La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 “Dottorati Innovativi con caratterizzazione Industriale”

UNIONE EUROPEA
Fondo Sociale Europeo

Università degli Studi di Sassari

Corso di Dottorato di ricerca in

Life Sciences and Biotechnologies

XXXIII Ciclo

La presente tesi è stata prodotta durante la frequenza del corso di dottorato in Life Sciences and Biotechnologies dell’Universita degli Studi di Sassari-XXXIII ciclo, nell'ambito della borsa di dottorato aggiuntiva del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI2014IT16M2OP005), Fondo Sociale Europeo, Azione I.1 “Dottorati Innovativi con caratterizzazione Industriale”

UNIVERSITY OF SASSARI

Department of Biomedical Sciences

Ph.D. Course in Life Sciences and Biotechnologies

XXXIII cycle

PhD course coordinator: Prof. Leonardo A. Sechi

Development of high throughput molecolar diagnostic tools to monitor sheep and potentially zoonotic infectious diseases on bulk milk

Tutor:

Prof.Marco Pittau

PhD student:

Eleonora Schianchi

Academic year 2019/2020

Table of contents

Eleonora Schianchi

Development of high throughput molecolar diagnostic tools to monitor sheep and potentially zoonotic infectious diseases on bulk milk

PhD School in Life Sciences and Biotechnologies
University of Sassari

Abstract

Ovine farming makes a considerable contribution to total milk production, particularly in Mediterranean regions, in Central and Eastern Europe, and in the Middle East. Diseases caused by viral and microbial pathogens are responsable for the economic damage in sheep production. The zoonotic diseasease are transmitted from animals to humans by direct contact or by contaminated milk, water, or the environment. Milk is a suitable matrice to detect pahogens involved in these infections. In Sardinia, animal farming can be considered one of the most important productive sectors. The aim of this project is the detection of microorganisms that cause some common diseases in Sardinia. Bulk Tank Milk (BTM) analysis was used to monitor health status in herds. In this project we developed qPCR assay for detecting and quantifying DNA of *Coxiella burnetii*, *Mycoplasma agalactiae (Mag)*, Maedi-visna virus (MVV) and *Mycobacterium avium* subspecies *paratubercolosis* (MAP) in BTM samples. The results showed the presence of these microorganisms in different farms in Sardinia. This project showed that qPCR is more sensitive than others diagnostic assay, suggesting that it may become the "gold standard" for the detection of several microorganisms from biological samples. qPCR presented high sensitivity, specificity and reproducibility and allowed to analyze a large number of samples in a short time. Furthermore, this molecular assay allows timely diagnosis of infections by detection of very low quantity of the microorganisms DNA. Early detection of pathogenic microorganisms is of primary importance to allow prophylactic measures aimed to contain diseases diffusion in animals, and to identify the presence of zoonotic agents that could represent a serious threat to human health.

Eleonora Schianchi

Development of high throughput molecolar diagnostic tools to monitor sheep and potentially zoonotic infectious diseases on bulk milk

PhD School in Life Sciences and Biotechnologies
University of Sassari

1. Introduction

Ovine species makes a considerable contribution to total milk production, particularly in Mediterranean regions, Central and Eastern Europe, and the Middle East (Bittante et al., 2016). Nowadays sheep milk production in the EU represents only 1.8 % of the annual milk production, mainly taking place in Greece, Spain, France, Italy, and Romania (2011: 92 %). Notably, two thirds of the world's sheep milk are produced in the Mediterranean area. Three million tons of sheep milk were produced in Europe in 2016, mainly used to produce milk products.

1.1 Mediterranean farming systems

The Mediterranean sheep productive cycle is related to the Mediterranean climate, which is represented by a moderate winter, seasonal precipitation in autumn and spring, and a relatively dry summer. Herbage growth, which is typically available in autumn and mostly in spring, is influenced by such a climate (Carta et al., 2009). The usual Mediterranean breeding system involves one lambing per year, with mature ewes mating in springtime and young ewes (approximately 20% of the flock) mating in early autumn. Mating is successful when their body weight is around 65–70% of normal adult weight. The lambing period for mature ewes occurs between October and December, because of the high availability of the natural pastures. Approximately 75% of young ewes conceive and lamb; the rest are successfully mated only the following spring and lambs at around 24 months of age during the mature ewes lambing season. The breeding of small ruminants is raised in Mediterranean areas providing a seasonal milk productivity that corresponds to that of summer season. Therefore, this period is characterized by unfavorable condictions regarding physiological aspects and nutritional factors (Bonanno et al., 2015).

1.1.1 Sheep farming in Sardinia

Sardinia has the highest concentration of dairy sheep in the Mediterranean (approximately 3 million head on 24,090 km2), and the Sarda sheep breed is the most emblematic dairy sheep breed found in the region

(Astruc et al., 2008; Bittante et al., 2016). Consistency of Sardinian sheep herds in 2019 was 3,019,108 sheep heads, with a decrease compared to 2016 (3.102.863) and 2018 (3.046.885). Nuoro territory holds the largest number of sheep heads (768.877, 25,5%), followed by the Sassari area (22.8%), Oristano (17.6%) and Cagliari (14.5%). During 2016-2019 period, Sassari province recorded the greatest decrease in heads number (-128.098), while Oristano showed the greatest increase (+19.805). Sassari is the district with the highest number of sheep heads (84.251). Data confirms that Sardinia is the leader in sheep and goat farming in Italy, with a number of animals breed of respectively 57.13% and 41.67% of the national total. Sheep milk (68.92%) and goat milk (57.30%) produced in Italy are harvested in Sardinia. The regional production of sheep and goat cheese is estimated around 60.000 tons, of which about 30.000 are DOP (Protected Designation of Origin) (Italian national statistical institute (Istat) and Food and Agriculture Organization (FAO). Farm sheep represent 75% of all Italian sheep, with semi-extensive farms based on natural passtures as their primary source of nutrition. Grazing occurs only during certain seasons and is dependent on the farm location and altitude. It can be concentrated in winter and spring, while in northern Italy and in the highlands in autumn or late spring. As a result, the milking season lasts between 150 and 250 days, except for factory farming. The dry period is greater than dairy cattle and goats (Odintsov Vaintrub et al., 2020). The Sarda sheep is a dairy-specific breed that has adjusted successfully to the difficult environment of Sardinia's farming, which is characterized by semi-extensive and semi-intensive farming systems (Macciotta et al., 1999; Carta et al., 2009). Dairy sheep farming in the Mediterranean area, which has distinctive characteristics due to its climate and cultural conditions, is one of the most significant sectors of extensive sheep farming. As a result, a variety of milk-specific local breeds have emerged, with yields more than double of the global average. The animals are grazed outside for 120-240 days in extended dairy sheep farming, while remaining in contact with the farmer for daily milking. The milking period can be used to collect data, which might contribute to feeding, breeding, and flock management, using specific technology solutions (Vaintrub et al., 2020). Homogeneity in milkability of the entire flock is also important for small ruminants, which are generally milked in groups. Furthermore, the presence of a single animal which requires a lot of time to milk slows down the operating rate and lengthens the total milking time. Machine milkability refers to a dairy ewe's purpose of

allowing a machine to remove milk from the mammary rapidly with minimal manual intervention. The whole udder empting is determined by physiological and anatomical aspects (Casu et al., 2008).

1.1.2 Livestock milk contamination

Exogenous contamination of milk and dairy products can be avoided or minimized by maintaining hygienic condictions in the stable, throughout the milking procedure, and during additional processing (EFSA BIOHAZ Panel, 2015; Gonzales-Barron et al., 2017; van den Brom et al., 2020). Hygiene in the stable during milking decreases pathogen transmission in the enviroment or from infected animals to healthy animals, reducing the number of animals potentially excreting pathogenic microorganisms in milk (van de Brom., 2020). Milk contamination can occur during milking, but it takes place also through bedding. In order to prevent milk-to-milk contamination, infected animals should be milked in separate groups, provided with clean bedding, following hygiene rules during milking, culling infected animals and vaccinating healthy ones. Even animals that do show clinical signs may shed microorganisms (van de Brom et al., 2020). Diseases caused by viral and microbial pathogens are responsable of the economic damage in sheep production. Sheep have greater prevalence and morbidity rates than other farmed animals, according to reports (Roger, 2008). It is impossible to produce sterile milk. Milk is used to search many infectious pathogens. General improvements in water sources, public health, hygiene, and pasteurization have essentially reduced microorganisms' transmission to human (Leedom, 2006). Based on data collected from 1993 and 2006, the Centers for Disease Control and Prevention (CDC) estimated that non-pasteurized milk and milk products had a 150-fold higher possibility to cause outbreaks and outbreak-related diseases per unit of product consumption (Langer et al., 2012; Boor, 2017). Animal illnesses can decrease farm gross income by up to 20%, with reductions being more visible and quantifiable during acute outbreaks. The economic impact of chronic diseases, either direct or indirect, on farm performance is frequently underestimated (Jarvis and Valdes-Donoso, 2018). Directly losses are mostly indicated by poorer milk outputs and less desirable milk composition, as well as a fertility reduction, increase exposure to diseases, testing and treatment costs. Local and international trade limitations may result in indirect loss (Whittington et al., 2019).

1.1.3 Zoonoses: diseases transmitted from animal to human

More than 60% of infectious diseases in humans are caused by pathogens that are shared with wild or domestic animals (associated with an increased risk for human disease onset) (Karesh et al., 2012). The rate of new and re-emerging zoonoses appears to be increasing over the last two decades. Some high-income countries can control zoonoses, but many low-income countries are unable to appropriately respond to current and emerging zoonoses (Klempner et al., 2004; Zinsstag et al., 2005). Different changes in land use, exploitation of natural resources, animal production systems, modern transportation, antimicrobial compounds use, and worldwide trade are the causes of the establishment of these pathogens in new ecological niches, such as in human (Woolhouse et al., 2006; Wolfe et al., 2007; Karesh et al., 2012). Zoonotic diseases are frequently classified by their way of transmission (for example, vector-borne or foodborne), microorganisms' type (microparasites, macroparasites, viruses, bacteria, protozoa, worms, ticks, or fleas), or degree of individual transmissibility (Lloyd-Smith et al., 2009). The zoonotic diseases are transmitted from animals to humans by direct contact or contaminated food, water, or by the environment (McArthur, 2019). The majority of zoonotic pathogens is transmitted to humans by direct contact with animals or indirectly through vectors which act as reservoirs (Gray et al., 1998). Transmission between animals can occur anywhere, in both urban and rural environments (Cross et al., 2019). Wildlife reservoirs sustain infective cycles in local livestock, constituting a serious problem to control zoonosis spread in pastoral communities due to the difficulties of reducing interactions between wildlife and livestock animals (Bengis et al., 2002). The CDC adopts a One Health strategy monitoring health and safety concerns and to discover how diseases spread across human, animals, and the environment by collaborating with health care providers, veterinarians, and ecologists (CDC, 2018). Collaboration with public health experts, who often use epidemiological approaches utilizing human case data, and disease ecologists, who typically utilize data from wildlife or livestock to simulate risk in humans, should be promoted. Modern disease ecology strategies could be particularly valuable in advancing predictions of new zoonosis emergence and transmission (Morse, 1995; Karesh et al., 2012).

