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Mycoplasma agalactiae and *Maedi visna virus*, two mastitogenic agents afflicting sheep dairy sector in Sicily: transmission mechanisms, innovative diagnostic methods, and prevention.

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The beauty of Science is to Make Thinks Simple

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INDEX

Abstract

In Sicily, sheep breeding is affected by two important diseases, Contagious agalactia (CA) and Maedi visna (MV), listed as priority diseases by the World Organization for Animal Health (OIE). Both etiological agents, Mycoplasma agalactiae (Ma) and Maedi visna virus (MVV), cause interstitial mastitis and a progressive sometimes sudden milk fall. The present PhD thesis aims to study unknown aspects of both diseases. First goal of the research was focused on the environmental risk linked to the persistence of Ma out of the host and alternative routes of transmission which probably justify the old way by the farmers to call this disease: "disease of the place". The results highlighted the persistence of the microorganism in different farm sites and in different environmental sources and the presence of asymptomatic carrier animals. Furthermore, the possible involvement of hematophagous parasites (ticks) as reservoirs of Ma was also investigated (1st contribution). Innovative molecular diagnostic methods for Ma detection were also developed and perfected, i.e. two Loop-Mediated Isothermal Amplification methods with two different target genes, an in-situ hybridization method useful to investigate pathology of the disease, and moreover an electrochemical device for the PCR-free detection of Ma, a novel approach for future rapid test (2nd contribution). The focused research on the viral disease caused by MVV, reported for the first time a genetic investigation at the E35K locus of the TMEM154 gene in Sicilian sheep breeds, to investigate their genetic susceptibility/resistance to the pathogen. The preliminary results showed that the protective K allele is present in low frequency in Valle del Belice breed, while in the two endangered breeds, Comisana and Barbaresca, was reported with good frequency (3rd contribution). Our results could be

a possible contribution for the implementation and development of new diagnostic tools and new genetic approach for future control and eradication strategies against CA and MV infections in Sicilian farming.

Sommario

In Sicilia, l'allevamento ovino è colpito da due importanti malattie, Contagious Agalactia (CA) e Maedi visna (MV), elencate come malattie prioritarie dall'Organizzazione Mondiale per la Salute Animale (OIE). Entrambi gli agenti eziologici, Mycoplasma agalactiae (Ma) e Maedi visna virus (MVV), causano mastite interstiziale e una progressiva, spesso improvvisa diminuzione di produzione di latte. La presente tesi di dottorato si propone di studiare aspetti sconosciuti di entrambe le malattie. Il primo obiettivo della ricerca è stato focalizzato sul rischio ambientale legato alla persistenza del Ma fuori dall'ospite e alle vie di trasmissione alternative che probabilmente giustificano l'antico modo degli agricoltori di chiamare questa malattia: "mal del sito". I risultati hanno evidenziato la persistenza del microrganismo in diversi siti di allevamento e in diverse fonti ambientali e la presenza di animali portatori asintomatici. Inoltre, è stato anche studiato il possibile coinvolgimento di parassiti ematofagi (zecche) come serbatoi di Ma (1° contributo). Sono stati inoltre sviluppati e perfezionati metodi diagnostici molecolari innovativi per il rilevamento di Ma, ovvero due metodi LAMP (Loop-mediated isothermal amplification) con due diversi geni target, un metodo di ibridazione in situ utile per indagare la patologia e inoltre un dispositivo elettrochimico per il rilevamento PCR-free di Ma, un nuovo approccio per futuri test rapidi (2 ° contributo). La ricerca focalizzata sulla malattia virale causata da MVV ha riportato per la prima volta un'indagine genetica al locus

E35K del gene TMEM154 in razze ovine siciliane, per indagare la loro suscettibilità/resistenza genetica al patogeno. I risultati preliminari hanno evidenziato che l'allele K protettivo è presente in bassa frequenza nella razza Valle del Belice, mentre nelle due razze a rischio, Comisana e Barbaresca, è stato segnalato con buona frequenza (3° contributo). I nostri risultati potrebbero essere un possibile contributo per l'implementazione e lo sviluppo di nuovi strumenti diagnostici e un nuovo approccio genetico per future strategie di controllo ed eradicazione contro le infezioni da CA e MV nell'agricoltura siciliana.

1. Contagious agalactia (CA) overview

1.1 Introduction

Contagious agalactia (CA) is a serious disease, primarily of livestock affecting milking sheep and goats and known for over 200 years (Loria & Nicholas, 2013). It is one of 117 diseases included as a notifiable disease listed in the diseases, infections, and infestations list of the World Organization for Animal Health (OIE) (https://www.oie.int/).

Although it is defined "agalactia", which refers to a marked decrease or even a complete loss of milk production, the symptomatology is not restricted to mammary glands but affects also, joints and eyes (the typical triad of symptoms), and, less commonly may appear abortion and respiratory disease (Bergonier, et al., 1997).

Historically, the etiological agent of CA, especially in sheep, is *Mycoplasma agalactiae* (Ma), while three other Mycoplasma species, most commonly affecting goats namely *Mycoplasma mycoides subsp. capri* (Mmc), *Mycoplasma capricolum subsp. capricolum* (Mcc), and *Mycoplasma putrefaciens* (Mp), have been added to the aetiological panel.

CA was first reported back in 1816 in Italy, where it was known as "mal del sito" ("disease of the place"), referring to its chronic persistence in an environment that allows the contamination of newly introduced herds (Loria & Nicholas, 2013).

Nowadays, it has been reported worldwide particularly in Mediterranean basin, as well as in ruminant breeding areas of the Middle East, Asia, North Africa, and South America (de Azevedo, et al., 2006; OIE 2018; Hosein, Saadati, Najimi, & Hassanpour, 2019).

The disease has a significant adverse effect on the farming economy in countries where small ruminant dairy production is important, due to drastic repercussions on milk production.

1.2 Current state of the art in diffusion and economic importance of CA

Nowadays, CA occurs wherever pastoralism and small ruminant dairy production are common, but it is almost undoubtedly underdiagnosed and underreported.

CA has been regularly observed in Europe, particularly in Mediterranean regions, as well as the Middle East, Asia, North Africa, and South America, through OIE notifications or scientific reports (Jaÿ & Tardy, 2019) but the OIE data are incomplete in order to calculate the real accurate prevalence because many regions do not report all outbreaks.

The disease is endemic in the Mediterranean basin where its diffusion is certainly favoured by traditional management as extensive farming, shared pasture, selection of breeds with high milk production, manual milking, mixed breeding of sheep and goats and uncontrolled exchange of animals between farms.

Its major economic impact, localised mainly in countries where small ruminant dairy husbandry, often subsisting on marginal land, represents the principal source of income for the individual farmers, who suffer very low consideration in the livestock industry (Nicholas, et al., 2008).

Estimating the economic impact of CA is a complex issue, and there are still not enough data to accurately estimate the economic consequences of CA in sheep and goats, also because small ruminant breeding is less profitable compared to the more lucrative cattle industry of Northern Europe.

CA is endemic in Sicily and after brucellosis is one of the most widespread problems in small ruminants Sicilian farms, causing about 40% of mastitis in sheep and goats.
Starting from 1932, inactivated vaccines were used to control the disease by the Istituto Zooprofilattico Sperimentale della Sicilia (Stazzi & Mirri, 1956).
However, Todaro et al., (2015) have quantified the losses in milk production following experimental infection with a pathogenic strain of Ma, carried out on 46 primiparous lactating ewes of the Valle del Belice breed. The affected animals showed a reduction in milk production of up to 17% and the analysis of milk composition showed a change of chemical parameters which affected the quality of cheese yield namely the percentages fat and protein. Discordant data about the effects of CA on milk composition, mainly the percentages of fat and protein, are present in the literature: some authors reported significant effects (Leitner, et al., 2003; Todaro, et al., 2015) while other authors didn't (De La Fe, et al., 2009; Fox, et al., 2003).
A more recent study (Loria, et al., 2018a) has estimated that the cost of one outbreak in mixed sheep/goat farms can range from 7 to 130 k€ depending on management and herd size, degree of exposure, the onset of disease in relation to the lactation period and capacity of the individual animal to resist the pathogen.

1.3 Clinical signs

In the Mediterranean countries, Ma is mainly related to dairy sheep husbandry, as it is considered the main aetiological agent of CA, while other species, such as *Mycoplasma mycoides subsp. capri* (Mmc), *Mycoplasma capricolum subsp. capricolum* (Mcc), and *Mycoplasma putrefaciens*

(Mp), are generally isolated from goat herds (Bergonier, Berthelot, & Poumarat, 1997; Chazel et al., 2010)
Under natural conditions, the most common infection routes for Ma are oral, respiratory or mammary routes. The incubation period of CA ranged from 6 to 30 days, and the disease may develop in an acute or more frequently, sub-acute/chronic syndrome. The course of the illness generally evolves in one month, but the chronic form can be observed for up to three months (Farina, etal., 2002). The acute syndrome is rare and is characterized by high fever, neurological signs, tremors and, in severe cases, can lead to death. The sub-acute form is typical of endemic areas and characterized by mastitis, keratoconjunctivitis and arthritis. Infection of the udders evolves as interstitial mastitis, initially characterized by swelling of the half udder, increased udders temperature and causes pain in milking, followed by a drastic decrease in milk production and quality (Nicholas, et al., 2008). However, after almost 2-3 weeks from the beginning of the infection, the majority of ewes excreting Ma in the milk recover to almost normal lactation levels (Todaro, et al., 2015). Pregnant animals may abort due to inflammation of the uterus or may give birth to non-viable offspring. Arthritis and keratoconjunctivitis are usually observed in 5-10% of cases (Guarda & Mandelli, 1989), particularly severe in young animals.
There are many factors influencing the severity of the disease: pastoralism, susceptible dairy breeds, prolonged lactation, quality and quantity of pasture available, and pathogenicity of the strains involved (Foglini, 1997).

1.4 Etiological agent

Mycoplasmas are one of the smallest self-replicating bacteria belong to the class Mollicutes which are characterized by

their lack of cell wall due to an inability to produce peptidoglycans. They have a small genome (0.58-1.4 Mbp) characterized by low G-C content (23%–40%) and limited to coding for proteins involved in metabolic and enzymatic activities (Parte, et al. 2011). Despite this, a gene family has been identified, that encodes phase-variable lipoproteins, generating surface diversity (Glew, et al., 2000). Mycoplasmas are very exacting, requiring media enriched with serum, yeast extract, peptone, and sterols for growth. Isolation of mycoplasmas by culture can be compromised by the overgrowth of other faster-growing bacteria; therefore, antimicrobials such as Thallium acetate or antibiotics are incorporated into the media. They are sensitive to osmotic shock, detergents, high temperatures, ultraviolet radiation and common disinfectants. Inoculated culture media is incubated for 7–10 days at 37°C and 5% CO_2, resulting in the growth of micro-colonies with a morphological appearance of "fried eggs", which are visible via light microscopy (Corrales, et al., 2007).This appearance is because of the central portion of the colony embedding itself into the agar surrounded by a zone of surface growth ((McVey, et al., 2013).

Mycoplasmas have evolved as "next-generation" pathogens with sophisticated biological properties that enable them to establish a complex interaction with their host (Rosengarten, et al., 2000).

Adhesion to host cells is considered an important virulence property and the first step for their successful colonization and subsequent damage of host tissues, as non-adhering mutants seem to be avirulent (Krause & Baseman, 1983; Razin & Jacobs, 1992). An important feature of mycoplasma infections is their ability to persist in the host for long periods, by evading the host immune system, through sophisticated

molecular systems of antigenic variability and host immunomodulation caused by the Vpma family of lipoproteins (Glew, et al., 2000; Glew, et al., 2002; Chopra-Dewasthaly, et al., 2017; Czurda, et al., 2017).
Strains of Ma were considered to be unusually homogenous (Solsona, Lambert, & Poumarat, 1996; Tola, et al., 1999), as a dominant subtype has disseminated within and among countries as France, Spain and Italy. However, importantly, in other areas genetically divergent strains exist, defining the geographical location of outbreaks (McAuliffe, et al., 2011; (Manso-Silvan et al., 2012; De la Fe, et al., 2012; Nouvel, et al., 2012; Tardy, et al., 2012; Ariza-Miguel, et al., 2013; Poumarat, et al., 2016).

1.5 Transmission

The disease is rapidly spread by contact between infected and healthy animals. The infection can also be indirectly transmitted via the hands of milkers or contaminated milking equipment (OIE, 2019).
Dissemination of the microorganisms into the environment occurs by means of ocular and nasal discharge, milk, faeces, urine and excretions from open joint lesions or the male genitourinary tract. Young animals are most commonly infected when suckling contaminated colostrum or milk. The manifestation of infection is observed early in spring when young animals are born, and females are in full lactation (Lambert, 1987; Kinde, et al., 1994; Corrales, et al., 2007). A further increase in the number of infected animals is recorded at the beginning of summer when young animals are susceptible to infection. The disease in a herd can persist for several months and often recurs during the next lactation or even in following several years (Madanat, et al., 2001; Loria, et al., 2013).

In areas where CA is endemic, as in Sicily, the clinical epidemiological situation is represented by chronically infected herds, with large numbers of asymptomatic carriers, able to further spread the infection and which therefore prove to be a danger to the flocks.

1.6 Diagnostic methods

Clinical suspicion of CA is generally based on an unexpected decrease in milk production in the herd followed by observation of characteristic clinical signs. The most common symptom is sub-acute mastitis with severe alterations in milk quality and quantity, change that is often present in many other bacterial (*Pasteurella, Staphylococcus, Streptococcus, E. coli* etc.) and viral (*Maedi-Visna virus*) mastitis and could be easily mistaken with for other diseases. For the isolation purpose of Ma, the most common clinical sample is milk, collected aseptically from the suspected udder or when conjunctivitis or arthritis are present, from affected mucosae (eye's or nasal secretions, joint fluids, abortions of vaginal secretions). Serum taken from whole blood is utilized for antibody detection, whereas DNA or antigen isolation could be considered in case of presence of septicaemia (high fever). Post-mortem tissue samples could also be analysed for antigen isolation or specific DNA determination (Jaÿ & Tardy, 2019).

The routine analysis method for isolating and identifying Ma in clinical samples is based on the microbiological culture in liquid or on solid media designed for its peculiar biochemical behaviour, which support the growth of mycoplasma colonies, after incubation at 37°C and 5% CO2, for 7–10 days (OIE Terrestrial Manual 2018 - Chapter 3.7.3). However, this method represents a time consuming and complicated process, due to the very slow growth of mycoplasmas.

The use of biochemical tests for Ma identification has little diagnostic value and gives results that are difficult to be interpreted, the biochemistry of M agalactiae can provide presumptive diagnosis because of film production and inability to ferment glucose or hydrolyse arginine.

Different serological tests have been developed to identify antibody induced by Ma. The Enzyme-Linked Immunosorbent Assay (ELISA), Complement Fixation Test (CFT), and Immunoblotting (IB) are the standard serological tests officially reported in the OIE manual diagnostic tests. Many studies have shown that ELISAs are more sensitive than the CFT assay (Lambert & Cabasse, 1989; Mega, et al., 1993).

Serological tests are used to detect the antibody responses elicited in both vaccinated and infected sheep (Tola, et al., 1997; Fusco, et al., 2007).

Growth inhibition test allows discrimination of an antibody response related to infection from the one induced by vaccination (Tola, et al., 1997), but it is very time-consuming. The western blot assay is extremely sensitive, but it does not allow the quantification of antibody level; so that ELISA is the test of choice among existing serological procedures because it has the potential for high sensitivity and specificity and in addition it is simple and allows testing of a large number of samples in a short period (Fusco, et al., 2007).

Nowadays, two commercial ELISA kits for the detection of serum antibodies against Ma are available (OIE Terrestrial Manual 2018 - Chapter 3.7.3). According to the literature, the diagnostic specificity (percentage of uninfected animals that test negative) of ELISA tests, is between 76% and 99% for tests using total antigens and between 97% and 100% for tests using fusion proteins (Pepin, et al., 2003; Kittelberger, et al., 2006; Fusco, et al., 2007; Campos, et al., 2009).

However, a good ELISA should be based on antigens, which are highly immunogenic and expressed during the entire course of infection in the highest possible number of field isolates (Kashoo, et al., 2011).
Nevertheless, the low diagnostic sensitivity of these tests may be caused by relatively low antibody responses in some animals, antigenic heterogeneity between strains of M. agalactiae (Solsona, et al., 1996; Glew, et al., 2000) cross-reactions to other Mycoplasma species due to the presence of common antigens.
Many studies have reported that P30, P48, P55, P40 and P80 are the immunodominant surface proteins of Ma (Fleury, et al., 2001; Tola, et al., 2001; Fleury, et al., 2002; (Santona, et al., 2002; Fusco et al., 2007).
The immunoreactivities and suitability of these proteins as diagnostic antigens have been demonstrated. Rosati, et al., (2000) developed an ELISA based on recombinant P48 that displayed a lower sensitivity in comparison to other conventional serological tests (Kittelberger, et al., 2006).
Fusco et al. (2007) examined the antibody response elicited in sheep during an experimental infection and identified two immunodominant and common antigens (P55 and P80) whose combination resulted in a more effective detection strategy against Ma. Indeed, P55 is a strongly immunoreactive protein and is very early expressed post-infection; still, it is not expressed by all strains. In contrast, P80 is a stable lipoprotein preserved among isolates from different Italian regions (Tola, et al., 2001) and it is always present during experimental infection.
Interest in Ma antigens identification is directed toward both developing better serological diagnosis tests and to the development of new vaccines.

Immunohistochemical techniques can also be used to identify Ma in infected tissue (Rodriguez, et al., 2002; Loria, et al., 2007). These techniques are especially useful for the post-mortem identification of these microorganisms. Their use can also be applied to evaluate the course of infection and the cell types involved.

The advances in molecular biology and biotechnology have provided rapid, sensitive and robust tests for identification of mycoplasmas including Ma.

Culture combined with serological tests and/or DNA amplification-based methods is the best strategy for overcoming the risk of presence of inhibitors in field samples (such as milk) and detecting CA in individual cases.

Several PCR-based methods specific for Ma have been developed. Some of these methods are based on the amplification of the 16S rRNA gene (Chávez González, et al., 1995; Johansson, etal., 1998 Königsson, et al., 2002; Bashiruddin, et al., 2005) or specific genes, like *uvrC* (Subramaniam, et al., 1998) and *mb-mp81* gene encoding the membrane protein P81 (Foddai, et al., 2005).

Denaturing gradient gel electrophoresis (DGGE) is a method to determinate the genetic diversity of microorganisms and also to detect and differentiate the majority of small ruminant's mycoplasmas, using universal primers for the V3 region of 16S rRNA (Muyzer, et al., 1993; McAuliffe, et al., 2003; McAuliffe, et al., 2005).

Nevertheless, this method concerns two-steps procedures that might be time consuming and laborious.

Nowadays, the use of real-time PCR has almost completely replaced the conventional PCR providing advantages of speed and sensitivity. This technique provides a quantification of the organism, higher specificity and analytical sensitivity and reduces the risk of cross-

contamination, at the same time, this technique is faster than conventional PCR, and it is totally automatic.
Several real-time PCRs specific to detect Ma have been developed (Lorusso, et al., 2007; Oravcová, et al., 2009), including a multiplex real-time-PCR method capable of discriminating etiological agents of CA (Ma vs M. mycoides cluster) (Becker, et al., 2012).
The sequencing of three Ma genomes (Sirand-Pugnet, et al., 2007; Nouvel, et al., 2010; Tardy, et al., 2012), led to the development of new molecular genotyping methods, such as multiple-locus variable number of tandem repeats (VNTR) or multilocus sequence typing (MLST) (McAuliffe, et al., 2008; (McAuliffe, et al., 2011).
Another molecular detection method called Loop-mediated Isothermal Amplification (LAMP) was recently developed and validated for direct detection of the Ma based on the P40 gene (Rekha, et al., 2015; Loria, et al., 2018b;Tumino, et al., 2020). The advantage of this method is that requires less time and equipment than PCR and it can potentially be used for tests in the field.

1.7 Eradication strategies, prevention and control

The control of CA in endemic areas should be considered a priority since this disease causes severe economic losses in small ruminants farming.
Current measures to control CA in affected areas have mainly based on the use of antibiotics and vaccines, with few changes over the last 20 years (Bergonier, et al., 1997).
Several studies have determined antibiotic resistance profiles of CA causing Mycoplasma spp. (Loria, et al., 2003; (Al-Momani, et al., 2006; Antunes, et al., 2007a; Antunes et al., 2007a; Antunes, et al., 2007b; Antunes, et al., 2008);

resistance may lead to failure of some antibiotic treatments (Gómez-Martín, et al., 2013).

Under field conditions, vaccines may prevent the appearance of new clinical signs and reduce mycoplasma excretion but are unable to prevent transmission of infection (De la Fe, et al., 2007; Agnone, et al., 2013a; Agnone, et al., 2013 b).

The low efficacy of currently available vaccines may be due to the lowest immunological effect of inactivated vaccine if compared to those attenuated by very aggressive chemical product (Formalin, Phenol) which delete the antigenic profile of the pathogen; to the multi-aetiological origin of the outbreaks nature of CA; to the evolutionary ability by the pathogen, to imitate the mammal's mucosal proteins to escape the host immune response.

