian strains was calculated to be 99.984%, whereas among Italian sequences only is 99.986%.

### Comparison of *B. bigemina* AMA-1 sequences among Italian and foreign strains

The comparison of the sequence of *B. bigemina* AMA-1 gene in the Italian strains to those coming from other Countries (Mexico-personal communication- and Argentina – GenBank AB481200) and to that one from a strain provided by VMRD Inc. (Pullman, WA, USA; lot V02064) demonstrated a high degree of similarity. The overall average of identity at the nucleotide level resulted 99.98%. The overall average of homology at the amino-acidic level was 99.99% (data not shown).

### Prediction of antigenic peptides

The 565-aminoacid sequence of the Italian mature protein (consensus sequence) was submitted to the Predicting Antigenic Peptides software to search for B-cells epitopes. Nineteen different peptides were identified by the software. Of these, 14 were conserved among Italian and Australian strains (data not shown), but the position of the peptides in the quaternary structure of the protein, their antigenicity and their real efficiency to induce protection against challenge, require further investigation.

### Discussion

The AMA-1 protein is an apically located protein that is shared by many apicomplexan organisms and that has a role in the host red blood cells invasion process. The AMA-1 family members exhibit significant common features and have been studied extensively to understand whether they could be considered as antigens to be included in a subunit vaccine. Studies based on synthetic peptides of PfAMA-1 have indicated that they elicit a specific immune response in humans naturally exposed to malaria parasites (Lal et al., 1996).

Antisera directed against BbAMA-1-derived peptides specifically reduced the *in vitro* invasion efficiency of *B. bovis*, indicating that AMA-1 is indeed located on the surface of merozoites and is accessible to antibodies (Gaffar et al., 2004).

*Babesia bigemina* AMA-1 protein, a newly identified surface antigen, has several common features with other AMA-1 family members and exhibits also a high degree of similarity with the orthologue gene of *B. bovis*. A comparison among Italian strains of *B. bigemina* demonstrated a high degree of both nucleotide and aminoacidic identity. Moreover, several putative antigenic peptides were detected and they could be used for investigation of the recognition by specific antibodies. These preliminary data provide basis for further study of the *B. bigemina* AMA-1 antigen and its possible inclusion in a subunit vaccine for control of bovine babesiosis.

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