1.2 Coxiella burnetii

Coxiella burnetii is the causative agent of Q fever, a zoonosis transmitted by animals. This pathogen infects different animal species and also humans. The main domestic reservoirs of *C. burnetii* are the ruminants, but also birds, rodents and arthropods (Babudieri and Moscovici, 1952). In addition, dogs and cats can also transmit the microorganism to human (Marrie et al., 1988, Buhariwalla et al., 1996). Q fever is widespread all over the world except in New Zealand (Woldehiwet, 2004). This disease is endemic and widespread in several countries although epidemic patterns have also been described with rural epidemics affecting large numbers of non-work-related individuals (Van den Brom et al., 2015). Between 2007 and 2009, in south Netherlands there were numerous outbreaks involving 4000 human cases (Vanderburg et al., 2014). These outbreaks revealed that Q fever is a real risk for public health. The presence of this microorganism in farm animals and therefore in foods originated from animals, such as milk, prompts to search *coxiella* in foods that could be a vehicle for this zoonosis. An important vehicle for human infection is unpasteurized milk. There is also a close correlation between the consumption of milk products and the onset of the disease in humans (Pexara et al., 2018).

1.2.1 Etiology of Coxiella burnetii

C. burnetii is a gram negative, obligate intracellular bacterium, charachterized by a small size (0.2-0.4 μm wide and 0.4-1 μm long), and it is resistant to high osmotic pressures. On the basis of its characteristic gene sequence, the genus coxiella belongs to the order of *Legionellales*, family *Coxiellaceae* with *Rickettsiella* and *Aquicella* (Gimenez, 1964; Seshadri et al., 2003; Eldin et al., 2017). Several studies showed the presence of different microorganism isolates, which have a genome that varies between 1.989.565 and 2.214.254 bp (Seshadri et al., 2003). Different studies confirmed the presence of five plasmids in *coxiella* (QpH1, QpRS, QpDG, QpDV, and plasmidless). They have been isolated from ticks, ruminats, and patients with acute Q fever. The first plasmid Qph1 was isolated from a tick (Samuel JE et al., 1983). Several studies by Hendrix et al. compared different strains of *C. burnetii* showing six distinct genomic groups related to acute and chronic disease (Hendrix et al., 1991). Genomic group I-III

strains own the plasmid QpH1 and have been isolated by individuals with acute Q fever, chiggers, cow milk, goat abortions, and ticks. Strains belong to Group IV, and containing QpRS plasmid, were isolated from cattle abortion and heart valves of patients with Q fever. The isolates of group V do not have plasmid but present plasmid-like sequences. These strains have been isolated from patients with Q fever endocarditis or hepatitis. Strains of group VI, found in rodents in the Utah desert, have an attenuated virulence and contain QpDG plasmid (Stoenner et al., 1959; Stoenner et al., 1960). The association between plasmid and disease has been confirmed by MST (Multispacer Sequence Typing) genotyping of 173 isolates. In this study, no correlation was found between the QpH1 plasmid and disease onset. In any case, a correlation was found between the plasmid QpDV (acute disease) and QpRS (chronic disease) (Glazunova et al., 2005). Beare et al.,2006 showed that QpDV plasmid is associated with new genomic groups, which are the VII and VIII. *Coxiella* has affinity for particular cells of the immune system, specialized phagocytes, which during the phagocytosis mechanism utilize receptor-ligand interactions (Flannagan et al., 2012). The bacterium can have two phases: virulent phase I and avirulent phase II. Antigenic modifications between these phases are due to a change in lipopolysaccharide (LPS) from smooth to rough. In the virulent phase LPS of *coxiella* inhibits immune response, enabling the phase I bacterium to survive and multiply in the host. (Van den Brom et al., 2015). *Coxiella* morphologically has three different forms: large-cell variants (LCV), small-cell variants (SCV) and small dense cells (SDC). The three forms diverge for their morphological and antigenic pattern and on the physical and chemical resistance (Heinzen et al., 1999). The SCV and the SDC are considered as resistant forms of the microorganism both in the host and in the environment. The causative agent is resistant to various conditions such as UV radiation, heat, desiccation, sonication, pressure (> 50 000 psi; 1 psi = 6.89 kPa) and osmotic and oxidative stress (Amano et al., 1984). This allows the infectious particles to resist up to 150 days in an extracellular environment. The morphological variant SCV or endospore is the form involved in the response by phagocytes in the early stage of infection and is associated with the consumption of contaminated food. The endospores can spread in the reproductive organs, such as the mammary glands, in fact they are secreted in milk of infected animals both with overt disease but also asymptomatic. They are also excreted by abortion, urine, and feces (Pexara et al., 2018). Different factors

can influence the maintenance of the infection in farmed animals, depending on manure management in all its phases, farming characteristics (size and density), and environmental conditions of the farm (EFSA 2010).

1.2.2 Epidemiology

Most common symptom in infected sheep and goats is abortion in late pregnancy. *Coxiella burnetii* is eliminated from infected animals through abortive material, milk, urine, faeces and birth fluids (Maurin and Raoult, 1999, Arricau Bouvery et al., 2003, Wouda and Dercksen, 2007). Infection in humans can be transmitted from infected animals by direct contact especially during abortion and birth, and by ingestion of non-pasteurized milk and dairy products (Tissot-Dupont et al.,2008). In humans, the disease occurs without obvious symptoms, or in acute and chronic forms. The main reservoirs of infection are farm and domestic animals that can transmit infection to humans by contaminated aerosols (Angelakis et al., 2010). Q fever usually manifests as an acute illness with flu-like symptoms and a high fever, although many individuals remain asymptomatic during infection. Full recovery is common after acute illness following antibiotic treatment. Despite this, some individuals may develop persistent forms ("chronic Q fever") such as endocarditis, hepatitis, lymphadenitis, myocarditis, osteomyelitis, and/or vascular infection (Vogiatzis et al., 2008; Eldin et al., 2017); (Fig 1).

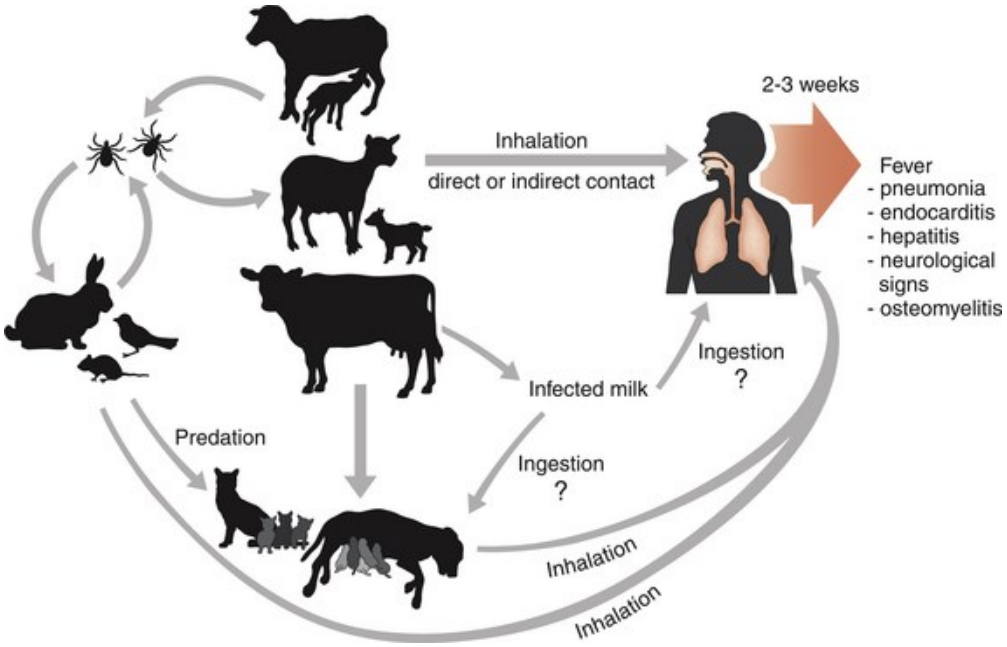


Fig. 1 *Coxiella burnetii transmission. When animals give birth, the organism is most commonly transmitted to humans. Infection in human occurs by direct contact with birth materials by indirect contact with organisms that persist in the environment. Most prevalent reservoirs of the microorganism for human infection are sheep, cattle, and goats, but infected cats and, less typically, dogs can also transmit the infection to humans. Ticks also carry the organism and are able to transmit it to wildlife, particularly rodents (such as small mice) and rabbits, as well as birds. When dogs and cats enter in contact with (or eat) wildlife species, they can be infected transmitting the microorganism to human. Consumption of contaminated milk could be a less effective form of transmission (Sikes and Norris, 2013).*

1.2.3 *C. burnetii* in Sardinia

In Sardinia *coxiella* causes economic losses due to abortions, infertility, and stillbirths in small ruminants (Masala et al., 2007). In the island *coxiella* has been isolated in ticks and humans (Chisu et al., 2021).

Each year in Sardinia this microorganism causes a hospitalization rate that corresponds to 11.9 cases per 10^5 inhabitants. The incidence of ovine abortion is very high, not only regarding the large number of heads but also due to the high animal density, breeding conditions (primitive herding practices and transhumance), and not suitable diagnosis and prophylaxis. The mortality of lambs and the missed lactation cause economic losses of about 10 million euros / year (Masala et al., 2004). In order to detect *coxiella* early in small ruminants, control and prevention measures play a key role (Field et al., 2000).

1.2.4 Laboratory diagnosis

For *coxiella* diagnosis, specific indirect methods are mainly used because isolation is not possible it with standard culture techniques. To date, serological techniques are the most used to detect *coxiella* infection. Currently, the detection of the DNA of this microorganism is carried out by qPCR in different clinical samples (blood, cardiac valves, or other surgical tissue biopsy specimens). The advantage of using these methods is the ability to detect the microorganism before seroconversion in patients with primary infection. A biosafety laboratory 3 (BSL3) is required to culture the microorganism. Analysis of the infected tissue can also be performed with immunohistochemistry techniques. In recent years, efforts have been made to improve the sensitivity of the different diagnostic techniques (Eldin et al., 2017). Several specific PCR tests have been developed for both animal and human samples for the detection of this etiological agent (Mallavia and Paretsky, 1967). Standard PCR assays are able to detect sequences of different plasmids, the 16S-23S RNA, the superoxide dismutase gene, the *com1* gene or the IS*1111* repetitive elements in human or animal samples (Ibrahim et al., 1997; Stein et al., 1997; Mediannikov et al., 2010). These methods have detection limits that depend by the number of bacteria, ranging from 10 to 10 ^{2}. The most commonly used method to detect *coxiella* is real-time PCR. The advantage of this method is that it takes less time than PCR and is able to quantify *coxiella* in the samples. The real-time PCR has a high sensitivity towards a target sequence (IS1111) which is an insertion element repeated a variable number of times in the genome of *C. burnetii* Nine Mile (Klee et al., 2006; Denison et al., 2007; Eldin et al., 2017).

1.2.5 Treatment, preventative measures, and surveillance in small ruminants

The preventive and therapeutic measures for small ruminants allow to decrease abortion rates, the spread of the bacterium and also the contamination of the environment. Other measures to limit the introduction of the infection are based on taking specific precautions before introducing animals into uninfected herds (Arricau Bouvery and Rodolakis, 2005). Considering that *coxiella* is an intracellular bacterium, is not clear its susceptibility to antibiotics. Several studies shown that in small ruminants somministration with two injections of oxytetracyclin (dose rate: 20 mg/ kg body weight) in the last month of pregnancy, decrease abortion rate. In each case, it was not possible to find beneficial effects on bacterial persistence and shedding (Berri et al.,2002; Arricau Bouvery and Rodolakis, 2005; Angelakis and Raoult, 2010). Vaccination can be used to prevent abortion and the spread of *coxiella*. The development of vaccines depends on the bacterial strain, and the most effective is the form of *coxiella* in phase I (Arricau Bouvery et al., 2005). It is also more effective if it is administered to uninfected animals before pregnancy (Hogerwerf et al., 2011; Van den Brom et al., 2013). In order to reduce the exposure of individuals to *coxiella* it is important to implement hygiene measures within the farms. Considering that the spread of *coxiella* occurs mainly during animal birth, it is important to implement hygiene rules in infected farms. Farmers and veterinarians, that are most exposed, should wear protective clothing even if it has been shown that this is not enough to prevent infection in humans (Whelan et al., 2011).