The multiple sources of infection and excretion of Ma through various body secretions lead to the rapid spread of disease. Thus, a rapid diagnosis is essential for the prevention and control of the spread of infection to susceptible animals and among flocks.

Ma infection could be prevented by adopting good management practices and following continuous surveillance monitoring for the pathogen. So-called “healthy carriers” as subclinically infected animals may also spread the infection; hence there is a need to utilize specific, sensitive and rapid diagnostic procedure for early detection (Jaÿ & Tardy, 2019).

2. Maedi Visna (MV) overview

2.1 Introduction

Maedi visna (MV) of sheep and arthritis/encephalitis (CAE) of goat are widespread viral diseases caused by closely related *Lentiviruses* belonging to the retroviridae family, result in direct losses in the major small ruminant producing countries worldwide (Kalogianni, et al., 2020)

The phylogenetic correlation between MV virus (MVV) and CAE virus (CAEV) shows explicit evidence of the existence of cross-species transmission between sheep and goats, for this reason, these strains have been generally called small ruminant lentiviruses (SRLV) (Shah, et al., 2004; Reina, et al., 2006; Leroux, et al., 2010; Souza, et al., 2015).

Both the diseases are characterized by a long incubation period and by a slowly progressive and subclinical infection, consisting in a chronic interstitial infiltration of mononuclear inflammatory cells in several organs, including lung, mammary gland, joints and central nervous system (Straub, 2004; Minguijón, et al., 2015; Gomez-Lucia, et al., 2018; OIE, 2019; Kalogianni, et al., 2020).

The clinical signs appear to be linked on the tropism of the small ruminant lentivirus strain, the species affected and the genetic background of each breed or animal. In general, depending on the severity of the lesions, one of the target organs is mainly interested, but it is not uncommon to find several of them affected in the same animal (Minguijón, et al., 2015; Gayo, et al., 2018; Gomez-Lucia, et al., 2018; Kalogianni, et al., 2020).

The primary route of infection is related to the horizontal transmission, mainly through to the respiratory secretions and milk or colostrum, which may contain infected monocytes

and macrophages. Vertical transmission (transplacental) and transmission via semen (Peterson, et al., 2008) are also possible but are less investigated from an epidemiological point of view, as well as, the role of the environmental contamination (e.g. faecal contamination of drinking water) in the transmission of the etiological agent has not been thoroughly investigated but is considered to be a risk factor (Blacklaws, et al., 2004; Peterhans, et al., 2004; Villoria, et al., 2013).

MV and CAE have been included by the World Organization for Animal Health (OIE) in the list of notifiable terrestrial and aquatic animal diseases, due to its economic impact on international trade of animals and their products. The increase of the prevalence of the disease, in the European continent, seems to have coincidence with the international movement of European breeds of the dairy small ruminant. However, there is still a scarcity of epidemiological data of the disease, and that represents a major obstacle for the implementation of disease control and eradication programs (OIE, 2019; Kalogianni, et al., 2020).

As there is no treatment against SRLV infections and all efforts for the development of vaccines have failed, controlled eradication programs remain the only approach to avoid SRLV infection in the small ruminant industry. Nevertheless, most of the programs used are based on serological tests, diagnosis of the infected animals at an early stage, culling of the latter and feeding kids with virus-free colostrum (Berriatua, et al., 2003; Reina, et al., 2009; Synge & Ritchie, 2010; Cirone, et al., 2019). However, these programs are expensive, time-consuming, and heterogeneous in terms of their planning and effectiveness.

Emerging research suggested that breed variability may influence susceptibility and resistance against SRLVs (Gomez-Lucia, et al., 2018).
The identification of genetic markers for resistance/susceptibility to SRLV infections may develop selective breeding plans in order to implement the eradication disease programs.
Different candidate loci have been previously reported to be associated with SRLV infection with various levels of statistical significance; however, only variants in the TMEM154 gene have resulted in multiple and replicated observation in order to be considered a validated genetic marker (Heaton, et al., 2012; Leymaster, et al., 2013; White & Knowles, 2013; Alshanbari, et al., 2014; Molaee & Luehken, 2018; Yaman, et al., 2019).

2.2 Current state of the art in diffusion and economic importance of the MV

MV counts as one of the 117 notifiable animal diseases, infections, and infestations listed by the World Organization for Animal Health (OIE) in 2020. However, no official map of MV prevalence worldwide is published; therefore, it is almost certainly underdiagnosed and underreported.
The examination of sheep milk production worldwide data reports that the disease should have increased with the enhanced tonnage and the international movement of European breeds of the dairy small ruminant (Table 2.1).
Historically, MV was described for the first time in 1939 in Iceland and was transmitted among several countries through the trading of breeding stocks (Blacklaws, et al., 2004). Nowadays the incidence of the MVV infection is globally differentiated and has been reported from most of Mediterranean Countries, Europe, United Kingdom, Canada,

United States and has recently been identified in Japan, while Iceland, Australia and New Zealand are considered MV-free regions (White & Knowles, 2013; Oguma, et al., 2014; Kalogianni, et al., 2020).
Figure 2.1 proposes a snapshot of MV distribution in these countries, according to OIE reporting (2015-2018).
The field observations suggest that the disease prevalence is higher in developed countries, which seems to be related to the management system and inefficient controls on sheep and goat imports.
But the OIE data are still insufficient to calculate accurate prevalence due to the partial data available.
The disease causes substantial production and economic losses due to its negative impact on milk production and on ewe productivity, lower lamb weights from older infected ewes, early culling or death of the infected animals, cost of the control programs and export restrictions (Keen, et al., 1997; Ploumi, et al., 2001; Arsenault, et al., 2003; Peterhans, et al. 2004; Reina, et al. 2009; Benavides, et al., 2013).

Table 2.1 Production of milk from sheep worldwide and its evolution between 2005 and 2018, for the first 15 producers in 2018.

Sheep milk production in Tons			
Year		**2018**	**2005**
Total worldwide		10631057 (+16%)	9152989
Europe		3168166 (+7%)	2950840
Fifteen biggest producers in 2018	Tunisia	1446271	253759
	Chad	1180276	20939
	China	1180276	1114930
	France	753819	255355
	Switzerland	647311	81169
	Qatar	626145	11652
	Somalia	544541	475000
	Iraq	524717	64837
	Spain	414000	407800
	Libya	381067	48212
	Slovakia	369378	412600
	Estonia	323758	60746
	India	316431	284459
	Algeria	304199	203000
	Afghanistan	208480	167059

Note: Data extracted from Food and Agriculture Organization of the United Nations (FAO).
http://www.fao.org/faostat/en/#data/QL.

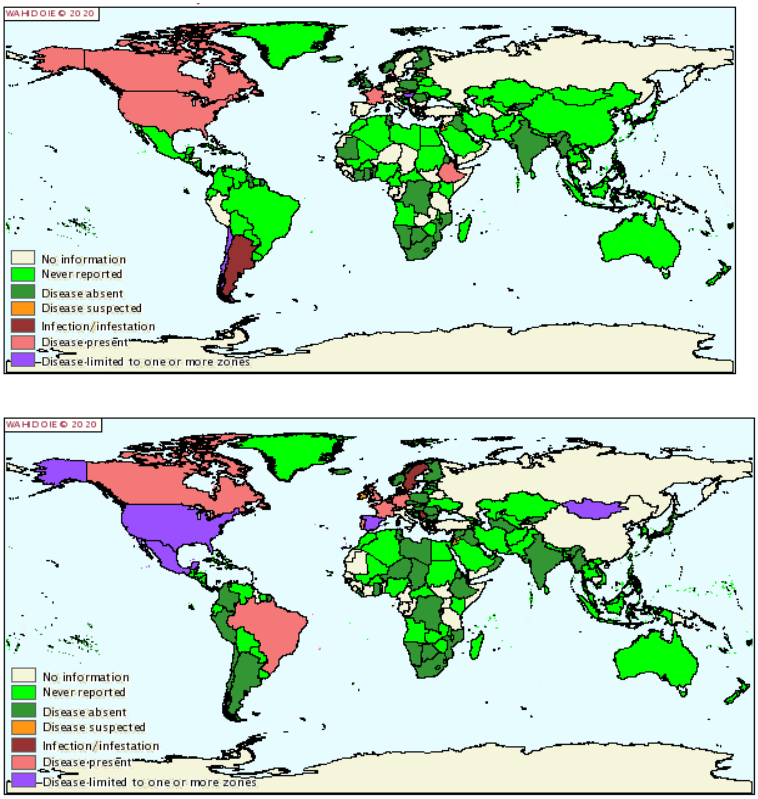


Figure 2.1 Maedi Visna distribution maps in 2005 and 2018 according to OIE reporting. Data extracted from the World Organisation for Animal Health [OIE] www.oie.int

2.3 Clinical signs

Typically, MVV's symptoms take several months or even years to develop, and in many cases are not evident or characteristic of the disease at its early stages (Kalogianni, et al., 2020). The organs mainly infected by MVV are the lungs, the mammary gland, the nervous system, and rarely also the joints. However, pneumonia and mastitis are the predominant clinical symptoms of MV.

The first signs of disease, generally, appear after age two and often include indurative mastitis (hard udder) and loss of body condition.

When the disease develops, the most common form may be associated with respiratory disease, due to chronic interstitial pneumonia, that increases the thickness of the alveolar septa and progressively reduces the air exchange capacity of affected lungs. The inter-alveolar septa are infiltrated with lymphocytes, monocytes, macrophages, and plasma cells, which contributes to lungs lesions. Another characteristic lesion is diffuse lymphoid hyperplasia responsible for the grey spots on the pulmonary pleural surface (Gomez-Lucia, et al., 2018; Kalogianni, et al., 2020).

The mammary disease is the second important form due to economic losses. It was observed that it is more frequent in mechanical milking and intensively reared animals (Barquero, et al., 2013).

Mastitis usually is interstitial, indurative, bilateral, and non-painful, with swollen mammary lymph nodes and decrease of milk production caused by the destruction of the acinar structure of the mammary gland due to the replication of the virus in macrophages or epithelial cells of the acini (Minguijón, et al., 2015; Gomez-Lucia, et al., 2018; Kalogianni, et al., 2020). Milk does not show changes in organoleptic characteristics, although increased cell counts

have been reported, mostly in goats (Paape, et al., 2001; Lipecka, et al., 2010; Asadpour, et al., 2014).
Nervous signs of the disease begin with the MVV infection of the central nervous system, the viral migration in monocytes and the infection of the perivascular macrophages. The main lesion is encephalitis, and the associated symptoms include progressive ataxia, progressive incoordination which can lead to loss of motor control, paresis and paralysis usually leading to recumbence, although the animal remains alert. Arthritis can also be present in MVV infection, mostly in goat. The syndrome is based on inflammations of the carpal, tarsal, metatarsal and metacarpal joints, leading to progressive marked lameness and involuntary culling of the animal.
In both, sheep and goats, only the respiratory and neurologic syndromes lead the animal to a cachectic stage and death, while arthritis and mammary syndrome cause several degrees of locomotive difficulty or a decreased milk production. (Minguijón, et al., 2015; Gomez-Lucia, et al., 2018; Kalogianni, et al., 2020).
Mostly, the severe lesions are present in lungs or mammary gland, but several cases have been reported that both organs have been affected with moderate or severe lesions (Benavides, et al.,2006; Gayo, et al., 2018) which may be related to the host immune response, genetic factors, viral strain.

2.4 Etiological agent

Both MVV and CAEV are RNA viruses that belong to the family retroviridae, genus *Lentivirus.* The genus *Lentivirus* includes many known viruses including the immunodeficiency viruses of humans (HIV), the immunodeficiency viruses of monkeys (SIV), the

immunodeficiency viruses of felines (FIV) and the immunodeficiency viruses of bovines (BIV), the equine infectious anaemia virus (EIAV) and the small ruminant lentiviruses (SRLV).

MVV and CAEV spherical virions measure between 90 and 120 nm and consist of a capsid surrounded by an envelope. The envelope is constituted by host-cell phospholipids bilayer in which the viral glycoproteins are inserted. The capsid surrounds the nucleocapsid and contains the viral RNA and the enzymes necessary for viral replication (Leroux, et al., 2010; Minguijón, et al., 2015; Gomez-Lucia, et al., 2018).

Retroviruses contain two linear molecules of RNA. Thanks to the reverse transcriptase the single-stranded RNA molecules are transformed into double-stranded DNA (dsDNA) and the retroviral genome (known as provirus when it is dsDNA) can be inserted into the genome of the host cell. The genomes of MVV and CAEV are between 8,400 and 10,000 nucleotides (nts) long, and they present the same basic genetic organisation described in all lentiviruses, that is two non-coding long terminal repeat (LTRs) at both ends containing three structural genes: *gag, pol*, and *env*.

Gag encodes the internal structural proteins, which protect the DNA (nucleocapsid, capsid and matrix). Gene *Pol* encodes the enzymes that are involved in replication and DNA integration, while *env* encodes the envelope proteins, that are the surface and the transmembrane glycoproteins. The surface glycoproteins are genetically variable determining the antigenic variability and the antibody response and also include the domains that are recognized by the cell receptors to allow entry into the host cell. The transmembrane glycoproteins are much more conserved

protein and have a fusion function between the viral and host-cell lipid membranes.

Accessory regulatory genes may be present, and the characteristics of the encoded proteins vary according to the host species (Leroux, et al., 2010; Minguijón, et al., 2015; Gomez-Lucia, et al., 2018).

The monocyte/macrophage and lymphocytes are the main target cell of Lentiviruses. However, in tissues, other additional cells target may also be infected, including epithelial cells in the mammary gland (Blacklaws, 2012).

According to the type of cell lineage in which lentiviruses replicate, a different immuno-response is triggered. Viruses that replicate in lymphocytes and macrophages (e.g. HIV) trigger an acquired immunodeficiency syndrome affecting lungs, central nervous system and gastrointestinal tract; as opposed of SRLV that infects the macrophages causing a slow disease in mammary gland, central nervous system, lung and joints and, not trigger the classical immunodeficiency syndrome (Leroux, et al., 2010; Minguijón, et al., 2015).

There is a large genetic diversity among the small ruminant lentiviruses. To date, a common ancestor seems to have been located in Turkey (Muz, et al., 2012), and the virus strains diversification appears to be associated with migration of sheep from the Middle East to Europe thousands of years ago. Small ruminant lentiviruses have been classified into five genetic groups, namely, A, B, C, D, and E. Genotypes A, B and E, originally described in sheep (A) or goats (B and E), may further be distributed into different subtypes (A1–A15, B1–B3, E1–E2) although many these strains have been found to infect both sheep and goats (Kalogianni, et al., 2020).

2.5 Transmission

The transmission mechanism of MV infection can occur by vertical and horizontal transmission with different significance. However, the lactogenic transmission has considered being the main route of disease transmission, through the ingestion of colostrum and milk from infected dams, allowing virions and infected cells to be absorbed by the lamb's intestine (Peterhans, et al., 2004; Bolea, et al., 2006).

Published evidence suggested that the transmission of the virus from the ewe to the lamb during pregnancy (transplacental) and the exposure to maternal body fluids and blood represents a source of infection, but the significance of transplacental viral transmission is difficult to assess and has not been fully clarified yet (Blacklaws, et al., 2004; Broughton-Neiswanger, et al., 2010).

Horizontal transmission of MVV mainly refers to the transmission through respiratory secretions containing infected monocytes, macrophages and dentritic cells (McNeilly, et al., 2008). In general, the airborne transmission of viral particles become significant in intensively reared flocks with long housing periods, low ventilation and close contact with infected animals (Blacklaws, et al., 2004; Peterhans, et al., 2004; Villoria, et al., 2013).

Regarding the possibility of sexual transmission, there are few evidences of virus proliferation in the genitals of infected rams (Peterson, et al., 2008) and this route of transmission need to be further investigated.

Small Ruminant Lentivirus has been detected in nasal secretions, saliva (Gudnadóttir & Pálsson, 1965), urine (Houwers, 1990). Transmission by contaminated milking equipment and by different environmental virus reserve (e.g. contaminated feed and pasture or drinking water

contaminated with faeces) has not been fully investigated (Blacklaws, et al., 2004; Peterhans, et al., 2004; Villoria, et al., 2013) and appears worthy of future study.
Identification and management of risk factors at the farm level are crucial to define a MVV control/eradication program. There are several risk factors that influence transmission of MVV between and within flocks.
The main risk factors that affect the likelihood of increase of horizontal transmission are flock size and stocking density, intensity of the farming system and age distribution (Leginagoikoa, et al., 2010; Pérez, et al., 2010; Barquero, et al., 2013; Junkuszew, et al., 2016; Michiels, et al., 2018).

2.6 Diagnostic methods

According to the World Organization for Animal Health (OIE), early and accurate diagnosis of MV disease is a critical parameter in terms of epidemiological research, control programs and small ruminant international trade.
The early diagnosis is arduous because the symptoms of the disease have an insidious onset and a slow progression; furthermore, many animals may never show clinical signs of the infection and, to date, no diagnostic test can be proposed as “gold standard” to determine the infection status of the animal, due to the genetic and antigenic variations of the MVV.
Current control and eradication programs are based on serological tests, mainly enzyme-linked immunosorbent assays (ELISAs) to detect antibodies against the virus.
Nevertheless, serological methods have several disadvantages that must be considered, in that the immune response to the disease requires a long time and the genetic variability of the virus may evade the host immune response (Ramírez, et al., 2013; Kalogianni, et al., 2020). Therefore,

there is a rate of infected animals that may be underdiagnosed, and consequently, the epidemiological situation remains underreported.

According to the World Organization for Animal Health (OIE) recommendations, the diagnostic techniques include viral isolation, serological test and molecular methods.

There are two approaches to the isolation SRLV: one for use with the live animal, and the second for use with necropsy tissues; but it is a long time and very laborious method that require expert staff, and it is not applicable for routine diagnosis.

Competitive ELISA methods using monoclonal antibodies, indirect ELISAs (I-ELISA) using purified whole virus preparations for antigen or recombinant protein have been developed and show different sensitivity and specificity values (OIE 2019).

In many laboratories, a combination of serological tests and PCR-based techniques are routinely used for determining the infection status of those animals considered doubts or false-negatives by serology. PCR-based methods permit to detect infected animals before seroconversion (Ramírez, et al., 2013; Kalogianni, et al., 2020).

Different conventional PCR, quantitative PCR and amplification based-methods (LAMP) have been performed to detect MVV infections in different animal samples (peripheral blood leukocytes, milk or mammary secretions, semen, synovial fluid and other tissues) (Minguijón, et al., 2015; OIE, 2019). However, an important issue in the use of PCR-based methods is the specificity and sensitivity that depends to multiple factors, such as variable viral load in the target sample, viral genetic heterogeneity, the choice of the amplified target viral region and cross-reaction between

SRLV (Ramírez, et al., 2013; Minguijón, et al., 2015; Kalogianni, et al., 2020).

2.7 Eradication strategies, prevention and control

Although multiple vaccines have been tried for SRLV, to date, there is neither an effective vaccine nor treatment against MVV (Reina, et al., 2013). The major obstacles for the development of an effective MV vaccine include the wide genetic variation of viral strains due to their continuous mutations and the necessity for the efficient and high antibody induction against the virus.

Therefore, control programs remain the only approach to contain the infection by MVV to spread in the small ruminant industry.

These programs have been performed in different countries, but often intermittently and with different approaches (Minguijón, et al., 2015; Kalogianni, et al., 2020).

The control strategies foresee:

1. Post-lambing management of lambs based on artificial suckling;
2. separation of seropositive and seronegative animals into two different groups within the farm in flocks with moderate seroprevalence while in flocks with high seroprevalence, the most efficient practice is the annual culling and replacement with uninfected animals,
3. acquiring animals from certified MVV-free farms;
4. quarantine of imported animals until the MV-status is determined;
5. regular cleaning and disinfection of facilities and equipment;
6. reduction of housing animals density and efficient ventilation;

7. using of rams MVV free for mating or semen collection;
8. breeding program focused on resistance to MVV (marked assisted selection).

However, all costs linked to the management of the control strategies are noteworthy, that may be impractical. For this reason, often only partial and incomplete programs have been followed that turned out to be not enough to the eradication disease goals.

2.8 The genetic basis of resistance/susceptibility to MV in sheep

Apart from the identification of risk factor and management of existing control programs, the identification of genetic markers of resistance seems promising to define eradication MV disease strategies.

One of the most well-known examples of marked assisted selection (MAS) for disease resistance is the case of the strong resistance to scrapie in sheep, conferred by specific genotypes at the prion gene, (*PRNP*) (Goldmann, 2008).

Recently, a genome-wide association study in North American sheep identified a single, major gene (transmembrane protein 154 gene, *TMEM154*) with missense variants significantly associated with host susceptibility to ovine lentivirus infection (Heaton, et al., 2012).

The biological function of *TMEM154* remains unknown for any species. However, the ovine *TMEM154* genomic assembly consists of seven *TMEM154* exons encoding for a precursor protein of 191 amino acids that is cleaved, between amino acid position 30 and 31, to a mature protein with 161 residues.

