Università degli Studi di Palermo

and

The Wenner-Gren Institute

INTERNATIONAL PhD COURSE IN IMMUNOPHARMACOLOGY

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

PhD student:

Annalisa Agnone

Dean:

Francesco Dieli, Professor

Tutor:

Guido Sireci, Professor

Co-tutor:

Carmen Fernández, Professor

Funded by: Istituto Zooprofilattico Sperimentale della Sicilia

Ciclo XXIII – SSD MED/04 - a.a. 2008-09

Table of contents

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[1]. 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[2]. Mollicutes phylogeny has been controversial for a long time. Thanks to the 16S rRNA sequence data Woese and co-workers[3] 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: *Mycoplasmatales*						
Family I: *Mycoplasmataceae*						
Genus I: *Mycoplasma*	102	580–1,350	23–40	Yes	Optimum growth at 37°C	Humans, animals
Genus II: *Ureaplasma*	6	760–1,170	27–30	Yes	Urea hydrolysis	Humans, animals
Order II: *Entomoplasmatales*						
Family I: *Entomoplasmataceae*						
Genus I: *Entomoplasma*	5	790–1,140	27–29	Yes	Optimum growth at 30°C	Insects, plants
Genus II: *Mesoplasma*	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: *Spiroplasmataceae*						
Genus I: *Spiroplasma*	33	780–2,220	24–31	Yes	Helical motile filaments; optimum growth at 30–37°C	Insects, plants
Order III *Acholeplasmatales*						
Family I: *Acholeplasmataceae*						
Genus: *Acholeplasma*	13	1,500–1,650	26–36	No	Optimum growth at 30–37°C	Animals, some plants, insects
Order IV: *Anaeroplasmatales*						
Family: *Anaeroplasmataceae*						
Genus I: *Anaeroplasma*	4	1,500–1,600	29–34	Yes	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Genus II: *Asteroleplasma*	1	1,500	40	No	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Undefined taxonomic status						
Phytoplasma	ND[b]	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[4], 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[4].

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"[1], 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*[5]. 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*[6], *M. pneumoniae*[7], *M. genitalium*[8], *M. gallisepticum*[9] and *M. synoviae*[10] 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[1]. For example, *M. penetrans* invasion of HeLa cells depends on the capacity of the cells to assemble actin microfilaments[11], and the entry of *M. gallisepticum* into the chicken embryo fibroblast is inhibited by the microtubule inhibitor nocodazole[9]. 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 α (TNF-α), interleukin 6 (IL-6) and IL-1][4,12]. 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-1β by mycoplasmas involves, unlike that by LPS, posttranscriptional events[12].

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)[13]. 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*[14] 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[12], 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.

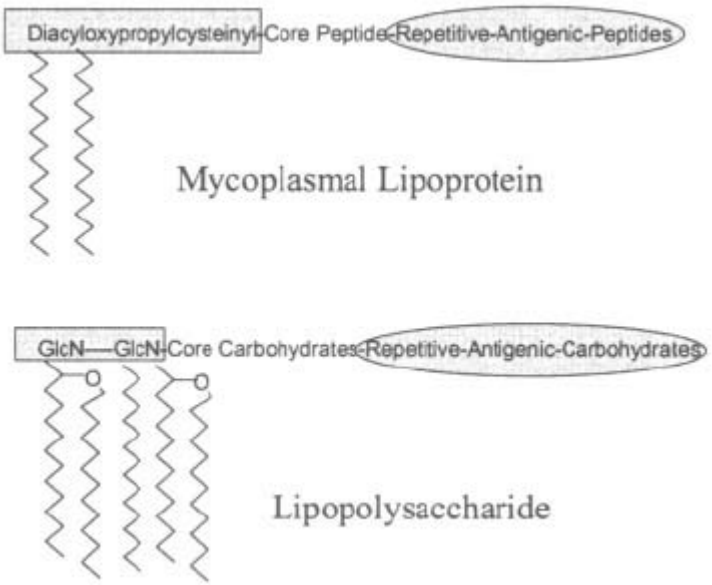


Figure 2: Structural similarities between Mycoplasmal lipoprotein and bacterial lipopolysaccharide. From Razin S, Herrmann R (2002), Molecular biology and pathogenicity of mycoplasmas, p. 457.

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 TNFα, IL-1, IL-6, nitric oxide and

oxygen radicals[15]. Thus proinflammatory cytokines, chemokines and prostaglandins[16,17] 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[18,19]

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[20]. 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[21]. 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[22]. Recently, the over expression of MHC class II molecules was also related to catarrhal bronchointerstitial pneumonia caused by *M. bovis* in cattle[23].

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 agalatia 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[24].

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[25]. *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[26].

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[27]. *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[27].

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[27,2]. They are represented as orthologues in other *Mycoplasma* species and it was hypothesized that they are related in adhesion to host cells[28], but the correlation between expression of variable surface proteins and adherence rates still seems controversial[29]. 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[30]. 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*[31], 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[32]. 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[33,34], 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*[35], and confirmed in our experience[36], the presence of antibodies is not detectable by serology until 25-30 days. In 2007 de la Fe and *et al*[37] 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* [38,39] 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[40,41]. In the study of the immune response elicited against *M. agalactiae* antigen, the attention was focused both in the memory cell populations ($CD45R0^+$– T lymphocytes), whose expansion was considered a consequence of the vaccination, and in the naïve cell populations ($CD45RA^+$ – T lymphocytes) that could be considered the reservoir of memory cells[42].

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[43,44,45,46,47].

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[48]. 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[49], 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[48], 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[50,51]. Nevertheless the real efficacy of currently circulating products (inactivated monovalent vaccines) is still doubtful in field conditions[37]. 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[52]), 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[,53,46,48], 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[54]. 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-γ 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-γ 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[36]. 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 1×10^6 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[37,48]. 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-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep

The expansion of antigen-specific IFN-γ positive lymphocytes was evaluated. It was initially sustained by $CD4^+$ T cells, that play a role around 15 days after infection. In a later phase of infection, CD8/IFN-γ double positive cells increase. γδ T-cells were not expanded at any analyzed time point. $IFN\gamma^+$ T cells disappear 60 days after infection, suggesting that antigen specific $IFN\gamma^+$ 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)[55]. The differences of pathogen excretion thought 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[56]; memory CD4+ Interferon-γ^+ T cells increased in the groups protected by these vaccines ($p<0.05$ when compared to unprotected groups). On the contrary, memory $CD8^+$ Interferon-γ^+ T cells increased in non-protected animals ($p<0.05$). $\gamma\delta^+$ Interferon-γ^+ 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 $CD4^+$ Interferon-γ^+ lymphocytes could induce protection from contagious agalactiae also activating other effector immune mechanisms such as antibody response and/or

antibody dependent cell cytotoxicity. $CD8^+CD45RO^+$ Interferon-γ^+ 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 $\gamma\delta^+$ T cells. Taken together, our results suggest that antigen-specific Interferon-γ^+ 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 $CD4^+$-IFN-γ^+ T-cells, followed by a later expansion of $CD8^+$-IFN-γ^+ T-cells, 30 days post infection.

- The immune monitoring of vaccinated and infected sheep suggests that timing of activation of $CD4^+$-IFN-γ^+ memory cells is a key step for the successful protection against *M. agalactiae*. Hereby we propone that $CD4^+$-IFN-γ^+-$CD45R0^+$ 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-γ 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.

References

[1] Razin S, Herrmann R (2002) Molecular biology and pathogenicity of mycoplasmas: Kluwer Academic/Plenum.

[2] Laurent-Xavier Nouvel MM, Pascal Sirand-Pugnet, Eveline Sagné, Michelle Glew, Sophie Mangenot, Valérie Barbe, Aurélien Barré, Stéphane Claverol, and Christine Citti (2009) Occurrence, Plasticity, and Evolution of the vpma Gene Family, a Genetic System Devoted to High-Frequency Surface Variation in Mycoplasma agalactiae. Journal of bacteriology 191: 10.

[3] Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Young, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of Mycoplasmas: basis for their classifycation. J. Bacteriol. 171:6455-6467.

[4] Razin S, Yogev D, Naot Y.Microbiol Molecular biology and pathogenicity of mycoplasmas. Mol Biol Rev. 1998 Dec;62(4):1094-1156.

[5] Lo SC, Hayes MM, Kotani H, Pierce PF, Wear DJ, Newton PB 3rd, Tully JG, Shih JW. Adhesion onto and invasion into mammalian cells by *Mycoplasma penetrans*: a newly isolated mycoplasma from patients with AIDS. Mod Pathol. 1993 May;6(3):276-80.

[6] Taylor-Robinson D, Davies HA, Sarathchandra P, Furr PM. Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy. Int J Exp Pathol. 1991 Dec;72(6):705-14.

[7] Baseman JB, Lange M, Criscimagna NL, Giron JA, Thomas CA. Interplay between mycoplasmas and host target cells. Microb Pathog. 1995 Aug;19(2):105-16.

[8] Baseman JB, Reddy SP, Dallo SF. Interplay between mycoplasma surface proteins, airway cells, and the protean manifestations of mycoplasma-mediated human infections. Am J Respir Crit Care Med. 1996 Oct;154(4 Pt 2):S137-44.

[9] Vogl G, Plaickner A, Szathmary S, Stipkovits L, Rosengarten R, Szostak MP. *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. Infect Immun. 2008 Jan;76(1):71-7. Epub 2007 Oct 22.

[10] Dusanić D, Bercic RL, Cizelj I, Salmic S, Narat M, Bencina D. *Mycoplasma synoviae* invades non-phagocytic chicken cells in vitro. Vet Microbiol. 2009 Jul 2;138(1-2):114-9. Epub 2009 Mar 4.

[11] Andreev J, Borovsky Z, Rosenshine I, Rottem S. Invasion of HeLa cells by *Mycoplasma penetrans* and the induction of tyrosine phosphorylation of a 145-kDa host cell protein. FEMS Microbiol Lett. 1995 Oct 15;132(3):189-94.

[12] Rawadi G, Roman-Roman S. Mycoplasma membrane lipoproteins induced proinflammatory cytokines by a mechanism distinct from that of lipopolysaccharide. Infect Immun. 1996 Feb;64(2):637-43.

[13] Quentmeier H, Schmitt E, Kirchhoff H, Grote W, Mühlradt PF. *Mycoplasma fermentans*-derived high-molecular-weight material induces interleukin-6 release in cultures of murine macrophages and human monocytes. Infect Immun. 1990 May;58(5):1273-80.

[14] Mühlradt PF, Kiess M, Meyer H, Süssmuth R, Jung G. Structure and specific activity of macrophage-stimulating lipopeptides from Mycoplasma hyorhinis. Infect Immun. 1998 Oct;66(10):4804-10.

[15] Gallily R, Kipper-Galperin M, Brenner T. *Mycoplasma fermentans*-induced inflammatory response of astrocytes: selective modulation by aminoguanidine, thalidomide, pentoxifylline and IL-10. 1999, Inflammation Dec;23(6):495-505

[16] Kaufmann A, Mühlradt PF, Gemsa D, Sprenger H. Induction of cytokines and chemokines in human monocytes by *Mycoplasma fermentans*-derived lipoprotein MALP-2. Infect Immun. 1999 Dec;67(12):6303-8.

[17] Mühlradt PF, Schade U.MDHM, a macrophage-stimulatory product of *Mycoplasma fermentans*, leads to in vitro interleukin-1 (IL-1), IL-6, tumor necrosis factor, and prostaglandin production and is pyrogenic in rabbits. Infect Immun. 1991 Nov;59(11):3969-74.

[18] Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem. 1999 Nov 19;274(47):33419-25.

[19] Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, Mühlradt PF, Akira S.J Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. Immunol. 2000 Jan 15;164(2):554-7.

[20] Stuart PM. Mycoplasmal induction of cytokine production and major histocompatibility complex expression. Clin Infect Dis. 1993 Aug;17 Suppl 1:S187-91.

[21] Stuart PM, Egan RM, Woodward JG. Characterization of MHC induction by *Mycoplasma fermentans* (incognitus strain). Cell Immunol. 1993 Nov;152(1):261-70.

[22] Frisch M, Gradehandt G, Mühlradt PF. *Mycoplasma fermentans*-derived lipid inhibits class II major histocompatibility complex expression without mediation by interleukin-6, interleukin-10, tumor necrosis factor, transforming growth factor-beta, type I interferon, prostaglandins or nitric oxide. Eur J Immunol. 1996 May;26(5):1050-7.

[23] Radaelli E, Luini M, Domeneghini C, Loria GR, Recordati C, Radaelli P, Scanziani E. Expression of class II major histocompatibility complex molecules in chronic pulmonary

Mycoplasma bovis infection in cattle. J Comp Pathol. 2009 Feb-Apr;140(2-3):198-202. Epub 2009 Jan 8.

[24] Robin Nicholas, Roger Ayling, Laura McAuliffe (2009). Mycoplasma Diseases of Ruminants. CABI.

[25] Nicholas, R.A.J. (2002) Improvements in the diagnosis and control of diseases of small ruminants caused by mycoplasmas. Small Ruminant Research 45, 145–149.

[26] Loria, G.R., Caracappa, S., Monteverde, P. and Nicholas, R.A.J. (2007) Infezione da *Mycoplasma agalactiae* in cervelli ovini: un nuova sito di infezione. *Large Animal Review* 13, 65–68.

[27] Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barré A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet. 2007 May 18;3(5):e75.

[28] Sachse K, Grajetzki C, Rosengarten R, Hänel I, Heller M, Pfützner H. Mechanisms and factors involved in Mycoplasma bovis adhesion to host cells. Zentralbl Bakteriol. 1996 Jun;284(1):80-92

[29] Thomas A, Sachse K, Dizier I, Grajetzki C, Farnir F, Mainil JG, Linden A. Adherence to various host cell lines of Mycoplasma bovis strains differing in pathogenic and cultural features. Vet Microbiol. 2003 Feb 2;91(2-3):101-13.)

[30] Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM. Characterization of P40, a cytadhesin of Mycoplasma agalactiae. Infect Immun. 2002 Oct;70(10):5612-21

[31] Rosati S, Pozzi S, Robino P, Montinaro B, Conti A, Fadda M, Pittau M. P48 major surface antigen of Mycoplasma agalactiae is homologous to a malp product of Mycoplasma fermentans and belongs to a selected family of bacterial lipoproteins. Infect Immun. 1999 Nov;67(11):6213-6

[32] McAuliffe L, Ellis RJ, Miles K, Ayling RD, Nicholas RA. Biofilm formation by mycoplasma species and its role in environmental persistence and survival. Microbiology. 2006 Apr;152(Pt 4): 913-22.

[33] Sanchis R, Abadie G, Lambert M, Cabasse E, Dufour P, Guibert JM, Pépin M. Inoculation of lactating ewes by the intramammary route with Mycoplasma agalactiae: comparative pathogenicity of six field strains. Vet Res. 2000 May-Jun;31(3):329-37.

[34] Fusco M, Corona L, Onni T, Marras E, Longheu C, Idini G, Tola S. Development of a sensitive and specific enzyme-linked immunosorbent assay based on recombinant antigens for rapid detection of antibodies against Mycoplasma agalactiae in sheep. Clin Vaccine Immunol. 2007 Apr;14(4):420-5. Epub 2007 Feb 7.

[35] Buonavoglia D, Fasanella A, Sagazio P, Tempesta M, Iovane G, Buonavoglia C. Persistence of antibodies to Mycoplasma agalactiae in vaccinated sheep. New Microbiol. 1998 Apr;21(2):209-12.

[36] La Manna MP, Agnone A, Villari S, Puleio R, Vitale M, Nicholas R, Sireci G: Expansion of intracellular IFN-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep. *Res Vet Sci* 2011, 91(3):e64-7. Epub 2011 Feb 26.

[37] de la Fe C, Assunção P, Saavedra P, Ramírez A, Poveda JB. Field trial of a combined vaccine against caprine contagious agalactia: humoral immune response in lactating goats. Vet J. 2007 Nov;174(3):610-5. Epub 2006 Dec 8.

[38] Castro-Alonso A, Rodríguez F, De la Fé C, Espinosa de Los Monteros A, Poveda JB, Andrada M, Herráez P. Correlating the immune response with the clinical-pathological course of persistent mastitis experimentally induced by Mycoplasma agalactiae in dairy goats. Res Vet Sci. 2009 Apr;86(2):274-80. Epub 2008 Aug 13.

[39] Castro-Alonso A, De la Fe C, Espinosa de los Monteros A, Rodríguez F, Andrada M, Poveda JB, Herráez P. Chronological and immunohistochemical characterization of the mammary immunoinflammatory response in experimental caprine contagious agalactia. Vet Immunol Immunopathol. 2010 Jul;136(1-2):43-54. Epub 2010 Feb 13.

[40] Akbar A, Terry L , Timms A, Beverly PCL, Janossy G. Loss of CD45RO and again of UCHL1 reactivity is a feature of primed T cells. *J. Immunol* . 1988 ; **140** : 2171 – 8.

[41] Uehara T, Miyawaki T, Ohta K *et al* . Apoptotic cell death of primed CD45RO+ T lymphocytes in Epstein-Barr virus-induced infectious mononucleosis . *Blood* 1992 ; **80** : 452 – 8.

[42] Gray D, Siepmann K, van Essen D *et a* . B-T lymphocyte interactions in the generation and survival of memory cells. *Immunol. Rev* . 1996 ; **150** : 45 – 61.

[43] Stazzi P., Mirri, A. (1956) Agalassia contagiosa degli ovini e dei caprini. In: Stazzi P., Mirri A. (Editors) Malattie infettive degli animali domestici, 11 edt. Palermo, Italy, pp. 881-891.

[44] Valenti, G. (1973) Agalassia contagiosa delle pecore e delle capre. Rivista di Zootecnia e Veterinaria 4, 329.

[45] Balbo, S.M. (1987) Igiene e profilassi negli allevamenti ovini e caprini. L'Italia Agricola 124, 196-197

[46] Consenti B, Montagna CO. Profilassi dell'Agalassia contagiosa degli ovi-caprini. Obiettivi Veterinari. 1999; 5, 31-33.

[47] León Vizcaíno L, Garrido Abellán F, Cubero Pablo MJ, Perales A. Immunoprophylaxis of caprine contagious agalactia due to Mycoplasma agalactiae with an inactivated vaccine. Vet Rec. 1995 Sep 9;137(11):266-9.

[48] Tola S, Manunta D, Rocca S, Rocchigiani AM, Idini G, Angioi A, Leori G. Experimental vaccination of against Mycoplasma agalactiae using different inactivated vaccine. Vaccine 1999; 17:2764-8.

[49] Stazzi P, Mirri A. Agalassia contagiosa degli ovini e dei caprini. In: Stazzi P., Mirri A. (Editors) Malattie infettive degli animali domestici, 11 edt. 1986. Palermo, Italy, pp. 881-891

[50] Bergonier D, De Simone F, Russo P, Solsona M, Lambert M, Poumarat F. Variable expression and geographic distribution of *Mycoplasma agalactiae* surface epitopes demonstrated with monoclonal antibodies. FEMS Microbiol Lett. 1996; 143(2-3):159-65

[51] de la Fe C, Assunção P, Rosales RS, Antunes T, Poveda JB. Characterisation of protein and antigen variability among *Mycoplasma mycoides* subsp. mycoides (LC) and *Mycoplasma agalactiae* field strains by SDS-PAGE and immunoblotting. Vet J 2006; 171(3):532-8

[52] Loria GR, Sammartino C, Nicholas RA, Ayling RD. In vitro susceptibilities of field isolates of *Mycoplasma agalactiae* to oxytetracycline, tylosin, enrofloxacin, spiramycin and lincomycin-spectinomycin. Res Vet Sci. 2003; 75(1):3-7.

[53] Buonavoglia D, Greco G, Quaranta V, Corrente M, Martella V, Decaro N. An oil-emulsion vaccine induces full-protection against Mycoplasma agalactiae infection in sheep. New Microbiol. 2008 Jan;31(1):117-23.