1.3 Mycobacterium avium subspecies paratuberculosis

Mycobacterium avium subsp. *Paratuberculosis* (MAP) causes paratuberculosis ('Johne's desease), a chronic bacterial disease affecting primarily domestic and wild ruminants (Windsor, 2015). The disease was first discovered in cattle in Europe during 1895. From that moment the disease has spread throughout the developed and developing countries, especially in the cattle dairy industry. The infection in small ruminants is also widespread, with sheep and goats being diagnosed in several countries, such as Australia, New Zealand, South Africa, United Kingdom, Norway, and Austria, as well as Mediterranean countries such as Greece, Spain, Portugal, Morocco, and Jordan (Hailat et al., 2010). *M. avium, M.*

hominissuis, M. silvaticum, and *M. paratuberculosis* are the four subspecies of *Mycobacterium avium*, each with a high degree of genetic similarity and varying degrees of infectivity in host species. One of the most important microorganisms in the livestock industry is *M. avium subsp. paratuberculosis* (MAP) that is considered a problem for world's milk heards (Hamilton et al., 2017). In a recent study it has been analyzed a correlation between MAP and Crohn's disease (CD) in humans. In addition, the disease causes economic losses and trade restrictions (Jarand et al., 2017). Jones disease, or animal paratuberculosis, is a persistent, contagious, and often lethal colon granulomatous infection. This microorganism was first detected in cattle, and then in sheep and goats. Most animals, especially domestic or wild ruminants, wild carnivores, laboratory animals, and nonhuman primates, can be colonized by tuberculosis bacilli (Waddell et al., 2008).

1.3.1 Etiology of *MAP*

Mycobacterium avium paratuberculosis belongs to the Mycobacterium avium complex (MAC), which is part of the Mycobacterium genus and Mycobacteriaceae family (Rathnaiah et al.,2017). Researchers at the University of Minnesota collaborated with the National Animal Disease Center in Ames, Iowa, to sequence the entire genome of Map strain K-10. In January 2004, the Map genome sequence was published and put in the GenBank database (accession no. AE016958). Map K-10 has a single circular sequence of 4.829.781 bp. A recent study compared the genome sequences of Map and three other Mycobacterium species (M. tubercolisis, M. leprae, and M. bovis), providing new information on the genetic basis of Map's mycobactin J dependency and slow development. Map has a thick waxy cell wall that contains 60% lipids, giving it acid fastness, hydrophobicity, and improved resistance to chemicals and physical processes such as the pasteurization (McNeil and Brennan, 1991; Grant et al., 1998; Wang et al., 2001; Primm et al. 2004). Mycobacteria is characterized by a lipid-rich cell wall causing slow growth due to nutrient uptake restrictions (Domingue and Woody, 1997). MAP can survive in the environment (up to 55 weeks) and in water sources for long periods (up to 48 weeks in dam water, and for 841 days in lake water microcosms) (Whittington et al., 2000; Pickup et al., 2005). In ruminants MAP

spread through mucosal barrier in the systemic circulation. To survive in the macrophages, MAP inhibits cell apoptosis, evades phagolysosome activity, avoids detection by modifying chemokine and cytokine signaling, and alters dendritic cell maturation. Given the limited lifespan of macrophages, cells will die in approximately 4 to 6 weeks releasing MAP into the local environment. Bacteria cross the intestinal lumen and are shed by feces or remain locally and start a new cycle. The microorganism and MAP antigen taken up by phagocytes, spreading in the lamina propria may disseminate to different sites in the intestine. Alternatively, they can enter in afferent lymph and migrate to the draining lymph node cause lymph node lesions or activation of T and B lymphocytes (Koets et al., 2015). Different antibacterial components, such as ROS (Reactive Oxygen Species) and nitric oxide, are not able to kill all mycobacterium avium species (Li et al., 2010; Schairer et al., 2012). The presence of the insertion element IS900, which occurs in 14–18 copies throughout the genome of MAP, distinguishes it from other Mycobacterium spp (McFadden et al., 1987; Green et al.,1989). Insertion sequence elements (ISEs) are short, mobile genomic elements that include genes involved in transposition. IS900, a member of the IS110 family of insertion sequences, was the first insertion sequence element discovered in mycobacteria (Green et al., 1989; Collins et al., 1993).

1.3.2 Epidemiology

The disease is spread predominantly by oral-fecal transmission route, and the main sources of infection are the infected animals' feces and milk. Furthermore, bacterial can be found in other body areas such as the womb, testicular parenchyma, and breast (Sweeney et al., 1992; Ayele et al., 2004; Whittington and Windsor, 2009). After infection, the disease might remain in a subclinical stage for years, with just a few animals displaying characteristic paratuberculosis clinical signs (Klinkenberg & Koets, 2015). Because of the long incubation period, the age profile within flocks influences the occurrence of clinical illness. While clinical paratuberculosis normally appears in adults, most individuals get infected during the first weeks of life after being exposed to MAP from older animals and eliminating the bacterium by their feces. It is also possible to get infected at a later age (Windsor, 2010; McGregor et al., 2012). Several studies suggested a role of MAP in human diseases, such as Inflammatory Bowel Disease (IBD) and

autoimmune illnesses (asthma, insulin-dependent diabetic mellitus, sarcoidosis, rheumatoid arthritis, multiple sclerosis, and celiac disease) (Grant, 2005; Naser and Thanigachalam, 2014). Based on clinical findings, different chronic gastrointestinal disorders with unknown aetiology are divided into three categories: ulcerative colitis, Crohn's disease, and inflammatory bowel disease (IBD) (Pierce, 2018). These bacteria are spread by draining water or rivers, and they can persist in both terrestrial and aquatic habitats for long periods of time, most likely inside amoebae and other protists (Pistone et al., 2012). The main sources of MAP transmission to humans and animals are water and milk (Grant et al., 2001). Furthermore, some studies have suggested that pasteurization may fail to remove MAP (Lund et al., 2002; Grant, 2003). MAP can be found in milk and colostrum as a result of fecal contamination of teats or shedding from the udder (Stabel & Lambertz, 2004); (Fig 2).

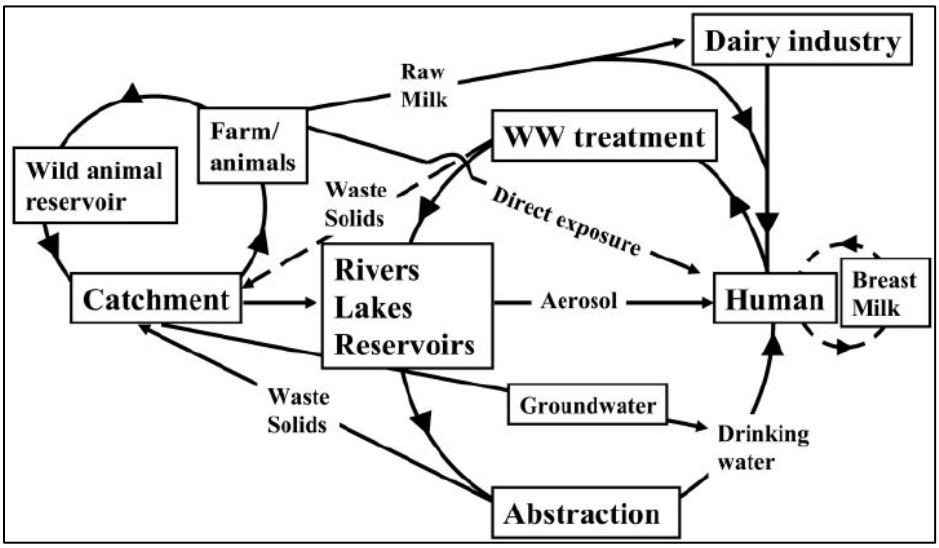


Fig 2. *Schematic representation of sources and reservoirs of infection of M. avium subsp. paratuberculosis in the environment in relation to human exposure. This model takes into account both the known and possible epidemiology of this bacterium to humans via dairy products, particularly milk. The main routs by which the microorganism is transmitted are represented by domestic livestock, wild animals. M. avium subsp. paratuberculosis accesses water through water shed runoff, with aerosols*

from contaminated rivers and water used for human consumption. All these ways are potential routes of exposure to pathogen in humans (Pickup et al., 2006).

1.3.3 Laboratory diagnosis

Isolation of the organism in culture remains the most efficient method for MAP detection. It can be isolated from different samples (milk, animal feces and blood), but the long doubling time of MAP in this procedure is slow and takes weeks to produce results. (Bower et al., 2010; Gilardoni et al., 2012). PCR assay allows the quick detection of the pathogen. (Vary et al., 1990; Moss et al., 1992; Millar et al., 1996; Grant et al., 1998; Englund et al., 1999; O'Mahony and Hill, 2002; Pillai and Jayarao, 2002; Fang et al., 2020). The most utilized molecular target for these assays is the insertion element IS900 (found in 14–18 copies in the *MAP* genome). However, IS900-like sequences have been found in non-*Map* isolates, which could give to a false-positive diagnosis (Cousins et al., 1999; Englund et al., 2002; Tasara et al., 2005; Mobius et al., 2008). As a result, other target elements such as f57, ISMav2 HspX, and locus 255 have been proposed (Poupart et al., 1993; Ellingson et al.,1998; Strommenger et al., 2001; Vansnick et al. 2004; Mobius et al. 2008). A variety of PCR assay (including real-time PCR) detect as *Map* target the single copy f57 gene (Tasara and Stephan, 2005; Tasara et al., 2005; Bosshard et al., 2006; Stephan et al., 2007; Schonenbrucher et al., 2008). The genetic similarity of *M. paratuberculosis* to other subspecies of the *M. avium complex* complicated early attempts to differentiate *M. paratuberculosis* using molecular biology techniques. *M. avium* subsp. *avium, M. avium* subsp. *paratuberculosis,* and *M. avium* subsp. *silvaticum* are all members of this complex, with *M. paratuberculosis* and *M. avium* sharing 97% of their genomes and 100% of the 16S rRNA gene (Thorel et al., 1990; Bannantine et al., 2003). *M. paratuberculosis* was detected by PCR in 52%–92% of Crohn's disease patients, compared to 5%–26% of controls, in subsequent studies using molecular-based approaches (Bull et al., 2003; Autschbach et al., 2005; Ellingson et al., 2000). In mycobacteriology, nucleic acid tests, which have a 99.2% accuracy and can detect 0.01 pg of DNA or 20–100 genomic copies, are significantly more appealing (Sanderson et al., 1992; Mobius et al., 2008). It is also difficult to detect *MAP* DNA in environmental or veterinary samples. Although molecular assays, such as PCR and real-time quantitative PCR, have the ability to

detect *MAP* DNA more quickly and precisely, and their detection performance is primarily dependent on the quality of the examined samples (Wells et al., 2006). Due to the similarities o*f M. paratuberculosis to M. avium* and other environmental mycobacteria, immunoassay tests are not really specific and sensible (Yakes et al., 2008; Huntley et al., 2005).