Heaton et al., (2012) identified five missense SNPs (L14H, T25I, D33N, E35K, T44M, N70I) and two frameshift

deletion polymorphisms (R4AD, E82YD) in the predicted signal peptide and the extracellular domain (exons 1 and 2). Conversely, nonsynonymous SNPs and frameshift polymorphisms were not observed in exons 3 through 7. The combinations of the eight ‘‘coding’’ polymorphisms gave rise to 8 haplotypes coding for as many isoforms. However, haplotypes 1, 2, and 3 have been the most frequent haplotypes found in U.S. sheep (Heaton, et al., 2012).

From a comparison of ovine TMEM154 amino acid residues with those in related mammalian species, haplotype 3, was identified as the most likely ancestral isoform in sheep.

The ancestral *TMEM154* haplotype 3 encodes glutamate (E) at position 35 and asparagine (N) at position 70. Haplotype 2 differed from the ancestral haplotype only by isoleucine (I) mutation at position 70. Haplotype 1 differed from haplotype 3 by a single amino acid substitution, the charged substitution, E35K.

Haplotype 2 or 3, both of which encode a glutamate amino acid residue at position 35 (E35) of the extracellular portion of TMEM154, have been associated with increased risk of SRLV infection. Conversely, ewes homozygous for haplotype 1, which encodes a lysine residue at position 35 (K35), have been strongly associated with less likely to become infected (Heaton, et al., 2012; Heaton, et al., 2013; Leymaster, et al., 2013; Sider, et al., 2013; Alshanbari, et al., 2014; Molaee, et al., 2019; Yaman, et al., 2019).

No difference was observed between haplotypes 2 and 3, suggesting N70I did not play a detectable functional role related to odds of infection with MVV.

Consequently, selecting for breeding stock with TMEM154 haplotype 1 with reduced susceptibility to ovine lentivirus infection could implement the eradication strategy.

EXPERIMENTAL PART

3. Presentation of the Research Program

In the previous sections, an overview of two important bacterial and viral diseases of ovine and caprine animals, notified from the World Organization for Animal Health (OIE), has been given.
Contagious agalactia (CA) and Maedi Visna (MV), have been known for a long time, yet they are still neglected diseases worldwide.
The difficulty of prevention and control of these diseases is related to their rapid spread, to the multiple sources of infection with both horizontal and vertical transmission, to the different forms of the disease from acute to sub-acute and chronic or asymptomatic and to the high mutation rate of the causative pathogens.
Therefore, rapid, accurate and early diagnostic tests for the detection of the causative pathogen are urgently needed, as well as the implementation of control strategies to minimize opportunities for the spread of the infections.

- The first disease object of study was the Contagious agalactia (CA), and the research activity was aimed to:
    1. investigating survival persistence and transmission mechanism of *Mycoplasma agalactiae* (Ma), in the small ruminant farm environment during two experimental trials;
    2. developing and validating of new molecular diagnostic methods for Ma detection.

- The second object of research focused on a viral disease caused by *Maedi-Visna virus* (MVV) was aimed to the genetic characterization of the Sicilian sheep breeds at the *TMEM154* gene in order to investigate their genetic susceptibility/resistance to the pathogen.

We think that the present work can be of great utility for the implementation of both the scientific knowledge on the various transmission route and of control strategies of both diseases.

4. EXPERIMENTAL PART: CONTAGIOUS AGALACTIA

4.1 First experimental contribution: investigating survival persistence and transmission mechanism of *Mycoplasma agalactiae* (Ma) in the small ruminant farm environment

4.1.1 Aim of the study

In Italy, CA is known as “mal di sito” (“site disease”) due to the ability of Ma to persist on-farm environment several weeks after an outbreak (Loria and Nicholas, 2013).

The organism’s ability to contaminate the environment and infect successive flocks suggests a role for environmental transmission (Bergonier, et al., 1997; McAuliffe, et al., 2006).

However, there is a lack of knowledge about the environmental risk of Ma, its persistence on-farm, and its resistance out of target species. Therefore, in order to investigate the survival and persistence properties of Ma in different environmental conditions and alternative mechanism of transmission of the disease, two longitudinal studies, were carried out on a small experimental farm in Sicily.

For this aim, different laboratory methods have been utilized to investigate Ma presence and its survival in two different seasons.

4.1.2 Material and methods

4.1.2.1Experimental model

The trial was organized in Sicily, in Sciacca district (Agrigento) in small group of sheep belonging an experimental farm.

The experimental infection was conducted twice: during the dry season (summer trial - from May 2017 to July 2017) and during the rainy season (winter trial - from February 2018 to May 2018).

A total of fourteen lactating sheep (Belice Valley breed, 12-24 months), seven ewes per trial, sampled from a disease-free area, and serologically negative for Ma were involved.

Following acclimatization, the ewes were infected with a wild type Ma strain via an ethically approved hand milking method. During the experimental test, the infected ewes were separated from the rest of the flock and confined in an area of about 250 m^2, where they were fed with hay and concentrates, according to the national code of practice for housing and care of animal used in scientific procedures (Ministry of Healthy Authorization. N. 283-2017).

Longitudinal sampling of milk, whole blood and mucosal swabs from the involved sheep, as well as a sampling of environmental sites around the farm, was performed over a minimum of seven weeks according to a scheduled plan. However, the winter trial was extended of 4 additional weeks, to investigate if even higher humidity values and lower temperatures could influence the survival of the etiological agent.

In addition, with the use of a weather station, climatic data were recorded over the duration of the trials.

During both the experiments, the clinical status of the ewes was carefully monitored.

The experimental infection was conducted according to an ethically approved protocol with authorization of the Italian Ministry of Health.

4.1.2.2 Sampling

During the two seasons of the trial, sampling of milk, mucosal swabs and environmental samples was performed twice a week for first three weeks and then weekly until the end of the trial, whereas, for ethical reasons, peripheral blood was only collected once a week.

In particular, for both the trials, the mucosal samples included: ocular, rectal, ear swabs and urine. Because during the summer experiment one sheep showed arthritis, a sample of joint fluid was collected and analysed. Furthermore, according to the results of the first trial, nasal swabs, and wool, were additionally included in the second investigation. The environmental samples included: water samples from the watering troughs ("water in" and "water out"), feeding troughs swabs, fence swabs, boot swabs from animal collecting area of the farm, boot swabs from grazing, boot swabs from milking area, muck heaps, faeces, milker hands swab and, only for the summer trial, ticks collection has been included.

Table 4.1.1 report the sampling plans of the experimental infection. During the summer trial were collected 70 milk samples, 49 serum samples, about 250 mucosal samples, about 100 environmental samples and 52 ticks collected from seven infected sheep.

The species, stage, and sex of the ticks were determined by microscopic examination based on the morphological keys reported in literature (Manilla et al., 1998; Walker et al., 2005).

During winter trial 98 milk samples, 77 serum samples, about 540 mucosal samples, about 140 environmental samples were collected.
All samples were transported to the laboratory under refrigerated conditions and processed immediately.

4.1.2.3 ELISA analysis

From the jugular vein, a total of 70 blood samples were collected; serum obtained by centrifugation at 1500 x g for 10 min was stored at 5±3°C for a maximum of 48 hours before testing. For detection of Ma antibodies in serum the «POURQUIER-ELISA *M. agalactiae*» ELISA kit (Pourquier ELISA M. agalactiae, Institut Pourquier) was used. It targets antibodies against a fusion protein equivalent to Ma P48 protein (Rosati, et al., 2000) and uses an anti-immunoglobulin G (IgG) conjugate. Normalized values (NV) based on optical density (OD) are given by the following formula: NV = (sample OD - negative control OD) × 100 (average OD of the positive control - average OD of the negative control). Results are interpreted as follows: negative when NV ≤ 50%, doubtful when NV is between 50-60% and positive when NV ≥ 60%.

Table 4.1.1 Sampling plans of the experimental infection on small group of fourteen sheep belonging an experimental farm, located in Sicily.

Summer trial (May 2017 - July 2017)		**Winter trial (February 2018 - May 2018)**	
Mucosal samples of seven Belice Valley sheep	**Environmental samples**	**Mucosal samples of seven Belice Valley sheep**	**Environmental samples**
Milk	Water In	Milk	Water In
Blood	Water out	Blood	Water out
Ocular swabs	Feeding troughs swabs	Ocular swabs	Feeding troughs swabs
Rectal swabs	Fence swabs	Rectal swabs	Fence swabs
Ear swabs	Boot swabs of animal collecting area	Ear swabs	Boot swabs of animal collecting area
Urine	Boot swabs of milking area	Urine	Boot swabs of milking area
Joint fluid*	Faeces	Nasal swabs**	Faeces
	Muck heaps	Wool**	Muck heaps
	Boot swabs of grazing		Boot swabs of grazing
	Milker hands swabs		Milker hands swabs
	Ticks*		

Note: * Additional sample in summer trial. ** Additional sample in winter trial

4.1.2.4 Microbiological analysis

Isolation of the pathogen was carried out according to OIE standard procedure (OIE, 2019). Briefly, the milk samples, mucosal swabs and environmental samples were incubated in tubes with 3 ml of mycoplasma medium (Oxoid® Mycoplasma Broth Base) after enrichment with yeast extract, porcine serum (at 10%) and antibiotics, until the stationary growth phase. Each sample was diluted in base 10 for subsequent 5 tubes (10-1 – 10-6) to eliminate the risk of contamination. All the broths were incubated at 37 °C with 10% CO_2 for over 4 days until evidence of growth (indicated by fine cloudiness or opalescence). So, the samples were plated (10 µl) on agar media (Oxoid® Mycoplasma Agar Base) and incubated at 37 °C with 10% CO_2 for 48-72 h. The agar medium was examined daily for the evidence of typical mycoplasma 'fried-egg' colonies using a stereomicroscope Nikon SMZ800N.

4.1.2.5 Molecular analysis

The DNA extraction was carried out from 1 ml broth culture of media inoculated with milk samples, mucosa swabs and environmental samples. In particular, the DNA from milk samples was extracted after 24h of enrichment in mycoplasma media while the other biological samples were processed after the evidence of mycoplasma-growth during the incubation.

DNA extraction was performed by commercial kit InstaGene™ Matrix according to the manufacturer's instructions of (Bio-rad). Tick's DNA was extracted from a total of nine tick pools: one pool from five sheep and two pools from the other two ewes.

4.1.2.6 Real-time PCR for p40 gene

A TaqMan real-time PCR targeting the *p40* gene (Oravcová, et al., 2009) was applied for specific detection of Ma DNA in milk, mucosa swabs, urine and environmental samples. The *p40* gene encoding an immunodominant adhesin that plays a key role in cytoadhesion of Ma. Bacterial adhesion is a key mechanism of mycoplasma virulence, and the protein P40 displays a strong and persistent signal in response to antibodies. According to Oravcovà et al., (2009) the amplification was performed in a 20 µl reaction volume including 2µl of DNA, 1X Sso Fast Probes Supermix (Bio-Rad Laboratories Srl) 300 nM for primers MAP40127F and MAP40235R and 200 nM for the 6-carboxyfluorescein [FAM]-labelled MAP40160P probe. Real-time PCR was conducted in a CFX96 Touch™ Real-Time PCR Detection System (Biorad) with the following program: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. In order to verify the absence of the PCR inhibitors in the DNA templates and to assess the PCR performance of each reaction, an exogenous internal positive control (IPC) was added and co-amplified in the same PCR reaction mixture, according to the manufacturer's instructions (TaqMan® Exogenous Internal Positive Control Reagents - VIC™Probe – Applied Biosystem).

4.1.2.7 PCR/Denaturing gradient gel electrophoresis (PCR/DGGE)

Samples showing growth in plate but at the same time negative or borderline real-time PCR ct (cycle threshold) values were subjected to DGGE analysis in order to identify the mycoplasma species grown in plate.

Amplification of the V3 region of the 16S RNA gene was performed according to McAuliffe (2005) using the universal

bacterial primer GC-341F and the mollicute-specific primer R543. The cycling conditions were: denaturation at 94°C for 5 min, followed by 30 cycles of 95 °C for 1 min, 56 °C for 45 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. The PCR amplicons were checked for correct amplification in 2% agarose gels stained with GelRedTM Nucleic Acid Gel Stain (Biotum). DGGE was performed using the DCode™ Universal Mutation System (Biorad). Samples (20 µl) were loaded onto 10% polyacrylamide/bis (30: 1) gels with denaturing gradients from 30 - 60% [where 100% is 7 M urea and 40% (v/v) deionized formamide] in TAE electrophoresis buffer (Severn Biotech). Electrophoresis was performed at 100 V at a temperature of 60 °C for 18 h. Gels were then stained with SBYR Gold (Cambridge BioScience) in TAE for 30 min at room temperature and visualized under UV illumination.
Figures 4.1.1 a) and b) report an overview of the processing scheme of the different types of samples.

4.1.2.8 Milk composition
Milk samples of winter trial were also analysed for fat, protein, casein and lactose percentages by infrared method (Combi-foss 6000, Foss Electric, Illerød, Denmark) and somatic cell count by cytological analysis with automatic flow cytometry equipment (Fossomatic 6000).

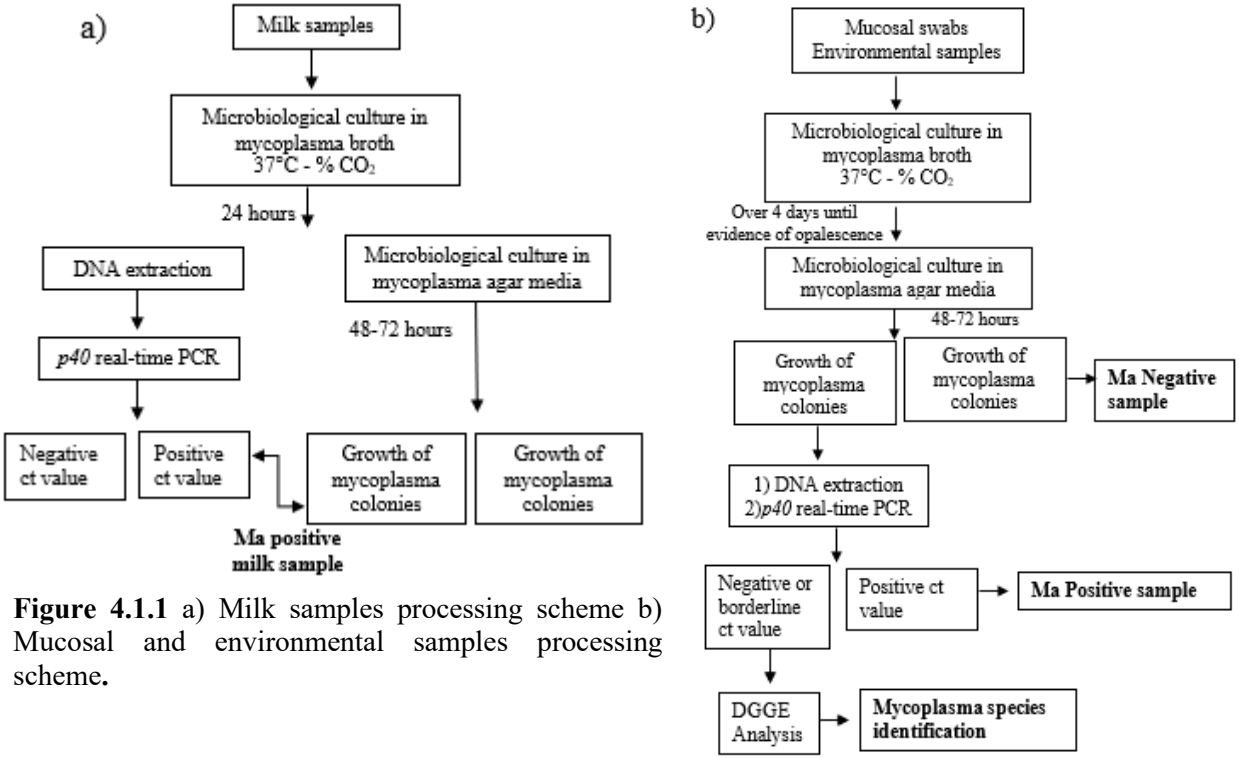


Figure 4.1.1 a) Milk samples processing scheme b) Mucosal and environmental samples processing scheme**.**

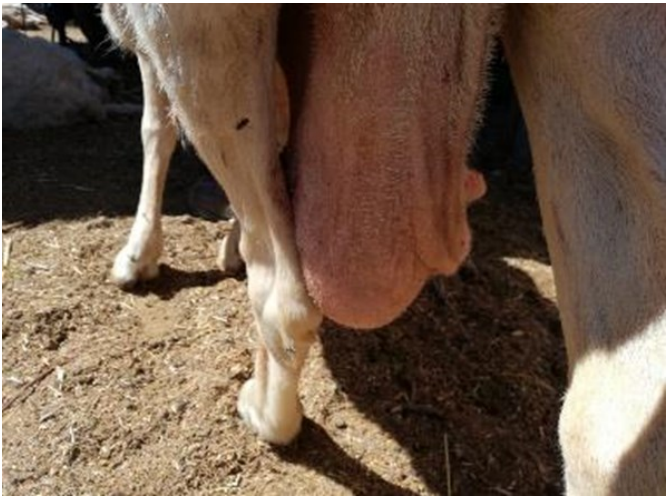
4.1.3 Results

4.1.3.1 Clinical status of sheep

From a clinical point of view, during the summer trial, the infection has occurred with a rapid spread of acute mastitis with a sudden alteration in the consistency of the milk that appeared discoloured, yellowish and containing abundant clots, as well as drastic decrease of milk production (Figure 4.1.2). In one sheep (sheep 4) of summer trial, multiple symptoms (acute mastitis and arthritis) occurred, while one ewe despite were excreting the microorganism in milk and showed Ma antibodies detection, never manifested any symptom.

During the winter trial, the acute mastitis occurred in three sheep with alteration of milk consistency and decline of production, two ewes showed mild mastitis, and two animals never manifested any symptom.

a)



b)

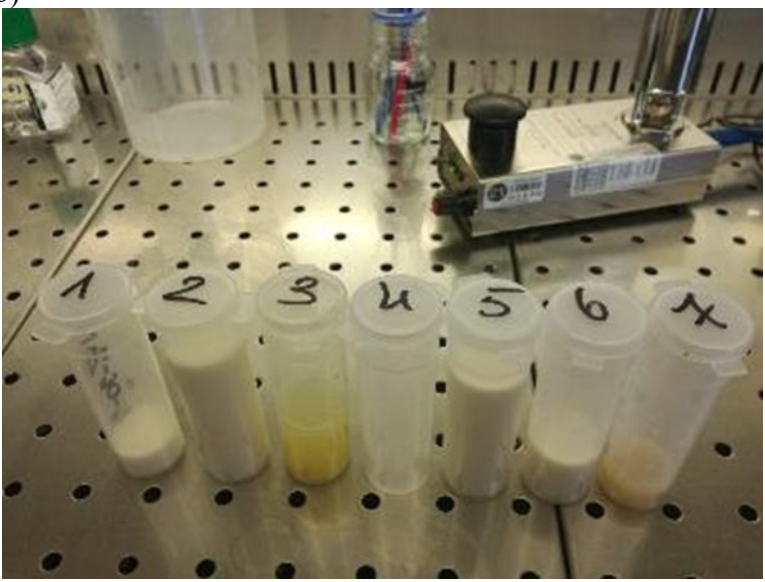


Figure 4.1.2 a) Marked atrophy of right udder caused by Ma infection. b) Milk samples of seven sheep involved in the experimental infection, in particular milk sample 3, 4 and 7 appear discoloured, yellowish, and containing abundant clots.

4.1.3.2 Ma detection in milk

In both trials, Ma was identified by culture and by real-time PCR in milk from the first week post-infection challenge; however, the course of infections has been different between the two experiments (Figure 4.1.3).

During the summer trial, the infection presented a slower progressive trend than the winter experiment in which Ma was rapidly excreted, and it was less constantly detected in milk samples (Figure 4.1.4).

In both the season and experimental infections some sheep were intermittently positive to the tests; especially in the winter experiment, but in which trial only two sheep

confirmed excretion of Ma in milk at the majority of scheduled sampling. Furthermore, it is worth highlighting that the sheep 1 and 2 (winter trial), which never presented clinical signs of infection, excreted the organism through just mucosal discharges and not in milk.