[54] OIE manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), chapter 2.7.5. Paris, France

[55] Agnone A, La Manna MP, Sireci G, Puleio R, Usticano A, Uzdemir U, Nicholas R, Chiaracane V, Dieli F; Di Marco V and Loria GR. Pathogen excretion and clinical symptoms as indicators for the comparative analysis of vaccines efficacy during Mycoplasma agalactiae infection. *Manuscript submitted*

[56] Agnone A[*], La Manna MP[*], Loria G, Puleio R, Villari S, Nicholas R, Guggino G, Sireci G. Timing of Activation of CD4 Memory Cells as a Marker of Protection for Contagious Agalactia in the Sheep**.** *Manuscript submitted*

Research in Veterinary Science xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Expansion of intracellular IFN-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep

M.P. La Manna [a,1], A. Agnone [a], S. Villari [b], R. Puleio [b], M. Vitale [b], R. Nicholas [c], G. Sireci [a,*], F. Dieli [a], G.R. Loria [b,1]

[a] Dipartimento di Biopatologia e Biotecnologie mediche e Forensi, Università di Palermo, Corso Tukory 211, 90131 Palermo, Italy
[b] Istituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi 3, 90100 Palermo, Italy
[c] Mycoplasma Group, Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey KT15 3NB, UK

ARTICLE INFO

Article history:
Received 26 November 2010
Accepted 25 January 2011
Available online xxxx

Keywords:
Agalactia
Mastitis
T cells
Peripheral blood mononuclear cells
$CD4^+$ cells
$CD8^+$ cells

ABSTRACT

A method to assess the expansion of antigen-specific intracellular IFN-γ 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-γ 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-γ positive lymphocytes in infected sheep was initially sustained by $CD4^+$ T cells at day 15 after infection, when antigen specific IgG start to be detectable, followed by CD8/IFN-γ double positive cells. γδ T-cells were not expanded at any time point analysed. $IFN\gamma^+$ T cells disappear 60 days after infection, suggesting that antigen specific $IFN\gamma^+$ 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.

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 $IFN\text{-}\gamma^+$ 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 1×10^6 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 $CD4^+$, $CD8^+$ and $TCR\gamma\delta^+$ T-lymphocytes positive for intracellular IFN-γ 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® solution (Cedarlane labs, Canada) and quantified by Trypan blue dye exclusion test. Cells were resuspended (1×10^6/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-γ staining, medium alone, as negative control, and

* Corresponding author. Address: Dipartimento di Biopatologia e Biotecnologie mediche e Forensi, Corso Tukory 211, 90100 Palermo, Italy. Tel.: +39 091 6555939; fax: +39 091 6555924.
E-mail address: sireci@unipa.it (G. Sireci).
[1] These authors equally contributed to the paper.

0034-5288/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2011.01.029

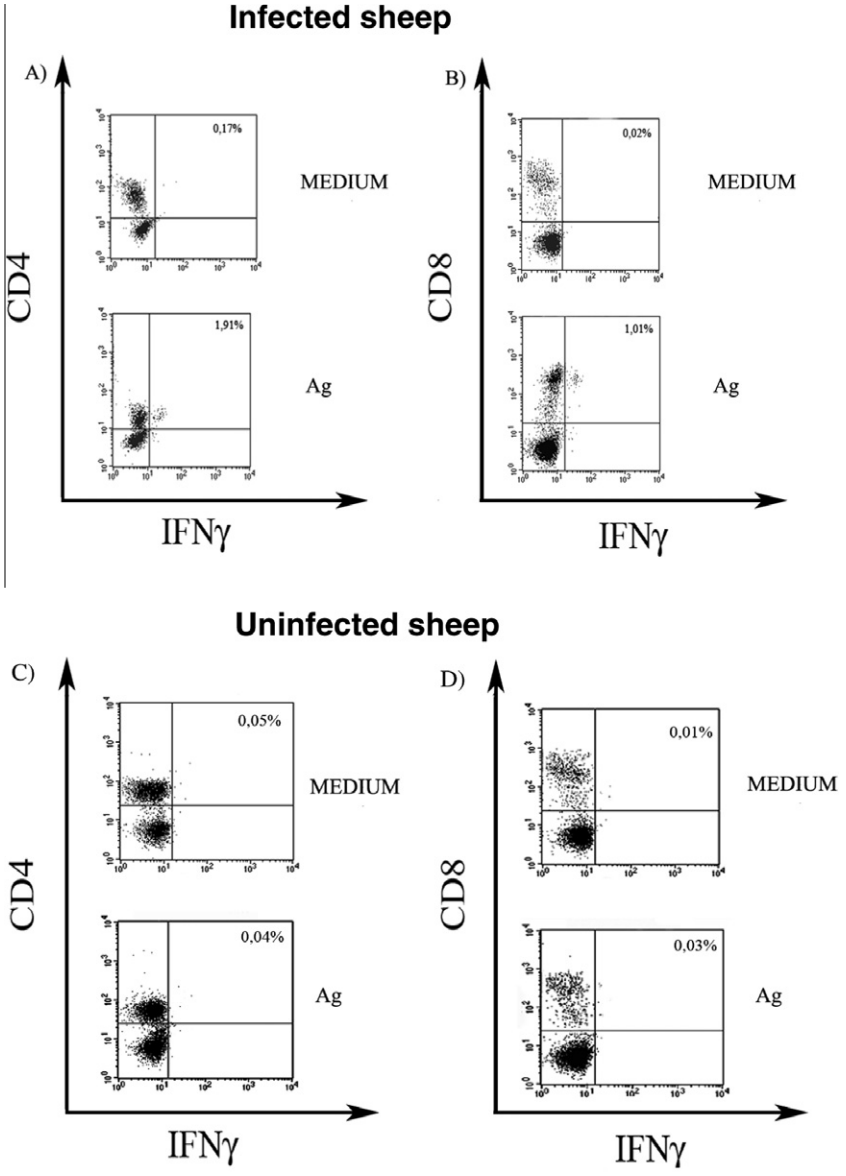


Fig. 1. Panels showed in the figure were obtained acquiring to the FACS-CAN (Becton Dickinson) 10000 events after gating lymphocytes. In infected sheep, panel of $CD4^+$ IFN-γ^+ were obtained 15 days after infection, $CD8^+$ IFN-γ^+ 30 days after the infection.

1×10^6 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 μM Monensin (Sigma–Aldrich, Italy) was added to each well to avoid IFN-γ release. PBMC were collected and pooled after 48 h of culture at 37 °C 5% CO_2 and flow-cytometric analyses were assessed. These assays were performed staining the cells with anti surface-FITC-labelled mAbs and anti-IFN-γ PE-labelled mAb at the concentration suggested by the suppliers in 100 μl PBS/0.1% sodium azide. Surface immunofluorescence was detected by anti-CD8 (FITC-labelled, clone CC63, mouse IgG2a, Serotec, UK), anti-γδ (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-γ 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 × 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.

Please cite this article in press as: La Manna, M.P., et al. Expansion of intracellular IFN-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep. Res. Vet. Sci. (2011), doi:10.1016/j.rvsc.2011.01.029

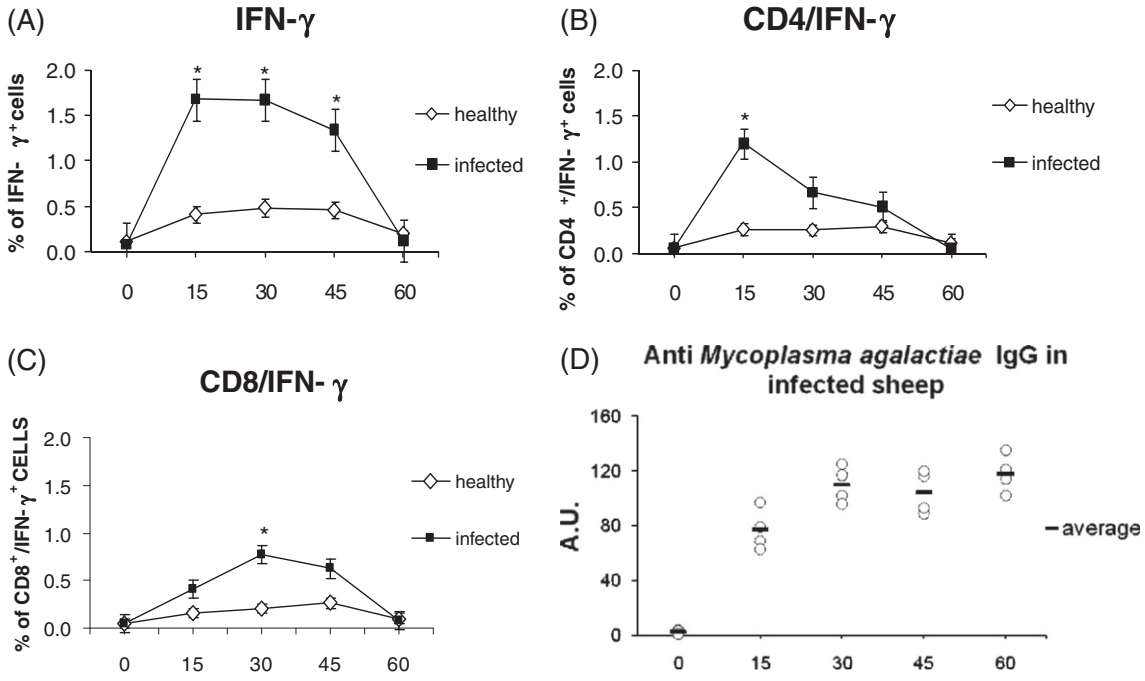


Fig. 2. PBMC from healthy (◇) and infected (■) ewes were obtained at the time points showed in the figure. The percentages of double positive cells were obtained analysing 10000 events on lymphocytes gate (Mann–Whitney *U* test * < 0.05). IgG from sera of infected ewes (○) were obtained at the points 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-γ^+ 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-γ.

As shown in Fig. 1A, CD4$^+$ IFN-γ^+cells increase, in infected sheep, from 0.17% to 1.91% at day 15 post infection while CD8$^+$ IFN-γ^+ 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-γ^+ 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-γ^+ T lymphocytes expansion was detected (Fig. 2B) while CD8$^+$/IFN-γ^+ T cells had a two fold increase 30 days after infection (Fig. 2C). From day 30, TCR$\gamma\delta^+$/IFN-γ^+ 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-γ 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-γ^+ T cells 15 days after infection and an antigen-specific CD8$^+$ IFN-γ^+ T cells increase at day 30, when CD4$^+$ IFN-γ^+ 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-γ^+ 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-γ^+ T cells. Probably, in the blood the increase of CD8$^+$ IFN-γ^+ 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-γ double positive cells and a later activation (4 weeks after infection) of CD8/IFN-γ double positive cells, the anti-MA immune response is mainly sustained by the immunoglobulin secretion. As the total percentages of IFN-γ^+ cells almost coincided with the CD4$^+$ IFN-γ^+ 15 days after infection or with CD8$^+$ IFN-γ^+ 30 days after infection, NK cells seem to be a minor component of MA-specific IFN-γ^+ 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-γ^+ 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-γ 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 IFNγ^+ cells expansion in the protective immune response to MA.

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

Acknowledgement

The experiments were supported by a grant to G.R.L. (Ricerca Corrente,-Ministero della Salute).

Please cite this article in press as: La Manna, M.P., et al. Expansion of intracellular IFN-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep. Res. Vet. Sci. (2011), doi:10.1016/j.rvsc.2011.01.029

References

Bergonier, D., Poumarat, F., 1996. Contagious agalactia of small ruminants: epidemiology, diagnosis and control. Review of Scientific Technology 15, 1431–1475.

Byrne, W., Mekey, B., McCormack, R., Egan, J., Ball, H., Sachse, K., 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. Veterinary Record 156, 767–771.

Castro-Alonso, A., De la Fe, C., Espinosa de los Monteros, A., Rodríguez, F., Andrada, M., Poveda, J.B., Herráez, P., 2010. Chronological and immunohistochemical characterization of the mammary immunoinflammatory response in experimental caprine contagious agalactia. Veterinary Immunology and Immunopathology 136, 43–54.

Da Massa, A.J., Wakenell, P.S., Brooks, D.L., 1992. Mycoplasmas of goats and sheep. Journal of Veterinary Diagnostic Investigation 4, 101–113.

Mohr, E., Cunningham, A.F., Toellner, K.-M., Bobat, S., Coughlan, R.E., Bird, R.A., MacLennan, I.C.M., Serre, K., 2010. IFN-{gamma} produced by CD8 T cells induces T-bet-dependent and independent class switching in B cells in responses to alum-precipitated protein vaccine. PNAS 107, 17292–17297.

Poumarat, F., Chazel, M., Tardy, F., Gaurivaud, P., Arcangioli, M.A., Le Grand, D., Calavas, D., 2009. VIGIMYC, le réseau national d'épidémio-surveillance des mycoplasmoses des ruminants, bilan 2003–2007. Bulletin Epidémiologique 31, 4–8.

Razin, S., Yogev, D., Naot, Y., 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiology and Molecular Biology Reviews 62, 1094–1156.

Rodríguez, F., Sarradell, J., Poveda, J.B., Ball, H.J., Fernández, A., 2000. Immunohistochemical characterization of lung lesions induced experimentally by Mycoplasma agalactiae and *Mycoplasma bovis* in goats. Journal of Comparative Pathology 123, 285–293.

Rodríguez, F., Fernández, A., Orós, J., Ramírez, A.S., Luque, R., Ball, H.J., Sarradell, J., 2001. Changes in lymphocyte subsets in the bronchus associated lymphoid tissue of goats naturally infected with different mycoplasma species. Journal of Veterinary Medicine B 48, 259–266.

Sarradell, J., Andrada, M., Ramírez, A.S., Fernández, A., Gómez-Villamandos, J.C., Jover, A., Lorenzo, H., Herráez, P., Rodríguez, F., 2003. A morphologic and immunohistochemical study of the bronchus associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*. Veterinary Pathology 40, 395–404.

Snapper, C.M., Peschel, C., Paul, W.E., 1988. IFN-γ stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. Journal of Immunology 140, 2121–2127.

Tajima, M., Yagihashi, T., Nunoya, T., Takeuchi, A., Ohashi, F., 1984. *Mycoplasma hyopneumoniae* infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. American Journal of Veterinary Research 45, 1928–1932.

Please cite this article in press as: La Manna, M.P., et al. Expansion of intracellular IFN-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep. Res. Vet. Sci. (2011), doi:10.1016/j.rvsc.2011.01.029

Elsevier Editorial System(tm) for The Veterinary Journal
Manuscript Draft

Manuscript Number:

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

Article Type: Original Article

Keywords: Vaccines comparison; Contagious Agalactia; Mycoplasma agalactiae

Corresponding Author: Dr Guido Ruggero Loria, DVM PhD

Corresponding Author's Institution: Istituto Zooprofilattico Sperimentale della Sicilia

First Author: Annalisa Agnone

Order of Authors: Annalisa Agnone; Marco P La Manna; Guido Sireci; Roberto Puleio; Antonella Usticano; Umit Uzdemir; Robin Nicholas; Valeria Chiaracane; Francesco Dieli; Vincenzo Di Marco; Guido Ruggero Loria, DVM PhD

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.

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

Annalisa Agnone [a,b]; Marco Pio La Manna [a]; Guido Sireci [a]; Roberto Puleio [b]; Antonella Usticano [b]; Umit Uzdemir [c]; Robin Nicholas [d]; Valeria Chiaracane [b]; Francesco Dieli [a]; Vincenzo Di Marco [b] and Guido Ruggero Loria [b,*]

[a] Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Università di Palermo, Corso Tukory, 211, Palermo, Italy

[b] Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Via Gino Marinuzzi, 3, Palermo, Italy

[c] Pendik Veterinary Control and Research Institute, Istanbul, Turkey

[d] Mycoplasma group, Veterinary Laboratories Agency, Woodham Lane, New Haw, Weybridge, Addlestone, UK.

* Corresponding author: Tel: +39 091 6565307

Email address: guidoruggero.loria@izssicilia.it (G.R. Loria)

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.

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

Introduction

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

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

In the last decade immunogenic proteins of *M. agalactiae* have been identified as suitable for vaccines against the disease (Tola et al., 1999) but their practical development to become 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 (Bergonier et al., 1996; de la Fe et al., 2006). Nevertheless the real efficacy of currently circulating products (inactivated monovalent vaccines) is still doubtful in field conditions (de la Fe et al., 2007).

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

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

The efficacy of vaccine against CA have been reported for some inactivated product (Tola et a1., 1999; Buonavoglia et al., 2008; Consenti et al., 1999), showing data on clinical efficacy (udder changes, milk changes, fever, general body conditions, arthritis, conjunctivitis, etc.) among different products. 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.

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

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

Materials and methods

Experimental design

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

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

Ethics

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 work was reliable using the animal facility of Istituto Zootecnico Sperimentale per la Sicilia (Palermo), where lactating sheep were managed according to traditional production management. The experiments were carried out with the authorization of the Italian Ministry of health (Decreto Ministeriale N° 101/2006 –A).

Clinical assessment

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

Mycoplasma cultures and PCR

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

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

Statistical analysis

The data of *M. Agalactiae* excretion in milk, ocular and nasal swabs were analysed considering the mean of CFU ± SD obtained by the determination of viable cells from the collected samples. The data were analysed with a statistical model of analysis of variance ANOVA. A risk analysis was also calculated separately analysing the probability for the animal to suffer of clinical symptoms, and the spreading of the pathogen in the environment (ocular and nasal swabs). These values were obtained using the Relative Risk and their I.C. 95%. 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 analyses were performed using Statistica 5.0 (StatSoft, Inc., 2010) and Openepi (Dean et al., 2011) software.

Results

Live and saponin vaccine conferred the best protection against clinical symptoms

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

(Stazzi and Mirri, 1986). The groups of “in contact” vaccinated sheep showed clinical signs of CA after the second week post challenge with highest incidence of mastitis 4 wpi (weeks post infection). At this time point a total of 10 sheep showed CA symptoms and 9 sheep were severely affected: 4belonging to control group, 4 to the commercial vaccine group and 1 to the farm vaccine group (Tab 2). In the first stage the disease was 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 was always involved showing from mild to marked enlargement in size. The presence in the udder of one of the above-mentioned changes was considered as “mastitis”. Two ewes only (8% of the total number) showed arthritis (1 ewe belonging to saponin inactivated vaccine group) and monolateral conjunctivitis (1 sheep belonging to control group). Arthritis involved all limbs, particularly visible in carpal and tarsal joints.

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

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

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

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

Microorganism excretion as a parameter of vaccine efficiency

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

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

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

Discussion

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

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

The sheep belonging to the live 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 CA symptoms. Saponin and farm 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.

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

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

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

Conflict of interest statement

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

Acknowledgments

This work was founded by Italian Ministry of Health (RC IZSSi 14/2008 and RC IZSSi 05/2010). Results are part of PhD project of Dr. Annalisa Agnone, funded by Istituto Zooprofilattico Sperimentale della Sicilia. The funders had no influence in study design and data analysis.

References

Bangham A.D., Horne R.W., Glauert A.M., Dingle J.T., Lucy J.A., 1962. Action of saponin on biological cell membranes. Nature 8 (196): 952 - 955.

Bergonier D., De Simone F., Russo P., Solsona M., Lambert M., Poumarat F., 1996.Variable expression and geographic distribution of *Mycoplasma agalactiae* surface epitopes demonstrated with monoclonal antibodies. FEMS Microbiology Letters 143(2-3), 159-165.

Buonavoglia D., Greco G., Quaranta V., Corrente M., Martella V., Decaro N., 2008. An oil-emulsion vaccine induces full-protection against *Mycoplasma agalactiae* infection in sheep. New Microbiology 31(1), 117-123.

Consenti B., Montagna C. O., 1999. Profilassi dell'Agalassia contagiosa degli ovi-caprini. Obiettivi Veterinari 5, 31-33.