1.3.4 Control of paratuberculosis

The control of paratuberculosis is based on population-level measures, such as culling of animals that shed *MAP*, implementing sanitary measures to prevent infection in neonatal/young stock, and prophylaxis by vaccine. Ruminant vaccination has been shown in controlled experiments to be able to minimize the clinical incidence of paratuberculosis, delaying the beginning of the disease, and reducing *MAP* fecal shedding, resulting in lower economic losses and *MAP* transmission (Bastida and Juste, 2011). Because of the capability of interfering with intradermal testing for bovine tuberculosis, vaccination is not utilized in the control of paratuberculosis in cattle (Serrano et al., 2017). In contrast, vaccination has been an important component in control in the Australian, Icelandic, and Spanish sheep industries, resulting in a large reduction in within-herd prevalence. Communication to farmers and veterinarians on control programs and monitoring farmers attitudes regarding the implementation of control measures, is a critical component of MAP control (Benedictus et al., 2000; Ritter et al., 2016).

1.4 *Mycoplasma agalactiae*

Contagious agalactia is a multi-organ illness that causes systemic infections and is thought to be one of the most devastating diseases in small ruminants. It is caused by mycoplasmas following Contagious Caprine Pleuropneumonia (CCPP) (Gómez-Martín et al., 2012; Gómez-Martín et al., 2013). *Mycoplasma agalactiae* is the principal etiological agent of this disease that especially affects goats and sheep, as well as a variety of wild animals. The disease rapid spread is ascribed to a number of causes, including primitive herding techniques, ineffective antibacterial drugs, and the implementation of few preventive measures. Following *M. mycoides* subsp. *mycoides* SC type, *M. agalactiae* has been the

second mycoplasma described. It was described in 1923, when Bridre and Donatien for the first time isolated the bacterium that causes contagious agalactia (CA) in goats (Bride and Donatien, 1923). Bridre and Donatien described CA as a disease characterized by mastitis, arthritis, and keratoconjunctivitis in sheep and goats caused by *M. agalactiae* (Briede and Donatien, 1925). The disease has been documented worldwide and causes high economic loss due to high morbidity in ovine populations (Edward et al., 1953; Poumarat et al., 2016). In addition, economic losses are due to reduced or suppressed milk supply, abortion, and high morbidity and mortality rates in adult sheep (Ariza-Miguel et al., 2012).

1.4.1 Etiology

M. agalactiae is a polymorphic bacterium with a genome of 1×10^{9} Da and a size ranging from 124 to 250 nm (Srivastava et al., 1982; Kumar et al., 2014). This microorganism develops colonies with dark centers that have a fried-egg appearance. *M. agalactiae* does not ferment glucose or hydrolyze urea or arginine, according to biochemical analysis (Lambert et al., 1987; Khan et al., Razin et al., 1998);(Fig 3).

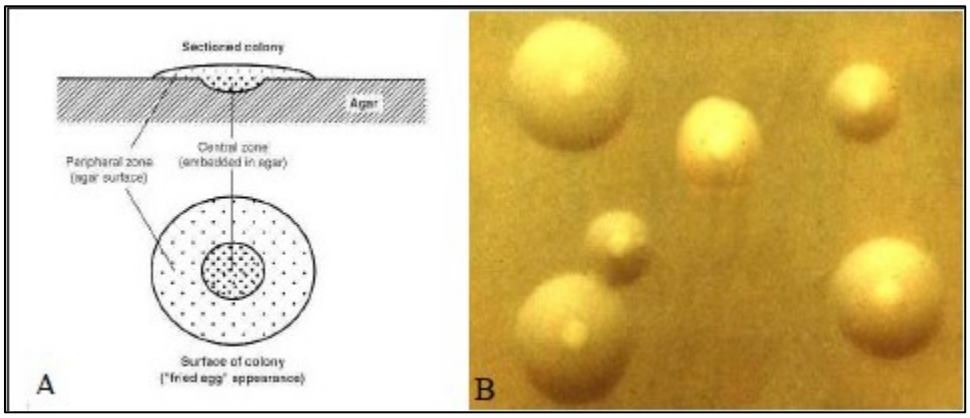


Fig. 3 *Morphology of a typical "fried-egg" mycoplasma colony. A: schematic representation; B: Mycoplasma agalactiae colonies on solid medium.*

To observe the colony characteristics, Giemsa staining from solid agar media is used to stain the mycoplasma colonies. Gram staining produces a pink tint in mycoplasma due to the lack of a cell wall (Srivastava, 1982; Kumar, 2014). *M. agalactiae* is extremely sensitive to high temperatures and can be inactivated in as little as 5 minutes at 60°C and in a minute at 100°C. It can also be deactivated by direct sunlight exposure during the hot summer months. At room temperature and in the refrigerator at 8°C, the organisms survival time varies from 1-2 weeks to 3-4 months, depending on other factors such as media pH. *M. agalactiae* can survive in both humid and cold conditions. At a temperature of 20°C, it can survive for 8 to 9 months. It is readly inactivated when exposed to UV radiation and dyes. Furthermore, disinfectants such as potassium hydrochloride, formalin, and chloramines can quickly destroy the organism (Bergonier et al., 1996). The absence of a cell walls and the presence of a trilaminar membrane, which mediates all contacts with the environment, distinguish the *Mycoplasma* genus. Membrane proteins are important for adhesion to host cell surface receptors and the development of an immune response (Tola et al., 1996). *M. agalactiae* is resistant to penicillum and its variants, like other mycoplasma species. It is susceptible to osmotic shock and detergents (Srivastava, 1982). Membrane proteins of mycoplasmas are important for their survival and pathogenicity, and those identified as antigens could be used to produce novel vaccines. Despite this, immunogenic membrane proteins from *M. agalactiae* have been discovered, including the P30 protein (Fleury et al., 2001), P40 cytoadhesion (MAG 2410) (Fleury et al., 2002), P48 protein (Rosati et al., 1999; Chessa et al., 2009), and MAG 5040 lipoprotein (Cacciotto et al., 2010). The complete genome sequence of *M. agalactiae* can be used to find new antigenic proteins for vaccines development (Sirand-Pugnet et al., 2007).

1.4.2 Epidemiology and transmission

CA was described for the first time in Sardinia in 1980 when sheep were imported (Tola et al., 1999). The spread of CA was improved in Sardinia because of several factors, including a large number of sheep heads (3.5 million milking sheep, or one-third of the total national stock), a high animal density per square kilometer (149/km^2 compared to 23/km^2 on the mainland), breeding conditions (primitive herding practices and transhumance), and insufficient diagnostic and preventive measures (Tola et al., 1999). As

the organism can survive at room temperature, it can spread quickly from sick to healthy animals. Auricular, ocular, and nasal secretions, feces, milk, and urine are the main sources of transmission of infection. Sexual transmission has been also described, and the microorganism can be transmitted also by contaminated utensils and milker hands. Vertical transmission occurs when infected colostrum or milk is consumed (Lamber, 1987; Kinde et al., 1994; Real et al., 1994; Kumar et al., 2014). In most situations, a flock with a chronic or persistent infection is detected for several months. Clinically positive animals are observed under favorable environmental conditions, such as during the hot and humid summer months. Young animals, malnourished, pregnant, or immunocompromised are more subjected to infection (Levisohn et al., 1991). Furhermore, microorganism excretion in milk have been reported in animals with mild or without clinical signs also after 8 years. As a result, asymptomatic carriers in herds are the major source of infection (da Massa and Brooks, 1991; Bergonier et al., 1996; Kumar et al., 2014).

1.4.3 Pathogenesis

Primary sites of clinical manifestation are alveoli of mammary glands, the mucosa of the respiratory tract, small intestine (Srivastava, 1982; Kumar et al., 2014). The organisms are disseminated through bloodstream to many organs, including the lungs, lymph nodes, eyes, mammary glands, joints, and tendons, resulting in various clinical symptoms (Razin et al., 1998; Kizil et al., 2006). Connective tissue involvement in the mammary glands causes early inflammation, which progresses to catarrhal or parenchymatous mastitis, resulting in agalactia (Srivastava, 1982). Mastitis-affected animals can transmit the disease to lambs through colostrum or milk (Bohach et al., 2021).

1.4.4 Clinical Signs

M. agalactiae can infect both male and female sheep and goats. Fever, anorexia, lethargy, and an inability to follow the herd are common clinical signs, followed by clinical symptoms based on the involvement of numerous organs such as the mammary glands, lungs, genitalia, joints, and conjunctiva (Razin et al., 1998). There have also been reports sporadic abortions in pregnant animals (Srivastava,

1982; Gil et al., 2003; Kizil et al., 2006). Importantly, whereas fever is common in acute infections and may be accompanied by nervous symptoms, both are uncommon in subacute and persistent infections Razin et al., 1998). Chronic involvement of joints and cornea losses result in lameness, inability to walk or stand, and blindness (Kumar et al., 2014).

1.4.5 Diagnosis

Clinical symptoms such as loss of milk production, mastitis, keratoconjunctivitis, and articular lesions are sufficient to make preliminary diagnosis. The clinical diagnosis is validated in laboratory by isolating and identifying the pathogen. *M agalactiae* can be detected in milk, auricular, ocular, vaginal, or nasal discharges, joints exudates, blood, and urine samples (Nicholas, 2002). The most used diagnostic tests are ELISA and PCR. ELISA tests allow the detection of specific antibodies, and it is a very symple, fast, and cheap method (Rosati et al., 1999, 2000; Robino et al., 2005; Alberti et al., 2008). The diagnosis, characterization, and differentiation of mycoplasmas, including M. agalactiae, have all been improved by the recent developments in molecular biology and biotechnology. PCR assay is widely used, and it appears to be a more sensitive method for identification (Bashiruddin et al., 2005). PCR techniques based on 16S rRNA (Bergonier et al., 1996) uvrC gene (Subramaniam et al., 1998), and multiplex PCR are routinely utilized and have a good diagnostic value (Greco et al., 2001). According to OIE recommendations, molecular detection based on the uvrC gene is critical. In both clinical and food microbiology, the real-time quantitative PCR technique can be employed to quantify the organism and is becoming increasingly popular for the diagnosis (Oravcová et al., 2008).

1.4.6 Control and Prevention

Infection can be avoided by implementing management practices and continous surveillance and monitoring for the pathogen. Subclinically infected animals can often spread the pathogen, and the use of a particular sensitive, and rapid diagnostic approach is necessary to detect it at an early stage. Suspected

animals should be isolated and monitored until further confirmation. Litter and other materials, such as discharges and aborted fetuses, should be disposed properly, and infected utensils should be sterilized.

1.4.7 Vaccines

Vaccination against *Mycoplasma agalactiae* in sheep causes both specific and nonspecific humoral and cellular responses. Although live attenuated vaccines (LAV) are more efficient and have been shown to provide better protection in ewes and their lambs than inactivated vaccines, they can cause a transitory infection with mycoplasma shedding through milk. Inactivated vaccines are significantly safer and have fewer side effects, but they provide less protection (Nicolet, 1994; Buonavoglia et al., 2012; Kumar et al., 2014). A DNA vaccine against CA was also developed, encoding P48 *Mycoplasma agalactiae*. It has been shown that this vaccine is able to induce an immunological response in BALB/c mice. In fact, IgG and IgG1 were identified in mice vaccinated with pVAX1/P48. In addition, proliferation of mononuclear cells of the spleen, levels of gamma interferon, interleukin-12, and interleukin-2 mRNAs were enhanced in immunized animals. Moreover, it has been proven that pVAX1/P48 vaccine can induce both T-helper 1 and T-helper 2 immune responses. For this reason, this vaccine can be considered a potential tool to control CA in sheep (Chessa et al., 2009).