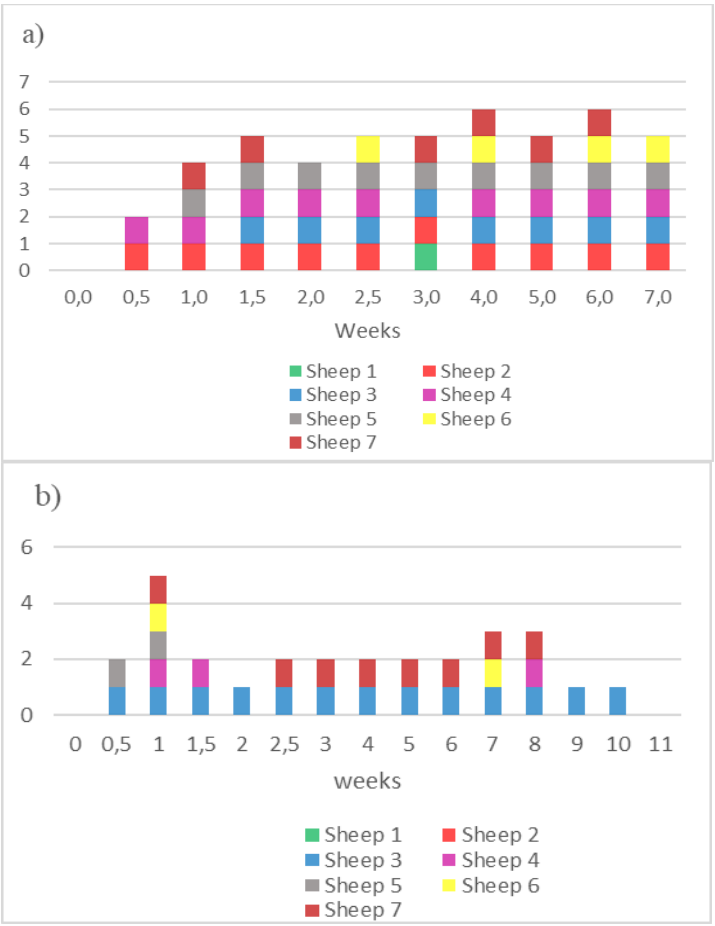


Figure 4.1.3 Ma detection in milk samples.
a) Summer infection trend; b) Winter infection trend

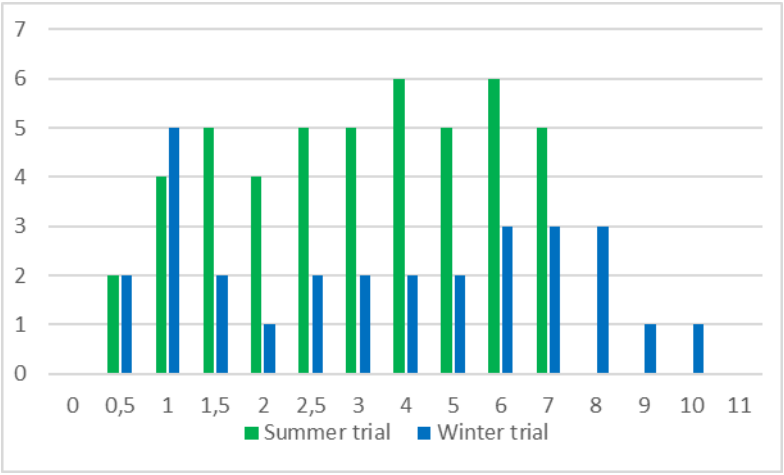


Figure 4.1.4 The number of sheep with Ma positive milk samples over the studies periods.

4.1.3.3 ELISA results

The disease progression, in both experimental infections, was confirmed by the presence of antibodies by the ELISA test. The presence of antibodies started to be detected in early-stage only in the summer trial (fist week post-infection), while in the winter study the antibody response was detected, for the first time, after about 14 days post-infection challenge. (Figure 4.1.5).

However, in the summer trial, only after 30 days, all ewes were showing antibody response to mycoplasma infection except for the sheep 1, which remained negative until 50 days post-infection start (Figure 4.1.5a).

In winter trial sheep 1, 4, and 6 seem that never presented an antibody response to Ma suggesting that it has been excreted before eliciting an immune response. Furthermore, the ELISA positivity of sheep 2 after about 30 days confirmed the infection status of this asymptomatic ewe (Figure 4.1.5b).

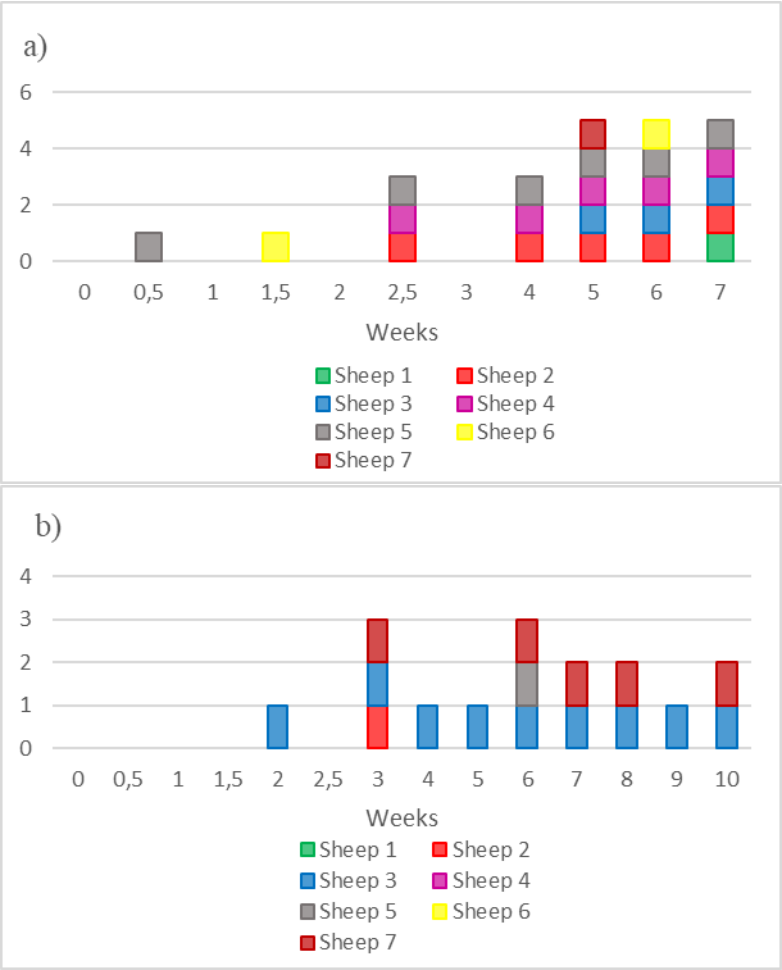


Figure 4.1.5 Ma antibodies detection by the ELISA test.
a) Summer trial; b) winter trial.

4.1.3.4 Ma detection in mucosa and environment

Rectal swabs were found to be Ma positive in 3 and 5 ewes in summer and winter trial, respectively, but this evidence was not constant in time (Figure 4.1.6).

Ma was detected for the first two weeks following infection in samples from eyes, nose, ears, urine, and wool only in winter study, excepting the ear swabs that were positive once also in summer study (Figure 4.1.7).

The joint fluid of sheep 4 (summer trial), that, during the experimental infection, manifested arthritis resulted positive to Ma (Figure 4.1.6 a).

The organism was identified intermittently from the different environmental site, in particular from milking area, faeces collected from the soil and muck heaps collected from the fields belonging the farm, in both trials. Only in the winter study, Ma was also detected in additional sites (feeding troughs, fence, animal collecting area and milker hands swab). Instead, Ma was never detected in the drinking water and in the grazing (swabs of the boot after walking in the pasture) (Figure 4.1.8).

Ma was also identified in two out of nine pools of ticks that comprised *R. bursa* adult females collected from two sheep positive for Ma, as resulted by the presence of antibodies and antigen excretion.

Morphological identification revealed that Ixodidae ticks included *Rhipicephalus bursa* (n=42), *Rhipicephalus sanguineus* (n=4) and *Haemaphysalis punctata* (n=6) and that the majority of *Rhipicephalus bursa* adult females were included in the two ticks pools mentioned above.

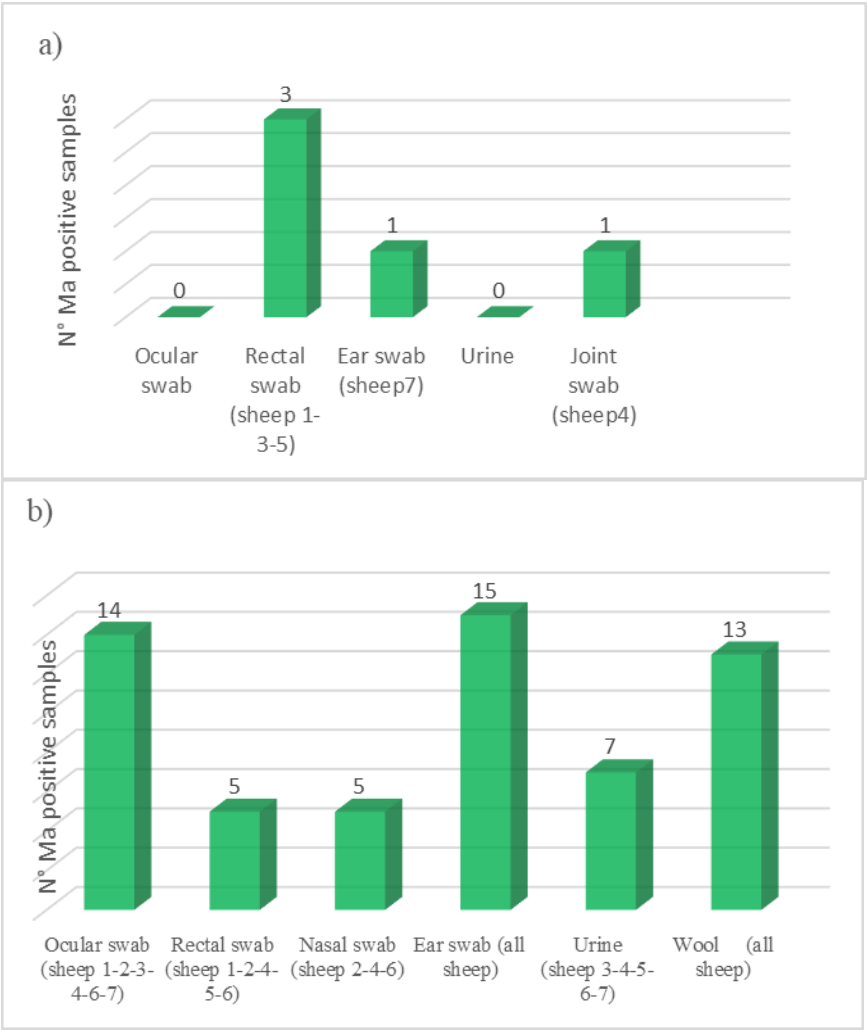


Figure 4.1.6 Ma detection in mucosal samples.
a) Summer trial; b) winter trial.

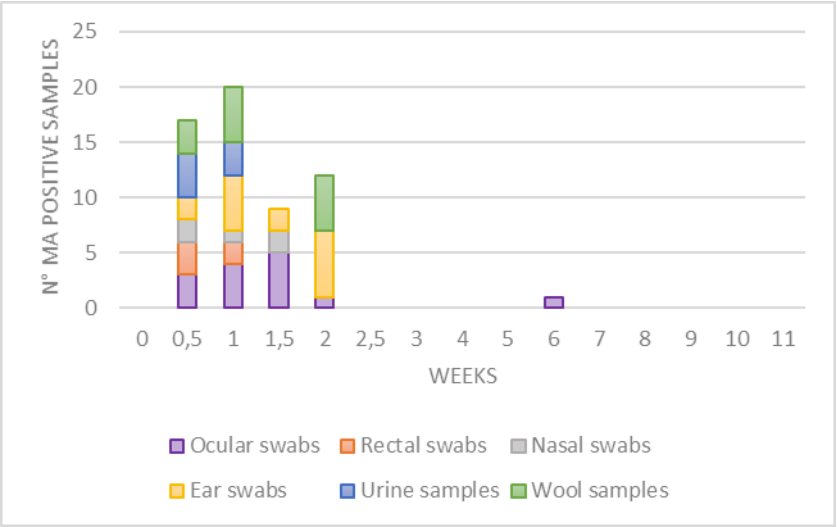


Figure 4.1.7 The number of Ma positive mucosal samples detected over the winter trial.

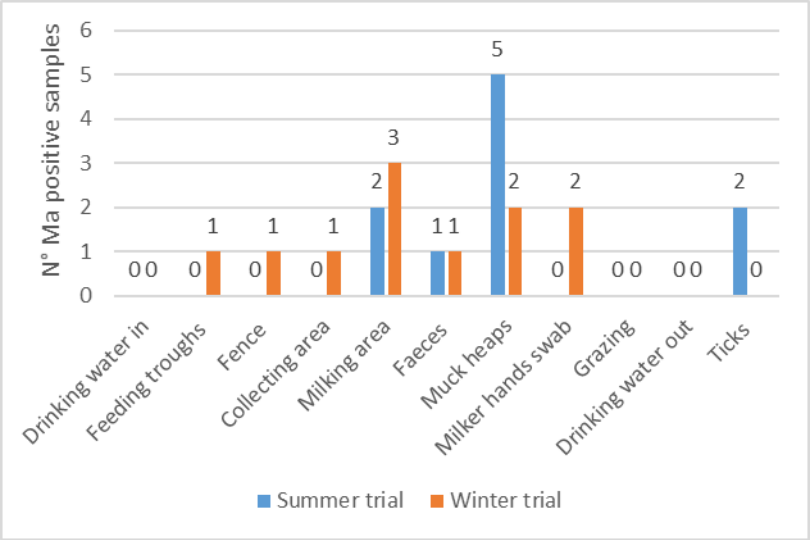


Figure 4.1.8. Environmental sample types in which Ma was detected over both trials.

The DGGE analysis (Figure 4.1.9) confirmed the positivity to Ma of some samples (ear swabs, rectal swab, nasal swabs faeces and muck heaps) (Table 4.1.2), resulted borderline in real-time PCR, in which the presence of other *Mycoplasma* species or PCR inhibitors has interfered negatively with the PCR amplification. *Mycoplasma arginini* has been detected in different mucosal swabs, urine and wool samples, especially in the winter trial, during which from nasal swabs *Mycoplasma ovipneumoniae* have also been isolated (Table 4.1.2). Concerning the doubtful results from environmental samples, DGGE tests identified the presence of *Acholeplasma laidlawii* and *axanthum*, the most common environmental contaminant belonging to Mollicutes Class (Table 4.1.2).

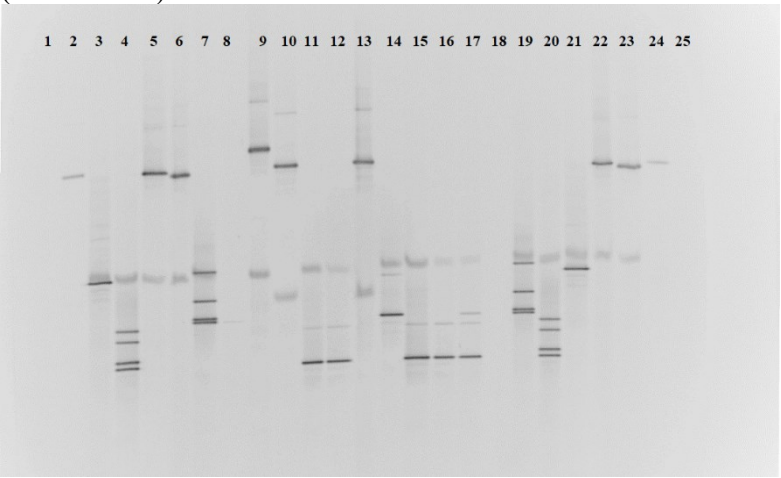


Figure 4.1.9 Mycoplasma species identification of mucosal and environmental samples by DGGE analysis. 1) and 25) negative controls; from 2 to 7 positive controls (*A. laidlawi; M. bovis; M. arginine; M. ovipneumoniae; M. conjunctivae; M. agalactiae); from 8 to 18 mucosal and environmental samples; from* 19 to 24 positive controls (*M. agalactiae; M.arginini; M. bovis; M. ovipneumoniae; M. conjunctivae; A. laidlawi*).

Table X.2 *Mycoplasma* species identification by DGGE analysis results for samples showing growth in plate but at the same time negative or borderline real-time PCR ct values.

	DGGE analysis results
Mucosal samples	
Ocular swabs	2 *M. agalactiae*
Rectal swabs	1 *M. agalactiae* 1 *M. agalactiae* + *M. arginine* 4 *M. arginine*
Ear swabs	1 *M. agalactiae* 1 *M. agalactiae* + *M. ovipneumoniae* 3 *M. arginine*
Urine	1 *M. arginine* 4 *Acholeplasma laidlawii*
Nasal swabs	1 *M. agalactiae* 1 *M. agalactiae* + *M. ovipneumoniae* 1 *M. agalactiae* + *M. arginine* 4 *M. ovipneumoniae* 3 *M. ovipneumoniae* + *M. arginine* 2 *M. arginine* 2 *Acholeplasma laidlawii*
Wool	2 *M. agalactiae* 1 *M. arginine* 4 *Acholeplasma laidlawii*
Environmental samples	
Fence swabs	1 *Acholeplasma laidlawii*
Boot swabs of animal collecting area	6 *Acholeplasma laidlawii*
Boot swabs of milking area	1 *M. agalactiae* 2 *Acholeplasma laidlawii* 1 *Acholeplasma axanthum*
Faeces	1 *M. agalactiae* 1 *Acholeplasma laidlawii*
Muck heaps	1 *M. agalactiae* 1 *Acholeplasma laidlawii*
Boot swabs of grazing	1 *Acholeplasma laidlawii*
Milker hands swabs	2 *M. agalactiae*

4.1.4 Discussion

It has been reported the ability of Ma to persist in the environment for several months after an outbreak of disease (Bergonier, et al., 1997; Loria & Nicholas, 2013) potentially enabling the maintenance of contamination for successive flocks.

The present challenge conducted in a pilot farm, reproducing natural disease transmission, allowed to identify key environmental sources from which viable Ma was recovered that may justify to the spread of the disease by indirect transmission.

The results showed that all symptomatic sheep have been affected by the subacute form of the disease showing mastitis, reduction of milk production and alteration of milk consistency with an increase of somatic cell count (SCC) in agreement with data reported in the literature (Bergonier & Poumarat, 1996; Gonzalo, et al., 2005; Todaro, et al., 2015; Tolone, et al., 2019).

In both trials, asymptomatic ewes have also been identified, that never presented clinical sign, despite they excreted the etiological agent through milk or mucosal discharges and presented and immune response to Ma.

The presence of asymptomatic carriers ("healthy carriers") was extensively reported, that detected the presence of Ma mainly in bulk tank milk or ear canal (Kheirabadi & Ebrahimi, 2007; De La Fe, et al., 2009; Gómez-Martín, et al., 2012; Tardy, et al., 2019). Disease transmission between farms may occur with the introduction of healthy carriers (Kinde, et al., 1994).

Our findings confirm that the main source of infection is due to Ma excretion in milk, thus milking practices play a highly significant role in indirect transmission, because responsible

for transferring the etiological agent from sick to healthy udders (Al-Momani, et al., 2008; Todaro, et al., 2015).
Excretion in milk has been detected in the early stage of the infection becoming intermittent with time (Ariza-Miguel, et al., 2012; Tardy, et al., 2019), especially in the winter trial, during which the rapid shedding of Ma occurred mostly by mucosal discharge. Therefore milk, ocular, nasal, and ear canal secretions represent not only the direct route of transmission among the animals but contribute to spreading Ma in the environment (Jay, et al., 2020).
Although not previously reported in sheep, in our experiment Ma has been detected intermittently in faeces and muck heaps, in agreement with previous studies that revealed the presence of the organism in goat's faeces (DaMassa, et al., 1987; Hasso & Al-Omran, 1994).
Furthermore, the multiple detections of the etiological agent in the different farm sites, especially during the winter trial, confirm the Ma persistence in the environment during the outbreak.
In fact, it is known that several *Mycoplasma* species including *Mycoplasma bovis, Mycoplasma putrefaciens, Mycoplasma cottewii* and *Mycoplasma agalactiae*, despite their poor mechanical resistance due to lack of cell wall, produce prolific biofilms that can contribute to mycoplasma persistence in the environment and inside the host leading to the chronicity of a disease (McAuliffe, et al., 2006).
Concerning the role of ticks in the mycoplasma transmission, further in-depth analysis are needed, but the positivity found in ticks collected from two clinical subjects for over four weeks would exclude the hypothesis of contamination occurred in the "septicaemic" phase by the parasite, suggesting the potential role of bloodsucking parasites in the maintenance and spread of CA.

In literature, the role of arthropods in *Mycoplasma* transmission has been confirmed for fleas and mites (Nayak & Bhownmik, 1990), and hemotropic mycoplasms were reported in lice from goats and in Ixodes ticks (Taroura, et al., 2005; Hornok, et al., 2012).

4.1.5 Conclusion

Small ruminant livestock is distributed worldwide, including in many less developed countries of the Mediterranean basin. CA has been known for a long time for its veterinary and moreover economic impact on traditional or intensive farming, yet it is still a neglected disease worldwide.

It is difficult to prevent and control CA due to its high morbidity are still unclear gaps aspects concerning multiple sources of infection, both horizontal and vertical transmission, and different clinical appearances of the disease from acute to sub-acute, and chronic and sometimes asymptomatic.

Our findings provide preliminary observations that can contribute to a better understanding of the complexity of the disease and its transmission. In particular, the excretion of the organism in faeces and its persistence on the environment together with asymptomatic carrier animals and involvement of hematophagous parasites as disease vectors or reservoirs of Ma, provide insight into potential mechanisms that enable CA to remain in a site for a long time and endemic in Mediterranean basin and in Sicily.