Contagious agalactia. In: OIE manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), 2008. Paris, France, 992-999.

de la Fe C., Assunção P., Rosales R.S., Antunes T., Poveda J.B., 2006.Characterization of protein and antigen variability among *Mycoplasma mycoides* subsp. mycoides (LC) and *Mycoplasma agalactiae* field strains by SDS-PAGE and immunoblotting.Veterinary Journal 171(3), 532-538.

de la Fe C., Assunção P., Saavedra P., Tola S., Poveda C., Poveda J.B., 2007. Field trial of two dual vaccines against *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. mycoides (large colony type) in goats. Vaccine 25(12), 2340-2345.

Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1.

Loria G.R., Sammartino C., Nicholas R.A., Ayling R.D., 2003. In vitro susceptibilities of field isolates of *Mycoplasma agalactiae* to oxytetracycline, tylosin, enrofloxacin, spiramycin and lincomycin-spectinomycin. Research in Veterinary Science 75(1), 3-7.

McAuliffe L., Ellis R.J., Ayling R.D., Nicholas R.A., 2003. Differentiation of Mycoplasma species by 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting. Journal of Clinical Microbiology 41(10), 4844-4847.

McAuliffe L., Ellis R.J., Lawes J.R., Ayling R.D., Nicholas R.A., 2005. 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating Mycoplasma species. Journal of Medical Microbiology 54 (Pt 8), 731-739.

Meynell G.G., Meynell E., 1970. Bacterial growth. In: Meynell G.G., Meynell E. (Editors) Theory and Practice in Experimental Bacteriology, Cambridge University Press, UK, pp 1-9

Nicholas R. and Baker S. in: Miles R, Nicholas R. Mycoplasma protocols. Humana Press, Totowa, New Jersey, 1998.

Nicholas R.A., Ayling R.D., McAuliffe L., 2009.Vaccines for Mycoplasma diseases in animals and man. Journal of Comparative Pathology 140(2-3), 85-96.

Postgate, J.R., 1969.Viable count and viability. Methods in Microbiology 1, 611-628.

Poveda J.B., Nicholas R.A.J., 1998. Serological identification of Mycoplasmas by growth and metabolic inhibition tests. In: Miles R.J. and Nicholas R.A.J. (Editors) Mycoplasma protocols, Methods in molecular biology. Vol. 104, Humana Books Press, Totowa, New Jersey, USA, pp. 105-111.

StatSoft, Inc. STATISTICA 5.0. 2010. Tulsa, OK (USA).

Stazzi P., Mirri A., 1986. Agalassia contagiosa degli ovini e dei caprini. In: Stazzi P., Mirri A. Malattie infettive degli animali domestici, 11 edt. Palermo, Italy, pp. 881-891.

Tola S., Manunta D., Rocca S., Rocchigiani A.M., Idini G., Angioi A., Leori G., 1999. Experimental vaccination of against *Mycoplasma agalactiae* using different inactivated vaccine. Vaccine 17, 2764-2768.

Table 1

Overview of the vaccine formulations used in this experiment

Group name	Vaccine	Number of animals	Producer	Inactivation procedure	Adjuvants
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	---	---	---

Animals were vaccinated with a proper dose of vaccines, following manufacturer instructions, and boosted after 30 days. Control group was not subjected to any vaccine injection.

Table 2

Percentages of sick animals in each group 4 weeks after infection

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

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

Table 3

Risk values in vaccinated and non-vaccinated sheep

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

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

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

Figure 1

Click here to download high resolution image

Agnone et al, Fig 1

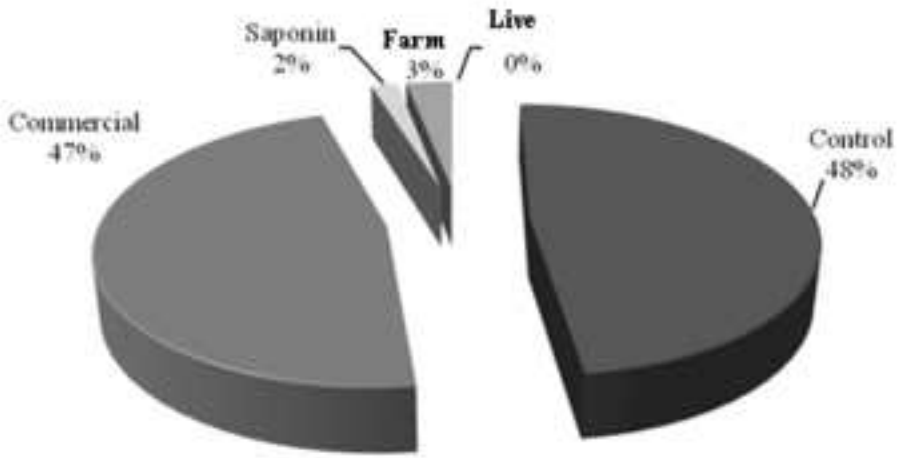


Figure 2
Click here to download high resolution image

Agnone et al, Fig 2

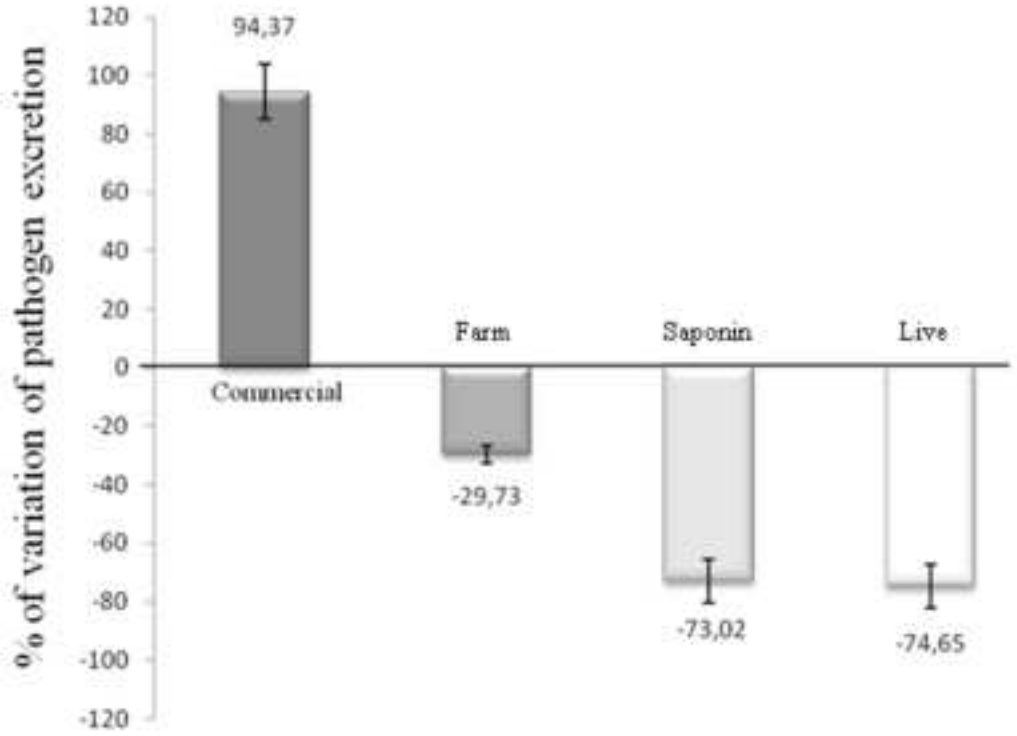
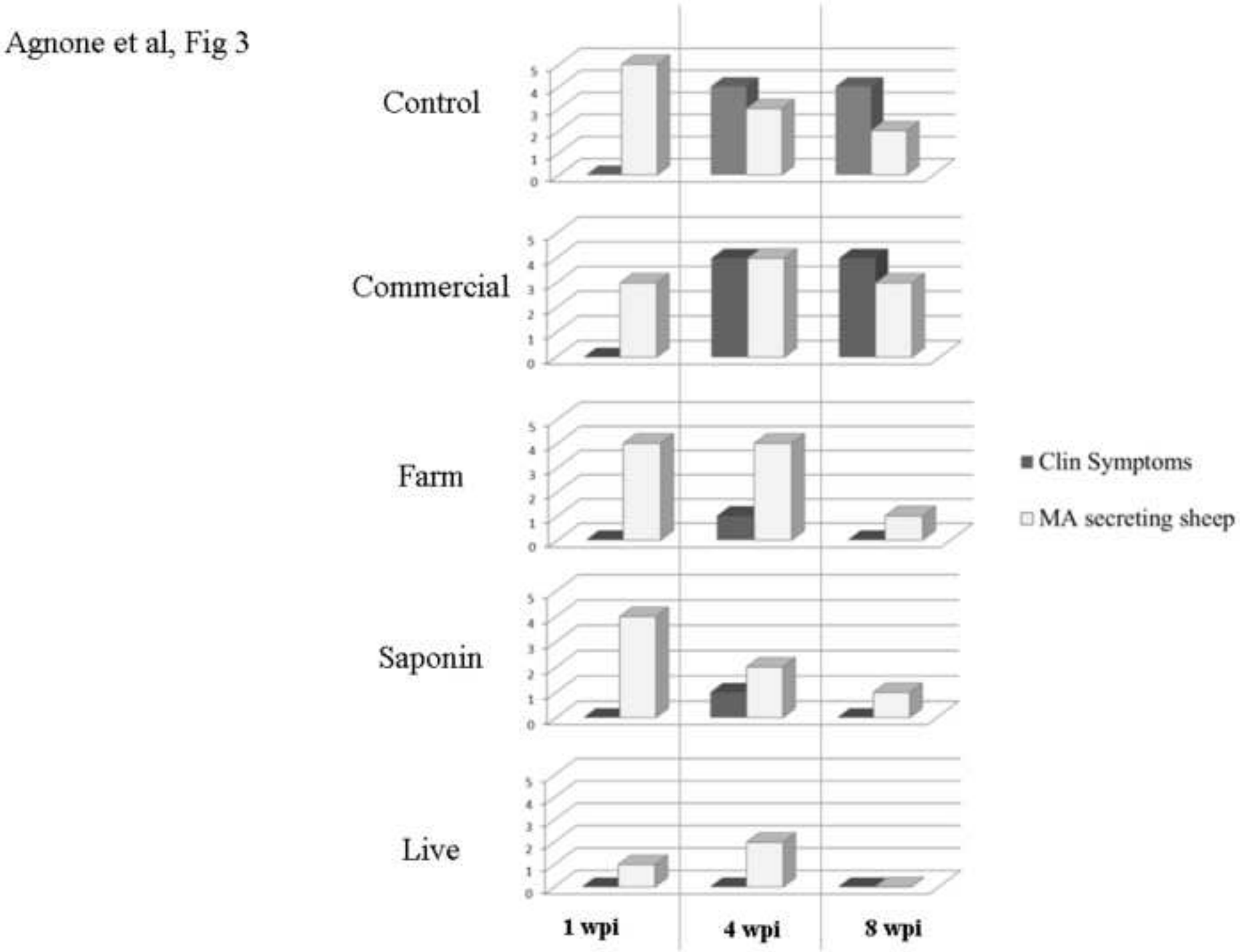


Figure 3

Click here to download high resolution image



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$^+$ memory cells as a marker of protection for Contagious Agalactia in sheep

Annalisa Agnone[1,2]*, Marco P La Manna [1]*, Guido R Loria[2], Roberto Puleio[2], Sara Villari[2], Robin A Nicholas[3], Giuliana Guggino[2], Guido Sireci[2§]

[1]Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Università di Palermo, Corso Tukory 211, Palermo, Italy

[2]Istituto Zooprofilattico Sperimentale della Sicilia, Via Gino Marinuzzi 3, Palermo, Italy

[3]Mycoplasma group, Animal Health and Veterinary Laboratories Agency, Weybridge. New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom.

*These authors contributed equally to this work

[§]Corresponding author

Email addresses:

AA: a.agnone@libero.it

MPLM: marcopio.lamanna@unipa.it

GRL: guidoruggero.loria@izssicilia.it

RP: roberto.puleio@izssicilia.it

SV: sara.villari@izssicilia.it

RAN: robin.nicholas@ahvla.gsi.gov.uk

GG: giuliana.guggino@unipa.it

GS: guido.sireci@unipa.it

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 $CD4^+$ Interferon-γ^+ T cells underwent an early expansion ($p<0.05$ when compared to unprotected groups), whilst memory $CD8^+$ Interferon-γ^+ T cells have increased in non-protected animals since 7 days post infection ($p<0.05$). $\gamma\delta^+$ Interferon-γ^+ 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 $CD4^+$ Interferon-γ^+ T cells, rather than $CD8^+$ or $\gamma\delta^+$ Interferon-γ^+ 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-γ^+ 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 Zooprophylactic Institutes (IZSs) 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).

$CD4^+$- $CD45R0^+$- IFN-γ^+ 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-γ was considered as a marker of cellular activation, as previously published [3, 19]. The data related to the cellular subsets of $CD4^+CD45R0^+$- $CD4^+CD45RA^+$- $CD8^+CD45R0^+$- and $CD8^+CD45RA^+$- and $\gamma\delta^+$ 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-□$^+$ cells, distinguishing between $CD4^+$ ($CD45R0^+$ or $CD45RA^+$) and $CD8^+$ ($CD45R0^+$ or $CD45RA^+$) and $\gamma\delta^+$ cells.

Data showed in figure 2A describe the relative percentages of CD45R0/CD45RA in gated $CD4^+$ IFN-γ^+ 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 $CD4^+CD45R0^+$ IFN-γ^+ 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 $CD4^+$- $CD45R0^+$- IFN-γ^+ 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 $CD4^+$- $CD45RA^+$- IFN-γ^+ 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 $CD4^+CD45R0^+$ IFN-γ^+ 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-γ^+ in the group 2 becomes significant only from day 30 post infection ($p<0.05$). Maximal absolute percentages of $CD4^+CD45R0^+$ IFN-γ^+ cells were observed 30 and 90 dpi in the group 3 ($p<0.05$).

$CD8^+$ $CD45R0^+$ IFN-γ^+ T-lymphocytes increase in sick animals

Figure 3A shows the relative percentages of CD45R0/CD45RA in gated $CD8^+$ IFN-γ^+ cells. In the group 1 high percentages of CD8 memory cells from day 15 to day 90 have been detected. $CD8^+$ $CD45R0^+$ IFN-γ^+ T-lymphocytes increase from day 0 to day 30 in the group 4. In the group 3, two peaks of $CD8^+$ memory IFN-γ^+ cells were observed at days 15 and 90. The higher percentages of $CD8^+$ $CD45R0^+$ IFN-γ^+ 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 $CD8^+$ $CD45R0^+$ IFN-γ^+ cells increase only at day 30 in absolute percentages.

γδ$^+$ IFN-γ$^+$ T lymphocytes quickly expand in a vaccinated and unprotected group

In the group 4 no significant expansion of γδ$^+$ IFN-γ$^+$ T lymphocytes was detected during all the time points of observation (Fig 4). The trend of γδ$^+$ IFN-γ$^+$ 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. γδ$^+$ IFN-γ$^+$ 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$^+$– T lymphocytes), whose timing of expansion was considered a consequence of the efficiency of vaccination, and in the naïve cell populations (CD45RA$^+$ – T lymphocytes) that could be considered the reservoir of memory cells [20]. It is well known that upon stimulation by specific antigen, naive 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-γ 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-γ$^+$ T cell subsets could be useful to monitor the T-cell mediated immune response during the infection. The T cell response of IFN-γ$^+$ 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$^+$ IFN-γ$^+$ cells followed by an expansion of CD8$^+$ IFN-γ$^+$ 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 $CD4^+$ $CD45R0^+$ IFN-γ^+, able to increase since day 7 after infection to day 90. The early increase of $CD4^+$ $CD45R0^+$ IFN-γ^+ 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 $CD8^+$ $CD45R0^+$ IFN-γ^+ 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 $CD4^+$ 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, $CD4^+$ $CD45R0^+$ IFN-γ^+ 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 $CD8^+CD45R0^+$ IFN-γ^+ 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 $CD4^+CD45R0^+$ IFN-γ^+ 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 γδ IFN-γ^+ T cells releasing mevalonate metabolites [28]. In control animals (group 1) two peaks of γδ IFN-γ^+ T cells were recorded, 7 dpi and 30 dpi. As γδ 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 γδ 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 $CD8^+$ cells. The peak of γδ IFN-γ^+ 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 γδ cells. The high amount of γδ IFN-γ^+ 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 γδ IFN-γ^+ 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-γ PE-labelled mAbs at the concentration suggested by the suppliers in 100 µl 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® reagent set (Serotec, U.K.) and then incubated with anti-IFN-γ 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 $CD4^+CD45R0^+$- $CD4^+CD45RA^+$- $CD8^+CD45R0^+$ and $CD8^+CD45RA^+$- 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.

Acknowledgements

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).

References

1. Bergonier D, Berthelot X, Poumarat F: **Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control**. *Rev Sci Tech* 1997, **16**: 848–873

2. OIE manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). Paris, France; 2008:992-999

3. La Manna MP, Agnone A, Villari S, Puleio R, Vitale M,Nicholas R, Sireci G: **Expansion of intracellular IFN-γ positive lymphocytes during *Mycoplasma agalactiae* infection in sheep.** *Res Vet Sci* 2011, **91**(3):e64-7.