1.5 Maedi-Visna: First Reports and epidemiology

Except in the United States, where the term "Ovine Progressive Pneumonia (OPP)" is used, Maedi-Visna (MV) is a terminology used globally to designate a disease caused by two viruses from the "small ruminant lentivirus" subgroup: maedi-visna virus (MVV) and caprine arthritis-encephalitis virus (CAEV). Recent epidemiological studies have discovered some common sheep and goat variants (Straub, 2004; Leroux et al, 2010) and co-infection have been reported (Straub, 2004). For these reasons, this cluster of viruses is now referred to as the small ruminant lentiviruses (SRLVs). Maedi–Visna virus (MVV) was the first member of the class of lentiviruses known as small ruminant lentiviruses (SRLV). Sigurdsson in 1954 was the first scientist to describe these "slow infections", which are distinct from

acute and chronic disease. According to his observations, these infections had a long incubation period in which the virus spread throughout the body without causing clinical signs, followed by severe symptoms and death occurs after several months or years. Maedi–Visna was first described in Iceland in 1939, where it was named "Purra Maedi" (dry dyspnoea in Icelandic) by herders to distinguish it from "Wota Maedi" (in Icelandic watery dyspnoea that is pulmonary adenomatosis). The disease was introduced in summer 1933 by the introduction of 20 apparently healthy Karakul sheep from Germany, that were introduced to improve the Icelandic native breed. According to Gislason (1947) epidemiological research, at least two of the rams were infected with MVV, resulting in epizootics in two distant parts of the island, with maedi (pneumonia) appearing about 6 years after the original introduction. The presence of MVV in flocks after the introduction of new animals could be tracked, and the pre-clinical incubation period has been estimated to be at least 2–3 years (Gislason, 1947; Sigurdsson, 1954; Straub, 2004; Blacklaws 2004). Later, it was determined that the same Karakul flocks are also responsible for pulmonary adenomatosis (wota maedi', or watery dyspnoea in Icelandic), paratuberculosis, and visna (which means "spinal cord" in Icelandic). After this first signalling, other reports on disease spread have been reported: Texel sheep from Holland introduces MVV in Denmark between 1955 and 1965 (Hoff-Jorgensen, 1978); by the same route from Denmark to Norway the disease was introduced between 1962–1970, causing Maedi-Visna in 104 flocks diagnosed in 1977; from Scotland to Canada in 1970 (Dukes et al, 1979); from Great Britain to Hungary in 1972; from Holland to France in 1956 (Cottereau et al, 1977); and from Sweden to Finland in 1981 (Sihvonen et al, 1999; Sihvonen et al, 2000). Caporale and colleagues described Maedi-Visna for the first time in Italy in 1983, describing MVV in two separate flocks in Central Italy (Abruzzo and Umbria). Later, Christodoulopoulos (2006) presented epidemiological data collected in Italy at the beginning of the 2000s, which revealed high prevalence levels across the country, ranging from 60 to 90%. Another report by Cirone et al in 2019 analysed 4800 serums from goats sampled from 1060 flocks in South Italy (Calabria and Apulia). He found a seroprevalences of 18,64% and 51,69% in animals and herd levels for SRLV, respectively. Pazzola et collegues in 2020, in a study which involved 1,079 Sarda sheep kept on 23 farms, obtained results

consistent with the study by Christodoulopoulos (2006): the prevalence of MVV-positive ewes was 43.6%, with only one farm out of 23 tested negatives for MVV.

1.5.1 Etiology

SRLV are related to human, simian, feline, and bovine immunodeficiency viruses, as well as horse infectious anaemia virus. The CAE virus and the MV virus have a tight association, and cross-species transmission is possible, especially when infected milk from one species is fed to the other (Pritchard and Dawson, 2000). The SRLV genome is a positive sense single-stranded RNA with three genes: GAG, POL and ENV. GAG gene is involved in the synthesis of structural protein: matrix (MA, p17), capsid (CA, p25), and nucleocapsid (NC, p14); POL gene encodes for enzymatic function involved in viral replication: reverse transcription and integration (protease (PRO), reverse transcriptase (RT), RNaseH, dUTPase, and integrase (INT); ENV gene encodes for envelope protein: trans-membrane proteins (TM, gp46); and surface subunit (SU, gp135) (Vigne et al, 1982; Desrosiers 2007); (Fig 4).

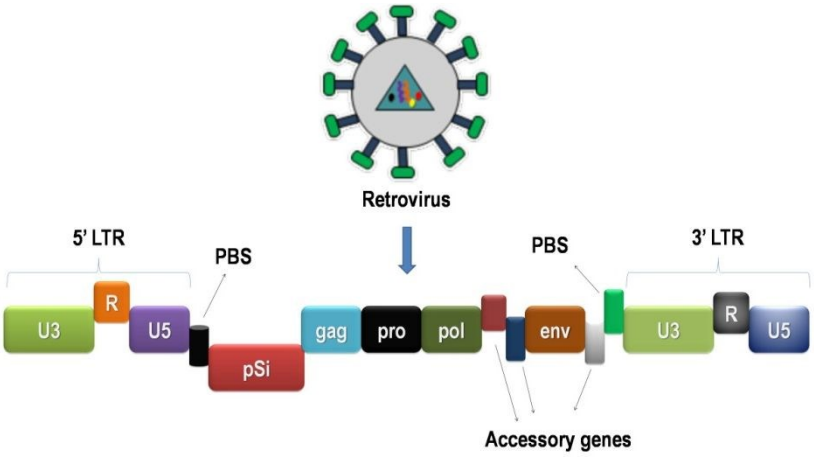


Fig. 4 *Retrovirus genome: gag, pol and env are the three structural genes that encode the proteins of the virus. The group-specific gene (gag), pol, and envelope (env) genes, which are flanked by the R regions and code for capsid, reverse*

transcriptase, integrase, protease, and envelope proteins, are the three important protein-coding genes. The 5′ and 3′ long terminal repeat (LTR) sequences, which they control gene expression, are used to identify retroviruses. Every LTR has two non-protein-coding regions, U5 at the 5′ end and U3 at the 3′ end, that encode regulatory elements. The R region is a short repetitive sequence at every end of the genome that ensures accurate end-to-end transfer in the expanding chain during reverse transcription (Saxena et al., 2016).

There are other auxiliary genes along the lentivirus genome: REV is a gene that permits unspliced and incompletely spliced viral mRNAs to be exported from the nucleus. (Toohey and Haase, 1994; Tiley et al, 1990). It is hypothesized that it could be also responsible for the formation of viral particles (Mazarin et al, 1990); another accessory gene called TAT, like to HIV-1 vpr, may be important in the nuclear localisation of the pre-integration complex (Villet et al, 2003); finally, the third accessory gene, named VIF, seems to be implicated in the inhibition of Apobec3 family members, like HIV-1 vif (Kristbjornsdottir 2004). Due to the large genetic variability, SRLVs are divided in five genotypes named from A to E, that differ from each other in 25–37% of their nucleotide sequences. Groups A and B are subdivided in subtypes A1–A15 and B1–B3, respectively. Group A consists of MVV strains, while group B consists of CAEV strains (Minguijon et al, 2015). The other three groups refer to strains which have been isolated from Norway (group C), Spain and Switzerland (group D) (Gjersetet al, 2006; Glaria et al, 2009), and Italy (group E). Group E is divided into two subtypes, E1 and E2, from northern Italy and Sardinia, respectively (Grego et al, 2009; Reina et al, 2010; Bertolotti et al, 2013). Subtypes A5 and A7, as well as groups C and D, have been isolated only in goats, while subtypes A2 seem to circulate only in sheep. Subtypes A1, A3, A4, A6, B1 and B2 have been found in both species (Olech et al, 2012). Recently, a new strain, named CRF01_ABSRLV, was isolated from Canadian goats. Probably this new strain was developed because of the recombination between of A2 and B1 subtypes (L'Homme et al, 2015).

1.5.2 Risk factors and pathogenesis

Susceptibility to the infection does not appear to be influenced by sex or age, while the breed could be a predisposing factor (Straub, 2004). The main route of transmission to lambs is colostrum or contaminated milk. The infection can also be transmitted by direct contact between animals (Dawson, 1980). MVV transmission between and within flocks is influenced by several risk factors. The close contact between animals and the likelihood of airborne transmission by respiratory droplets and coughing, favouring horizontal transmission according to flock size, animal density, and intensive farming. (Pérez et al, 2010; Lago et al, 2012; Michiels et al, 2018; Schaller et al, 2000; Junkuszewa et al, 2016; Shuaib et al, 2010; Blacklaws, 2012). The increased secretions in animals, co-infected of Jaagsiekte sheep retrovirus (JRSV) enhances the probability of MV transmission (Perez, 2010; Blacklaws, 2004). Poor cleaning and disinfection of milking equipment (Blacklaws, 2004; Minguijón, 2015), the use of the same needle or unsterilized surgical instruments on several animals, poor farm cleanliness, and sharing pastures with other flocks are all significant risk factors for MVV horizontal transmission. (Lago et al., 2012; Shuaib et al., 2010). Seroprevalence has also been shown to be greater in mixed-species flocks (sheep and goats), probably due to cross-species transmission of various SRLV strains (Lago et al., 2012; Alba et al., 2008). SRLVs show a tropism for several cells: monocyte/macrophage and dendritic cells, epithelial cells in the mammary gland, endothelial cells, and dendritic cells in the nervous system (Narayan et al., 1982; Narayan et al., 1983 Ryan et al., 2000; Lerondelle et al., 1999; Stowring et al., 1985). Transplacental transmission of the infection is debatable, whereas transit of lamb during partorition appears to be a route of infection (Blacklaws et al., 2004; Broughton-Neiswanger et al., 2010). The infection is spread over the mucosal barrier via macrophages and dendritic cells, and it is transmitted by colostrum or respiratory secretions (direct contact or emission of aerosolized droplets) (Blacklaws et al., 2004; McNeilly et al., 2008; McNeilly et al., 2007; Niesalla et al., 2008). Infected macrophages can reach myeloid stem cells in the bone marrow, allowing the provirus to integrate and infect the animal for the rest of its life (Gendelman et al, 1985; Grossi et al, 2005). The virus disseminates into the lymph nodes through the dendritic cells, and with macrophages spread into bloodstream allowing systemic infection (Ryan et al, 2000; McNeilly et al, 2008). Immature macrophages carry the virus into tissues where these cells mature and allow virus replication (Peluso et al., 1985; Haase., 1986). Virus replication is slow, so the number of

infected blood cells in bloodstream is quite low (McNeilly et al., 2008). Immune responses to viral replication and antigens generate chronic inflammation (Haase, 1986). The MV virus can survive in an inactive form (integrated provirus) inside monocytes and macrophages for a long time, and clinical signs are not always present (Blacklaws, 2012). Some factors such as age, the coexistence of multiple pathologies and environmental stress favor the moltiplication of the virus in animals, causing clinical manifestations of the disease. The infected animals seroconverted approximately 6 months after infection. Histopathological abnormalities in one or more target organs, such as the lung, central nervous system, mammary glands, and joints, may occur after a period of 1-2 years. The onset of histological lesions does not always coincide with the onset of symptoms, and the animal can stay in a preclinical state for months or years. Many sheep remain asymptomatic carriers for the rest of the life, and only small percentage of seropositive animals (25–30%) show clinical signs. The course of the disease is predictable once clinical signs appear, and it is ultimately fatal (Christodoulopoulos, 2006).