4.2 Second experimental contribution: developing and validating of new molecular diagnostic methods for the Ma detection

4.2.1 Validation of Loop-Mediated Isothermal Amplification (LAMP) Field Tool for Rapid and Sensitive Diagnosis of Contagious agalactia in Small Ruminants

The following work has been already published as:
Tumino, S., Tolone, M., Parco, A., Puleio, R., Arcoleo, G., Manno, C., Nicholas, R. A. J., & Loria, G. R. (2020). Validation of Loop-Mediated Isothermal Amplification (LAMP) field tool for rapid and sensitive diagnosis of Contagious Agalactia in small ruminants. *Animals*, 10, 509.

4.2.1.1 Introduction

Today in the Middle East and countries in the Mediterranean basin, the management of Contagious agalactia (CA) is one of the highest priorities for sheep and goat farming due to the severe losses in milk production, increased lamb mortality, cost of veterinary assistance and the difficulty of eradicating the infection once established in a herd.
Early diagnosis is essential for the rapid and effective management of the disease in order to avoid the spread of the infection to the whole flock; therefore, rapid, precise, and low-cost methods for pathogen detection are urgently needed.
Traditionally, according to OIE guidelines (OIE, 2019), the current diagnosis of CA is based on the isolation of causative mycoplasmas from affected animals, which are further identified by biochemical, serological or molecular tests such

as PCR-based methods. The isolation of the mycoplasma in selective enrichment media represents a laborious and time-consuming process, as mycoplasmas grow very slowly. Furthermore, serological tests such as the enzyme immunoassay or complement fixation test may be ineffective in the first stage of the disease, which may result in false-negative cases because the antibodies are detectable only after 10-15 days from infection (Buonavoglia, et al., 1999). During the past decades, a number of polymerase chain reaction (PCR) and real-time quantitative PCR-based assays have been widely applied for rapid detection of Ma (Tola, et al., 1997; McAuliffe, et al., 2005; Lorusso, et al., 2007; Oravcovà, et al., 2009; Becker, et al., 2012).

Nevertheless, disadvantages for PCR and real-time PCR such as the presence of inhibitors normally in milk and the requirement for trained staff and well-equipped laboratories limit their utilization as rapid tests and in-field practice. More recently, new molecular diagnostic tools have been developed to provide higher sensitivity, specificity and to reduce time and costs. Loop-Mediated Isothermal Amplification (LAMP) is an innovative and economic gene amplification tool based on its ability to amplify a target gene with high efficiency under isothermal conditions, unlike PCR, in the range of 60 to 65 °C (Notomi, et al., 2000).

In comparison to the conventional PCR and real-time PCR, the LAMP technique is less sensitive to inhibitors present in biological samples. It does not require temperature cycling and can be performed using a simple heating device such as water bath; therefore, it can potentially be used for tests in the field (Mori & Notomi, 2009). LAMP is more specific and faster than PCR and real-time PCR because it employs four oligonucleotide primers, namely FIP (forward inner primer), BIP (backward inner primer), F3 (forward primer) and B3

(backward primer) to recognize six different regions of the target gene. Two extra primers, LF (loop forward) and LB (loop backward) are also incorporated in order to accelerate the amplification of reaction as well as enhance the specificity (Nagamine, et al., 2002). LAMP has been shown to be a sensitive and specific method for the detection of veterinary pathogens (Hill, et al., 2008; Trangoni, et al., 2015; Sheet, et al., 2016; Ashraf, et al., 2018).
The detection of Ma by LAMP was first reported by Rekha et al. (2015). However, the method for the detection of Ma in milk, the most common sample received by laboratories, has not yet been validated on field samples.
The aim of this work is to validate a LAMP test, previously developed by Loria et al., (2018b) for the detection of Ma in sheep milk samples in order to confirm both its effectiveness and robustness as a diagnostic tool and its potential practical use as a rapid and cheap field test.

4.2.1.2 Material and methods

4.2.1.2.1 Samples Preparation

The NCTC reference strain of Ma (NCTC 10123) and three wild strains of Ma (Sc 123/4; Pa 116/20; Pa 49/19), previously isolated and identified by the OIE Reference Laboratory for CA at the Istituto Zooprofilattico Sperimentale della Sicilia, were used in this study for the artificial contamination of milk. The wild strains of Ma have been isolated from milk of sheep and goats affected by interstitial mastitis. In detail, the etiological agent has been identified by standard laboratory approaches according to the World Organization for Animal Health microbiological isolation (OIE, 2019) and denaturing gradient gel

electrophoresis (DGGE) analysis (McAuliffe, et al., 2005) of DNA extracted from 1 ml of milk.
Ma strains were grown in modified Hayflick's medium and after incubation at 37 °C in 5% CO_2 for 72 hrs, the broths were then stored at −80 °C. Ma cultures at exponential growth were serially diluted, and colony-forming units (CFU) were determined using standard procedures (Rodwell et al., 1983). To assess the limit of detection (LOD) of the assay, pasteurized milk, previously checked to be DNA mycoplasma-free by DGGE analysis, was contaminated with Ma and serial dilutions were made in the range of 10^7 to 10 CFU/ml, according to Oravcovà et al., (2009). Overall, 60 previously pasteurized milk samples were spiked with a serial dilution of different wild strains of Ma to compare sensitivity between real-time PCR and LAMP. In addition, 30 different positive milk samples collected from Sicilian outbreaks and 20 negative samples were included in the test. The use of mycoplasma isolates from sheep and goats milk samples provided the highest level of clonal diversity for test specificity validation, and the study results are valid for the source population and target population. Therefore, a total of 110 samples of milk (90 positives and 20 negatives) were used to investigate the efficacy of the new method. The genomic DNA used for the real-time PCR was extracted from 1 ml of positive and negative milk samples, using the InstaGene Matrix (Bio-Rad laboratories, Hercules, CA, USA) according to the manufacturer's instructions and our standard laboratory approach. For the Lamp assay, the DNA extraction was performed from 100 µl of the milk sample, using a single step and an incubation at room temperature, using the reagents supplied for the kit. The conduct and reporting of each test were done blind to the other test results.

4.2.1.2.2 TaqMan Real-Time-PCR

We have compared the LAMP assay to a well-established real-time-PCR method. A TaqMan real-time PCR assay was run on a Bio-rad CFX96 real-time PCR detection system and it was performed to amplify the *p40* gene, using MAP40127F and MAP40235R primers together with a 6-carboxyfluorescein [FAM]-labelled MAP40160P probe, according to Oravcovà et al., (2009).

An exogenous internal positive control (IPC) was incorporated into the PCR reaction mixture in order to assess the absence of the PCR inhibitors, according to the manufacturer's instructions (TaqMan® Exogenous Internal Positive Control Reagents—VICTMProbe—Applied Biosystem, Foster city, CA).

4.2.1.2.3 Lamp Assay

The LAMP assay was carried out using the ICGENE device (Enbiotech Group s.r.l., Palermo, Italy). It is composed of a portable instrument and a kit developed for specific detection of Ma. The system is composed of a real-time fluorometer with the automatic interpretation of results by the direct visualization of the sigmoid curve in a tablet, using a specific application (Enbiotech Group s.r.l., Palermo, Italy).

Six primers targeting the *p40* gene (Rekha et al., 2015) were used (Table 4.2.1.1). Three µL of the extracted DNA samples were used to obtain the specific amplification of the target in a final volume of 55 µL, including 22 µL of LAMP mix (Enbiotech, Palermo, Italy) containing freeze-dried primer and a master mix with reagents useful to carry out the test and 30 µL of mineral oil. All reagent compositions are protected by trade secret. The optimal conditions for amplification were obtained at a temperature of 64 °C for 60 mins. Positive and negative DNA control included in the kit were also used. The

positivity was assessed graphically by the direct visualisation of the sigmoid curve on the device display.

Table 4.2.1.1 Primer sequences for Loop-Mediated Isothermal Amplification (LAMP) for detection of Ma

Primers	Length (bp)	Primer Sequence (5'–3')
F3	21	GGTTTATTAACTGCGTCATCA
B3	19	CAACAGTTGCATTCGTCTT
FIP	46	ACCTTATCACCATTATCTTGTGGATCA
		GTGCCTTTATTAGCTCGTA
BIP	46	AGCATTAGGTGAAGTTGTCAAAAATA
		TTGAGCTTGCTTCAGGAATT
LF	24	GTGAATTTTCGTTCTTATCATCAC
LB	26	ACAAATCTAGGTGAAATAGTATTACC

4.2.1.2.4 Statistical Analysis

Statistical analyses were performed using the caret package in R (Kuhn, 2008). Sensitivity (Se) was calculated as the proportion of samples identified as positive by the assay. Specificity (Sp) was calculated as the proportion of negative test results obtained among healthy controls. We also carried out a comparison of accuracy in terms of prevalence (P), positive predictive value (PPV) and negative predictive value (NPV) for real-time-PCR and LAMP methods. The study was performed in compliance with the Standards for Reporting of Diagnostic accuracy (STARD) statement (Bossuyt, et al., 2015).

4.2.1.3 Results and Discussion

For the evaluation of performance characteristics of the LAMP assay, results were compared to those of the real-time PCR. As expected, the results showed that no positive signal occurred for any of the negative milk samples for both tests, confirming the specificity of the primer set for Ma reported in the literature (Rekha, et al., 2015; Loria, et al., 2018).

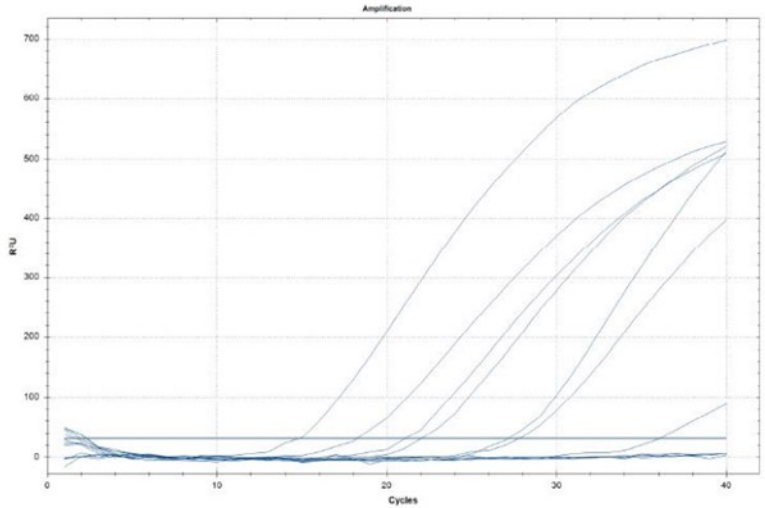
The LAMP assay was able to detect Ma in 81 of 90 positive milk samples vs 69 positive samples detected by real-time PCR; therefore, LAMP technology was found to be more sensitive than real-time PCR with a sensitivity of 90% (95% CI 0.84–0.96) and 77% (95% CI 0.59–0.95), respectively (Table 4.2.1.2). Although the sample size for test validation was not calculated a priori, considering 110 samples it was possible to obtain a test power of 90% (sensitivity).

Table 4.2.1.2 Comparison of real-time PCR and LAMP results.

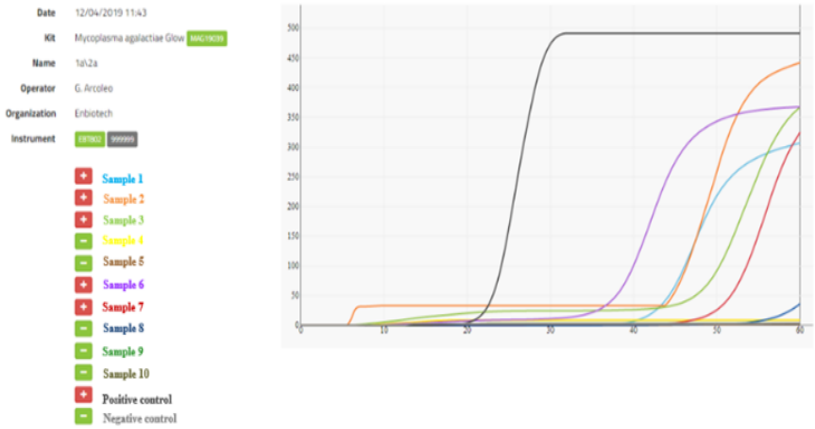
Test	TP	FP	FN	TN	P (%)	Se (95%CI)	Sp (95%CI)	PPV	NPV (95% CI)
Real-time PCR	69	0	21	20	0.82	0.77 (0.59–0.95)	1	1	0.49 (0.44–0.54)
LAMP	81	0	9	20	0.82	0.90 (0.84–0.96)	1	1	0.69 (0.65–0.73)

TP: true positive; FP: false positive; FN: false negative; TN: true negative; P: Prevalence; Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value

The sensitivity of LAMP assays and real-time PCR was determined in terms of CFU by making 10-fold serial dilutions of milk contaminated with Ma. Real-time PCR was able to detect up to the level of 10^4 CFU/ml, while LAMP could detect up to 10^3 CFU/ml, indicating that LAMP was 10-fold more sensitive than real-time PCR. The processing time of the LAMP assay was within 45 minutes from the beginning of the amplification, rather more than the 55 min required for the real-time PCR (Figure 4.2.1.1). The NPV of the LAMP test was higher than the real-time PCR method (0.69% vs 0.49%).



(a)



(b)

Figure 4.2.1.1 (a) Amplification curves obtained from real-time PCR and (b) LAMP assay.

Current diagnostic methods, using sheep and goats milk samples, include a variety of Mac-specific PCR-based assays (Jaÿ, et al., 2019). In literature, these methods all extensively reported to have a high sensitivity and accuracy with a detection limit between 10 to 350 CFU/ml (Tola, et al., 1997; Lorusso, et al., 2007; Oravcovà, et al., 2009; Becker, et al., 2012) a quite high level in comparison to our detection limits for both the methods (real-time PCR and LAMP). Furthermore, our LAMP LOD was not as good as those reported by other LAMP assays for the detection of other veterinary mycoplasma spp. (Rekha, et al., 2015; Ashraf, et al., 2018; Zhang, et al., 2019). However, it is known that performances of molecular diagnostic methods highly depend on the DNA extraction methods used and their efficiency to remove the natural inhibitors, which are present in the milk samples (Tatay-Dualde, et al., 2015; Tardy, et al., 2019).

Furthermore, the PCR-based assays require specific and expensive reagents, instruments and special precautions; thus, they are not suitable for application use in the field, at great distances from diagnostic laboratories. On the other hand, The LAMP system with the mini portable instrument is inexpensive, requires little equipment and technical support, and is not space consuming. In terms of turnaround time speed, the total time to detection, including the DNA extraction step, was only 1 hr and 20 min using the ICGENE device, in comparison with 2 hrs of real-time PCR procedure. Moreover, the real-time PCR price per test (in euro, excluding Value-Added Tax) was estimated to be 24 € compared to 15 € (excluding Value-Added Tax) of the LAMP test price.

Recently, a variety of isothermal amplification methods have been developed in the molecular diagnosis of a range of diseases and play a significant role in monitoring and controlling the spread of local epidemics in several countries (Li, et al., 2017). Each of these existing techniques has advantages and disadvantages. However, it is known that the indirect detection methods like turbidity or also the colour change resulting from the use of hydroxynaphthol blue (Goto, et al., 2009; Soleimani, et al., 2013) could be challenging to see by the naked eye. Other methods require opening the tube after amplification, such us running product on a gel and the addition of fluorescent dye (SYBR Green), causing carry-over contamination and detection of false-positive results (Iwamoto, et al., 2003; Parida, et al., 2008; Lau, et al., 2010). In our experience, the best solution to avoid the risk for amplicon contamination is to use fluorescent dyes, detectable by amplification curves in real-time.

In our study, the ICGENE device has the advantage of being a portable device detecting the fluorescence emitted from the

sample in real-time with the automatic interpretation of final results, observing the amplification curves on the tablet screen. Therefore, it is suitable for use directly in the field, and it does not require any process after thermal incubation, reducing the risk of environmental cross-contamination.

4.2.1.4 Conclusions

The results obtained confirmed that the LAMP assay is faster and has more sensitivity (90% vs 77%) than the real-time PCR method. In conclusion, the LAMP portable device could be a potential field test, because it does not need the use of expensive laboratory equipment, does not require qualified staff and it is not affected by contamination.

Undoubtedly, speed, easiness and cost-effectiveness of the LAMP assay make it a promising and effective diagnostic tool in the field level for controlling Ma infection.

4.2.2 Development of a Loop-mediated isothermal amplification (LAMP) assay for the detection of Ma genetically divergent strains: from DNA sequence analysis, through designing LAMP method to test clinical and environmental samples.

The following work has been conducted alongside the OIE reference laboratory for CA at the Animal and Plant Health Agency (APHA) in the UK. A manuscript is going to be submitted for publication.

4.2.2.1 Introduction

One of the main features of mycoplasma genomes is their fast and dynamic evolution, with one of the highest bacterial mutation rates, resulting in the rapid emergence of new strains (Citti, et al., 2018; (Faucher, et al., 2019).

This aspect has relevant implications on the specificity of PCR-based diagnostic methods requiring their regularly re-validate on newly circulating strains.

An isothermal amplification assay, potentially is used for tests in the field, was recently developed for direct detection of the Ma based on the *p40* gene (Rekha, et al., 2015), and validated for commercial development in Italy (Loria, et al., 2018; Tumino, et al., 2020).

However, testing different Ma strains, which have been shown by other genetic characterisation methods to be divergent, they have been undetected using the *p40* gene-based amplification methods (Rekha, et al., 2015; Oravcovà, et al., 2009). The present study aimed to develop a LAMP assay, targeting a different gene to detect Ma associated with infection by a comprehensive range of genotypes. The assay

was evaluated for the detection of Ma in sheep milk and other clinical samples to confirm performance and robustness as a diagnostic tool and further to assess its potential for identifying Ma in environmental samples.

4.2.2.2 Materials and methods

4.2.2.2.1 Field and reference strains

A total of 19 Ma strains were used in the present study, including the Ma reference strain (NCTC 10123). The strains were selected based on Multilocus Sequence Typing (MLST) type (McAuliffe, et al., 2011) and whole-genome sequence information to ensure representations of genomically similar and divergent strains. In addition, 13 strains identified as belonging to other CA causative organisms [*Mycoplasma putrefaciens* (Mput), *M. mycoides subsp. capri* (Mmc), *M. mycoides subsp. capricolum* (Mcc)] and strains representing other Mycoplasma and mollicute species (including *M. conjunctivae, M. arginini, M ovipneumoniae, M. bovis* and *Acholeoplasma laidlawii*) were included (Table 4.2.2.1).

All isolate, previously cloned to single colonies, were revived in Eaton's broth medium (Nicholas & Baker, 1998) with incubation at 37°C in a modified atmosphere (5% CO_2) handled according to local standard operating procedures.

Besides, a total of 126 field DNA samples (ear, eye, nasal, rectal's swabs, milk and environmental samples) provided by the OIE reference laboratory for CA of the Istituto Zooprofilattico Sperimentale della Sicilia and tested previously using other culture and PCR-based approaches, were examined.

Table 4.2.2.1 Panel of strains used in the optimization and determination of specificity of the LAMP assay

Species	**Strain**	**Origin**
M. agalactiae	NCTC 10123 (PG2)	Spain
M. agalactiae	112SR14	Wales
M. agalactiae	L9	Spain, Gran Canaria
M. agalactiae	Mon 14	Mongolia
M. agalactiae	35	Not known, German archive
M. agalactiae	2290-87	France
M. agalactiae	4941	Portugal
M. agalactiae	215-86	France
M. agalactiae	C7985	Portugal
M. agalactiae	C3487	France
M. agalactiae	97-95	Spain
M. agalactiae	1659-99	Spain
M. agalactiae	40F15	Sicily (IT)
M. agalactiae	281F03	Spain
M. agalactiae	44F15c	Sicily (IT)
M. agalactiae	45F13	Italy (North)
M. agalactiae	46F13	Italy (North)
M. agalactiae	249F03	Sardinia (IT)
M. agalactiae	227F03	Greece
M. putrefaciens	NCTC 10155 (KS1)	USA
M. mycoides subsp. capri	GM12	USA
M. mycoides subsp. capri	50F07	Pakistan
M. mycoides subsp. capri	23F09	Sicily (IT)
M. mycoides subsp. capricolum	10276	France
M. mycoides subsp. capricolum	15316	France
M. mycoides subsp. capricolum	4156	France
M. mycoides subsp. capricolum	California Kid	USA
M. bovis	NCTC 10131 (PG45)	USA
M. arginini	NCTC10129	
M. conjunctivae	NCTC10147	
M. ovipneumoniae	NCTC 10151	
Acholeoplasma laidlawii	NCTC 10116 (PG8)	

4.2.2.2.2 Genomic DNA extraction

DNA from each cloned isolate was extracted from 1 ml of culture using a Maxwell® DNA purification kit (A51010, Promega UK, Southampton, United Kingdom), on a Maxwell® 16 Bench-top DNA extraction system, following the kit manufacturer's instructions. 16S rRNA gene PCR-DGGE (McAuliffe, et al., 2005) was used to re-confirm the identity of the isolates. The DNA concentration and purity were measured with the Qubit Fluorimeter using the Qubit ds-DNA HS Assay Kit (Life Technologies, Carlsbad, CA).