4. Buonavoglia D, Greco G, Quaranta V, Corrente M, Martella V, Decaro N: **An oil-emulsion vaccine induces full-protection against *Mycoplasma* agalactiae infection in sheep**. *New Microbiol* 2008, **31**(1):117-123

5. Castro-Alonso A, De la Fe C, Espinosa de los Monteros A, Rodríguez F, Andrada M, Poveda JB, Herràez P: **Chronological and immunohistochemical characterization of the mammary immunoinflammatory response in experimental caprine contagious agalactia**. *Vet Immunol Immunopathol* 2010, **136**(1-2):43-54

6. Buonavoglia D, Greco G, Corrente M, Greco MF, D'Abramo M, Latronico F, Fasanella A, Decaro N: **Long-term immunogenicity and protection against *Mycoplasma agalactiae* induced by an oil adjuvant vaccine in sheep**. *Res Vet Sci* 2009, **88**(1):16-19

7. Nicholas RA, Ayling RD, McAuliffe L: **Vaccines for *Mycoplasma* diseases in animals and man**. *J Comp Pathol* 2009, **140**(2-3):85-96

8. Nicholas RA, Ayling RD: ***Mycoplasma bovis*: disease, diagnosis, and control**. *Res Vet Sci* 2003, **74**(2):105-112

9. Nouvel LX, Marenda M, Sirand-Pugnet P, Sagné E, Glew M, Mangenot S, Barbe B, Barré A, Claverol S, Cicci C. **Occurrence, Plasticity, and Evolution of the vpma Gene Family, a Genetic System Devoted to High-Frequency Surface Variation in *Mycoplasma agalactiae*.** *J Bacteriol* 2009, **191**:4111–4121

10. Bergonier D, De Simone F, Russo P, Solsona M, Lambert M, Poumarat F: **Variable expression and geographic distribution of *Mycoplasma agalactiae* surface epitopes demonstrated with monoclonal antibodies.** *FEMS Microbiol Lett* 1996, **143**:159-65

11. de la Fe C, Assunção P, Rosales RS, Antunes T, Poveda JB: **Characterization of protein and antigen variability among *Mycoplasma mycoides* subsp. mycoides (LC) and *Mycoplasma agalactiae* field strains by SDS-PAGE and immunoblotting.** *Vet J* 1996, 171(3):532-538

12. Tola S, Manunta D, Rocca S, Rocchigiani AM, Idini G, Angioi PP, Leori G: **Experimental vaccination of against *Mycoplasma agalactiae* using different inactivated vaccine**. *Vaccine* 1999, **17**:2764-2768

13. Miles R, Nicholas R: **Mycoplasma protocols.** Edited by Miles R, Nicholas R. Totowa, New Jersey: Humana Press; 1998

14. de la Fe C, Assunção P, Saavedra P, Tola S, Poveda C, Poveda JB **Field trial of two dual vaccines against Mycoplasma agalactiae and Mycoplasma mycoides subsp. mycoides (large colony type) in goats.** Vaccine. 2007, **25**(12):2340-2345

15. Poveda JB, Nicholas RAJ: **Serological identification of Mycoplasmas by growth and metabolic inhibition tests**. In *Mycoplasma protocols, Methods in molecular biology Volume 104* edited by Miles RJ and Nicholas RAJ, Totowa, New Jersey, Humana Books Press; 1998: 105-111

16. McAuliffe L, Ellis RJ, Ayling RD, Nicholas RA: **Differentiation of Mycoplasma species by 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting**. *J Clin Microbiol* 2003, **41**(10):4844-4847

17. Addae MM, Tetteh JK, Ishiwada N, Komada Y, Yamaguchi S, Ofori-Adjei, Kamiya H, Akanmori BD: **High CD4/CD45RO+ and CD8/CD45RO+ frequencies in children with vaccine-modified measles**. *Pediatr Int* 2006, **48**(5):449-453

18. de la Fe C, Assunção P, Saavedra P, Tola S, Poveda C, Poveda JB: **Field trial of two dual vaccines against *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. mycoides (large colony type) in goats**. *Vaccine* 2007, **25**(12):2340-2345

19. Dedieu L, Totte P, Rodrigues V, Vilei EM, Frey J. **Comparative analysis of four lipoproteins from *Mycoplasma mycoides* subsp. mycoides Small Colony identifies LppA as a major T-cell antigen** *Comp Immunol Microbiol Infect Dis* 2010. **33**(4):279-290

20. Gray D, Siepmann K, van Essen D, Poudrier J, Wykes M: **B-T lymphocyte interactions in the generation and survival of memory cells**. *Immunol Rev* 1996, **150**:45–61

21. Akbar A, Terry L, Timms A, Beverly PCL, Janossy G: **Loss of CD45RO and again of UCHL1 reactivity is a feature of primed T cells**. *J Immunol* 1988, **140**:2171–2178

22. Uehara T, Miyawaki T, Ohta K, Tamaru Y, Yokoi T, Nakamura S, Taniguchi N: **Apoptotic cell death of primed CD45RO+ T lymphocytes in Epstein-Barr virus-induced infectious mononucleosis**. *Blood* 1992, **80**:452–458

23. Scacchia M, Sacchini F, Filipponi G, Luciani M, Lelli R, Tjipura-Zaire G, Di Provvido A, Shiningwane A, Ndiipanda F, Pini A, Caporale V, Hübschle OJ: **Clinical, humoral and IFNgamma responses of cattle to infection with *Mycoplasma mycoides* var. mycoides small colony and attempts to condition the pathogenesis of the infection**. *Onderstepoort J Vet Res* 2007, **74**(3):251-263

24. Caccamo N, Meraviglia S, La Mendola C, Guggino G, Dieli F, Salerno A: **Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens**. *J Immunol* 2006, **177**(3):1780-1785

25. Naess LM, Oftung F, Aase A, Wetzler LM, Sandin R, Michaelsen TE: **Human T-cell responses after vaccination with the Norwegian group B meningococcal outer membrane vesicle vaccine**. *Infect Immun* 1998, **66**(3):959-965

26. Totté P, Rodrigues V, Yaya A, Hamadou B, Cisse O, Diallo M, Niang M, Thiaucourt F, Dedieu L: **Analysis of cellular responses to *Mycoplasma mycoides* subsp. mycoides small colony biotype associated with control of contagious bovine pleuropneumonia**. *Vet Res* 2008, **39**(1):8.

27. Totté P, Mather A, Reslan L, Boublik Y, Niang M, Du Plessis D, Dedieu L: **Identification of *Mycoplasma mycoides* subsp. mycoides small colony genes coding for T-cell antigens**. *Clin Vaccine Immunol* 2010, **17**(8):1211-1216

28. Eberl M, Hintz M, Jamba Z, Beck E, Jomaa H, Christiansen G: ***Mycoplasma penetrans* is capable of activating V gamma 9/V delta 2 T cells while other human pathogenic mycoplasmas fail to do so**. *Infect Immun* 2004, **72**(8):4881-4883

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-γ$^+$ T cells during the observation period

The figure 2A shows the relative percentages of $CD4^+CD45R0^+IFN\text{-}\gamma^+$ and $CD4^+CD45RA^+IFN\text{-}\gamma^+$ T-lymphocytes in $CD4^+IFN\text{-}\gamma^+$ cells. In panel B the absolute percentages of $CD4^+CD45R0^+IFN\text{-}\gamma^+$ are shown. It can be noted that the comparison of the percentage of activated $CD4^+CD45R0^+$ 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 $CD8^+CD45R0^+IFN\text{-}\gamma^+$ and $CD8^+CD45RA^+IFN\text{-}\gamma^+$ T-lymphocytes gated on $CD8^+IFN\text{-}\gamma^+$ cells. The comparison of the absolute percentages of $CD8^+CD45R0^+$ IFN-γ^+ 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-γ positive γδ T-cells trend in vaccinated and control groups

The mean percentages of IFN-γ positive γδ 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

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

Agnone et al, Fig 1

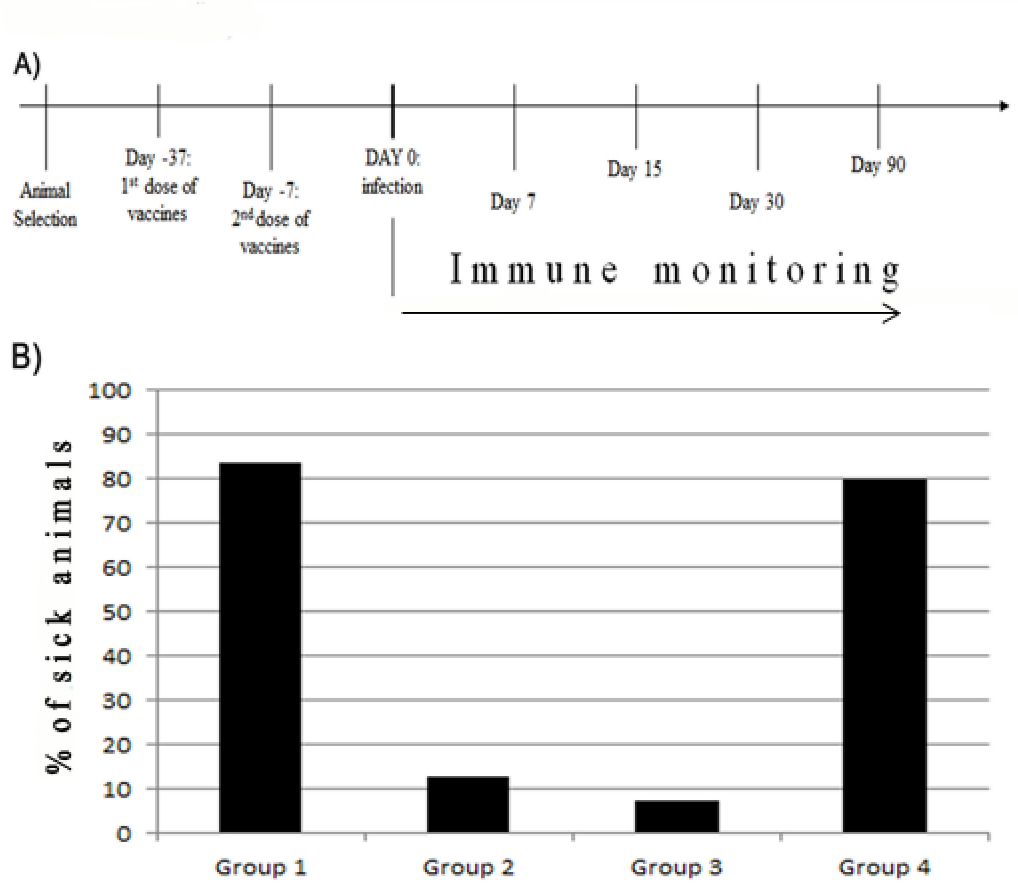


Figure 1

Agnone et al, Fig 2

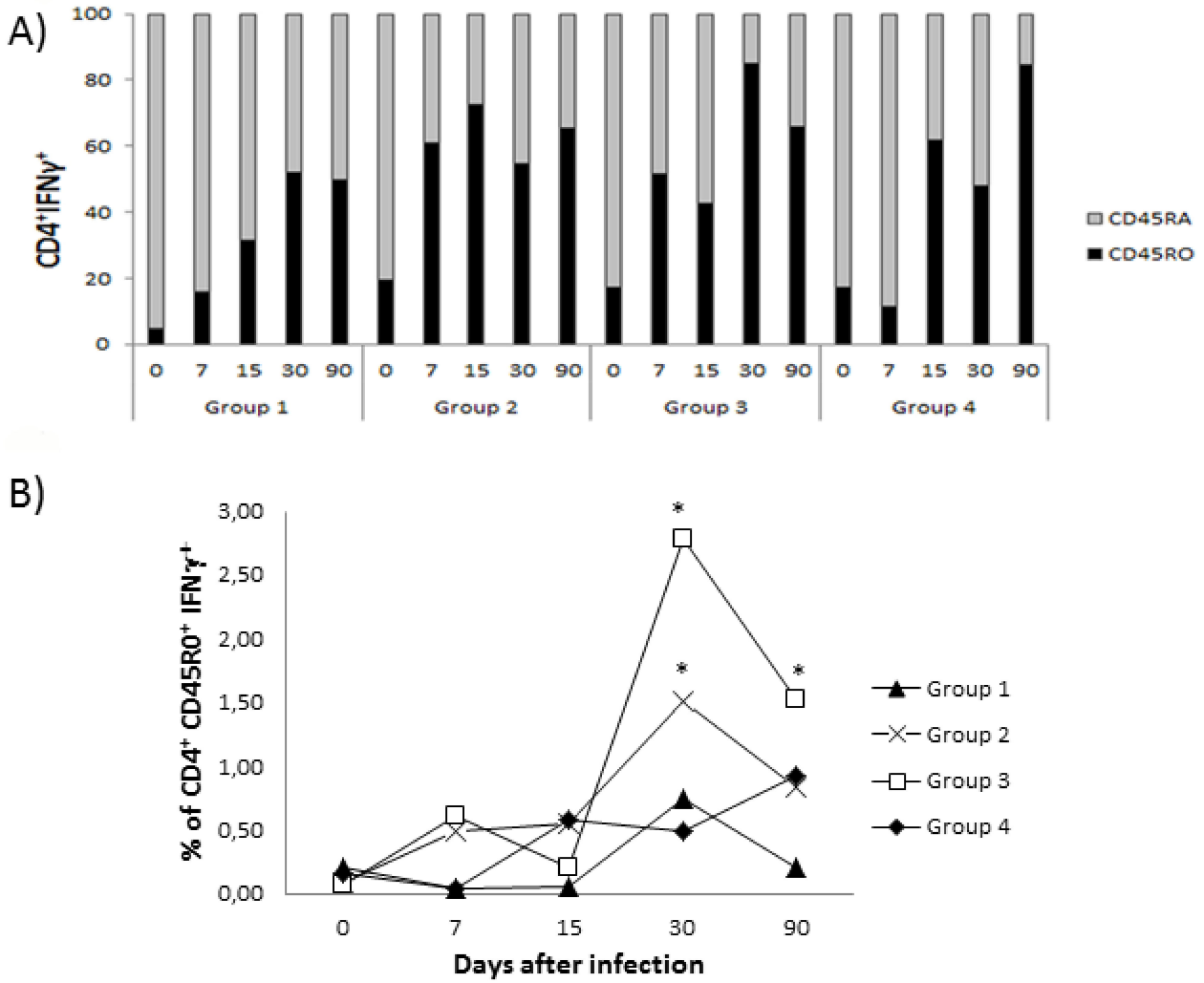


Figure 2

Agnone et al, Fig 3

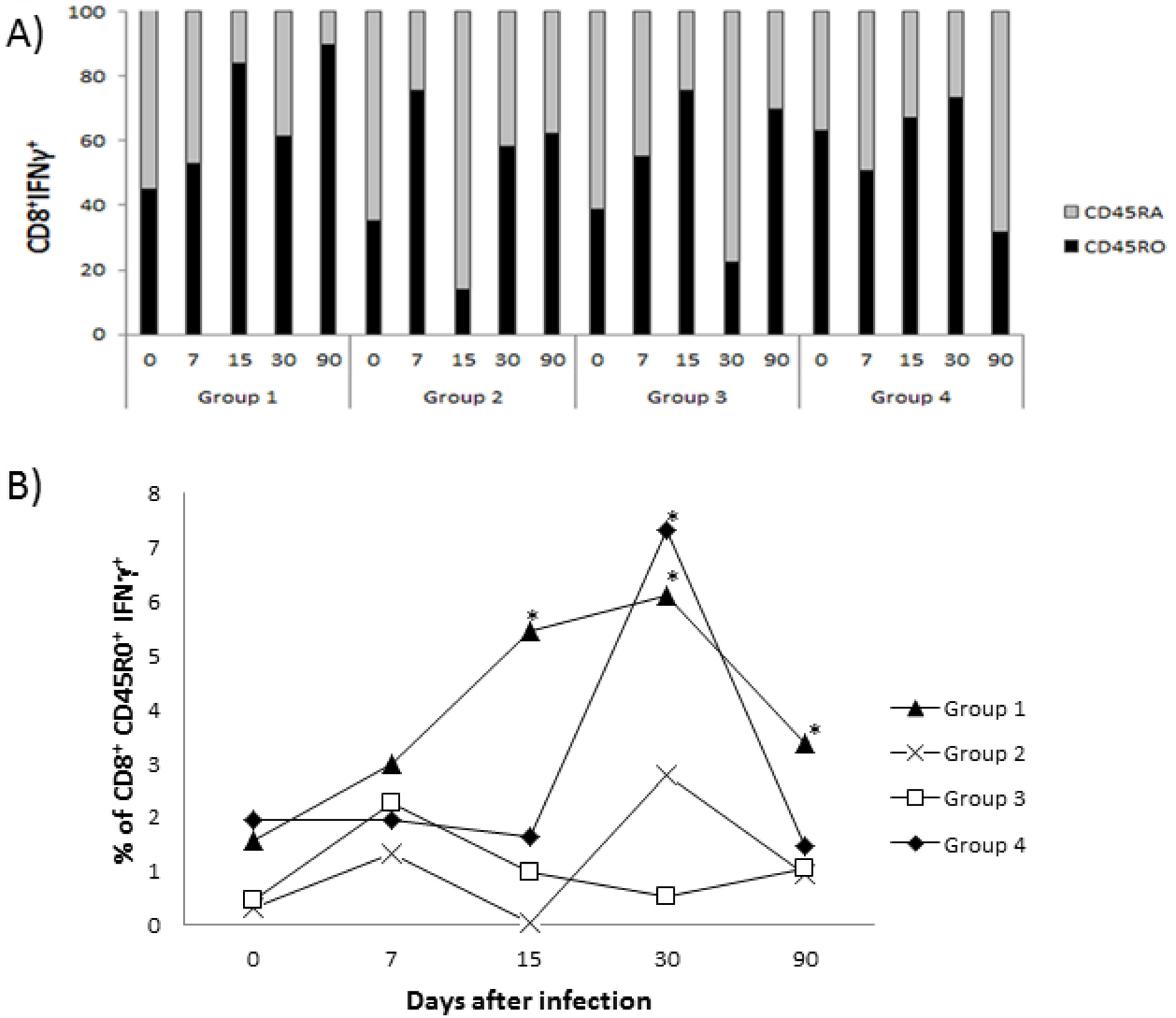


Figure 3

Agnone et al, Fig 4

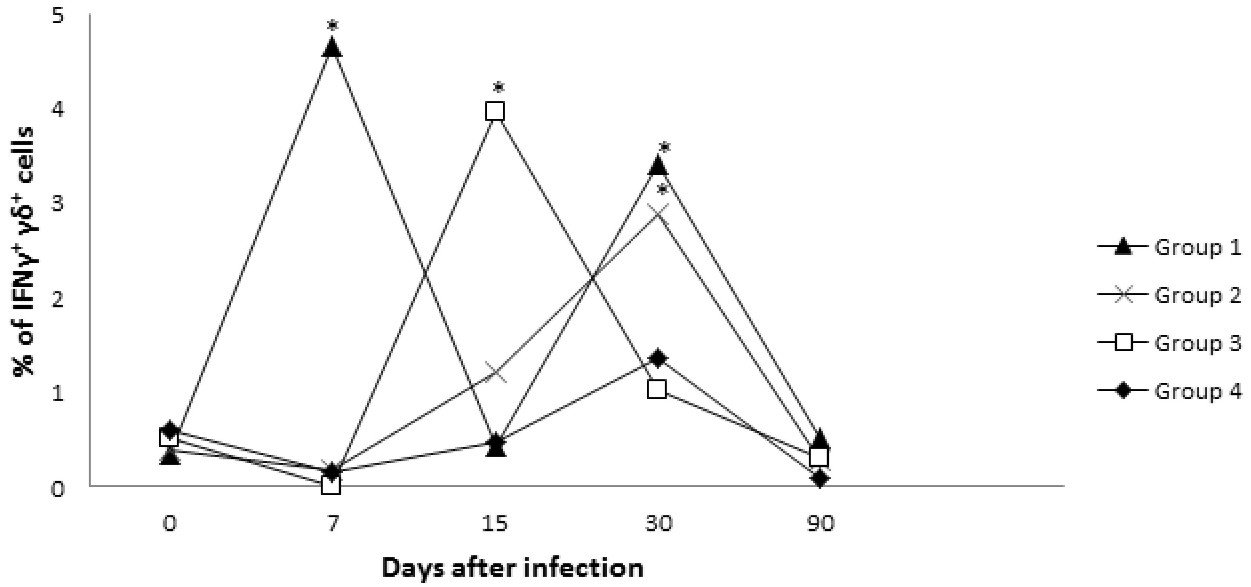


Figure 4

Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 768789, 8 pages
doi:10.1155/2012/768789

Review Article

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

Annalisa Agnone,[1] Alessandra Torina,[2] Gesualdo Vesco,[2] Sara Villari,[2] Fabrizio Vitale,[2] Santo Caracappa,[2] Marco Pio La Manna,[1] Francesco Dieli,[1] and Guido Sireci[1]

[1] *Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi (DiBiMeF), Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy*
[2] *Istituto Zooprofilattico Sperimentale della Sicilia, Via Gino Marinuzzi 3, 90129 Palermo, Italy*

Correspondence should be addressed to Guido Sireci, guido.sireci@unipa.it

Received 12 July 2011; Revised 4 November 2011; Accepted 7 November 2011

Academic Editor: Antonio Cascio

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-γ, together with IL12, determines Th1 induction [6]. IL6 and IL1 are necessary for Th17 production, while the role of TGFβ needs still to be deeper investigated [7, 8]. Finally, activated naïve CD4 T cells stimulated by TGF-β 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 hatche 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-1α, IL-6, IL-10, and TGF-β, but not IL-12 and TNF-α [18]. Prototypical immune responses are characterized by increased lymphoproliferation of $CD4^+$ and $CD8^+$ 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-γ 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-γ production decreases. On the contrary IL-10 concentration decreases with the age of infected puppies while IFN-γ 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-γ 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

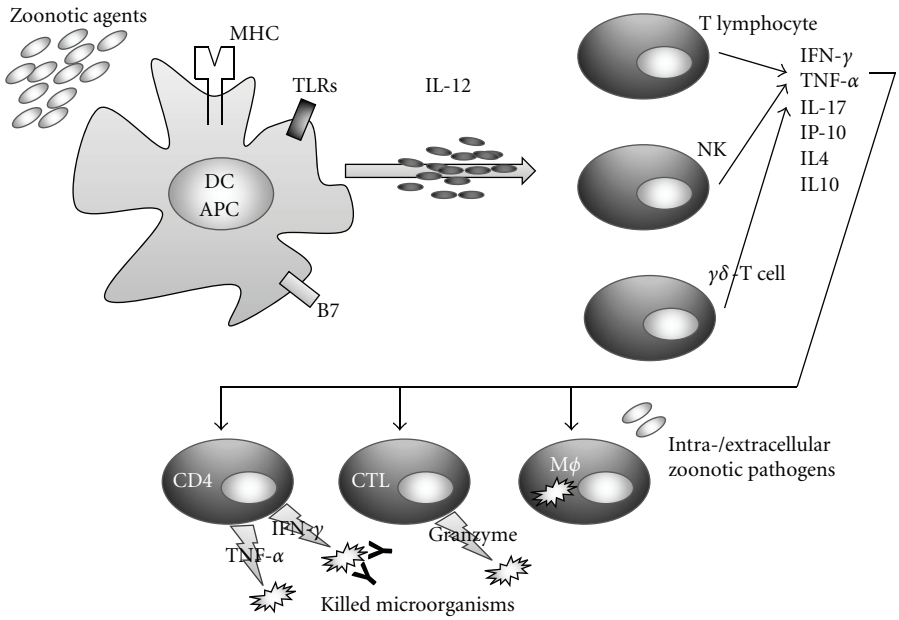


Figure 1: Schematic network of cells and molecules in response to zoonotic agents. An "oversimplified" scenario constituted by various cells and molecules involved both in binding of epitopes derived from pathogens and in the effector mechanisms hereby represented. APCs bind zoonotic derived epitopes 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 $CD4^+$ proliferative T-cell response was observed at the early stage of infection, and IFN-γ, 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-α mRNAs [27].