1.5.3 Clinical signs

The viral strain, the host immunological response, and the host genetic profile for virus resistance or susceptibility are factors influencing clinical manifestation of SRLV infection (Reina et al, 2013; Gayo et al, 2018). Lesions of SRLVs infection in tissues and organs are caused both by the immune response to the viral antigens and the viral replication (Blacklaws, 2012). The tissues and organs target of MVV are lungs, mammary gland, nervous system, and joints. (Blacklaws, 2012; Minguijon et al., 2015). The most common clinical manifestations of MVV in sheep are pneumonia and mastitis (Blacklaws, 2012). Lymphocytic hyperplasia can also manifest itself in the liver, heart, and kidneys (Angelopoulou et al., 2006; Brellou et al., 2007). Multiple-organ infection can occur as the disease progresses. However, the severity of lesions depends on organs involvment (Minguijon et al, 2015). Dyspnoea and elevated respiratory rate are two clinical symptoms (Minguijon et al, 2015, López, and Martinson, 2017); ataxia, weakness in the hind limbs, incoordination, paresis, are signs of the neurological system (Blacklaws, 2012; Minguijon et al., 2015). Virus can induce an indurative non-suppurative interstitial mastitis

(Blacklaws, 2012; Minguijon et al., 2015); more rarely, signs of arthritis may appear (Blacklaws, 2012, Christodoulopoulos, 2016; Gomez-Lucia et al., 2018, Minguijon et al., 2015).

1.5.4 Diagnosis

A timely and accurate diagnosis of MV is required for effective disease control and eradication in ovine flocks. Serological tests currently used to make diagnoses are mainly enzyme-linked immunosorbent assays (ELISAs) to detect specific antibodies against the virus (Kaba et al., 2013). Seropositive animals are considered infected and must be removed from the flock. There are significant barriers in disease eradication, such as immune response to disease, and seroconversion takes a long time resulting in animals that became carriers of virus (Hüttner et al., 2010; Azkur and Aslan, 2011). Available assays for detection of antibodies are agar gel immunodiffusion (AGID) test, radioimmunoprecipitation assay (RIPA), Western blotting (WB) and ELISA. PCR is used for detection of pro-viral DNA. Peripheral blood mononuclear cells are used for viral detection (Reina et al, 2009; Herrmann-Hoesing, 2010). Advantage of PCR is to detect infection prior to the seroconversion. Serological tests may be used in conjunction with PCR to solve the problem caused by lack of specific universal primers (Peterhans et al., 2004; Reina et al., 2009; Herrmann-Hoesing, 2010). Nested-PCR techniques and Real-Time PCR increase method sensitivity and specificity; nonetheless, they are less used (Reina et al., 2009; Herrmann-Hoesing, 2010., Carrozza et al., 2010). Until today, no vaccine or cure are available against MV.

2. Aim of the project

Animal farming can be considered one of the most important productive sectors of the Sardinia Island. In Sardinia, about 3.5 million sheep are bred, representing more than 40% of the national population with regional agricultural turnover (25%). Animal infectious disease can decrease the gross income. This can be observed especially during acute outbreaks. The economic impact of chronic diseases on farm earning is underestimated. Directly losses are mostly indicated by poorer milk outputs and less desiderable milk composition. Early identification of pathogenic microorganisms is of primary importance in order to plan prophylactic measures suitable to control animal but also to identify the presence of zoonotic agents potentially dangerous to human health. This project has been financed by the PON Programma Operativo Nazionale "Ricerca e Innovazione" in collaboration with NUREX S.r.l.
The project aimed to the detection of microorganism that cause some common diseases in Sardinia and are difficult to detect by classic routine analysis. Bulk Tank Milk (BTM) analysis were used for monitoring health status in herds. BTM analysis is less expansive, more convenient, and faster than testing samples from individual animals or groups of sheep. The aim of this study is: i) standardization and validation of new diagnostic methods for the detection of pathogens in sheep's milk; ii) epidemiological analysis of collected data according to different parameters (etiological agents, type and characteristics of the farms and geographical location); iii) implementation of operative protocols in order to reduce infections caused by the selected microorganisms. The research was targeted to different microorganisms that cause pathologies of considerable economic and health impact. The selected pathogens were: *Coxiella burnetii*, *Mycobacterium avium* sub. *Paratuberculosis* (Map), *Mycoplasma agalactiae* (Mag), *Small Ruminant Lentivirus* (SRLV).

3. Materials and Methods

3.1 Sample collection

The milk samples were collected from different herds of sheep from various geographical areas of Sardinia. BTM samples were taken from the Tank of the companies registered with ARAS (Regional Breeders Association of Sardinia) and transported in cool boxes to the laboratory (Department of Veterinary Medicine, infectious diseases section, University of Sassari), where they were tested for the identification of the pathogens of interest. A total of 1000 unpasteurized sheep milk samples were collected from dairy herds in Sardinia between 2014 and 2015. The samples were stored at -20°C for DNA extraction. The samples were taken in a sterile condition, kept a 4°C during transport. The samples were immediately transfered to the diagnostic laboratory and were stored at -20°C without using any preservatives.

3.1.1 Isolation of milk somatic cells

- Falcon tubes containing 20 ml of milk were centrifuged for 15 min at 3500 g at 4 °C;
- 1 mL of skim milk was transferred to 1.5 mL microtubes and frozen at -20 °C;
- Supernatant was subsequently discarded;
- Cellular pellets were washed twice with Phosphate Buffered Saline (PBS) and resuspended in 0.2 mL of PBS and stored at -20 °C;

3.1.2 DNA extraction from BTM

DNA extraction was performed on 1000 BTM samples with an automated nucleic acid extraction method using an automatic high-throughput "robot" developed by Nurex s.r.l., based on the use of paramagnetic

beads with high affinity for nucleic acids. The microscopic paramagnetic beads bind with different nucleic acids molecules. The commercial kit used for the isolation of total nucleic acids is the MagMAX Total Nucleic Acid Isolation Kit (Life technologies). The first phase is characterized by the homogenization of the sample with zirconia beads (present inside the bead tubes, supplied in the kit) by the use of TissueLyser (Qiagen). Process takes 15 minutes and uses a frequency of 30Hz in a solution of guanidinium thiocyanate, which causes a rapid release of nucleic acids and simultaneously inactivates the nucleases. Purification of DNA was performed by the automatic extractor. The samples were eluited with isopropanol and a resin containing the paramagnetic beads is added. Beads / nucleic acids complexes were immobilized with magnet fingers and washed to remove proteins and other contaminants. Finally, DNA was eluted in a small volume (≈ 50µl) of low salt concentration buffer, or in sterile MilliQ H2O. The DNA obtained was subjected to quantitative and qualitative evaluation by means of a UV-visible spectrophotometer (Biophotmeter, Eppendorf) which allowed the determination of both the concentration and the degree of purity of our DNA (A260 / A280 and A260 / A230).

3.1.3 Preparation of plasmid standards

PCR profile details are shown in the following table:

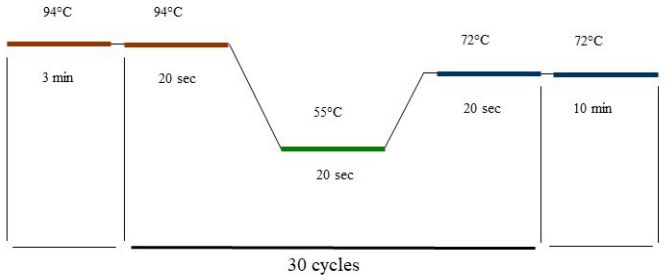
Reagent	Volume
CoralLoad buffer 10X	5µL
Dntp 10 mM	1µL
Primer forward	1.5µL
Primer reverse	1.5µL
Taq DNA polymerase	0.25µL
Template DNA	variable
H_2OMQ	to 50 µL

Composition of the reaction mix of PCR (QIAGEN Kit)

C. burnetii specific PCR primers were designed to amplify an 86 bp portion of the IS1111. Primers were:

- cox_FOR (5'-GATAGCCCGATAAGCATCAAC-3')
- cox_REV (5'-GCATTCGTATATCCGGCATC-3')

The following reaction profile was used: initial denaturation: 3 minutes at 94°C; 30 cycles of: denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds, extension to 72 ° C for 20 seconds and final extension to 72°C for 10 minutes.

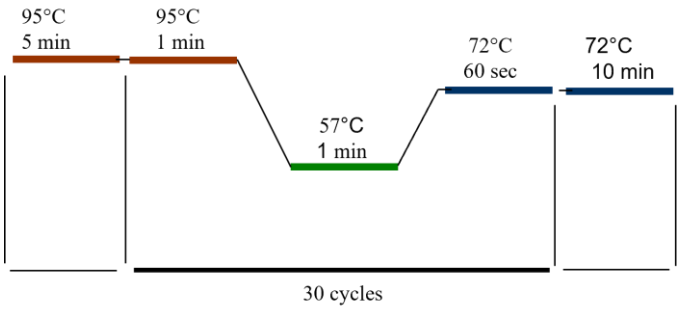


Schematic representation of the PCR protocol with primers cox_FOR+cox_REV

To detect Mycoplasma agalactiae (Mag), primers were:

- FS1 (5'-AAAGGTGCTTGAGAAATGGC-3')
- FS2 (5'-GTTGCAGAAGAAAGTCCAATCA-3');

The following reaction profile was used: initial denaturation 5 minutes at 95°C; 30 cycles of denaturation at 95°C for 60 seconds, 57°C for 60 seconds, 72°C for 60 seconds and 72°C for 10 minutes.



Schematic representation of the PCR protocol with primers FS1+FS2

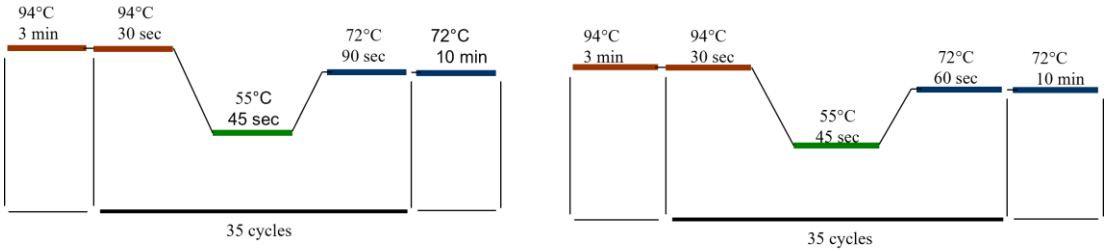
To detect maedi-visna virus (MVV) the specific nested-PCR primers were designed to amplify an 816 bp portion of primers targeting a conserved region of the gag gene mapped into the reference sequence CAEV-Cork (Genbank accession number M33677) between nucleotides 1070-1801 (Grego et al.,2007). In the first PCR primers used to amplify DNA fragments (1320 bp) were:

- GAG F1 (5'-TGGTGARKCTAGMTAGAGACATGG-3')
- GAG R1 (5'-CATAGGRGGHGCGGACGGCASCA-3');

The following reaction profile was used: initial denaturation 3 minutes at 94°C; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension to 72°C for 90 seconds and final extension to 72°C for 10 minutes. The samples were re-amplified after the first PCR to increase the sensitivity of the reaction. In the second PCR primers were used to amplify DNA fragments (816 bp) were:

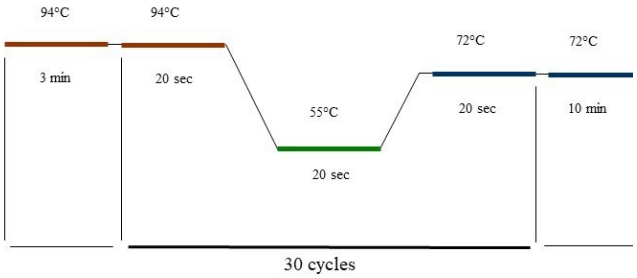
- GAG F2 (5'-CAAACWGTRGCAATGCAGCATGG-3')
- GAG R2 (5'-GCGGACGGCASCACAGG-3')

The following reaction profile was used: initial denaturation 3 minutes at 94°C; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension to 72°C for 60 seconds and final extension to 72°C for 10 minutes.