4.2.2.2.3 Primer design

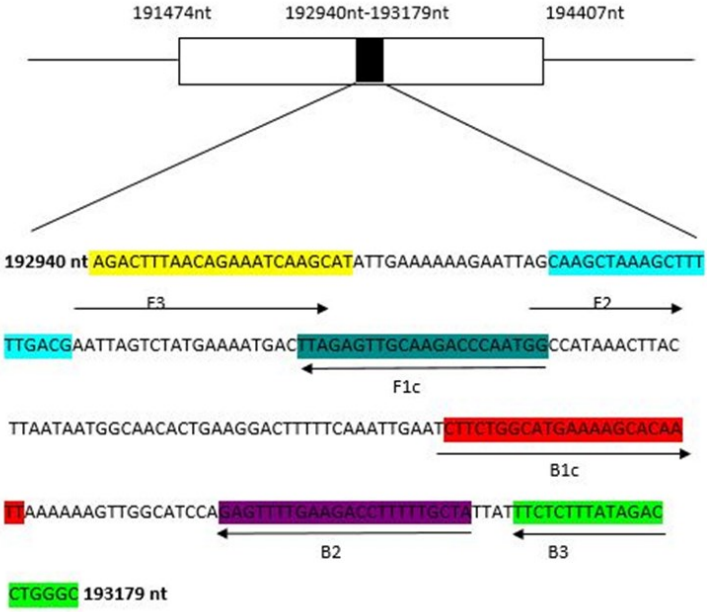
A set of four LAMP primers were designed targeting a region of the *dnaE* gene, which encodes the DNA polymerase III subunit alpha, essential for bacterial replication. This region had been selected based on the alignment of *dnaE* sequences from 68 different Ma strains, compared with the reference Ma PG2 (GenBank Acc. Num. CU179680.1) to identify the conserved regions using (MegaPro, DNASTAR software). LAMP primers were designed using the online primer design software PrimerExplorerV5 (http://primerexplorer.jp/lampv5e/index.html).

All primers sequences for LAMP are listed in Table 4.2.2.2 and the binding regions of target sequences are shown in Figure 4.2.2.1. The in-silico specificity of primers was assessed by BLASTn (http: // www. Ncbi.nlm.nih.gov/BLAST/).

Table 4.2.2.2 Primer sequences for LAMP.

Primer	Type	Sequence (5'- 3')
F3	Forward outer	AGACTTTAACAGAAATCAAGCAT
B3	Backward outer	GCCCAGGTCTATAAAGAGAA
FIP	Forward inner (F1C-F2)	CCATTGGGTCTTGCAACTCTAACAA GCTAAAGCTTTTTGACGA
BIP	Backward inner (B1c-B2)	CTTCTGGCATGAAAAGCACAATTTA GCAAAAAGGTCTTCAAAACTC

Figure 4.2.2.1 Location of loop-mediated isothermal amplification primers in the *dnaE* gene of Ma PG2 (Acc. Num. CU179680.1).



4.2.2.2.4 Optimisation of LAMP reaction conditions

The LAMP reaction conditions were optimised incubating at the temperature range from 65 to 69 °C for a different time (from 40 min to 60 min). The temperature of the melting curve analysis was 68.5 – 95 °C ramping at 0.02 °C per sec. The concentration of primers F3/B3 (0.2, 0.4, 0.6 µM) and FIP/BIP (0.8, 1.6, 2 µM) was also optimized.

4.2.2.2.5 Reaction conditions for LAMP

The optimized LAMP reaction was performed in a final volume of 25 µL, which contained: 15µl of Isothermal Master mix (ISO-001, OptiGene, Horsham, West Sussex, United Kingdom), 0.4 µM each of primer F3 and B3, 1.6 µM each of FIP and BIP and 4 µl of DNA template. The mixture was incubated at optimized conditions of 68.5 °C for 1 min (40 cycles) followed by an annealing step from 95 to 68.5 °C with 0.02-degree increments per second.

4.2.2.2.6 Detection of LAMP products

The LAMP amplicon was detected both the level of fluorescence in real-time (FAM channel) and by gel electrophoresis on a 2 % agarose gel in order to observe the typical ladder pattern of bands under ultraviolet light. The Ladder 100 kb (Promega) was incorporated as standard.

4.2.2.2.7 Specificity and sensitivity of LAMP assay

Nineteen Ma strains were used to evaluate the specificity of the LAMP assay, including 7 Ma strains known to be genetically divergent as determined by whole genome sequencing and MLST (data not shown). In addition, other *Mycoplasma* spp. (*Mycoplasma putrefaciens* (Mput), *M.*

mycoides subsp. capri (Mmc), *M. mycoides subsp. capricolum* (Mcc), *Mycoplasma bovis, M. ovipneuomoniae, M. arginini, M. conjunctivae*) and the ubiquitous *Acholeoplasma laidlawii* (Table 1) were analysed. We, also tested some DNA mixed samples comprising Ma with other *Mycoplasma* spp. (Mmc, Mcc, *M. ovipneuomoniae, M. arginini, M. conjunctivae, M. bovis*) in 1:4 ratio respectively in order to verify if the presence of exogenous DNA interfered with the detection of the low concentration of target DNA.
To evaluate the sensitivity of the developed real-time LAMP assay, genomic DNA of Ma reference strain (NCTC 10123) was tested in 10-fold serial dilutions from 4×10^3 pg/µL to 4 fg/µL.

4.2.2.3 Results

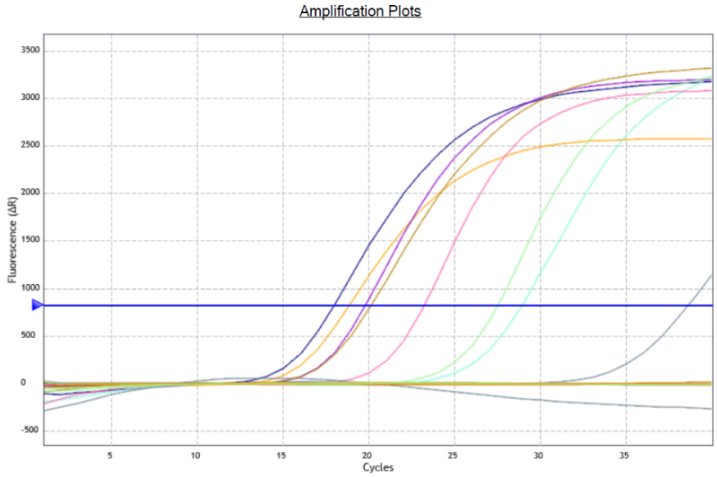
4.2.2.3.1 Optimization of LAMP reaction conditions
The optimal results for real-time LAMP assay were obtained at the primer concentration of 0.4 µM for each of the outer primers and 1.6 µM for each of the inner primers.
In fact, the reaction of amplification with lower primer concentrations resulted both slower and with lower signal intensity, while no significant differences were observed using higher primer concentration.
The reaction temperature of 68.5ºC was considered the optimal temperature, and the optimal duration time of the LAMP reaction was found to be 40 min. The temperature of the melting curve analysis was from 95 to 68.5 °C with a 0.02 decrement per second.

4.2.2.3.2 Detection of LAMP products
The LAMP products were detected using the real-time fluorimetry, with 6-carboxyfluorescein (FAM) as a

fluorescent dye, captured at 516 nm using the Aria MX (Agilent Technologies) (Figure 4.2.2.2a). Melting curve analysis showed no significant difference between *dnaE* LAMP products of the panel of Ma strains tested. The melting temperature of *dnaE* gene-specific amplicon was 82.94 °C (±0.34 °C) (Figure 4.2.2.2b), whereas those belonging to other ruminant mycoplasma species tested ranged from 70.60 °C to 80.40 °C.

Agarose gel electrophoresis using 2% pre-cast gels (Sigma Aldrich) was also used for confirming LAMP products looked visually as expected. The target sequences amplified by the LAMP assay showed the typical ladder pattern of bands due to the formation of stem-loop structures of amplified DNA of various stem lengths (Figure 4.2.2.3).

a)



b)

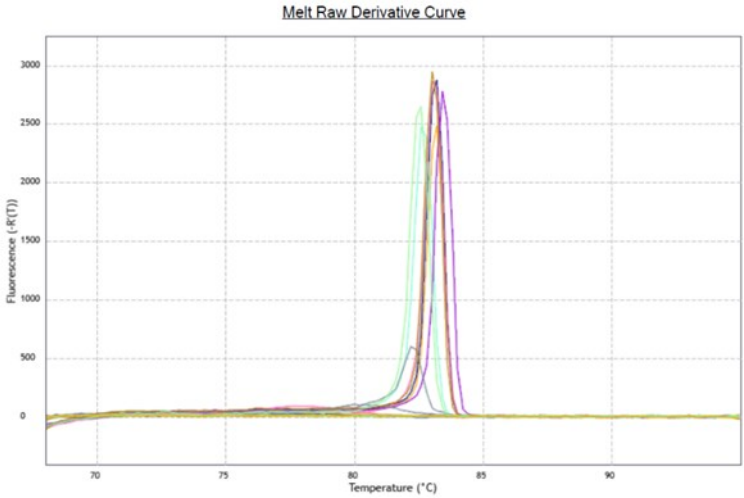


Figure. 4.2.2.2 a) Amplification plot b)) Melting curves of Ma *dnaE* Lamp

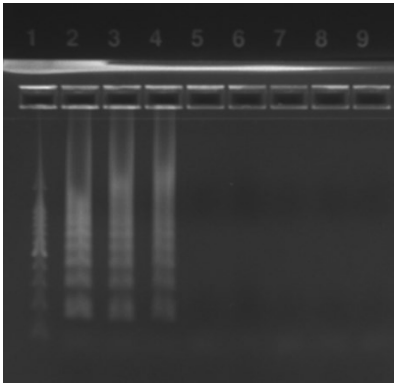


Figure. **4.2.2.3**- *dnaE*-LAMP assay electrophoretic pattern: 1) 100 bp ladder, 2) *Ma* NCTC, 3) *Ma* NCTC, 4) *Ma* 165-99, 5) *Mmc* GM12, 6) *Mcc* 4156, 7) *Mmc* 26F09, 8) *Mput*, 9) blank.

4.2.2.3.3 Specificity and sensitivity of LAMP assay

The LAMP assay was found to be specific for the Ma isolates, including strains determined to be genomically divergent. One genetically divergent strain (2290-87, of French origin) showed a high ct (cycle threshold) value (38.70), but the derived melt curve was as expected for *Ma* isolates. No fluorescence signal in real-time, corresponding with isothermal amplification and no typical ladder pattern of bands was observed for LAMP products from isolates of other *Mycoplasm*a species (Figure 4.2.2.2a – Figure 4.2.2.3). Furthermore, the assay was also able to detect the target DNA in all mixed DNA samples tested. This indicated high analytical specificity of established LAMP assay for the selective detection of Ma at least for pure isolates, even though the time of reaction was extended to 1 hr. The DNA concentration of the serial dilutions ranged from 4×10^3 pg/µL to 4 fg/µL. The lowest analytical sensitivity of the LAMP assay was recorded as 4 fg/µL.

4.2.2.3.4 Field samples

To evaluate the diagnostic efficiency of LAMP assay for use in clinical and environmental samples, a total of 126 DNAs prepared from a selection of culture enriched samples (ear, eye, nasal, rectal swabs, milk and environmental samples) were tested. All samples had previously been tested using culture and PCR-based approaches; 80 of the samples were also identified by PCR-DGGE. All samples previously determined to be Ma positive by other methods were confirmed by LAMP assay. However, in contrast, other samples that had been identified as borderline values by the real-time PCR (ct values between 33 and 36), were identified as positive by the LAMP assay. Investigation of the melt curves produced for such samples indicated a product with

the expected melting temperature, but apparently at low concentration. Furthermore, 20% of 80 samples investigated by PCR-DGGE and identified negative for Ma but containing other *Mycoplasma* or related mollicute species (*M. arginini, M. ovipneumoniae, Acholeplasma laidlawii*) showed acceptable LAMP ct values (ct values between 25 and 30). While, 9% of samples identified to be other *Mycoplasma* spp. showed high LAMP ct values (ct values between 31 and 36) and an ear sample from which Ma and *M. ovipneumoniae* were confirmed present by PCR-DGGE, had very borderline LAMP ct value.

4.2.2.4 Discussion

One of the difficulties in controlling the diffusion of CA infection is represented by the import of healthy carrier animals, which without exhibiting clinical manifestations of the disease represent a potential risk of spreading infection in those countries where the disease is absent (Ariza-Miguel, et al., 2012; Alves, et al., 2013; Prats-van der Ham, et al., 2017; Tardì, et al., 2019). Furthermore, the existence of asymptomatic carriers contributes to underestimating the incidence of CA.

Therefore, to strengthen the control and prevention of disease, rapid, low cost, easy to manipulation and reliable approaches for early diagnosis of disease and point of care tests for on-site suspected outbreaks screening for Ma, are required. Currently, there are several methods available for Ma identification and the detection of the immune response to the etiological agent, as the conventional culture, serological tests, biochemical identification, and PCR-based methods (OIE, 2019). To date, different Ma specific PCR assays methods have been developed and assessed (Tola, et al., 1997; Greco, et al., 2001; McAuliffe, et al., 2005;

Lorusso, et al., 2007; Oravcovà, et al., 2009; Becker, et al., 2012), but require expensive equipment and regular revalidation due to the high mutation rate in mycoplasma genomes (Jaÿ, et al., 2020).

An attractive alternative to traditional methods of Ma detection is LAMP, which is an isothermal amplification simple to set.

A previously developed LAMP for the Ma detection, targeting the *p40* gene (Rekha, et al., 2015) has been under commercial development and validation (Loria, et al., 2018, Tumino, et al., 2020) to provide results in the field in about 1 hour. In the present study, we previously tried the published primers, but they failed in repeatability and the detection of some of Ma strains tested. The alignment of Ma *p40* sequences (MegaPro; DNASTAR software) of Ma panel tested, confirmed sequence divergence affecting the chosen primer locations. Consequently, in this study, we developed a new LAMP test, for the detection of Ma, based on a conserved region of the *dnaE* gene. The LAMP reaction is carried out under isothermal conditions at 68.5 °C, and a positive result was observed within 40 min.

The amplification products were easily observed by fluorescence that gives a positive signal as an amplification curve, overcoming the disadvantages of naked eye methods (Rekha, et al., 2015) as adding fluorescent dye (SYBR Green) after the thermal incubation, that could cause aerosol pollution and false-positive results.

The positives results were confirmed by the melting peaks generated, that represent the specific amplified product. As further confirmation, the LAMP products were visualized by agarose gel electrophoresis, and they were consistently matching with a typical LAMP ladder-like pattern.

Our LAMP assay was able to specifically identify all Ma strains tested, including the genetically divergent strains, but it did not amplify other *Mycoplasma* and *Acholepasma* species including *M. bovis*, which is phylogenetically related to Ma. The LAMP test also detected the Ma DNA in mixed DNA samples, showing that the presence of non-target DNA in the sample does not interfere with the efficiency of DNA target amplification.

In the literature, the detection limits for LAMP assays are expressed in different units [number of colony-forming units (CFU), number of cells or quantity of DNA], making a comparison of the sensitivities of assays very hard.

In the present study, the analytical sensitivity of the LAMP assay for detecting Ma DNA amounted to up to 4 fg/ µl, showing a similar or higher sensitivity compared to the LAMP assays published previously for Ma and other Mycoplasma species and Bacteria of veterinary interest (Ohtsuki, et al., 2008; He, et al., 2014; Rekha, et al., 2015; Sheet et al., 2016; Ashraf et al., 2018; Ehtisham-Ul-Haque, et al., 2017).We also evaluated the LAMP assay efficiency for the detection of Ma in clinical and environmental samples, previously tested by real-time PCR and PCR-DGGE.

The LAMP assay confirmed the positive samples for Ma identified by both the methods, real-time PCR and DGGE. However, 20% of the samples identified to be other *Mycoplasmas* spp. by PCR-DGGE and borderline in real-time, resulted positive in LAMP. These finding could be linked to the fact that they are mixed DNA samples of Ma and other Bacteria or *Mycoplasma* spp., which may have interfered with standard amplification in PCR-DGGE, while the low Ma DNA concentration has been weakly detected by real-time PCR (high ct value) but it has been clearly observed and confirmed by the melting peak of the LAMP assay.

Nevertheless, 9% of samples (nasal swab, ear swabs, milker hands swab, faeces, rectal swabs and wool) identified to be other *Mycoplasma* spp. showed very borderline LAMP ct values (high ct values between 31 and 36).
These findings emphasized the difficult to interpret of certainly doubtful samples with high ct values, also in relation to the PCR-DGGE identification. Different reasons could be related to these discrepancies: the efficiency of the extraction method to remove amplification inhibitors present in the sample (Tatay-Dualde et al., 2015), the type of biological sample, and the related difficulties in processing the sample, e.g. faeces and rectal swabs generally present large amounts of contamination and require the sample filtration (OIE, 2019). These aspects could have some implications where there are low numbers of the Ma cells to be recovered, and consequently in the low target DNA concentration extracted. On the other hand, the use of several media containing selective components inhibits the contamination of mycoplasma enrichment by the sample flora, but the DNA of other microorganisms persist and could interfere with the detection of the Ma target sequence.

4.2.2.5 Conclusion

In conclusion, even if the developed LAMP method gave promising results in terms of detection of genetically divergent circulating strains and seemed to have a greater sensibility when compared to real-time PCR and PCR-DGGE, any questionable result (borderline ct value) related to the field-samples represents weakness.
Therefore, further analysis is required aimed at validating the test with milk samples which today represent the primary biological sample type for surveillance programs.

4.2.3 Detection of *Mycoplasma agalactiae* by in situ hybridisation and characterisation of inflammatory infiltrates by immunohistochemistry in sheep udders

The following work has been presented as scientific contribution at the Congress of the veterinary pathology and veterinary clinical pathology 2019:
R. Puleio, S. Tumino, L. Condorelli, A. Parco, M. Tolone, A. Tamburello and G.R. Loria. Histological patterns related to *Mycoplasma agalactiae* mastitis. *J. Comp. Path*. 2020, Vol. 174, 157-198.

4.2.3.1 Introduction

In situ hybridization (ISH) is a technique that allows for precise localisation of a specific segment of nucleic acid within a histologic section. The underlying basis of ISH is that nucleic acids if preserved adequately within a histologic specimen, can be detected through the application of a complementary strand of nucleic acid to which a reporter molecule is attached. Visualisation of the reporter molecule allows localising DNA or RNA sequences in tissue samples and environmental samples (Levsky, J, & Singer, 2003).
This study describes an *in situ* hybridisation (ISH) method to detect *Mycoplasma agalactiae* (Ma) in infected tissue and characterisation of the inflammatory infiltrates in sheep mastitis. Single-molecule visualisation in individual cells is achieved through use of a novel probe design strategy and a hybridization-based signal amplification system to simultaneously amplify signals and suppress background (Wang, et al., 2012).

4.2.3.2 Material and methods

Laboratory investigations, which are culture and real-time PCR according to OIE guidelines, have been previously carried out on milk and tissues samples from slaughtered sheep in order to confirm the disease in the target organs.
Therefore, Udders and supra-mammary lymph nodes were collected for histological and immunohistochemical (IHC) examination.
RNA *in situ* hybridisation was performed using the RNAscope kit (Advanced Cell Diagnostics Inc., Hayward, CA, USA) according to the manufacturer's instructions. The RNAscope probe used was designed to detect the mRNA expression of the *p40* gene (GenBank Accession number: AJ344229) encoding an adhesin that displays a strong role in the virulence of Ma.
For further characterisation of the inflammatory infiltrates, the expression of Ma antigen, MHCII, CD3 and CD79 lymphocytes was investigated by IHC.

4.2.3.3 Results

Sample tissues taken from sheep infected by Ma were identified by culture and real-time PCR methods. Histological findings showed an interstitial monocytic infiltrate in the acute phase, while in an advanced stage of the disease a persistence of monocytic infiltration with marked atrophy of secretory tissue associated with monocyte infiltration and interstitial fibrosis was also observed.
ISH for Ma was positive in the lumen of the acini and ducts (Figure 4.2.3.1), while interstitial monocytic cells were positive for MHC-II and CD3 antigen.
CD3-positive cells, in particular, were located in the interstitial tissue around alveoli and ducts, while MHC-II was more reactive in the central areas of interstitial infiltrates.