The opportunistic parasite *Toxoplasma gondii* belongs to the phylum apicomplexa. Feline acts as definitive hosts in its life cycle, while mammalians, 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 $CD8^+$ T-cells response, sustained also by $CD4^+$cells expansion, is mounted [31]. The role of $CD4^+$ in the activation of $CD8^+$ has been demonstrated in mice [32], where the generation of optimal numbers of antigen specific $CD8^+$ effector T cells was found to require $CD4^+$ 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-γ production. The IFN-γ production is sustained by $\gamma\delta$-T lymphocytes [33] that help $CD4^+$ and $CD8^+$T cells to restrict parasite growth until the emerging of the complete adaptive response. It has been recently demonstrated that the $CD8^+$ 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 $CD8^+$ 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, $V\delta2^+$ $\gamma\delta$ 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 $CD4^+$ (Th1) phenotype characterized by IFN-γ secretion while in susceptible mice the dominant response is a $CD4^+$ (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-γ, 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 leishmaniases, 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-γ 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-γ 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-γ 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-γ 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-γ^+ cells were within the $CD4^+$ 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 $CD8^+$ 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 $\gamma\delta$, $CD8^+$, $CD4^+$, 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. $CD4^+$ and $CD8^+$ T cells are key components of anti-mycobacterial immunity [56, 57]. Both IFN-γ 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-γ [60]. The severity of *M. tuberculosis* infection may be detected by measuring $CD4^+$ and $CD8^+$ 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 $CD4^+$ T cells and their naïve, effector, and late differentiated memory subsets [61], with a drop in all the three phenotypic populations. Similarly, $CD8^+$ 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 6 kDa early secretory antigenic target of *Mycobacterium tuberculosis*) are recognized with high frequency by $CD8^+$ T lymphocytes of naturally infected cattle, thus confirming a role of ESAT-6 specific $CD8^+$ T cells in the response to *M. bovis*. Nevertheless, the number of IFN-γ-positive CD8-negative cells was larger than that of IFN-γ^+ $CD8^+$ T cells, indicating that IFN-γ^+ $CD8^+$ 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-α, 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 Vγ9Vδ2 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. Vγ9Vδ2 T cells are specifically stimulated by Brucella to secrete TNF-α, important for the autocrine activation of macrophage functions, IFN-γ, and other cytokines [69]. *In vitro*, Vγ9Vδ2 T cells exhibit a strong cytotoxicity against Brucella-infected cells. Vγ9Vδ2 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 Vγ9Vδ2 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 Vγ9Vδ2 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 Vγ9Vδ2 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 Vγ9Vδ2 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.

References

[1] A. Seimenis, "Zoonoses: a social and economic burden," *Eastern Mediterranean Health Journal*, vol. 4, no. 2, pp. 220–222, 1998.

[2] K. E. Jones, N. G. Patel, M. A. Levy et al., "Global trends in emerging infectious diseases," *Nature*, vol. 451, no. 7181, pp. 990–993, 2008.

[3] OIE (World Organisation for Animal Health), "Cost of national prevention systems for animal diseases and zoonoses in developing and transition countries," Final Report, 2009.

[4] E. M. van Leeuwen, J. Sprent, and C. D. Surh, "Generation and maintenance of memory CD4(+) T Cells," *Current Opinion in Immunology*, vol. 21, no. 2, pp. 167–172, 2009.

[5] C. Dong, "Genetic controls of th17 cell differentiation and plasticity," *Experimental and Molecular Medicine*, vol. 43, no. 1, pp. 1–6, 2011.

[6] J. Zhu and W. E. Paul, "CD4 T cells: fates, functions, and faults," *Blood*, vol. 112, no. 5, pp. 1557–1569, 2008.

[7] M. Veldhoen, R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger, "TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells," *Immunity*, vol. 24, no. 2, pp. 179–189, 2006.

[8] N. J. Wilson, K. Boniface, J. R. Chan et al., "Development, cytokine profile and function of human interleukin 17-producing helper T cells," *Nature Immunology*, vol. 8, no. 9, pp. 950–957, 2007.

[9] W. Chen, W. Jin, N. Hardegen et al., "Conversion of peripheral CD4+CD25-naive T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3," *Journal of Experimental Medicine*, vol. 198, no. 12, pp. 1875–1886, 2003.

[10] S. Baron, Ed., *Medical Microbiology*, chapter 78, University of Texas Medical Branch at Galveston, Galveston, Tex, USA, 4th edition, 1996.

[11] A. K. Abbas, A. H. Lichtman, and S. PilIai, *Cellular and Molecular Immunology*, chapter 13, Saunders Elsevier, 6th edition, 2007.

[12] R. A. Goldsby, T. J Kindt, and B. A. Osborne, *Kuby Immunology*, 4th edition.

[13] Y. J. Guo, K. Y. Wang, and S. H. Sun, "Identification of an HLA-A$*$0201-restricted CD8(+) T-cell epitope encoded within Leptospiral immunoglobulin-like protein A," *Microbes and Infection*, vol. 12, no. 5, pp. 364–373, 2010.

[14] P. P. Chieffi, S. V. dos Santos, M. L. de Queiroz, and S. A. Z. Lescano, "Human toxocariasis: contribution by Brazilian researchers," *Revista do Instituto de Medicina Tropical de Sao Paulo*, vol. 51, no. 6, pp. 301–308, 2009.

[15] D. Despommier, "Toxocariasis: clinical aspects, epidemiology, medical ecology, and molecular aspects," *Clinical Microbiology Reviews*, vol. 16, no. 2, pp. 265–272, 2003.

[16] J. F. Magnaval, L. T. Glickman, P. Dorchies, and B. Morassin, "Highlights of human toxocariasis," *Korean Journal of Parasitology*, vol. 39, no. 1, pp. 1–11, 2001.

[17] G. Rubinsky-Elefant, C. E. Hirata, J. H. Yamamoto, and M. U. Ferreira, "Human toxocariasis: diagnosis, worldwide seroprevalences and clinical expression of the systemic and ocular forms," *Annals of Tropical Medicine and Parasitology*, vol. 104, no. 1, pp. 3–23, 2010.

[18] E. Kuroda, Y. Yoshida, B. E. Shan, and U. Yamashita, "Suppression of macrophage interleukin-12 and tumour necrosis factor-α production in mice infected with Toxocara canis," *Parasite Immunology*, vol. 23, no. 6, pp. 305–311, 2001.

[19] A. Torina, S. Caracappa, A. Barera et al., "Toxocara canis infection induces antigen-specific IL-10 and IFNγ production in pregnant dogs and their puppies," *Veterinary Immunology and Immunopathology*, vol. 108, no. 1-2, pp. 247–251, 2005.

[20] P. R. Torgerson, K. Keller, M. Magnotta, and N. Ragland, "The global burden of alveolar echinococcosis," *PLoS Neglected Tropical Diseases*, vol. 4, no. 6, article e722, 2010.

[21] J. Eckert and P. Deplazes, "Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern," *Clinical Microbiology Reviews*, vol. 17, no. 1, pp. 107–135, 2004.

[22] D. A. Vuitton and B. Gottstein, "Echinococcus multilocularis and its intermediate host: a model of parasite-host interplay," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 923193, 14 pages, 2010.

[23] I. Emery, M. Liance, E. Deriaud, D. A. Vuitton, R. Houin, and C. Leclerc, "Characterization of T-cell immune responses of echinococcus multilocularis-infected C57BL/6J mice," *Parasite Immunology*, vol. 18, no. 9, pp. 463–472, 1996.

[24] F. A. Rojo-Vazquez, J. Pardo-Lledias, M. F. Hunefeld et al., "Cystic echinococcosis in Spain: current situation and relevance for other endemic areas in Europe," *PLoS Neglected Tropical Diseases*, vol. 5, no. 1, article e893, 2011.

[25] H. Wen, T. Aji, and Y. M. Shao, "Diagnosis and management against the complications of human cystic echinococcosis," *Frontiers of Medicine in China*, vol. 4, no. 4, pp. 394–398, 2010.

[26] S. Fauser and P. Kern, "T-Lymphocyte cytokine mRNA expression in cystic echinococcosis," *Acta Tropica*, vol. 64, no. 1-2, pp. 35–51, 1997.

[27] J. Torcal, M. Navarro-Zorraquino, R. Lozano et al., "Immune response and *in vivo* production of cytokines in patients with liver hydatidosis," *Clinical and Experimental Immunology*, vol. 106, no. 2, pp. 317–322, 1996.

[28] M. Munoz, O. Liesenfeld, and M. M. Heimesaat, "Immunology of Toxoplasma gondii," *Immunological Reviews*, vol. 240, no. 1, pp. 269–285, 2011.

[29] J. McAuley, K. M. Boyer, D. Patel et al., "Early and longitudinal evaluations of treated infants and children and untreated historical patients with congenital toxoplasmosis: the Chicago collaborative treatment trial," *Clinical Infectious Diseases*, vol. 18, no. 1, pp. 38–72, 1994.

[30] E. Y. Denkers and R. T. Gazzinelli, "Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection," *Clinical Microbiology Reviews*, vol. 11, no. 4, pp. 569–588, 1998.

[31] I. A. Khan, K. A. Smith, and L. H. Kasper, "Induction of antigen-specific human cytotoxic T cells by Toxoplasma gondii," *Journal of Clinical Investigation*, vol. 85, no. 6, pp. 1879–1886, 1990.

[32] M. Tsuji, P. Mombaerts, L. Lefrancois, R. S. Nussenzweig, F. Zavala, and S. Tonegawa, "$\gamma\delta$ T cells contribute to immunity against the liver stages of malaria in $\alpha\beta$ T-cell-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 1, pp. 345–349, 1994.

[33] K. A. Jordan, E. H. Wilson, E. D. Tait et al., "Kinetics and phenotype of vaccine-induced CD8+ T-cell responses to Toxoplasma gondii," *Infection and Immunity*, vol. 77, no. 9, pp. 3894–3901, 2009.

[34] R. Bhadra, H. Guan, and I. A. Khan, "Absence of both IL-7 and IL-15 severely impairs the development of CD8+ T cell response against Toxoplasma gondii," *PLoS ONE*, vol. 5, no. 5, Article ID e10842, 2010.

[35] R. McLeod, M. O. Beem, and R. G. Estes, "Lymphocyte anergy specific to Toxoplasma gondii antigens in a baby with congenital toxoplasmosis," *Journal of Clinical and Laboratory Immunology*, vol. 17, no. 3, pp. 149–153, 1985.

[36] J. H. Yamamoto, A. L. Vallochi, C. Silveira et al., "Discrimination between patients with acquired toxoplasmosis and congenital toxoplasmosis on the basis of the immune response to parasite antigens," *Journal of Infectious Diseases*, vol. 181, no. 6, pp. 2018–2022, 2000.

[37] T. Hara, S. Ohashi, Y. Yamashita et al., "Human Vδ2+ $\gamma\delta$ T-cell tolerance to foreign antigens of Toxoplasma gondii," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 10, pp. 5136–5140, 1996.

[38] A. Pavli and H. C. Maltezou, "Leishmaniasis, an emerging infection in travelers," *International Journal of Infectious Diseases*, vol. 14, no. 12, pp. e1032–e1039, 2010.

[39] World Health Organization, "Control of the Leishmaniases Report of a meeting of the WHO Expert Committee on the control of the Leishmaniases," World Health Organization, Geneva, Switzerland, 2010.

[40] M. T. M. Roberts, "Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment," *British Medical Bulletin*, vol. 75-76, no. 1, pp. 115–130, 2005.

[41] Y. Vanloubbeeck and D. E. Jones, "The immunology of Leishmania infection and the implications for vaccine development," *Annals of the New York Academy of Sciences*, vol. 1026, pp. 267–272, 2004.

[42] E. Mougneau, F. Bihl, and N. Glaichenhaus, "Cell biology and immunology of Leishmania," *Immunological Reviews*, vol. 240, no. 1, pp. 286–296, 2011.

[43] L. Manna, S. Reale, E. Viola et al., "Leishmania DNA load and cytokine expression levels in asymptomatic naturally infected dogs," *Veterinary Parasitology*, vol. 142, no. 3-4, pp. 271–280, 2006.

[44] L. Manna, S. Reale, E. Picillo, F. Vitale, and A. E. Gravino, "Interferon-gamma (INF-γ), IL4 expression levels and Leishmania DNA load as prognostic markers for monitoring response to treatment of leishmaniotic dogs with miltefosine and allopurinol," *Cytokine*, vol. 44, no. 2, pp. 288–292, 2008.

[45] G. M. Santos-Gomes, R. Rosa, C. Leandro, S. Cortes, P. Romão, and H. Silveira, "Cytokine expression during the outcome of canine experimental infection by Leishmania infantum," *Veterinary Immunology and Immunopathology*, vol. 88, no. 1-2, pp. 21–30, 2002.

[46] A. R. Bharti, J. E. Nally, J. N. Ricaldi et al., "Leptospirosis: a zoonotic disease of global importance," *The Lancet Infectious Diseases*, vol. 3, no. 12, pp. 757–771, 2003.

[47] F. Xue, H. Dong, J. Wu et al., "Transcriptional responses of Leptospira interrogans to host innate immunity: significant changes in metabolism, oxygen tolerance, and outer membrane," *PLoS Neglected Tropical Diseases*, vol. 4, no. 10, article e857, 2010.

[48] C. A. Bolin, J. A. Cassells, R. L. Zuerner, and G. Trueba, "Effect of vaccination with a monovalent Leptospira interrogans serovar hardjo type hardjo-bovis vaccine on type hardjo-bovis infection of cattle," *American Journal of Veterinary Research*, vol. 52, no. 10, pp. 1639–1643, 1991.

[49] B. M. Naiman, S. Blumerman, D. Alt et al., "Evaluation of type 1 immune response in naïve and vaccinated animals following challenge with Leptospira borgpetersenii serovar Hardjo: involvement of WC1(+) $\gamma\delta$ and CD4 T cells," *Infection and Immunity*, vol. 70, no. 11, pp. 6147–6157, 2002.

[50] W. C. Brown, T. F. McElwain, G. H. Palmer, S. E. Chantler, and D. M. Estes, "Bovine CD4+ T-lymphocyte clones specific for rhoptry-associated protein 1 of Babesia bigemina stimulate enhanced immunoglobulin G1 (IgG1) and IgG2 synthesis," *Infection and Immunity*, vol. 67, no. 1, pp. 155–164, 1999.

[51] B. M. Naiman, D. Alt, C. A. Bolin, R. Zuerner, and C. L. Baldwin, "Protective killed Leptospira borgpetersenii vaccine induces potent Th1 immunity comprising responses by CD4 and $\gamma\delta$ T lymphocytes," *Infection and Immunity*, vol. 69, no. 12, pp. 7550–7558, 2001.

[52] A. Lowanitchapat, S. Payungporn, A. Sereemaspun et al., "Expression of TNF-α, TGF-β, IP-10 and IL-10 mRNA in kidneys of hamsters infected with pathogenic Leptospira," *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 33, no. 5, pp. 423–434, 2010.

[53] M. Lipp and G. Müller, "Shaping up adaptive immunity: the impact of CCR7 and CXCR5 on lymphocyte trafficking," *Verhandlungen der Deutschen Gesellschaft für Pathologie*, vol. 87, pp. 90–101, 2003.

[54] WHO, "Global tuberculosis control," WHO Report, The Russian Federation, WHO, Geneva, Switzerland, 2005.

[55] J. M. Rocco and V. R. Irani, "Mycobacterium avium and modulation of the host macrophage immune mechanisms," *International Journal of Tuberculosis and Lung Disease*, vol. 15, no. 4, pp. 447–452, 2011.

[56] C. S. Aagaard, T. T. K. T. Hoang, C. Vingsbo-Lundberg, J. Dietrich, and P. Andersen, "Quality and vaccine efficacy of CD4+ T cell responses directed to dominant and subdominant epitopes in ESAT-6 from Mycobacterium tuberculosis," *Journal of Immunology*, vol. 183, no. 4, pp. 2659–2668, 2009.

[57] K. Hoebe, E. Janssen, and B. Beutler, "The interface between innate and adaptive immunity," *Nature Immunology*, vol. 5, no. 10, pp. 971–974, 2004.

[58] N. Caccamo, S. Meraviglia, C. la Mendola, G. Guggino, F. Dieli, and A. Salerno, "Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens," *Journal of Immunology*, vol. 177, no. 3, pp. 1780–1785, 2006.

[59] Y. Tsukamoto, M. Endoh, T. Mukai et al., "Immunostimulatory activity of major membrane protein II from Mycobacterium tuberculosis," *Clinical and Vaccine Immunology*, vol. 18, no. 2, pp. 235–242, 2011.

[60] S. Davoudi, M. Rasoolinegad, M. Younesian et al., "CD4+ cell counts in patients with different clinical manifestations of tuberculosis," *Brazilian Journal of Infectious Diseases*, vol. 12, no. 6, pp. 483–486, 2008.

[61] D. S. S. Rodrigues, E. A. S. Medeiros, L. Y. Weckx, W. Bonnez, R. Salomão, and E. G. Kallas, "Immunophenotypic characterization of peripheral T lymphocytes in Mycobacterium tuberculosis infection and disease," *Clinical and Experimental Immunology*, vol. 128, no. 1, pp. 149–154, 2002.

[62] F. Vitale, S. Reale, E. Petrotta et al., "ESAT-6 peptide recognition by bovine CD8+ lymphocytes of naturally infected cows in herds from southern Italy," *Clinical and Vaccine Immunology*, vol. 13, no. 4, pp. 530–533, 2006.

[63] N. Caccamo, G. Guggino, S. A. Joosten et al., "Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection," *European Journal of Immunology*, vol. 40, no. 8, pp. 2211–2220, 2010.

[64] C. Agrati, E. Cimini, A. Sacchi et al., "Activated Vγ9Vδ2 T cells trigger granulocyte functions via MCP-2 release," *Journal of Immunology*, vol. 182, no. 1, pp. 522–529, 2009.

[65] B. S. A. Kabeer, A. Raja, B. Raman et al., "IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy," *BMC Infectious Diseases*, vol. 11, p. 135, 2011.

[66] T. Buzgan, M. K. Karahocagil, H. Irmak et al., "Clinical manifestations and complications in 1028 cases of brucellosis: a retrospective evaluation and review of the literature," *International Journal of Infectious Diseases*, vol. 14, no. 6, pp. e469–e478, 2010.