Schematic representation of the nested-PCR protocol with primers GAGF1+GAGR1; GAGF2+GAFR2

To detect *M. avium* subsp. *paratuberculosis* (MAP) specific PCR primers were designed to amplify a 108 bp portion of the insertion element IS900, (present as 14–18 copies in Map genome). Primers were: IS900_F (5'- GATGGCCGAAGGAGATTG-3') and IS900_R (5'-CACAACCACCTCCGTAACC-3'), with the following reaction profile: initial denaturation 3 minutes at 94°C; 30 cycles of: denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds, extension to 72 ° C for 20 seconds and final extension to 72°C for 10 minutes.



Schematic representation of the PCR protocol with primers IS900_F+IS900_R

3.1.4 Agarose gel electrophoresis

2% agarose gels were prepared in 1X TAE buffer. After boiling GelRed® (BIOTUM, Italian Chemical Society, Rome) was added to final 1X concentration and mixed. GelRed® was used for analytical visualization of DNA fragments. DNA samples supplemented with loading buffer were loaded into wells together with 1kb plus DNA ladder (Invitrogen). Agarose gel electrophoresis was performed at 80V, and DNA was visualized with the GelDoc EZ® system (Biorad).

3.1.5 Cloning and sequencing

Two positive samples were purified with the kit DNA Clean & concentrator™ (Zymo Research, Italia, Cat n° D4033) with the following protocol: five volumes of DNA Binding Buffer have been added to the PCR products; the mix was transferred to a provided column in a collection tube and was centrifuged for 30 seconds at 16,000×g. One sample was quantified with the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific). Purified samples were cloned into the plasmid pCR®2.1 vector (Invitrogen, Italia cat. N° K450002) (Fig 5).

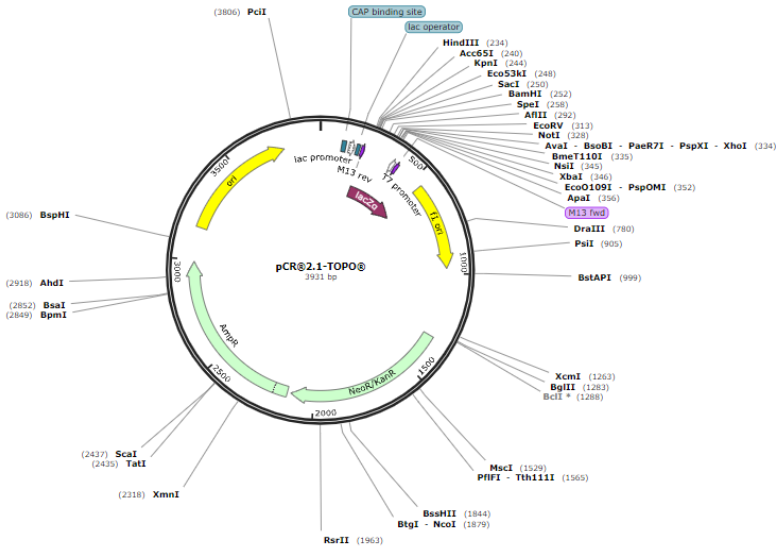


Fig.5 *pCR®2.1 vector (Invitrogen)*

- **First day.** Each amplified was subjected to a ligation reaction as shown in the table:

Reagent	Volume
Fresh PCR product	0.5-4 µL
Salt Solution	1µL
Water	Add to a total volume of 5 µL
TOPO vector	1µL
Final volume	**6µL**

Ligation mix

The reaction mix was incubated for 5 minutes at room temperature (22°C to 23°C).

- **Second day.** Transformation of chemically competent Escherichia coli cells (One shot TOP10 strain, Invitrogen) following the protocol provided by the kit. The tubes containing the ligation products were briefly centrifuged and placed on ice; the vials containing 50 µl of competent cells (One Shot ® Competent cells) were thawed on ice; 2 µl of ligation product were pipetted into the vials, mixed gently and incubated for 30 minutes on ice; the cells were subjected to thermal shock: 30 sec at 42 ° C and immediately transferred to ice; 250 µl of S.O.C. were added medium (previously brought to room temperature) with each vial. The vial was shaken (200 rpm) at 37°C for 1 hour. Two plates of LB agar containing ampicillin (100 µg / ml) were seeded for each cell vial. The plates were incubated at 37 ° C overnight.

- **Third day.** 2 colonies were selected for each plate. These were grown in 3 ml of LB with ampicillin (100 µg / ml) at 37 ° C overnight at 225 rpm.
- **Fourth day.** Plasmid was extracted from liquid medium cultures by purification using the ZuppyTM Plasmid miniprep Kit cat n ° D4020. 600 µL of bacterial culture grown in LB medium was added to a 1.5 ml microcentrifuge tube. The bacterial culture was centrifuged for 30 seconds at maximum speed. The supernatant was discarded, and the bacterial cell pellet was resuspended in 600 µL of H2OMQ. 20 µL of 7X Lysis Buffer (Blue) was added and mixed by inverting the

tube 4-6 times. 350 µL of cold Neutralization Buffer (Yellow) was added and the sample was inverted 2-3 times to ensure complete neutralization. The samples have been centrifuged at 11,000 – 16,000 x g for 2-4 minutes. The supernatant (~900µL) has been transferred into the provided Zymo-Spin™ IIN column. The column was placed in a collection tube and have been centrifuged for 15 seconds. Then it was eliminated the flow-through and the column was put back into the collection tube and was added 200 µL of Endo-Wash Buffer. They have been centrifuged foe 30 seconds and then was added 400 µl of Zyppy™ Wash Buffer to the column. After 1-minute centrifugation they were transferred into a 1.5 ml microcentrifuge tube. Into the column matrix was added 25 µL of H2OMQ and the samples have been left for 1 minute at room temperature. They were then centrifuged for 30 seconds to elute the plasmid DNA. The obtained plasmid minipreps were subjected to enzymatic digestion with EcoRI to verify the presence of the corresponding band. The following digestion mix was used: (20000U / ml) EcoRI HF 0.3µL, 10X cutsmart 3 µL, plasmid about 500 ng, H2OMQ up to 30 µL. The digestion product was subjected to electrophoretic running in 2% agarose gel. Then the ultraviolet light DNA bands were visualized using GelRed® Nucleic Acid Gel Stain (BIOTUM, Società Italiana Chimici, Rome) as a nucleic acid intercalator. The sample showing the expected size insert was sent to BMR Genomics s.r.l. of Padova for sequencing using the universal primers M13 Forward (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3'). A quantity of plasmid equal to 400-500 ng was placed in a 200 µL eppendorf. The plasmid was completely dried at 65 ° C and shipped. The sequences obtained in this study was processed with Chromas 2.2 software (Technelysium, Helensvale, Australia) and aligned with CLUSTALX version 2.0 (Larkin et al., 2007) to obtain the type sequences. These typical sequences were compared with the sequences deposited on the GenBank database by means of the BLASTN program (http://blast.ncbi.nlm.nih.gov/, Altschul et al., 1990).

3.1.6 Real time PCR

Real-time PCR, or qPCR (quantitative PCR), allows you to quantify the PCR product at each amplification cycle in real time. A quantitative analysis of the amount of initial DNA template is thus carried out with the emission of fluorescence emitted by fluorophores at each amplification cycle. These probes equipped with a high-energy fluorophore (reporter) and a fluorophore inhibitor (quencher).

3.1.7 Standard DNA construction

After sequencing the plasmids containing the inserts of our interest, the recombinant plasmids specific for each microorganism analyzed were used to construct the standard curve.

3.1.8 qPCR setup and standard curve

For each microorganism (*C. burnetii*, Mag, MVV and MAP) the real-time PCR was performed with specific primers and probes. β-actin was used as the housekeeping gene. Serial dilutions from 1×10^9 copies to a single copy of the sequence were used to establish a standard curve. The qPCR was run on three replicates of each dilution to establish a measure of intra-assay variation and single replicates of each dilution.

3.1.9 Real-Time PCR assay

To detect *C. Burnetii*, Real time PCR assay was performed to amplify an 86 bp target of the IS1111 sequence using the primers cox_FOR (5'-GATAGCCCGATAAGCATCAAC-3'and reverse (cox_REV: 5'-GCATTCGTATATCCGGCATC-3'), and the cox probe (5'-FAM-TCATCAAGGCACAAT-3') (Panning et al., 2008). Sensitivity for this qPCR was determined to be 100 copies of the target sequence per microliter. Cycle threshold was obtained with scalar dilutions of pCR2.1/IS1111 plasmid.

To detect Mag primers and probes were designed complementary to the coding region for the P48 lipoprotein of Mag; MAG48 FORWARD (5'-TTCAGGAACACCTCAAGCTACTACA-3'), MAGP48 REVERSE (5'-TGAACCAGCAACAGGGTAAGAA-3') and MAGP48 probe (5'-FAM-TAACTCTGTGGTTAAAGCT-3'). Cycle threshold was obtained with scalar dilutions of pCR2.1/MAGP48 plasmid. For MVV detection, specific primers and probes were design to amplify a conserve regions of gag / pol gene using v3.0 Software (Applied Biosystems). A further set of primers and probe was designed to target ruminant β-actin encoding gene as the internal control of the reaction. Sensitivity for this qPCR was determined to be 100 copies of the target sequence per microliter. To detect MAP, Real time PCR assay was performed to amplify 108 bp target of the sequence using the primers IS900_F: (5'-GATGGCCGAAGGAGATTG-3') and IS900_R (5'-CACAACCACCTCCGTAACC -3') and IS900 probe (5'-FAM-ATTGGATCGCTGTGTAAGGACACGT -3'). Sensitivity for this qPCR was determined with scalar dilutions from pCR2.1/IS900 plasmid. For each microorganism the standard curve (Cycle threshold vs copy number) was obtained through scalar dilutions of the plasmid containing the target gene in H2O milli Q, subsequently amplified in triplicate for each dilution. The ct values corresponding to the different dilutions were thus obtained. Luna® Universal qPCR Master Mix kit (New England Biolabs) with the following protocol was used:

COMPONENTS	20 µL REACTION	FINAL CONCENTRATION
Luna Universal qPCR Mix	10 µL	1X
Forward primer (10 µM)	0.5 µL	0.25 µM
Reverse primer (10 µM)	0.5µL	0.25 µM
Template DNA	variable	<100 ng
Nuclease-free Water	to 20 µL	

Composition of the reaction mix (Luna Universal qPCR Master Mix Kit)

3.1.10 Quantitative Real-Time PCR

The amplifications were performed with real-time thermal cycler CFX96 (Bio-Rad) with the following program: 1 minute at 95°C, 40 cycles for denaturation at 95°C for 15 seconds and extension at 60 °C for 30 seconds. The plasmids constructed for each microorganism with the sequence of the target gene inside are used as standard construct the standard curve for the calculation of copy number.