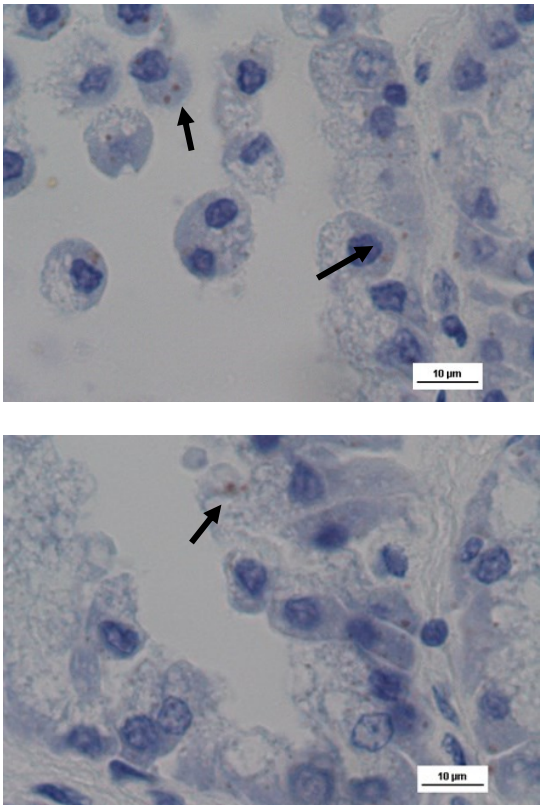


Figure 4.2.3.1 RNAscope detection of RNA FFPE udder section of a sheep infected by Ma. Chromogenic staining (DAB) (brown) indicates in situ hybridisation with a RNAscope probes B-*M.agalactiae* p-40. Scale Bar: 10µm

4.2.3.4 Conclusions

ISH for the detection of Ma rRNA in Formalin-Fixed Paraffin-Embedded (FFPE) tissues showed high efficacy and specificity in order to detect the pathogen in target cells. IHC showed rare lymphocytic infiltrates in subclinical mastitis, indicating a chronic, persistent infection, which could be reactivated by some immunodeficiency conditions.

4.2.4 PCR-free detection of Mycoplasma agalactiae DNA by Au decorated NiO nanowall disposable electrodes

This report is part of an article accepted for publication in ACS Applied Materials & Interfaces.
Urso M., Tumino S., Bruno E., Bordonaro S., Marletta D., Loria G.R., Shacham-Diamand Y., Priolo F., Mirabella S. (2020). Ultrasensitive electrochemical impedance-based detection of Mycoplasma agalactiae DNA by low-cost and disposable Au decorated NiO nanowall electrodes. ACS Applied Materials. Interfaces.12, 44, 50143–50151.

4.2.4.1 Introduction

According to the International Union of Pure and Applied Chemistry, a biosensor is a compact analytical device incorporating a bioreceptor associated with a physicochemical transducer that detects specific biochemical reactions by electrical, thermal or optical signals.
The bioreceptor interacts with the target analyte, while transducer converts this interaction into an electronic signal (Wu, et al., 2019). As bioreceptor, different components of biological origin, such as enzymes, nucleic acids, antibodies or whole cells, are used (Chambers, Arulanandam, Matta, Weis, & Valdes, 2008).
Sensors based on nucleic acids are gaining increasing attention, mainly due to their comparable sensitivity and reproducibility to PCR technologies (Petralia, et al., 2017).
DNA sensors are based on the detection of variations in the physical-chemical properties of a sensing element upon hybridization between an immobilized ssDNA probe and the complementary sequence. This approach ensures high selectivity because of the specificity of DNA base pairing (Zhang, et al., 2006). Various strategies have been developed

to detect DNA hybridization. As a result, a wide variety of DNA sensors have been reported, including optical, piezoelectric and electrochemical transducers (Arugula, et al.,2014).

Among the different devices developed so far, electrochemical sensors are very advantageous due to their robustness, miniaturization, sensitivity and fast response along with low cost/energy/mass characteristics and great potential as devices intended for commercialization (Zhang, et al., 2007; Wei, Lillehoj, & Ho, 2010). The working electrode is a core component of any electrochemical sensor, and for DNA sensors, is where probe ssDNA is immobilized via physical absorption, self-assembly or covalent bonds (as in the case of thiolated probe ssDNA and Au electrodes). Upon hybridization of probe ssDNA with the complementary sequence, an electrochemical signal is generated whose amplitude defines the sensitivity of the sensor (Zhang, et al., 2006). Various electrochemical techniques have been used to enhance the hybridization signal, including voltametric, amperometric, coulometric and impedimetric (Daniels, et al., 2007). Among them, electrochemical impedance spectroscopy (EIS) is considered a powerful technique to sensitively detect electrochemical signal variations caused by DNA hybridization (Park, et al., 2009; Le, et al., 2015; Bahadır, et al., 2016).

Despite PCR is nowadays considered the gold standard method for detection of the viral or bacterial genome in clinical diagnosis, this methodology is still quite laborious requiring complex, expensive instrumentations, qualified personnel and specialized laboratories.

These aspects represent a severe limitation for large scale screening and early diagnosis, especially in the developing

countries due to poor clinical laboratory infrastructures and cost constraints.
In this context, one of the most challenging research goals is represented by the development of in-field diagnosis devices based on biosensors. In veterinary disease, the rapid and specific diagnosis of infection can significantly reduce the occurrence of outbreaks and the consequent economic losses. In the Mediterranean countries, CA represents one of the main causes of infection in small ruminants. In rural marginal areas and especially in many developing countries where CA is present, outbreaks may occur hundreds of miles from the nearest diagnostic laboratory; therefore, portable, miniaturized devices that can be used directly in the field by veterinaries are required to allow a massive and rapid diagnostic screening.
In the literature, previous authors developed several DNA-based biosensors for pathogens microorganism related to livestock and foodborne (Vidic, et al., 2017; Wu et al., 2019). In the present work, conducted in collaboration with the researchers of the department of physics of the University of Catania (UNICT), we performed on the successful application of a novel nanostructured electrode for the PCR-free electrochemical detection of Ma DNA, which seems promising for its application in the direct on-field diagnostic analysis.
The nanostructure electrode has been developed and fabricated by the department of physics of UNICT, while Di3A department of UNICT and OIE reference laboratory for Contagious agalactia of Sicily (Istituto Zooprofilattico Sperimentale della Sicilia) have supplied the Ma DNA extracted from infected sheep milk and performed real-time PCR analysis.

4.2.4.2 Material and methods

4.2.4.2.1 DNA extraction from milk samples and real-time PCR

Positive Ma milk sample was collected from a sheep with clear symptoms of CA, affected by interstitial mastitis, alteration in the consistency of the milk and clear decline of milk production. The presence of Ma in the milk sample was confirmed by microbiological identification and real-time PCR at the OIE Reference Laboratory for Contagious agalactia at the Istituto Zooprofilattico Sperimentale della Sicilia, according to the World Organization for Animal Health guidelines (OIE Terrestrial Manual 2018 - Chapter 3.7.3). As a negative control, a milk sample collected from a sheep previously checked to be Ma-free was included.

The genomic DNA was extracted from 1 ml of milk of infected and healthy sheep, using a Chelex-based InstaGene Matrix (Bio-Rad laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The cell lysis phase occurs by boiling in the presence of InstaGene matrix, which absorbs the cell lysis products. Briefly, 1 ml of the milk sample was centrifuged at 12000 rpm for 3 min, the supernatant was removed, and after addition of InstaGene Matrix, the suspension was incubated at 56°C for 25-30 minutes, followed by heating at 100 °C for 12 min. After final centrifugation at 13000 rpm for 3 min, the supernatant containing the extracted DNA was collected and stored at 20°C. A TaqMan Real-time PCR, targeting *p40* gene, was performed according to Oravcovà et al., (2009). In order to measure the amount of Ma DNA in 2 µl of DNA, serial tenfold dilutions of the recombinant plasmid containing the fragment of DNA target gene (109 bp of *p40* gene), in a concentration ranging from 2 to 200000 copies per reaction were tested (each concentration was tested in duplicate).

Prior to hybridisation, DNA obtained from infected and healthy sheep were heated up to 90-95°C to denature the DNA. Then, hybridisation with target DNA from milk samples was obtained by immersion in a 2.5 ml solution at 40°C, made by diluting 20 µl of the milk sample in 2480 µl of 0.1 M PBS (pH 7).

4.2.4.2.2 Development of Ma electrochemical sensor

The electrode was composed of Au decorated NiO nanowalls grown by low-cost methods, on which a ssDNA probe specific for Ma was immobilised. The ssDNA probe sequence (5'-GAT GAT AAG AAC GAA AAT TCA CAA A-3'), based on *p40* target gene, was chosen according to Oravcovà et al., (2009) due to its high specificity for Ma detection and consisted of the 28bp sequence modified with a thiol on 5' end and a cyanine (Cy3) dye on 3' end (Hylabs, 8990 Da molecular weight).

Thiolated DNA was used to obtain a robust binding on Au nanoparticles surface, while modification with Cy3 dye on 3'end, observed by a confocal microscope with a ~510 nm excitation wavelength, was used to verify the successful ssDNA probe immobilization on Au decorated NiO nanowalls.

The electrode was tested with the synthetic ssDNA complementary (target DNA) at different concentrations (0.2, 1 and 1.5 µM), after a prehybridization step with a 30 pb non-specific ssDNA sequence to improve electrode stability saturating the further adsorption sites, in order to prevent false positives during sensing.

Then, the hybridization between the probe and target DNA was detected by monitoring the electrochemical impedance.

All tests were performed on two independently prepared electrodes, and EIS measurements were repeated three times

for each electrode in order to test a preliminary electrode reproducibility.

4.2.4.3 Results and discussion

Figure 4.2.4.1 shows the schematic diagram reporting electrode fabrication of the novel DNA sensor based on Au decorated NiO nanowalls. The electrode sensor consisted of NiO nanowalls, synthesized by aqueous methods and thermal annealing on conductive substrates and Au decoration, by electroless deposition.

Ma thiolated ssDNA probe was immobilized onto Au decorated NiO nanowalls and a prehybridization step with non-specific DNA was performed to saturate further adsorption sites.

The hybridization with target DNA was evaluated as the variation of electrode impedance by electrochemical impedance spectroscopy (EIS) measurements.

The electrode successfully reacted to the different concentration of synthetic target ssDNA (from 0.2 to 1.5 μM) with high reproducibility and stability.

Figure 4.2.4.2 shows the typical frequency-dependent impedance Z (|Z| and Phase(Z) vs frequency) recorded before and after hybridization.

At 0.1 Hz the largest differences between the samples are observed and at increasing concentrations of target DNA, |Z| increases while Phase(Z) does not change significantly, especially in the low-frequency region.

It is worth noting that |Z| and Phase(Z) measured three times at 1 Hz for the two independently prepared electrodes after hybridization with 1 μM, as well as for other target DNA concentrations, are similar, confirming the high reproducibility and stability of the electrode and DNA detection.

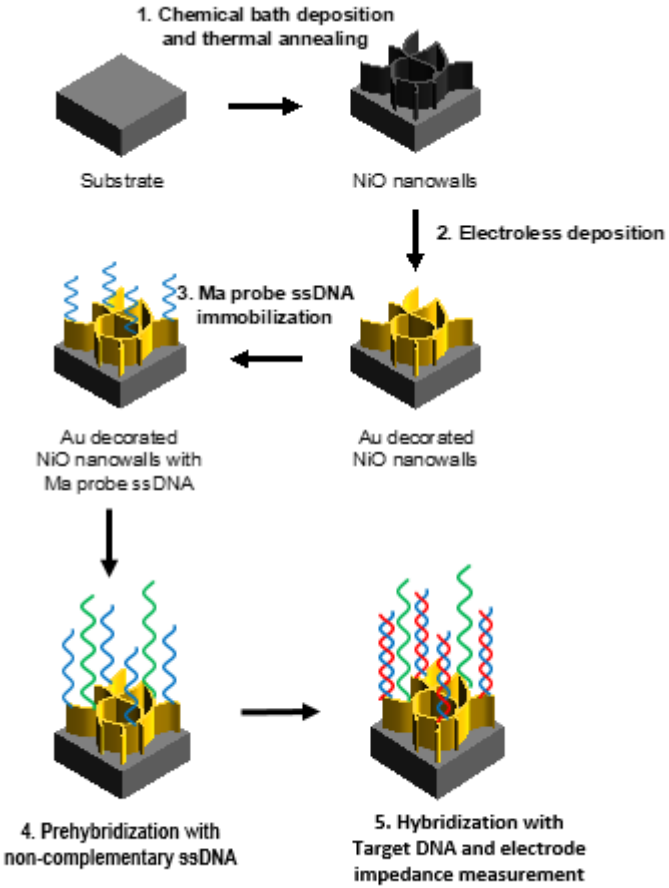


Figure 4.2.4.1 Schematic diagram of the DNA sensor based on Au decorated NiO nanowalls.

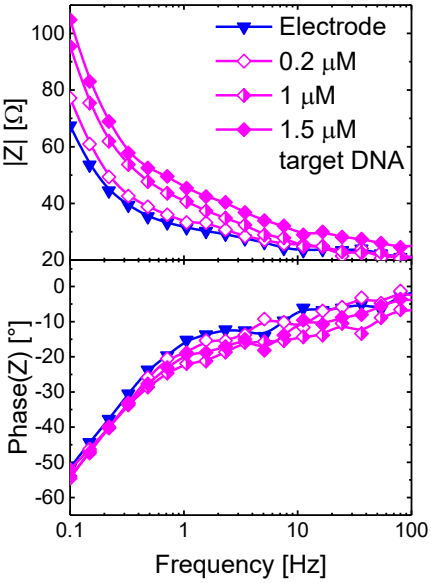


Figure 4.2.4.2 Bode plots of the electrode after fabrication (blue down triangles) and hybridization with different concentrations of synthetic target DNA (0.2 μM magenta open diamonds, 1 μM magenta half diamonds and 1.5 μM magenta diamonds) showing |Z| and Phase(Z) vs frequency, as obtained by EIS measurements.

Therefore, to validate the on-field applicability of the proposed electrode for Ma DNA detection, the DNA extracted from the milk of healthy and infected sheep have been tested.

Real-time PCR was used to quantify the amount of Ma DNA in the milk of the infected sheep. Serial 10-fold dilutions of plasmid DNA (ranging from 2 to 200000 copies per reaction) and corresponding cycle threshold values were used to plot a standard curve.

The linearity of calibration curves represented by the regression coefficient (R^2) showed values close to 1 (0.998),

indicating that the assay was highly linear (Figure 4.2.4.3 a-b).
Based on this calibration, the limit of detection (LOD) of real-time PCR resulted to 20 copies of target gene per reaction (Table 4.2.4.1) and Ma DNA concentration detected by the electrode has been about 53 ± 2 copy number µl-1 (Figure x.x c), almost approaching the LOD of our real-time PCR analysis, which is similar to those reported in other studies (Lorusso et al., 2007; Oravcovà et al., 2009; Becker et al., 2012).

Table 4.2.4.1 Real-time detection of Ma in the milk sample

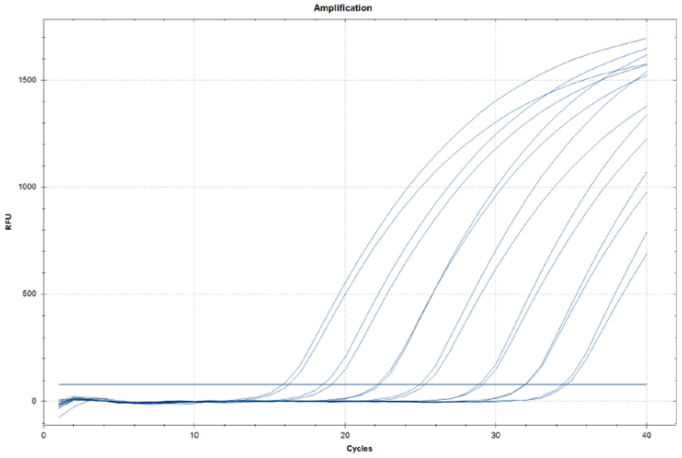
Ma DNA [a]	**Copy number/reaction**[b]	**Ct Mean ± SD** [c]
Stock	200000	22,83 ± 0.37
10^{-1}	20000	24.81 ± 0.70
10^{-2}	2000	27.04 ± 1.39
10^{-3}	200	28.86 ± 0.73
10^{-4}	20	32.27± 0.60
10^{-5}	2	NA

[a] Tenfold serial dilution of the Ma DNA extracted from positive milk.
[b] Copy number of DNA target gene.
[c] Ct mean of Ma DNA tested in duplicate; SD: standard deviation; NA: not applicable

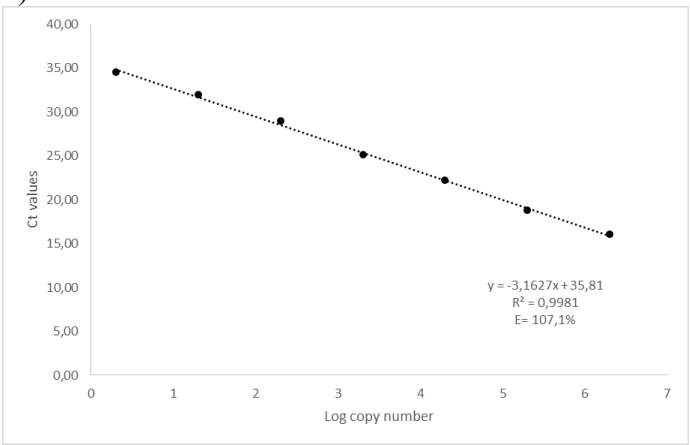
The test with DNA extracted from healthy sheep milk gave a null response (green column in Figure 4.2.4.3(c)) as it does not contain Ma DNA, but rather exogenous DNA.
The test with DNA extracted from infected sheep milk was successful (red column in Figure 4.2.4.3(c)) it gave a positive response (~40%), proving that DNA hybridization is very

specific and effective despite the natural Ma DNA is much longer than the synthetic one.

a)



b)



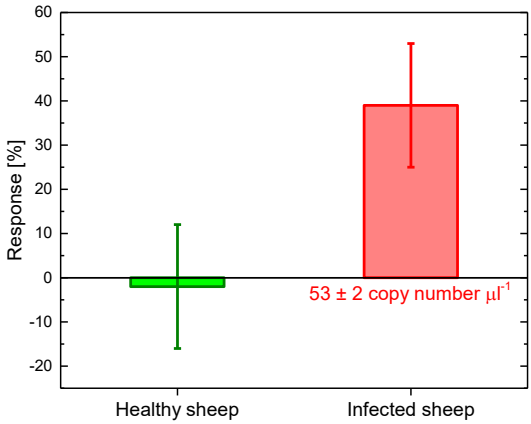


Figure 4.2.4.3. Amplification plot (a) and linear regression curve (b) of the Ma real-time PCR assay generated by using tenfold serial dilutions of standard Ma DNA and the corresponding cycle threshold (ct) values (black squares). Each dilution was tested in duplicate and the error bars are of the same size as the symbols. The coefficient of determination (R^2), the slope value (-3,162) of the regression curve and the PCR Efficiency (E=107%) were calculated. (c) Electrode response to DNA extracted from healthy (green column) and infected sheep milk (red column).

4.2.4.4 Conclusions

In this work, the response to DNA extracted from infected sheep milk in concentration as low as 53 ± 2 copy number µl-1 and the absence of response to DNA from healthy sheep milk demonstrate the great potential of the proposed electrode for low-cost, rapid, on-field and PCR-free Ma DNA detection.

However, to be recommended by the World Animal Health Organization as a Ma detection method, further inter-laboratories tests are required in order to be validated the specificity sensitivity and reproducibility of the device.

5. EXPERIMENTAL PART: MAEDI VISNA

5.1 Third contribution: Genetic characterization at *TMEM154 E35K* gene in Sicilian sheep breeds and estimation of the RR on the risk of MVV infection: preliminary results

The following work was conducted in collaboration with AGRIS Sardegna and Department of Agricultural, Food and Forestry Sciences (University of Palermo).

5.1.1 Introduction

In Sicily, dairy production coming from sheep livestock represents an important economic income, particularly for those disadvantaged rural areas, in which alternative economic activities are limited by the harsh environment. After Sardinia, Sicily is the second Italian region for the number of sheep reared, counting about 758.000 animals (BDN-dell'Anagrafe Zootecnica, https://www.vetinfo.it/j6_statistiche). Nowadays several sheep flocks of different breeds graze in the island. Although sheep resulting often from crossbreeding, four native sheep breeds are still reared in Sicily: Barbaresca Siciliana (BAR), Comisana (COM), Pinzirita (PIN), and Valle del Belice (VDB) (Figure 5.1.1.1).

The Barbaresca is an ancient Sicilian fat-tail sheep originated from substitution crossings between Tunisian Barbary rams from North Africa and the local Pinzirita breed (Bigi and Zanon 2008).