[67] J. Dornand, A. Gross, V. Lafont, J. Liautard, J. Oliaro, and J. P. Liautard, "The innate immune response against Brucella in humans," *Veterinary Microbiology*, vol. 90, no. 1–4, pp. 383–394, 2002.

[68] A. Bertotto, R. Gerli, F. Spinozzi et al., "Lymphocytes bearing the $\gamma\delta$ T cell receptor in acute Brucella melitensis infection," *European Journal of Immunology*, vol. 23, no. 5, pp. 1177–1180, 1993.

[69] F. Ottones, J. Liautard, A. Gross, F. Rabenoelina, J. P. Liautard, and J. Favero, "Activation of human Vγ9Vδ2 T cells by a Brucella suis non-peptidic fraction impairs bacterial intracellular multiplication in monocytic infected cells," *Immunology*, vol. 100, no. 2, pp. 252–258, 2000.

[70] S. Bessoles, M. Ni, S. Garcia-Jimenez, F. Sanchez, and V. Lafont, "Role of NKG2D and its ligands in the anti-infectious activity of Vγ9Vδ2 T cells against intracellular bacteria," *European Journal of Immunology*, vol. 41, no. 6, pp. 1619–1628, 2011.

Elsevier Editorial System(tm) for Ticks and Tick-Borne Diseases
Manuscript Draft

Manuscript Number:

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

Article Type: Original Research Article

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

Corresponding Author: Dr Alessandra Torina,

Corresponding Author's Institution:

First Author: Alessandra Torina

Order of Authors: Alessandra Torina; Annalisa Agnone; Valeria Blanda; Angelina Alongi; Rosalia D'Agostino; Santo Caracappa; Anna Marino; Vincenzo Di Marco; Josè de la Fuente

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.

Suggested Reviewers: Katherine M Kocan
Katherine.Kocan@okstate.edu
Dr Kokan has a very deep knowledge on Anaplasma and on diagnostic methods. She has a very long experience and a number of related publications.

Edmour Blouin
Edmour.Blouin@okstate.edu
His long expertize in the field is attested by a number of related publications

Opposed Reviewers:

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

Alessandra Torina[a*], Annalisa Agnone[b§], Valeria Blanda[a§], Angelina Alongi[a], Rosalia D'Agostino[a], Santo Caracappa[a], Anna M.F. Marino[a], Vincenzo Di Marco[a] and Josè de la Fuente[c]

[a]Istituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi n.3, 90123 Palermo, Italy.

[b]Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi – Università di Palermo – Corso Tukory n.211, 90134 Palermo, Italy.

[c]Instituto de Investigación en Recursos Cinegéticos IREC-CSIC-UCLM-JCCM, Ronda e Toledo s/n, 13005 Ciudad Real, Spain.

[§]AA and VB contributed equally to this work

*Corresponding author:

Alessandra Torina

Istituto Zooprofilattico Sperimentale della Sicilia

Via Gino Marinuzzi, 3 - 90129 Palermo

Tel. 00390916565360, Fax 00390916565361

E-mail: alessandra.torina@izssicilia.it; alessandra.torina@libero.it

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 *Anaplama* 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 ΔG values were lower than -2 kcal/mol and the ΔG 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 $MgCl_2$ 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 µl. The obtained PCR products were visualized by UV lamp in a 1.5% agarose gel containing 0.1µg/ 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 $MgCl_2$, 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 µl.

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. o*vis (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.

Acknowledgments

This work was supported by the Italian Ministry of Health (IZSSi 07/08 and IZSSi 09/09).

References

Aktaş, M., Altay, K., Dumanlı, N., 2005. Development of a polymerase chain reaction method for diagnosis of *Babesia ovis* infection in sheep and goats. Vet Parasitol 133: 277–281.

Aubry, P., Geale, D.W., 2011. A review of bovine anaplasmosis. Transbound Emerg Dis. 58(1): 1-30.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F.G., Inoue, N., Alhassan, A., Kanno, T., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2002. Detection of natural infection of *Boophilus microplus* with *Babesia equi* and *Babesia caballi* in Brazilian horses using nested polimerase chain reaction. Vet Parasitol 107: 351-357.

Brayton, K.A., Palmer, G.H., Brown, W.C., 2006. Genomic and proteomic approaches to vaccine candidate identification for *Anaplasma marginale*. Expert Rev Vaccines 5: 95-101.

Carret, C., Walas, F., Carcy, B., Grande, N., Prècigout, E., Moubri, K., Schetters, T.P., and Gorenflot, A., 1999. Babesia canis canis, *Babesia canis vogeli*, *Babesia canis rossi*: Differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J Eukaryot Microbiol, 46(3): 298-303.

D'Oliveira, C., van der Weide, M., Habela, M.A., Jacquiet, P., Jongejan, F., 1995. Detection of *Theileria annulata* in blood samples of carriers by PCR. J Clin Microbiol 33: 1665-2669.

de la Fuente, J., Atkinson, M.W., Hogg, J.T., Miller, D.S., Naranjo, V., Almazán, C., Anderson, N., and Kocan, K.M., 2006. Genetic characterization of *Anaplasma ovis* strains from Bighorn Sheep in Montana. J Wildl Dis 42(2): 381–385.

de la Fuente, J., Atkinson, M.W., Naranjo, V., Fernández de Mera, I.G., Mangold, A.J., Keating, K.A., and Kocan, K.M., 2007. Sequence analysis of the *msp4* gene of *Anaplasma ovis* strains. Vet Microbiol 119: 375–381.

de la Fuente, J., Lew, A., Lutz, H., Meli, M.L., Hofmann-Lehmann, R., Shkap, V., Molad, T., Mangold, A.J., Almazán, C., Naranjo, V., Gortázar, C., Torina, A., Caracappa, S., García-Pérez, A.L., Barral, M., Oporto, B., Ceci, L., Carelli, G., Blouin, E.F., Kocan, K.M., 2005c. Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. Anim Health Res Rev 6: 75–89.

de la Fuente, J., Massung, R.F., Wong, S.J., Frederick, K.C., Lutz, H., Meli, M., von Loewenich, F.D., Grzeszczuk, A., Torina, A., Caracappa, S., Mangold, A.J., Narajo, V., Stuen, S., and Kocan, K.M., 2005b. Sequence analysis of the msp4 gene of *Anaplasma phagocytophilum* strains. J Clin Microbiol 43(3): 1309-1317.

de la Fuente, J., Naranjo, V., Ruiz-Fons, F., Höfle, U., Fernández de Mera, I.G., Villanúa, D., Almazán, C., Torina, A., Caracappa, S., Kocan, K.M., Gortázar, C., 2005a. Potential vertebrate reservoir hosts and invertebrate vectors of *Anaplasma marginale* and *A. phagocytophilum* in central Spain. Vector-Borne and Zoonotic Diseases 5: 390-401.

de la Fuente, J., Ruiz-Fons, F., Naranjo, V., Torina, A., Rodríguez, O., Gortázar, C. 2008. Evidence of *Anaplasma* infections in European roe deer (*Capreolus capreolus*) from southern Spain. Research in Veterinary Science 84: 382-386.

de la Fuente, J., van den Bussche, R.A., Prade, T.M., Kocan, K.M., 2003. *Anaplasma marginale* msp1α Genotypes evolved under positive selection pressure but are not markers for geographic isolates. J Clin Microbiol 41(4): 1609-1616.

Dumler, J.S., Barbet, A.C., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa Y., and Rurangirwa, F.R., 2001. Reorganization of the genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol 51: 2145–2165.

Dunning Hotopp, J.C., Lin, M., Madupu, R., Crabtree, J., Angiuoli, S.V., Eisen, J., Seshadri, R., Ren, Q., Wu, M., Utterback, T.R., Smith, S., Lewis, M., Khouri, H., Zhang, C., Niu, H., Lin, Q., Ohashi, N., Zhi, N., Nelson, W., Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sundaram, J., Daugherty, S.C., Davidsen, T., Durkin, A.S., Gwinn, M., Haft, D.H., Selengut, J.D., Sullivan, S.A., Zafar, N., Zhou, L., Benahmed, F., Forberger, H., Halpin, R., Mulligan, S., Robinson, J., White, O., Rikihisa, Y., Tettelin, H., 2006. Comparative genomics of emerging human ehrlichiosis agents. PLoS Genet 2: e21.

Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G,M, 1993. Multiplex polimerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet Parasitol 50: 69-81.

Harrus, S., Waner, T., Aizenberg, I., Foley, J.E., Poland, A.M., and Bark, H., 1998. Amplification of Ehrlichial DNA from Dogs 34 Months after Infection with *Ehrlichia canis*. J Clin Microbiol 36(1): 73-6.

Hornok, S., Hofmann-Lehmann, R., Fernández de Mera, I.G., Meli, M.L., Elek, V., Hajtós, I., Répási, A., Gönczi, E., Tánczos, B., Farkas, R., Lutz, H., de la Fuente, J. 2010. Survey on blood-sucking lice (Phthiraptera: Anoplura) of ruminants and pigs with molecular detection of *Anaplasma* and *Rickettsia* spp. Veterinary Parasitology 174: 355-358.

Hornok, S., Micsutka, A., Fernández de Mera, I.G., Meli, M.L., Gönczi, E., Tánczos, B., Mangold, A.J., Farkas, R., Lutz, H., Hofmann-Lehmann, R., de la Fuente, J. 2011. Fatal bovine anaplasmosis in a herd with new genotypes of *Anaplasma marginale, A. ovis* and concurrent haemoplasmosis. Research in Veterinary Science, in press.

Inokuma, H., Fujii, K., Matsumoto, K., Okuda, M., Nakagome, K., Kosugi, R., Hirakawa, M., Onishi T., 2002. Demonstration of *Analasma* (*Ehrlichia*) *platys* inclusions in pheripheral blood platelets of a dog in Japan. Vet Parasitol 110: 145-152.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Garcia-Garcia, J.C., 2004. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. Parasitology 129: S285–S300.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The natural history of *Anaplasma marginale.* Vet Parasitol 167(2-4): 95-107.

Krier, J.P., Ristic, M., 1963. Anaplasmosis. VII. Experimental *Anaplasma ovis* infection in whitetailed deer (*Dama virginiana*). Am J Vet Res 24: 567–572.

Kuttler, K.L., 1981. Infection of splenectomized calves with *Anaplasma ovis*. Am J Vet Res 42(12): 2094-6.

Kuttler, K.L., 1984. *Anaplasma* infections in wild and domestic ruminants: A review. J Wildl Dis 20: 12–20.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G., 2007. ClustalW and ClustalX version. Bioinformatics 23: 2947–2948.

Muhlnickel, C.J., Jefferies, R., Morgan-Ryan, U.M., Irwin, P.J., 2002. *Babesia gibsoni* infection in three dogs in Victori. Aust Vet J 80(10): 606-10.

OIE (World Organisation for Animal Health), 2008. Chapter 2.4.1. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th ed., pp. 601– 602. Paris, France.

Persing, D.H., Mathiesen, D., Marshall, W.F. Telford SR, Spielman A., Thomford J.W., Conrad P.A., 1992. Detection of *Babesia microti* by polymerase chain reaction. J Clin Microbiol. 30(8): 2097-103.

Stuen, S., Nevland, S., Moum, T., 2003. Fatal cases of tick-borne fever (TBF) in sheep caused by several 16S rRNA gene variants of *Anaplasma phagocytophilum*. Ann NY Acad Sci 990: 443-434.

To, H., Kako, N., Zhang, G.Q., Otsusa, H., Ogawa, M., Ochiai, O., Nguyen, S.V., Yamaguchi, T., Fukushi, H., Nagaoka, N., Akiyama, M., Amano, K., and Hirai, K., 1996. Q Fever Pneumonia in Children in Japan. J Clin Microbiol. 34(3): 647-51.

Torina, A., Alongi, A., Naranjo, V., Estrada-Peña, A., Vicente, J., Scimeca, S., Marino, A.M.F., Salina, F., Caracappa, S., and de la Fuente, J., 2008. Prevalence and genotypes of *Anaplasma* species and habitat suitability for ticks in a Mediterranean ecosystem. Appl Environ Microbiol 74 (24): 7578–7584.

Tzianabos, T., Anderson, B.E., McDade, J.E., 1989. Detection of *Rickettsia rickettsii* DNA in clinical specimens by using polymerase chain reaction technology. J Clin Microbiol 27(12): 2866-8.

Zaugg, J.L, 1987. Experimental infections of *Anaplasma ovis* in pronghorn antelope. J Wildl Dis 23: 205–210.

Zaugg, J.L., 1988. Experimental anaplasmosis in mule deer: Persistence of infection of *Anaplasma marginale* and susceptibility to *A. ovis*. J Wildl Dis 24: 120–126.

Zaugg, J.L., Goff, W.L., Foreyt, W., Hunter, D.L., 1996. Susceptibility of elk (*Cervus elaphus*) to experimental infection with *Anaplasma marginale* and *A. ovis*. J Wildl Dis 32: 62–66.

Table 1. PCRs used for pathogen detection

Assay	Primers	Target	Amplicon size (bp)
A. ovis/A. marginale[7]	MSP43 / MSP45	*msp4*	851
Anaplasma spp.[30]	16SANAF / 16SANAR	16S rRNA	467
A. phagocytophilum[9]	MSP4AP5 / MSP4AP3	*msp4*	849
A. platys[20]	EPLAT-5 / ERB2	16SrRNA	780
Ehrlichia canis[17]	ECC / ECB	16SrRNA	480
Rickettsia conorii[33]	Tz 15-19/Tz 16-20	17KDa	246
Coxiella burnetii[31]	Q3 / Q5	htpB	501
Babesia ovis[1]	Bbo-F /Bbo-R	ssu rRNA	549
Babesia bovis[16]	BOF/BOR	RAP 1	356
Babesia bigemina[16]	BiIA / BiIB	RAP 1	278
Babesia canis[5]	PIROA / PIROB	ssrRNA	400
Babesia gibsoni[27]	PIRO A1 /Piro B	18SrRNA	450
Babesia caballi[3]	Bc48R3 / Bc48F1	48KDa rhoptry protein	530
Babesia microti[29]	BAB 1 / BAB 4	ssrRNA	238
Theileria equi[3]	EMA5 / EMA6	merozoite antigen 1	268
Theileria annulata[6]	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 $5x10^{-8}$ 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