The Pinzirita originates from the Asian or Syrian sheep of the Sanson, precisely from the Zackel strain, and is considered a native breed of Sicily.
The Comisana breed derives from sheep breeds of the Southern Mediterranean area and its name came from "Comiso", a town developed from livestock productions located in Ragusa Province.
The Valle del Belice breed originates in ’80 from hybridization among the native Pinzirita, Comisana breeds and some Sarda rams. The selective interbreeding has given rise to a new biotype of sheep, which combines the attitudinal and morphological characteristics typical of the three original breeds.
They produce milk mainly addressed to the production of traditional raw milk cheeses and ricotta, often approved, and protected by "Slow Food Presidium" (www.slowfoodfoundation.com) and PDO (protected designed of origin). (Since) their ability to adapt and to produce in marginal areas they provide an invaluable animal resource for present and future needs.
Unfortunately, according to the Domestic Animals Diversity-Information System (DAD-IS) FAO database, nowadays much of these precious sheep breeds are recognized as endangered or in critical risk status, excepted for the Valle del Belice breed (Figure 5.1.1.2).

a)

b)

c)

d)

Figure 5.1.1.1 Sicilian sheep breeds: a)Barbaresca – b)Pinzirita – c) Comisana –d) Valle del belice

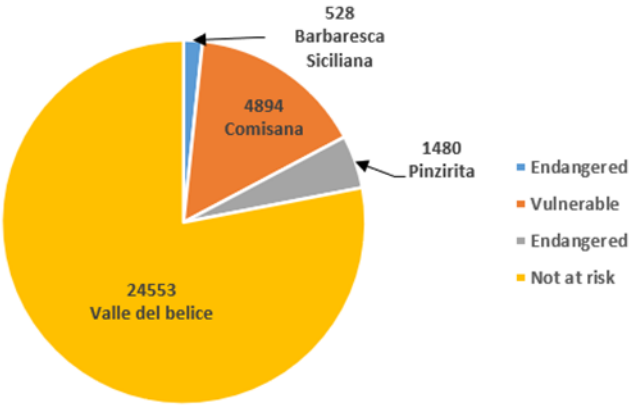


Figure 5.1.1.2 Populations data of Sicilian sheep breeds and their local rick status. Note: Data extracted from DAD-IS, 2019

These breeds are worth of safeguarding because of their economic role in rural communities but also because they are generally resistant to the most common infectious diseases.
Maedi Visna (MV) is a multisystemic disease of small ruminant characterized by persistent and slow lentiviral infection, consisting in a chronic interstitial infiltration of mononuclear inflammatory cells in several organs, such and lung, mammary gland, joints and central nervous system (Straub, 2004; Minguijón, et al., 2015; Gomez-Lucia, et al., 2018; OIE, 2019; Kalogianni, et al., 2020).
The etiological agents of MV is *Maedi visna virus*, a *Lentiviruses* belonging to the retroviridae family, that is highly prevalent in most sheep-producing countries, including the USA, Canada, most European countries, India, China, Japan, and multiple countries across both South America and Africa (White & Knowles, 2013; Oguma, et al., 2014; Kalogianni, et al., 2020).

Like other lentiviral diseases, there is no treatment and vaccines against MV are ineffective.
A number of candidate loci have been previously reported to be associated with small ruminant lentiviruses (SRLV) infection with varying levels of statistical support (Herrmann-Hoesing et al., 2008; White et al., 2009; Larruskain et al., 2010; White et al., 2013).
However, a genome-wide association study in North American sheep identified a single, major gene (transmembrane protein 154 gene, *TMEM154*) with missense variants, in the exons 1 and 2, significantly associated with host susceptibility to MV infection (Heaton et al., 2012).
The biological function of TMEM154 protein remains unknown in sheep and in other mammalian species as well.
The ovine *TMEM154* gene consists of seven *TMEM154* exons encoding for a precursor protein of 191 amino acids that is cleaved to a mature protein of 161 residues.
Searching for genetic variation in *TMEM154* gene, Heaton et al. (2012), described three haplotypes at protein levels, named haplotypes 1–3; phylogenetic analysis showed haplotype 3 was ancestral in ruminants.
Specifically, polypeptide variants that contain glutamic acid (E) at position 35 and asparagine (N) at position 70 (haplotype 3), or E35 and isoleucine (I) at position 70 (haplotype 2), were associated with increased susceptibility to the MVV, whereas protein variant that contains lysine (K) at position 35 and asparagine (N) at position 70 (haplotype 1), were associated with reduced susceptibility.
Because there is no information about genetic susceptibility to *Maedi visna virus* (MVV) in the Sicilian sheep breeds, the present study was aimed to:

A. genetic characterizing at *TMEM154 E35K* gene in three out of the four autochthonous sheep breeds, in

order to provide preliminary data about the frequencies of the protective allele (K);

B. investigating the association of *TMEM154 E35K* allele/genotype with MVV serological status for the purposes estimating the effect of the risk allele (E) on the risk of MVV infection.

A. Genetic characterization at TMEM154 E35K

5.1.2a Material and methods

5.1.2.1a Sampling

A total of 235 animals (149 rams, 86 ewes) from 29 flocks, across 7 provinces (Agrigento, Enna, Caltanissetta, Catania, Palermo, Trapani and Ragusa) of Sicily (South-Italy) were collected between 2019 and 2020 (Table A.1). The sample included the following Sicilian sheep breeds: Valle del Belice (VDB), Comisana (COM) and Barbaresca (BAR).

As this set of samples was used to estimate TMEM154 E/K allele frequencies within breeds, the sampling was carried out including all males per flock and from 2 to 25 sheep per flock according to the herd's size, in order to minimize closely related animals.

Peripheral blood samples were collected by veterinarians, using vacuum vials with EDTA anticoagulant, and stored frozen until further analysis.

Table A.1 Samples collected from Sicilian ovine breeding flocks.

Breed	**Sample (*n*)**			**Flocks (n)**
		Rams	Ewes	
Valle del Belice	118	104	14	20
Comisana	91	31	60	7
Barbaresca	26	14	12	3

5.1.2.2a DNA Extraction

Genomic DNA was obtained from peripheral blood leukocytes by using the Quick-DNA™ Miniprep Plus Kit (Zymo, Irvine, CA, USA) following the protocol provided by the manufacturer.

DNA quantity and quality were measured by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

5.1.2.3a TMEM154 Genotyping

The TaqMan allelic discrimination assay was used for genotyping a single nucleotide polymorphism (SNP) in the coding region of *TMEM154* gene (*Ovis aries* chromosome 17, rs408593969 g.4857244 G>A), resulting in the substitution of the ancestral glutamic acid (E) with lysine (K) at position 35 of the mature protein. For this purpose, primers and dual-labelled allele-specific oligonucleotide probes were designed. The SNP of interest is localized approximately in the middle of the sequence. Primers and TaqMan probes sequences have been kindly provided from AGRIS (Sardinia). Amplicon sizes, primer and probe sequences, are listed in Table A.2.

The TaqMan allelic discrimination assay was performed using Bio-rad CFX96 real-time PCR detection system in a total volume of 20µl containing 1x SsoAdvancedTM Probes® Supermix (Bio-Rad Laboratories), 500 nM of primers, 250 nM of each probe, and ~150 ng of DNA. The real-time PCR program consisted of an DNA polymerase activation and DNA denaturation step (3 min at 95°C), followed by 40 amplification cycles, of denaturation for 20 s at 95°C and annealing-elongation for 30 s at 60°C.

The fluorescent signals (Fam and Hex) were detected in real-time during the annealing-elongation step.
Each assay included 9 samples of known genotypes as positive controls (AA, AG, GG) representing all combinations of the polymorphism and 2 no-template control. Positive controls were sequenced to confirm the presence of the polymorphisms described. Genotypes of unknown samples were considered reliable only if the results for all reference samples were correct.

5.1.2.4a SNPs screening in TMEM154 by sequencing
A preliminary PCR amplification and sequencing, of a region including exons 2 and 3 of TMEM154 gene according to Heaton et al., (2012) were carried out in a total of 70 samples in order to check the presence of potential new SNPs in the Sicilian breeds that could have interfered which the TaqMan allelic discrimination. PCR products (771 bp) were sequenced by Applied Biosystems 3500 genetic analyzer using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., USA) at Department of Agricultural, Food and Forestry Sciences (University of Palermo).

5.1.2.5a Statistical analysis
Genotypic and allelic frequencies were calculated, and the Chi-squared test was used to test whether the population deviated from Hardy–Weinberg equilibrium.

Table A.2 Primers and probes sequence used for TMEM154 genotyping and sequencing

Primers – probes sequence (5'-3')	Amplified region	Amplicon size(bp)	Purpose
5'-GTCTCCATGACAAGTCTCAATTTTGT-3' (forward) 5'-GCTTAGGGCCTCTGACTCTTCA-3' (reverse) Probe allele K 5'-6FAM-AGGACACA**A**AACTGT- BHQ1-3' Probe allele E 5'-HEX-AGGACACA**G**AACTGT- BHQ1-3'	Exon 2	117bp	determination of nucleotide substitution g.4857244 G>A, resulting in amino acid substitution E35K,
5'-GCTCCATTTATGTTCAATCA-3' (forward) 5'-GAGATGGAAGCTGTGTGTTTC-3' (reverse)	Exon 2-3	771 bp	amplification and sequencing for verification of genotyping results

Bold letters: nucleotide positions leading to allele specific binding of probe.

5.1.3a Results

All sampled animals were successfully genotyped for the amino acid substitution at position 35 of TMEM154 using the TaqMan allelic discrimination high-throughput method.

For every probe, relative fluorescence unit values were measured every cycle and after background normalization plotted against the cycle number. The obtained amplification plot is shown in Figure A.1.

The genotyping results were confirmed by sequencing analysis (Figure A.2) verifying no new SNPs were found in target E35K *TMEM154* region.

Furthermore, in all samples sequenced, the locus N70I resulted monomorphic for asparagine (N). These data show that the haplotype 3 and 1 are predominant in sample tested, representative of the Sicilian sheep breeds.

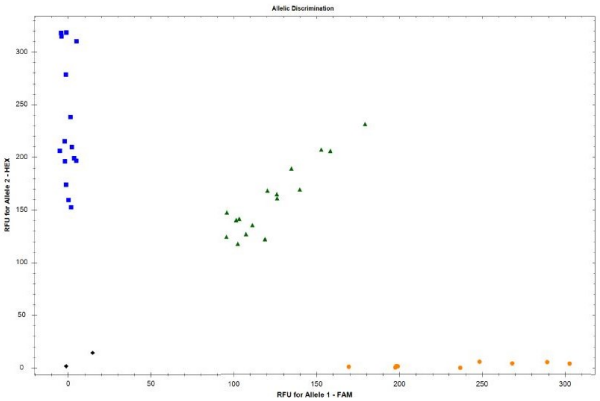


Figure A.1 Allelic Discrimination plot of *TMEM154* E35K genotyping showing a clear separation between the signals derived from allele1 (allele K) or allele2 (allele E). The homozygous KK is shown in orange and the homozygous EE is shown in blue, while the green triangles represent the heterozygous carriers (EK). In black the NTC (no template control).

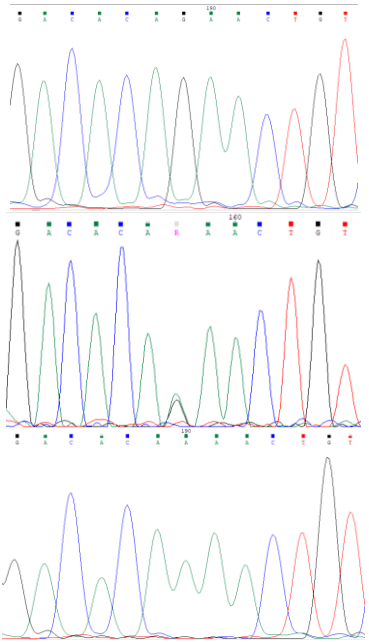


Figure A.2 Sequencing for verification of genotyping results. From top to bottom: homozygous GG (EE), heterozygous (AG) (KE) and homozygous AA (KK).

In the whole sample of 235 animals from 29 flocks, the putative protective allele (K) at amino acid position 35 of *TMEM154* was observed at frequencies of 34 % (Table A.3). Deviation from the Hardy–Weinberg equilibrium was only observed considering all breeds set (*p-value* 0.006), whereas no deviation was found for genotype frequencies in VDB and BARB breed subsets and COM breed subset narrowly missed the significance threshold.
In the breed subset the putative protective K allele was less frequent than the putative risk allele E especially in VDB (21%), whereas COM (46%) and BAR (50%) showed a more balanced distribution of the two alleles Table A.3).

Table A.3 *TMEM154 E/K* allele and genotype frequencies in Sicilian ovine breeds tested

Breed	**N**	***TMEM154 E35K* genotype frequency**			***TMEM154 E35K* allele frequency**		**Chi-squared test p-value**
		EE	**EK**	**KK**	**E**	**K**	
Valle del Belice	**118**	76 (0.64)	35 (0.30)	7 (0.06)	0.79	0.21	0.284
Comisana	**91**	31 (0.34)	37 (0.41)	23 (0.25)	0.54	0.46	0.085
Barbaresca	**26**	6 (0.23)	14 (0.54)	6 (0.23)	0.50	0.50	0.694
Total	**235**	113 (0.48)	86 (0.37)	36 (0.15)	0.66	0.34	0.006

5.1.4a Discussion

This study supplies novel information on the frequencies of the putative protective *TMEM154 K35* allele and relative genotype for selection against MV susceptibility in autochthonous Sicilian sheep breeds.

Variants of ovine transmembrane protein 154 gene (*TMEM154*) have shown to play a central role in susceptibility to MVV infection (Heaton et al., 2012). Haplotypes 1, 2, and 3 are most common worldwide (Heaton et al., 2013) and sheep with 1 or 2 copies of either haplotype 2 or 3, both of which encoding a glutamate amino acid residue at position 35 (E35), have a greater risk of MVV infection than sheep with haplotype (K35) (Heaton et al., 2012).

In VDB breed the allele E resulted predominant in agreement with the results of recent studies conducted in, German, Iranian and Turkish ovine breeds (Molaee, et al., 2018;

Molaee et al., 2019; Yaman et al., 2019). In particular, the very low frequency of a protective allele (K) in the VDB reflects the results recently obtained for Sarda sheep breed (www.ruminantia.it/selezione-per-la-resistenza-genetica-alla-maedi-visna-negli-ovini-opportunita-e-problematiche).
The obtained data suggested that this low frequency of the protective allele (K), both in Valle del Belice and Sarda breeds, is probably related to their genetic relationship and to the strategies of genetic selection for the milk traits carried out in these highly productive dairy breeds.
In contrast, the protective K allele is present in the Sicilian sheep breeds, in particular in COM and BARB breeds with relatively high frequencies, but further investigation is needed in a larger sample including more flocks, in order to avoid the effect of inbreeding within the flocks.
Generally, natural and uncontrolled mating is the common practice for local sheep breeds, with a minimal exchange of animals among flocks, resulting in an increase of inbreeding within the flock (Mastrangelo, et al., 2012).
In fact, previous studies carried out at genomic level and based on run of homozygosity (ROH) pattern analyses reported a recent inbreeding or strong population bottlenecks for the Barbaresca and Valle del Belice breeds (Ciani, et al., 2014; Mastrangelo, et al., 2017; Mastrangelo, et al., 2018).
These preliminary findings show how Sicilian autochthonous breeds could serve as a resource for the selection of resistance to MVV. However, any breeding program should consider the endangered status of each breed to preserve the genetic variability and the biodiversity, also when dealing with disease control.

5.1.5a Conclusion

This study reported for first time, a genetic investigation at the *E35K* locus in three Sicilian autochthonous sheep breeds reared in traditional management systems and in marginal areas. The two endangered breeds COM and BARB showed a good frequency of the protective allele K at *E35K* locus that could resulted useful for the natural protection against MVV infection.

Our findings could help for the development of future control and eradication strategies of MV infection in Sicilian sheep farming.

B. Estimating the effect of the risk allele (E) on the risk of MVV infection

5.1.2b Material and methods

5.1.2.1b Sampling

A total of 94 animals (56 rams, 38 ewes) from 8 flocks, across 4 provinces of Sicily (South-Italy) were collected between 2019 and 2020.

The sample included 20 rams of Valle del Belice (VDB) breed collected from a farm located in the province of Agrigento, 22 rams and 26 ewes of Comisana (COM) breed collected from 4 farms distributed in provinces of Enna, Agrigento, and Palermo, and 14 rams and 12 ewes of Barbaresca (BAR) Siciliana breed collected from 3 farm in Agrigento and Caltanissetta.

No clinical signs of MV disease was reported at the sampling and no epidemiological data about MVV infection status was available for the samples set.

Peripheral blood samples were collected by veterinarians, using vacuum vials with and without EDTA anticoagulant and stored frozen until further analysis.

5.1.2.2b Serological testing for MV status

The plasma was separated by centrifugation at 3000 g for 10 min and stored at −20 °C. Plasma samples were shipped to the laboratory of Virological Diagnostic Area (Istituto Zooprofilattico Sperimentale della Sicilia) for serological testing with an enzyme-linked immunosorbent assay (ELISA) (IDEXX CAEV-MVV p28 Ab Screening, IDEXX Laboratories Italia S.r.l), according to the manufacturer's instructions.

According to the guidelines of the used ELISA kit, the cut-off value is defined based on the corrected optical density (OD) at a wavelength of 450 nm ratio of sample to positive control (S/P).

5.1.2.3b DNA Extraction and TMEM154 Genotyping

Genomic DNA was obtained from peripheral blood leukocytes by using the Quick-DNA™ Miniprep Plus Kit (Zymo, Irvine, CA, USA) following the protocol provided by the manufacturer.

DNA quantity and quality were measured by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

All samples were genotyped for *TMEM154 E35K* by TaqMan allelic discrimination assay. Details on the genotyping methods were described in the previous section (section A).

5.1.2.4b Statistical analysis

Statistically significant differences in the distribution of allele and genotype frequencies between groups of serologically MV negative and positive samples were tested by Fisher's exact test.

The relative risk (RR) to be serologically MV positive (in a MV affected flock) was estimated for animals carrying one and/or two copies of the putative susceptible allele (risk factor) with the method of Altman et al., (1990) using the following equation: $RR = \frac{a/(a+b)}{c/(c+d)}$

Where a is the number of serologically MV positive individuals carrying the risk factor, b is the number of serologically MV negative individuals carrying the risk factor, c is the number of serologically MV positive individuals carrying no risk factor, and d is the number of serologically MV negative individuals carrying no risk factor.

5.1.3b Results

A proportion of 18 out of 94 (19%) Sicilian sheep samples were found to be MVV positive by ELISA diagnosis, by the detection of antibodies against p28 protein which enters the composition of the viral capsid.

Three of four farms of COM breed were seronegative flocks while the fourth one was among those with the highest seroprevalence (74%). Regarding VDB breed the 20% of sampled animals resulted seropositive and among the BARB sheep sampled only one ram resulted seropositive.

All sampled animals were successfully genotyped for the amino acid substitution at position 35 of TMEM154 using the TaqMan allelic discrimination high-throughput method.

No significant association with the serological MVV status was found for the putative protective and risk genotypes of the TMEM154 gene applying Fisher's exact test (Table B.1). The relative risk (RR) to be serologically MV positive was calculated for sheep carrying one or two copies of the putative risk allele (*TMEM154*: E at position 35) and compared to that of sheep carrying no risk allele.
In the breed subgroups, the absence of observations in serologically MV positive or negative groups produced large confidence intervals. Therefore, this analysis was only done considering all sheep set.
No significant difference in the RR to be serologically MV positive for sheep with one or two E alleles compared to those without E allele was observed, with an RR only of 0.91 (Table B.1).

N sheep subset	**MV status (n sheep)**	***TMEM154* E35K genotype frequency**		**p-value**	**RR**	**95%CI**
		KK	**EK/EE**			
In total (94)	Negative (76)	14 (0.18)	62 (0.82)	0.5835	0.91	From 0.295 to 2.784
	Positive (18)	3 (0.17)	15 (0.83)			

Table B.1 *TMEM154* E/K allele and genotype frequencies in serologically MV positive and negative sheep (VDB 20; COM 48; BARB 26) with results from Fisher's test and relative risk (RR) analyses. CI: confidence interval

5.1.4b Discussion

Multiple observations on more than 2,900 sheep representing 11 breeds were reported about the strong evidence for a consistent association between the risk allele (E) and the high risk of MVV infection (Heaton et al., 2012).

The relative risk level estimated for sheep carrying one or two E alleles in the analysed sample in this study (RR = 0.91) falls within the RR range reported by previous studies in Iranian and German sheep flocks (RR from 0.48 to 2.34), being more similar to Kermani breed (Molaee et al., 2018; Molaee et al., 2019).
Therefore, in order to provide adequate power in testing for the statistical significance it is necessary to find flocks with a "moderate" level of infection (e.g., not all or most sheep should be positive or negative) and a balanced ratio of sheep carrying the genotypes susceptible and resistant. Of course, such conditions are hard to find in field conditions and require time and screening of a large sample size.
However, Heaton et al., (2012) have been shown that the KK genotype is not fully protective reporting that about 26% of sheep carrying two K alleles were seropositive in Nebraska sheep. Several factors may influence the proportion of seropositive sheep with this genotype such as viral strength, the dose of exposure, route of infection, additional host risk factors (e.g., animal crowding, coinfection with another disease), presence of different MVV strain(s) or subtype(s) (Heaton et al., 2012; Sider et al., 2013; Alshanbari et al., 2014).

5.1.5b Conclusion

This study presents the first association of TMEM154 E35K allele/genotype with MVV serological status in three Sicilian sheep breeds for the purposes estimating the effect of the risk allele (E) on the risk of MVV infection. Further investigation is needed to validate this preliminary data, therefore further samplings from other flocks, are necessary to provide a more accurate estimation of the MVV susceptibility and seroprevalence of these breeds.

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