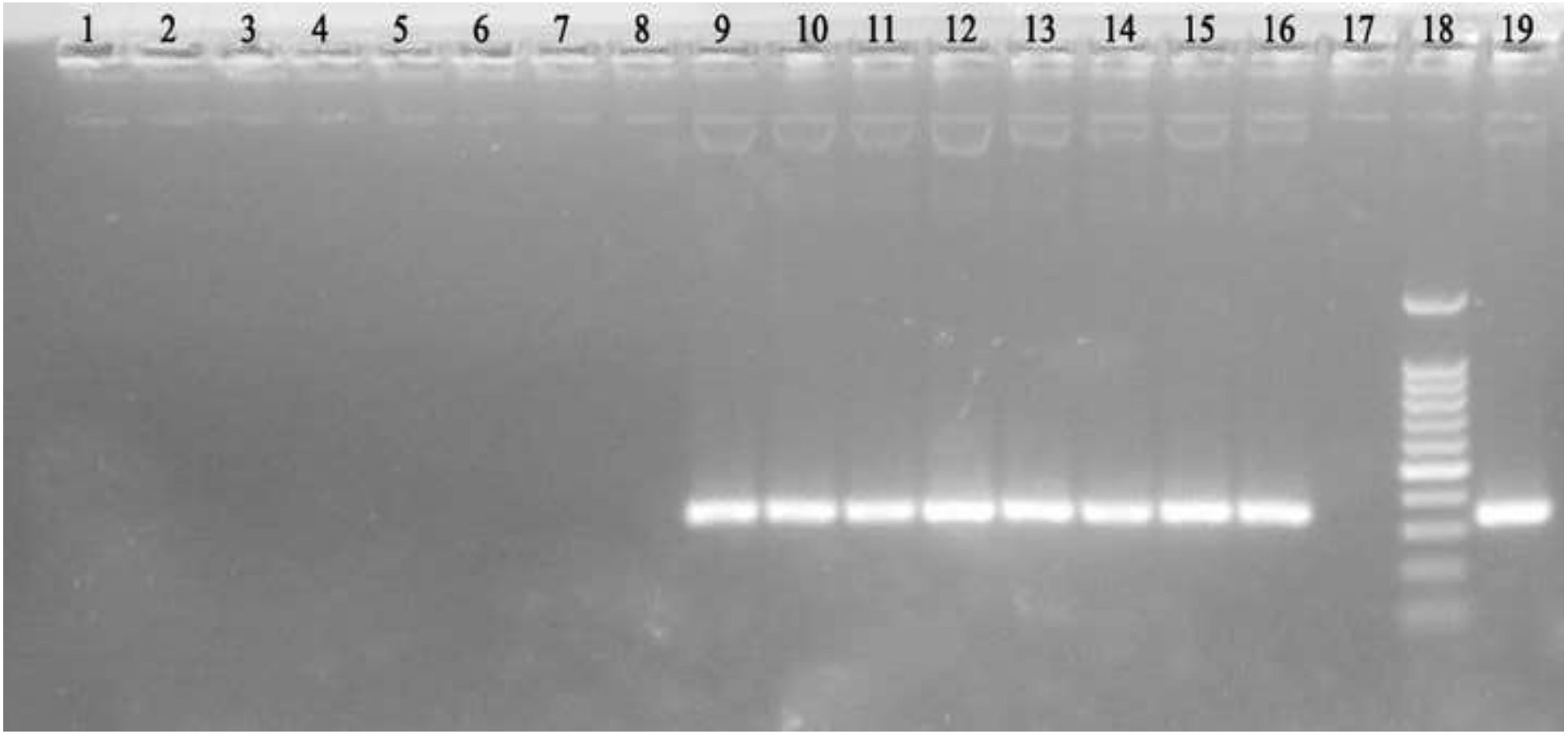


Figure 2

Click here to download high resolution image

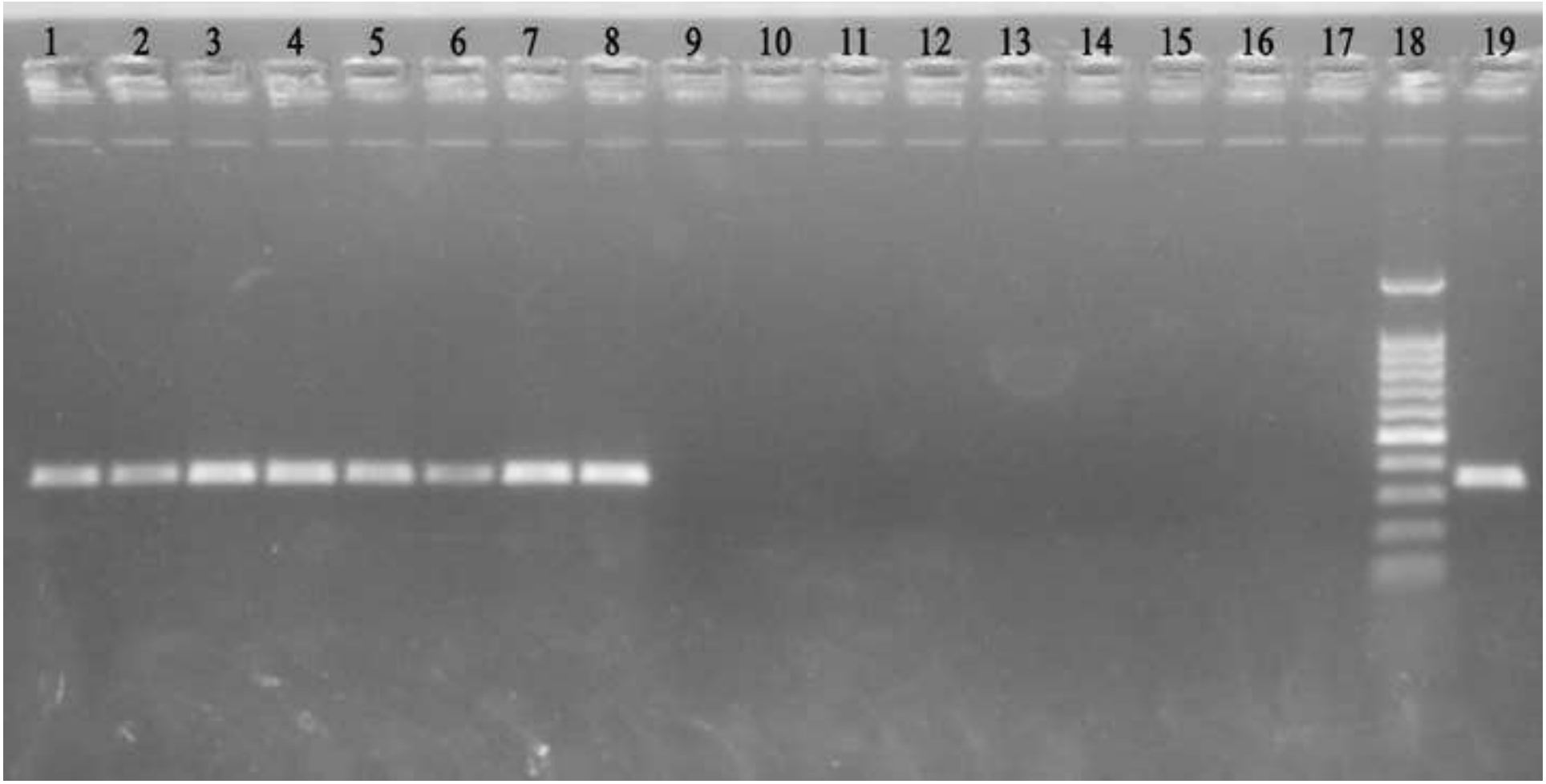
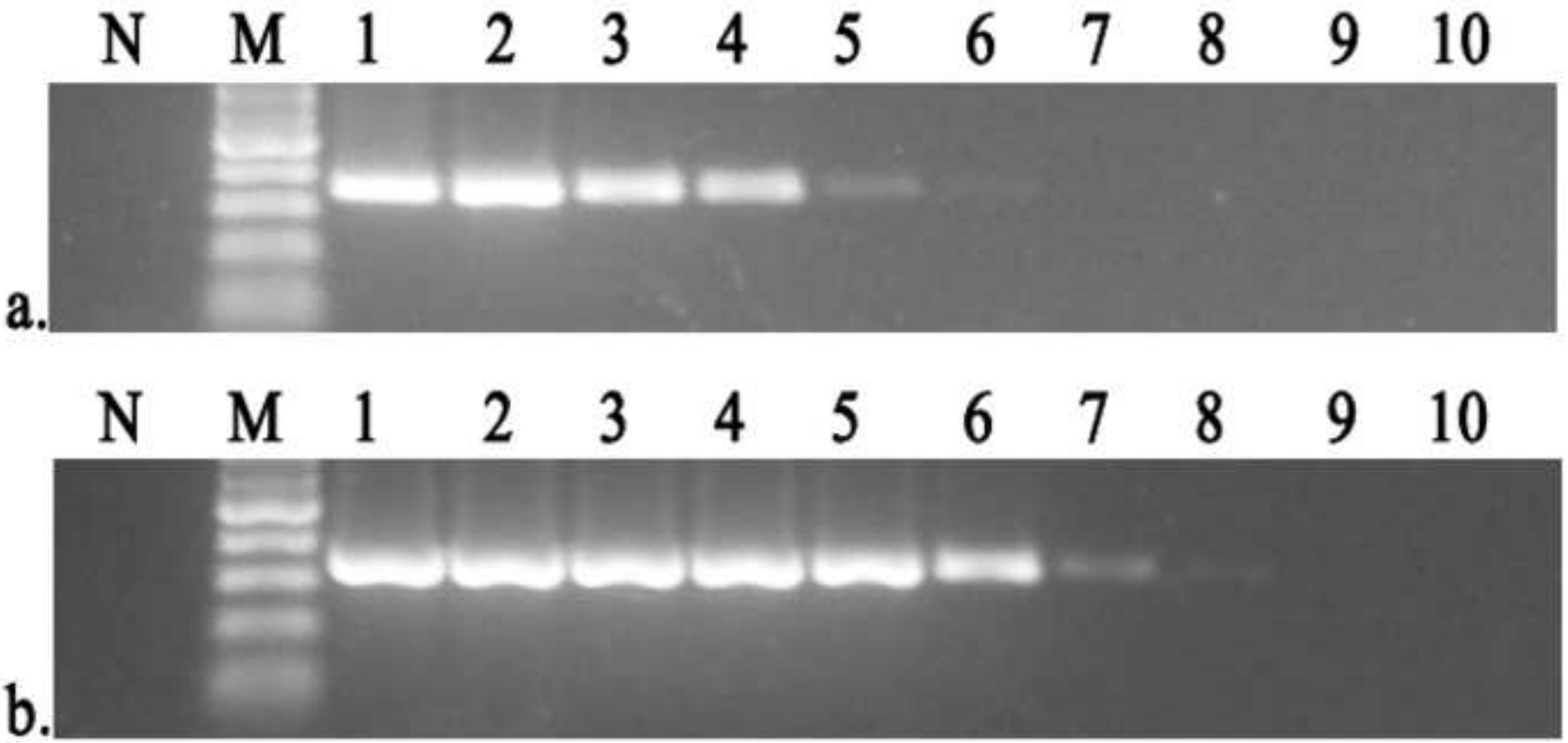


Figure 3

Click here to download high resolution image



Elsevier Editorial System(tm) for Ticks and Tick-Borne Diseases
Manuscript Draft

Manuscript Number:

Title: Prevalence of Anaplasma, Ehrlichia, Rickettisa and Coxiella in different tick species

Article Type: Original Research Article

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

Corresponding Author: Dr Alessandra Torina,

Corresponding Author's Institution: Istituto Zooprofilattico Sperimentale della Sicilia

First Author: Alessandra Torina

Order of Authors: Alessandra Torina; Santo Caracappa; Guido Sireci; Angelina Alongi; Salvatore Scimeca; Rosalia D'Agostino; Valeria Blanda; Annalisa Agnone; Vincenzo Di Marco

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, Rickettisa 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.

Suggested Reviewers: Katherine Kocan
Katherine.Kocan@okstate.edu
She has been working on tick borne diseases for a long time.

Victoria Naranjo
MVictoria.Naranjo@uclm.es
Dr Naranjo has a good expertize on ticks and tick borne parasites

Opposed Reviewers:

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

Alessandra Torina[a]*, Santo Caracappa[a], Guido Sireci[b], Angelina Alongi[a], Salvatore Scimeca[a], Rosalia D'Agostino[a], Valeria Blanda[a], Annalisa Agnone[b], Vincenzo Di Marco[a]

[a]Istituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi n.3, 90123 Palermo, Italy.

[b]Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi – Università di Palermo – Corso Tukory n.211, 90134 Palermo, Italy.

Corresponding author:

Alessandra Torina

Istituto Zooprofilattico Sperimentale della Sicilia

Via Gino Marinuzzi, 3

90129 Palermo, Italy

Phone: 0039 091 6565360

Fax: 0039 091 6565361

alessandra.torina@libero.it

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

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, Rickettisa* 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, Rickettisa , Coxiella,* Italy

Introduction

Ticks (Acari: Ixodidae) are important parasites present in almost every geographic zone in the world. Ticks are vectors of (viral, bacterial, and protozoal) pathogens that cause disease in humans and animals worldwide (de la Fuente et al., 2008). The deep knowledge of the incidence and prevalence of the tick species involved in the transmission, as well as their geographical distribution, would be very important for the control of tick-borne diseases (TBDs). Several species of ticks are indigenous in Italy and have been reported to infect companion and production animals as well as humans.

A number of Spotted fever group Rickettsiae is associated with ticks and these pathogens can be transmitted both in trans-ovarian and trans-stadial way. The main vectors are ticks belonging to *Dermacentor*, *Rhipicephalus*, *Haemaphysalis* and *Ixodes* genera.

Although Mediterranean spotted fever (MSF) due to *Rickettsia conorii* was thought for a long time to be the only tick-borne rickettsial disease present in Europe, five more spotted fever rickettsiae have been described as emerging pathogens in the last decade (Parola et al., 2004). Sicily is the Italian region with a high number of TBDs cases, probably due to particular climatic aspects. TBDs diagnosed in Sicily are mainly theileriosis, anaplasmosis, babesiosis, and other rickettsioses, besides bacterial and virus diseases (Caracappa et al., 1999). Every year, several outbreaks due to *Theileria*, *Babesia* or *Anaplasma* infections in cattle are recorded. Imported breeds are the most susceptible.

Sicily represents a typical Mediterranean ecosystem to study tick infestations and the prevalence of endemic tick-borne pathogens (Torina et al., 2008a). Several studies have characterized the prevalence of tick-borne pathogens in vertebrate hosts in Sicily (de la Fuente et al., 2005a,b, 2006; Torina et al., 2007, 2008b). However, limited data are

available on the presence and prevalence of these pathogens in ticks (de la Fuente et al., 2005a; Torina et al., 2008b, 2010).

The aim of the study was to analyze tick species and pathogen interactions present in Sicily. In this study, we have analyzed ticks collected in Sicily in the last seven years.

Materials and methods

Ticks were collected in Sicily, during the period 2004-2010 by dragging vegetation or directly removed from hosts. A total number of 5349 PCRs were performed in ticks samples collected from infested animals (3703) or questing ticks (1646). All specimens were identified by morphological characters using standard taxonomic keys for Italian Ixodidae (Manilla, 1998) and kept alive for at least one week. It was previously demonstrated that ticks are able to clean the organisms by day 4 after being removed from a highly parasitized host (de la Fuente et al., 2001), ticks were thus disinfected and stored in 70% ethanol until rinsed with distilled water, and cut in sterile tubes prior to the processing. DNA from ticks was extracted using TriReagent (Sigma, St. Louis, MO, USA) following manufacturers recommendations. The DNA was resuspended in sterile distilled water and stored at -20°C until used.

PCR with specific primer sets were used for detection of *Anaplasma* spp (Stuen S, 2003), *A.marginale (*de la Fuente J, 2003), *A.phagocytophilum (*de la Fuente J, 2005c), *Rickettsia* spp. (Tzianabos T, 1989), *Ehrlichia* sp (Ulrike G, 1996) and *Coxiella burnetii* (To H, 1996) as previously described with 0.1–10 ng of DNA using 10 pmol of each primer and the Ready-To-Go PCR beads (Amersham, Piscataway, NJ, USA).

Reactions were performed in an automated DNA thermal cycler for 35 cycles. The PCR products were analyzed in 1% agarose gels to check the size of amplified fragments. Control reactions were performed without the addition of DNA in the reaction to rule

out contaminations during PCR. Positive PCR products for *A.marginale* were sent to Macrogen Inc. (Seoul, Korea) to confirm by sequencing the specificity of the reactions. Prevalence was calculated for each pathogen in different tick species. Data reported were referred to at least fifty ticks tested for each pathogen.

Results

After the identification step, ticks were classified as follows: *Rhipicephalus (Boophilus) annulatus, Dermacentor marginatus, Haemaphisalis sulcata, Hae. punctata, Hyalomma lusitanicum, Hy. marginatum, Ixodes ricinus, Rhipicephalus turanicus, Rh. bursa, Rh. sanguineus.* In Figure 1 are reported the tick species and pathogen interactions for *Anaplasma, Ehrlichia,* and *Rickettsia* species and for *Coxiella burnetii.*

The pathogen *Anaplasma* spp was found in all the ticks 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 mainly present in *Hy. marginatum* (8,9%) and *Hae. punctata* (6,3%).

Discussion

During the last decade several studies on tick infesting animals have been carried out in Italy (Garippa and Sanna 1990; Di Todaro et al. 1999; Genchi and Manfredi, 1999; Georges et al. 2001; Cringoli et al. 2002; Beninati et al. 2004, 2005; Rinaldi et al. 2004). In most cases, the above mentioned studies are related to occasional tick collections, i.e. 2–3 moth periods, and/or to one host only.

Our study was conducted on ticks collected in Sicily, especially in the area near Palermo, where MSF, mainly due to *R. conorii*, has been endemic for long time.

In a previous study, the clinical data of 176 MSF patients in a period ranging from 1996 to 1999 were analysed (Picciotto et al., 2000). It was showed that an occupational risk exists among small farmers, but that there were also environmental risks for subjects living in rural areas due to contact with infected dogs or other animals. In Sicilian ticks (n.150) collected in 2001 and 2002 on livestock (Beninati et al. 2004, Beninati et al. 2005) and analysed by polymerase chain reaction (PCR), was evidenced the presence of *R. slovaca* in *D. marginatus* and in *Hae. punctata* and *R. aeschlimannii* and *R. africae* in *Hy. marginatum*.

The pathogenic role of *R. slovaca* in humans has been shown in France (Raoult et al., 2002; Cazorla et al., 2003). It could be thus argued that our tick investigation has been an important stimulus to implement studies for better understanding human TBDs transmission in Sicily.

The tick species identified in this study have been previously associated with pathogen transmission. Recent surveys carried out in Sicily on haemoparasites transmitted by ticks to cattle have shown a very high prevalence of mixed infections: *Babesia* spp. and/or *Theileria* spp. and/or *Anaplasma* spp. (Loria et al. 1999; Greco et al. 2000; Sparagano et al., 2000; Georges et al.. 2001) or *Anaplasma*, *Ehrlichia* and *Rickettsia*

spp. were detected in *H. marginatum* ticks collected from cattle (Torina et al., 2010). Some species sampled can also transmit *Rickettsia* to humans and animals. In fact, *R. conorii*, causative agent in Europe of Mediterranean Spotted Fever (MSF), was detected in *R. sanguineus*, but also in *R. turanicus* and in *D. marginatus* (Torina et al 2010). *R. slovaca*, responsible for the human disease known as tick-borne lymphadenopathy is found in *D. marginatus* and other spotted fever group rickettsiae are present in *H. marginatum* (Beninati et al. 2005).

Ticks in Sicily play an important role in the transmission of vector-borne diseases for humans and animals.

We confirmed that *Rickettsia* infection is common in ticks found in Sicily. In fact, the presence of *Rickettsia* was detected in all the eight tick species found in the area, and that *D. marginatus* could play a relevant role in *Riclettsia* transmission rather than *R. sanguineus that* shows high prevalence but is a less anthropophilic tick.

The high prevalence in the ticks species tested for *Anaplasma* and *Rickettsia* pathogens could be due to the high number of pathogens belonging to the two genera circulating in Sicily while *Coxiella Burnetii* and *Ehrlichia* have lower prevalence.

The characterization of tick-borne pathogens in ticks suggested differences in the role that different tick species play in the pathogen life cycle and transmission. This information is important for epidemiological studies of tick-borne pathogens in Sicily and to evaluate the risks associated with pathogen transmission to humans and animals.

Acknowledgements

The paper was supported by IZSSi 07/08, Italian Ministry of Health. Authors would like to thank Rosa Filippi and Franco Ferraro for their technical support.

References

Beninati, T., Genchi, C., Torina, A., Caracappa, S., Bandi, C. and Lo, N., 2005. Rickettsiae in ixodid ticks, Sicily. Emerg. Infect. Dis. 11(3): 509–511.

Beninati, T., Lo N., Torina, A., Caracappa, S., Bandi C., Piccolo, G., Franceschi, A. and Caracappa, S. 1999. Livestock production and animal health in Sicily, Italy. Parassitologia 41(1): 17–23.

Cazorla, C., Enea, M., Lucht, F. and Raoult, D. 2003. First isolation of Rickettsia slovaca from a patient, France. Emerg. Infect. Dis. 9(1): 135.

Cringoli, G., Otranto, D., Testini, G., Buono, V., Di Giulio, G., Traversa, D., Lia, R., Rinaldi, L., Veneziano, V. and Puccini, V. 2002. Epidemiology of bovine tick-borne diseases in southern Italy. Vet. Res. 33: 421–426.

de la Fuente, J., Estrada-Pen˜a, A., Venzal, J.M., Kocan, K.M. and Sonenshine, D.E., 2008. Overview: ticks as vectors of pathogens that cause disease in humans and animals. Front. Biosc. 13, 6938–6946.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., McEwen, B.R., Clawson, D. and Kocan, K.M., 2001: Major surface protein 1a effects tick infection and transmission of the ehrlichial pathogen Anaplasma marginale. Int. J. Parasitol. 31, 1705–1714.

de la Fuente, J., Massung, R.B., Wong, S.J., Chu, F.K., Lutz, H., Meli, M.L., von Loewenich, F.D., Grzeszczuk, A., Torina, A., Caracappa, S., Mangold, A.J., Naranjo,

V., Stuen, S., Kocan, K.M., 2005c. Sequence analysis of the msp4 gene of Anaplasma phagocytophilum strains. J. Clin. Microbiol. 43, 1309–1317.

de la Fuente, J., Torina, A., Caracappa, S., Tumino, G., Furlà, R., Almaza´n, C. and Kocan, K.M., 2005a. Serologic and molecular characterization of Anaplasma species infection in farm animals and ticks from Sicily. Vet. Parasitol. 133, 357–362.

de la Fuente, J., Torina, A., Naranjo, V., Caracappa, S, Vicente, J., Mangold, A.J., Vicari, D., Alongi, A., Scimeca, S. and Kocan, K.M., 2005b. Genetic diversity of Anaplasma marginale strains from cattle farms with different husbandry systems in the Province of Palermo, Sicily. J. Vet. Med. B 52, 226–229.

de la Fuente, J., Torina, A., Naranjo, V., Nicosia, S., Alongi, A., La Mantia, F. and Kocan, K.M., 2006. Molecular characterization of Anaplasma platys strains from dogs in Sicily, Italy. BMC Vet. Res. 2, 24.

de la Fuente, J., van den Bussche, R.A., Prade, T.M. and Kocan, K.M., 2003. Anaplasma marginale msp1α Genotypes evolved under positive selection pressure but are not markers J.Clin.Microb., p. 1609-1616

Di Todaro, N., Piazza, C., Otranto, D. and Giangaspero, A., 1999. Ticks infesting domestic animals in Italy: current acarological studies out in Sardinia and Basilicata regions. Parassitologia 41(1): 39–40.

Garippa, G. and Sanna, E. 1990. Ixodidi di frequente riscontro nei mammiferi dell'Asinara. Parassitologia 32: (1): 117–118.

Genchi, C. 2004. Molecular characterization of Spotted Fever Group Rickettsiae in Ixodid ticks from Sicily. Parassitologia 46(1): 161.

Genchi, C. and Manfredi, M.T. 1999. Tick species infesting ruminants in Italy: ecological and bio-climatic factors affecting the different regional distribution. Parassitologia 41(1): 41–45.

Georges, K., Loria, G.R., Riili, S., Greco, A., Caracappa, S., Jongejan, F. and Sparagano, O. 2001. Detection of haemoparasites in cattle by reverse line blot hybridation with a note on the distribution of ticks in Sicily. Vet. Parasitol. 99: 273–286.

Greco, A., Loria, G.R., Dara, S., Luckins, T. and Sparagano, O. 2000. First isolation of Trypanosoma theileri in Sicilian cattle. Vet. Res. Commun. 24(7): 471–475.

Loria, G.R., Riili, S., Vitale, F., Greco, A. and Sparagano, O. 1999. Clinical and laboratory studies on theileriosis outbreaks in Sicily, Italy. Parassitologia 41(Suppl. 1): 63–67.S. Manilla, G., 1998: Fauna D'Italia. Acari: Ixodida. Calderini, Bologna

Munderloh Ulrike, G., Madigan, John E., Dumler, J. Stephen, Goodman, Jesse L., Hayes, Stanley F., Barlough, Jeffrey E., Nelson, Curtis M. and Kurtti, Timothy J. 1996

March. Isolation of the Equine Granulocytic Ehrlichiosis Agent, Ehrlichia equi, in Tick Cell Culture. J Clin Microbiol. 34(3): 664–670.

Parola, P. 2004. Tick-borne rickettsial diseases: emerging risks in Europe. Comp. Immunol. Microbiol. Infect. Dis. 27(5): 297–304.

Picciotto, D., Provenzali, A., Sorrentino, S., Vitale, F., Vesco, G., Caracappa, S. and Verso, M.G. 2000. Rickettsia infections in Sicily and mass media: attention not entirely groundless. Med. Lav. 91(5): 494–500.

Raoult D., Lakos, A., Fenollar, F., Beytout, J., Brouqui, P. and Fournier, P.E. 2002. Spotless rickettsiosis caused by Rickettsia slovaca and associated with Dermacentor ticks. Clin. Infect. Dis. 34(10): 1331–1336.

Rinaldi, L., Otranto, D., Veneziano, V., Milillo, P., Buono, V., Iori, A., Di Giulio, G. and Cingoli, G. 2004. Cross-sectional survey of ticks (Acari: Ixodidae) in sheep from an area of the southern Italian Apennines. Exp. Appl. Acarol. 33(1–2): 145–151.

Sparagano, O., Loria, G.R., Gubbels, M.J., De Vos, A.P., Caracappa, S. and Jongejan, F. 2000. Integrated molecular diagnosis of Theileria and Babesia species of cattle in Italy. Ann. N. Y. Acad. Sci. 916: 533–539

Stuen, S., Nevland, S. and Moum, T. 2003. Fatal cases of tick-borne fever (TBF) in sheep caused by several 16S rRNA gene variants of Anaplasma Phagocytophilum Ann. N.Y. Acad. Sci. 990: 443-434

To, H., Kako, N., Zhang, G.Q., Otsusa, H., Ogawa, M., Ochiai, O., Nguyen Sa, V., Yamaguchi ,T., Fukushi, H., Nagaoka, N., Akiyama, M., Amano, K. and Hirai ,K. 1996. Q Fever Pneumonia in Children in Japan. J.Clin.Microb. p.647-651.

Torina, A., Alongi, A., Naranjo, V., Estrada-Pen˜a, A., Vicente, J., Scimeca, S., Marino, A.M.F., Salina, F., Caracappa, S. and de la Fuente, J. 2008a. Prevalence and genotypes of Anaplasma species and habitat suitability for ticks in a Mediterranean ecosystem. Appl. Environ. Microbiol. 74, 7578–7584.

Torina, A., Alongi, A., Naranjo, V., Scimeca, S., Nicosia, S., Di Marco, V., Caracappa, S., Kocan, K.M. and de la Fuente, J. 2008b. Characterization of Anaplasma infections in Sicily, Italy. Ann. N. Y. Acad. Sci. 1149, 90–93.

Torina, A., Alongi, A., Scimeca, S., Vicente, J., Caracappa, S. and de la Fuente, J. 2010. Prevalence of Tick-Borne Pathogens in Ticks in Sicily Transboundary and Emerging Diseases. 57: 46–48

Torina, A., Vicente, J., Alongi, A., Scimeca, S., Turla´, R., Nicosia, S., Di Marco, V., Caracappa, S. and de la Fuente, J. 2007. Observed prevalence of tick- borne pathogens in domestic animals in Sicily, Italy during 2003–2005. Zoonoses Public Health 54, 8–15

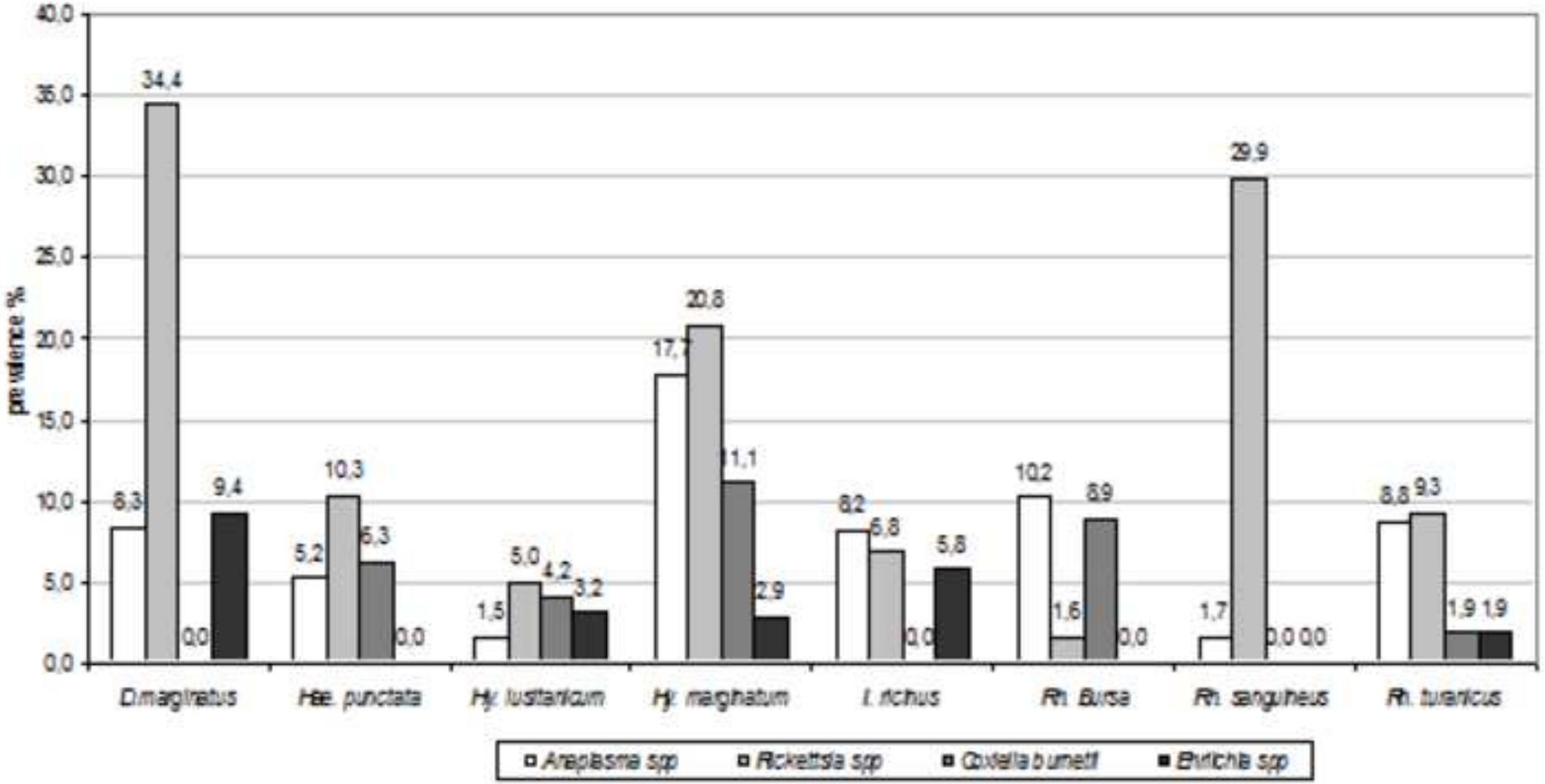
Tzianabos, T, Anderson, BE. and McDade, JE. 1989. Detection of Rickettsia rickettsii DNA in clinical specimens by using polymerase chain reaction tecnology J.Clin.Microb. Dec. 2866-2868.

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.

Figure

Click here to download high resolution image



Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

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

A. Torina[1], A. Agnone[1,2], G. Sireci[2], J. J. Mosqueda[3], V. Blanda[1,4], I. Albanese[4], M. La Farina[4], A. Cerrone[5], F. Cusumano[1] and S. Caracappa[1]

[1] National Reference Centre for *Anaplasma*, *Babesia*, *Rickettsia* and *Theileria* (CRABaRT), Istituto Zooprofilattico Sperimentale Della Sicilia ''A.Mirri'', Palermo, Italy
[2] Dipartimento di Biopatologia e Metodologie Biomediche, University of Palermo, Palermo, Italy
[3] Universidad Autonoma de Queretaro, Queretaro, Mexico
[4] Dipartimento Biologia Cellulare e Dello Sviluppo, University of Palermo, Palermo, Italy
[5] Istituto Zooprofilattico Sperimentale Del Mezzogiorno, Portici (NA), Italy

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

Correspondence:
Annalisa Agnone. Dipartimento di Biopatologia e Metodologie Biomediche, University of Palermo, Via Gino Marinuzzi 3, 90129 Palermo, Italy. Tel.: +393297313456; Fax : +390916565361;
E-mail: a.agnone@libero.it

Received for publication June 29, 2009

doi:10.1111/j.1865-1682.2010.01118.x

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) 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 preformed.

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 (Figueroa 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 *Eco*RI, 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 helixes 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.

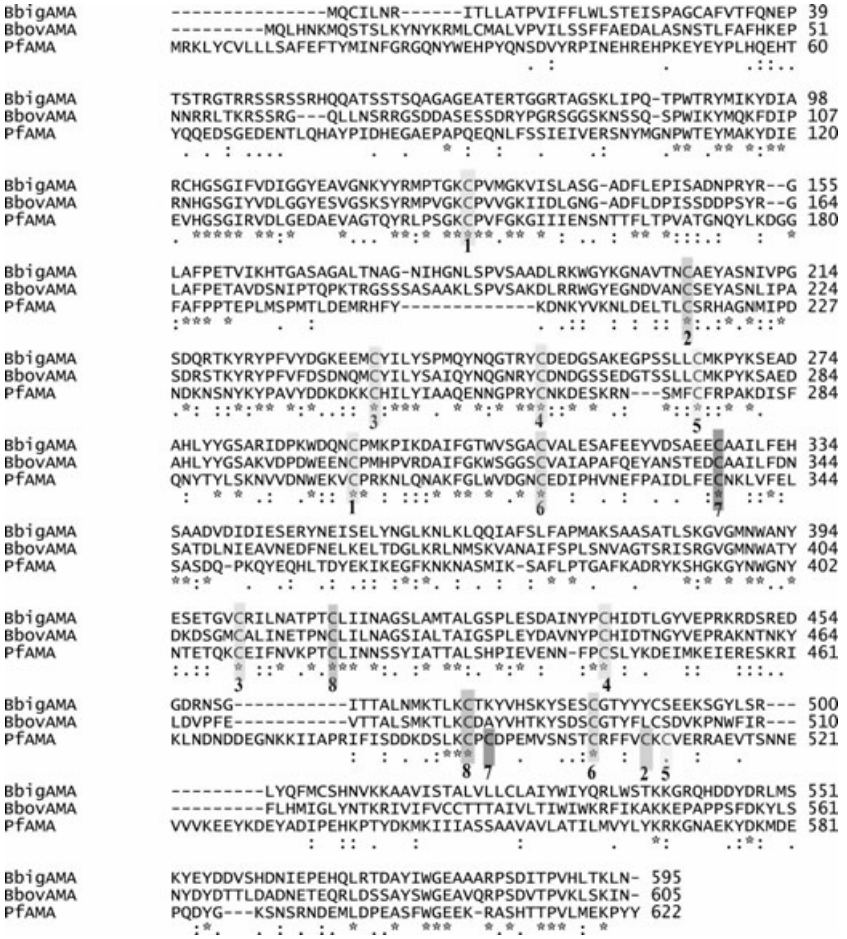


Fig. 1. Multiple-sequence alignment of AMA-1 proteins of *B. bigemina* (BbigAMA), *B. bovis* (BbovAMA) and *Plasmodium falciparum* (PfAMA). ''*'' means that the residues in that column are identical in all sequences in the alignment. '':'' means that conserved substitutions have been observed, ''.'' 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 aminoacidic 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.

Acknowledgements

This research was financed by INCO project no. 003691 and IZSSi 02/2007. The results are part of the PhD projects of Annalisa Agnone and Valeria Blanda. We are grateful to the Wellcome Trust Sanger Institute for the sharing of the *Babesia bigemina* genome sequencing data.

References

Deans, J. A., A. M. Knight, W. C. Jean, A. P. Waters, S. Cohen, and G. H. Mitchell, 1988: Vaccination trials in rhesus monkeys with a minor, invariant, Plasmodium knowlesi 66 kD merozoite antigen. *Parasite Immunol.* 10, 535–552.

Escalante, A. A., H. M. Grebert, S. C. Chaiyaroj, M. Magris, S. Biswas, B. L. Nahlen, and A. A. Lal, 2001: Polymorphism in the gene encoding the apical membrane antigen-1 (AMA-1) of *Plasmodium falciparum*. X. Asembo Bay Cohort Project. *Mol. Biochem. Parasitol.* 113, 279–287.

Ferre, F., and P. Clote, 2005a: DiANNA: a web server for disulfide connectivity prediction. *Nucleic Acids Res.* 33, W230–W232.

Ferre, F., and P. Clote, 2005b: Disulfide connectivity prediction using secondary structure information and diresidue frequencies, 2005. *Bioinformatics* 21, 2336–2346.

Figueroa, J. V., L. P. Chieves, G. S. Johnson, and G. M. Buening, 1993: Multiplex polimerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. *Vet. Parasitol.* 50, 69–81.

Finn, R. D., J. Tate, J. Mistry, P. C. Coggill, J. S. Sammut, H. R. Hotz, G. Ceric, K. Forslund, S. R. Eddy, E. L. Sonnhammer, and A. Bateman, 2008: The Pfam protein families database. *Nucleic Acids Res.* 36, D281–D288.

Gaffar, F. R., A. P. Yatsuda, F. F. Franssen, and E. de Vries, 2004: Erythrocyte invasion by *Babesia bovis* merozoites is inhibited by polyclonal antisera directed against peptides derived from a homologue of *Plasmodium falciparum* apical membrane antigen 1. *Infect. Immun.* 72, 2947–2955.

Gasteiger, E., A. Gattiker, C. Hoogland, I. Ivanyi, R. D. Appel, and A. Bairoch, 2003: ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 31, 3784–3788.

Hodder, A. N., P. E. Crewther, M. L. S. M. Matthew, G. E. Reidi, R. L. Moritzi, R. J. Simpsoni, and R. F. Anders, 1996: The disulfide bond structure of plasmodium apical membrane antigen-1. *J. Biol. Chem.* 271, 29446–29452.

Kumar, S., J. Dudley, M. Nei, and K. Tamura, 2008: MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.* 9, 299–306.

Lal, A. A., M. A. Hughes, D. A. Oliveira, C. Nelson, P. B. Bloland, A. J. Oloo, W. E. Hawley, A. W. Hightower, B. L. Nahlen, and V. Udhayakumar, 1996: Identification of T-cell determinants in natural immune responses to the *Plasmodium falciparum* apical membrane antigen (AMA-1) in an adult population exposed to malaria. *Infect. Immun.* 64, 1054–1059.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins, 2007: ClustalW and ClustalX version. *Bioinformatics* 23, 2947–2948.

Latitha, P. V., S. Biswas, C. R. Pillai, and R. K. Saxena, 2008: Immunogenicity of a recombinant malaria vaccine candidate, domain I + II of AMA-1 ectodomain, from Indian *P. falciparum* alleles. *Vaccine* 26, 4526–4535.

Mosqueda, J., J. Ramos, A. Falcon, A. Agnone, A. Torina, J. Figueroa, A. Alvarez, and R. Bautista, 2008. Identification and molecular characterization of an antigen of *Babesia bigemina* with homology to the apical membrane antigen 1 (AMA-1) of Apicomplexa. VI International Conference on Ticks and Tick-borne Pathogens. Book of proceedings, pp. 234.

Peterson, M. G., V. M. Marshall, J. A. Smythe, P. E. Crewther, A. Lew, A. Silva, R. F. Anders, and D. J. Kemp, 1989: Integral membrane protein located in the apical complex of *Plasmodium falciparum*. *Mol. Cell. Biol.* 9, 3151–3154.

Tamura, K., J. Dudley, M. Nei, and S. Kumar, 2007: MEGA4: molecular Evolutionary Genetics Analysis software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.

Tress, M. L., P. L. Martelli, A. Frankish, G. A. Reeves, J. J. Wesselink, C. Yeats, P. I. Olason, M. Albrecht, H. Hegyi, A. Giorgetti, D. Raimondo, J. Lagarde, R. A. Laskowski, G. López, M. I. Sadowski, J. D. Watson, P. Fariselli, I. Rossi, A. Nagy, W. Kai, Z. Størling, M. Orsini, Y. Assenov, H. Blankenburg, C. Huthmacher, F Ramírez, A. Schlicker, F. Denoeud, P. Jones, S. Kerrien, S. Orchard, S. E. Antonarakis, A. Reymond, E. Birney, S. Brunak, R. Casadio, R. Guigo, J. Harrow, H. Hermjakob, D. T. Jones, T. Lengauer, C. A. Orengo, L. Patthy, J. M. Thornton, A. Tramontano, and A. Valencia, 2007: The implications of alternative splicing in the ENCODE protein complement. *Proc. Natl. Acad. Sci. USA* 104, 5495–5500.

Xia, X. 2000. *Data Analysis in Molecular Biology and Evolution*. Kluwer Academic publishers, Boston, pp. 276.

Xia, X., and Z. Xie, 2001: DAMBE: data analysis in molecular biology and evolution. *J. Hered.* 92, 371–373.

Yokoyama, N., M. Okamura, and I. Igarashi, 2006. Erythrocyte invasion by Babesia parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. *Vet. Parasitol.* 138, 22–32.

Acknowledgements

I would like to thank all the people, friends, colleagues, professors, collaborators that made all of this possible.

Un ringraziamento speciale va a Mamma, Papà, Marcy, Cla e Nonnini, che mi hanno sostenuto e sopportato dal primo giorno all'ultimo, chi da vicino, chi dall'alto. Un abbraccio forte alla piccola Chiaretta, deliziosa *cura* di tutti i malanni, che riesce a risollevarmi anche nei momenti di più cupa disperazione, ricordandomi perché vale la pena continuare a lottare anche quando tutto sembra perduto.

Grazie agli amici e ai colleghi dell'Istituto Zooprofilattico che hanno condiviso con me le piccole e grandi gioie e piccole e grandi sconfitte.

Grazie ai fantastici coniugi Sireci, il CAPO e la CAPA, che mi hanno adottato nella loro piccola famiglia credendo in me e dandomi tanta fiducia; sanno riportarmi a terra quando i miei progetti volano troppo in alto, e sanno spronarmi, quando necessario, a non sottovalutare le mie capacità.

Grazie al Prof Dieli e a tutto il collegio dei *validissimi* docenti del nostro dottorato. So che è stata dura gestire questo esperimento del corso di Dottorato Internazionale, ma voi lo avete fatto egregiamente.

Grazie alla piccola, grande Valeria, con la quale per tanto tempo siamo state una eccezionale "macchina da laboratorio": un unico ingranaggio con due teste e quattro mani che funzionava alla perfezione. La partner ideale al lavoro, una carissima amica fuori dalle mura dell'Istituto (e durante le pause pranzo!).

Grazie a Marco, che mi ha guidata con pazienza e straordinaria professionalità, senza il quale sarebbe stato impossibile portare avanti questo progetto e aiutare le nostre piccole amiche "belanti".

Grazie a Guido, architetto, mente e coordinatore del progetto, comunque pronto a scendere *in campo* e prendersi cura, insieme al caro Roberto, delle piccole pecore in tutte le condizioni atmosferiche, anche a suon di cornetti e caffè!!!

Thanks a lot to Carmen, Robin and Roger, who gave me the opportunity to visit their labs, took care of me during all my stay, and allowed me to go on with my projects.

Dulcis in fundo, last but not least, grazie dolce Amore mio, musa delle mie ispirazioni, linfa vitale delle mie giornate, forza generatrice delle mie azioni, unica ragione per cui mi alzo al mattino. Sei uno splendido compagno di vita.