4. Results

4.1 Real-time PCR (*C. Burnetii*)

A total of 806 samples of BTM have been examined in qPCR for detection of *C. Burnetii* DNA. The samples analyzed were from different areas of Sardinia. Bulk tank milk samples representing 79 herds result positive for the presence of *C. Burnetii* DNA. Cycle threshold vs copy number was obtained with scalar dilutions ranging from 100 to 100000 copies/µl of pCR2.1/IS1111 plasmid. Samples with a cycle threshold (CT) value less than or equal to 36.5 were considered positive (Fig 6).

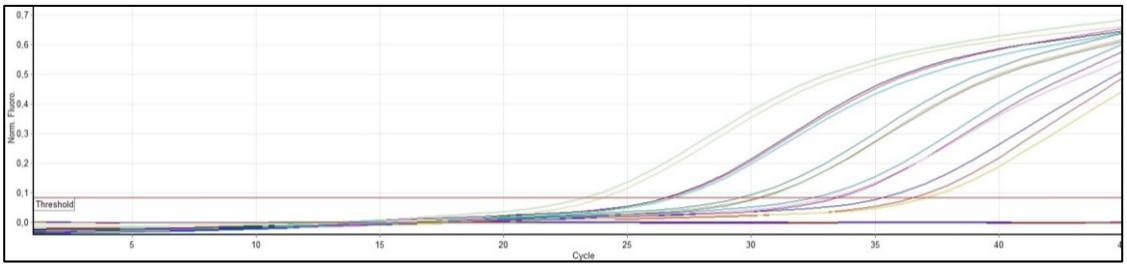


Fig.6 *Standard curve obtained by amplifying scalar dilutions of pCR2.1/ IS1111 with cox_FOR/cox_REV and cox_probe.*

4.1.2 Interpretation of the data

The sample is defined as compliant if an amplification curve is found similar to that observed in the positive control. Samples are tested in duplicate.

Each sample was evaluated individually in the replicates following the scheme following: two positivity

= positive sample; two negativities = negative sample; one positivity and one negativity = doubtful sample and repetition of the amplification.

4.1.3 Geographic distribution of positive herds

Herds positive for shedding of *C. burnetii* DNA were identified in different areas of Sardinia (Tab.1). The number of positive samples was highest in the center of Sardinia (50%) than in North (13.6%) and South (2.5%). Out of 806 tested samples, the total number of *coxiella* positives in all the geographical areas considered was 79 samples (9,8%). The number of positive samples out of the 806 tested divided by geographical area is as follows: North Sardinia (3,6%), Center Sardinia (4,6%) and South Sardinia (1,6%) (Tab.1; Fig 7).

	Positive samples / n° geogr. area	Positive samples/ n° tot. Samples
North	29/214 (13.6%)	29/806 (3,6%)
Center	37/74 (50%)	37/806 (4.6%)
South	13/518 (2.5%)	13/806 (1.6%)
Total	**79/806 (9.8%)**	

Tab.1 *Geographic distribution of positive samples a coxiella in dairy herds of Sardinia.*

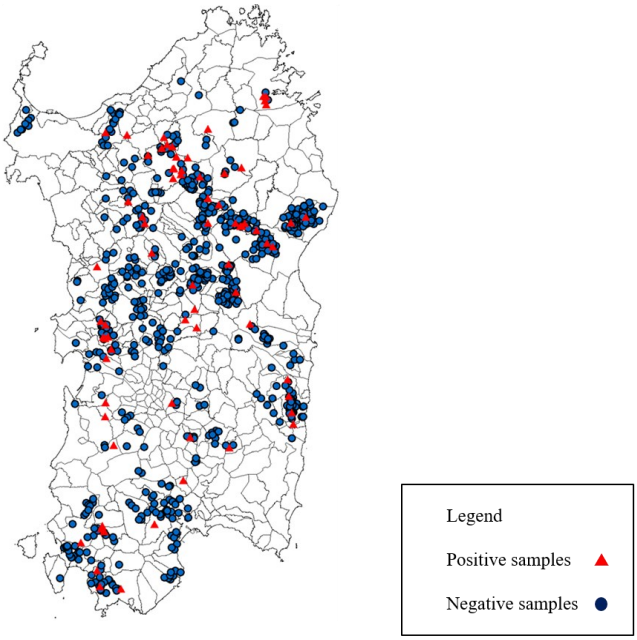


Fig.7 *Geographic Information System (GIS). Geographic distribuiton of coxiella samples milk positive and negative*.

Some of the positive samples were tested in PCR have confirmed results obtained in qPCR. The IS1111 insertion sequence appareance was confirmed by its amplification from samples (Fig 8).

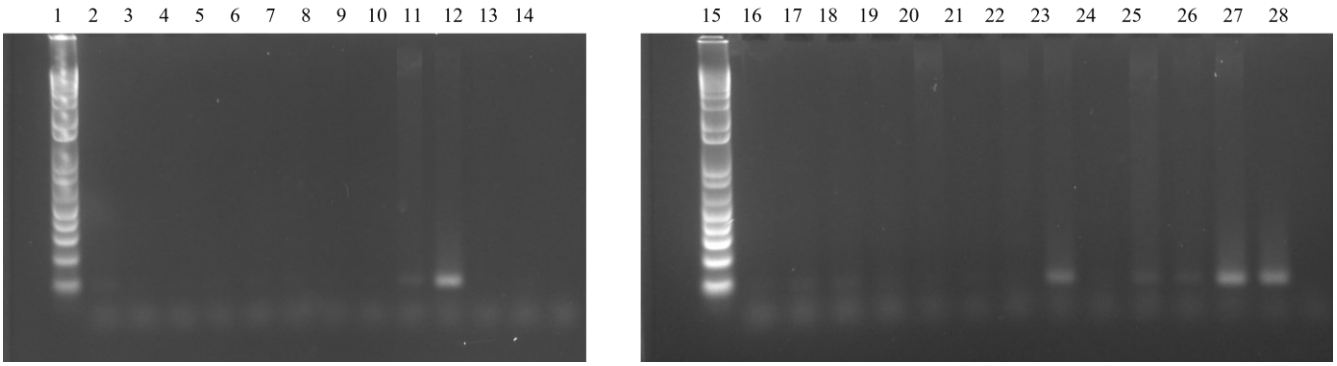


Fig.8 *Agarose gel**:** amplification of IS1111 insertion sequence (86bp).**1**: 1kb plus DNA ladder;**2-14**: BTM samples;**15:1**kb plus DNA ladder;**16-27**: BTM samples; **28**: positive control.*

Samples were sent for sequencing to BMR Genomics s.r.l. (Padova). The analysis of sequence (45 nucleotides length) with BLASTN showed a 100% similarity with Coxiella burnetii, confirming the specificity of the PCR tool:

- cox1_col2_F CTTGCATAATTCATCAAGGCACCAATGGTGGCCAATTTAAATTGT

4.2 Real-time PCR (*M. agalactiae*)

A total of 1000 samples of BTM have been examined in qPCR for detection of Mag DNA. Bulk tank milk samples representing 65 herds were positive for the presence of Mag DNA. Cycle threshold vs copy number it was obtained with scalar dilutions ranging from 10 to 1.000.000 copies/µl of pCR2.1/Mag48 plsmid. Samples with a cycle threshold (CT) value less than or equal to 36.9 were considered positive (Fig 9).

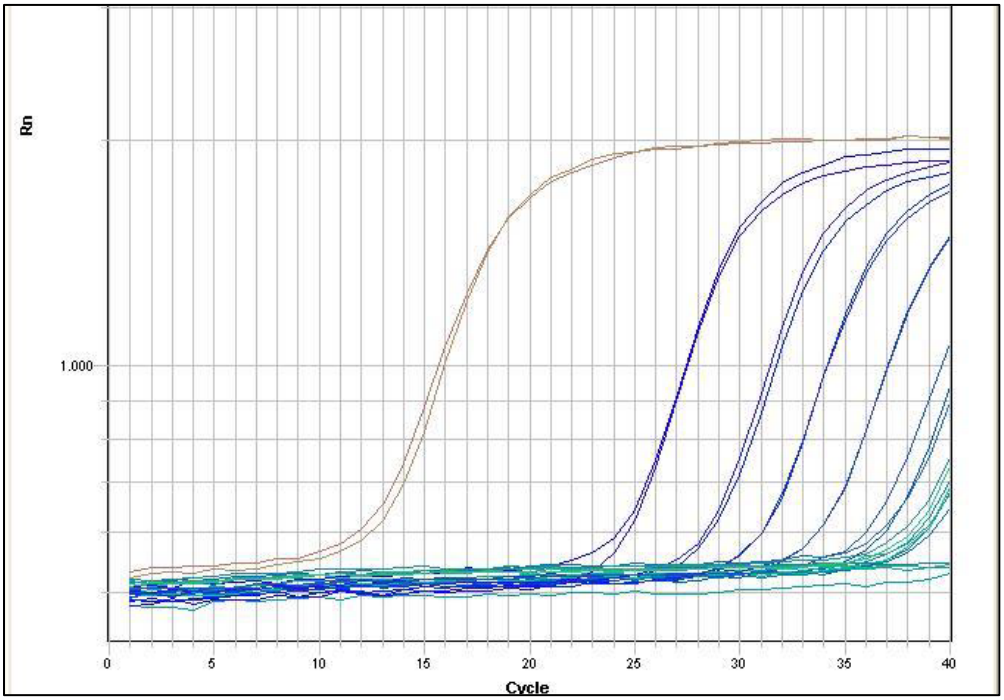


Fig.9 *Standard curve obtained by amplifying scalar dilutions of pCR2.1/P48 with MAG48 FORWARD/ MAGP48 REVERSE and MAGP48 PROBE.*

4.2.1 Interpretation of the data

The sample is defined as compliant if an amplification curve is found similar to that observed in the positive control. Samples are tested in duplicate. Each sample was evaluated individually in the replicates following the scheme following: two positivity = positive sample; two negativities = negative sample; one positivity and one negativity = doubtful sample and repetition of the amplification.

4.2.2 Geographic distribution of positive herds

Herds positive for shedding of Mag DNA were identified in different areas of Sardinia (Tab.2). The number of positive samples was highest in the center of Sardinia (16.8%) than in North (4.6%) and South

(1.9%). Out of 1000 tested samples, the total number of Mag positives in all the geographical areas considered was 65 samples (6.5%). While the number of positive samples out of the 1000 tested divided by geographical area is equal to: North Sardinia (1%), Center Sardinia (4,5%) and South Sardinia (1,3%) (Tab.2).

	Positive samples / n° geogr. area	Positive samples/ n° tot. Samples
North	10/214 (4.6%)	10/1000 (1%)
Center	45/268 (16.8%)	45/1000 (4.5%)
South	10/518 (1.9%)	13/1000 (1.3%)
Total	**65/1000 (6.5%)**	

Tab.2 *Geographic distribution of positive samples a Mag in dairy herds of Sardinia.*

Some of the positive samples were tested in PCR have confirmed results obtained in qPCR. The MAGP48 sequence appareance was confirmed by its amplification from samples (Fig 10).

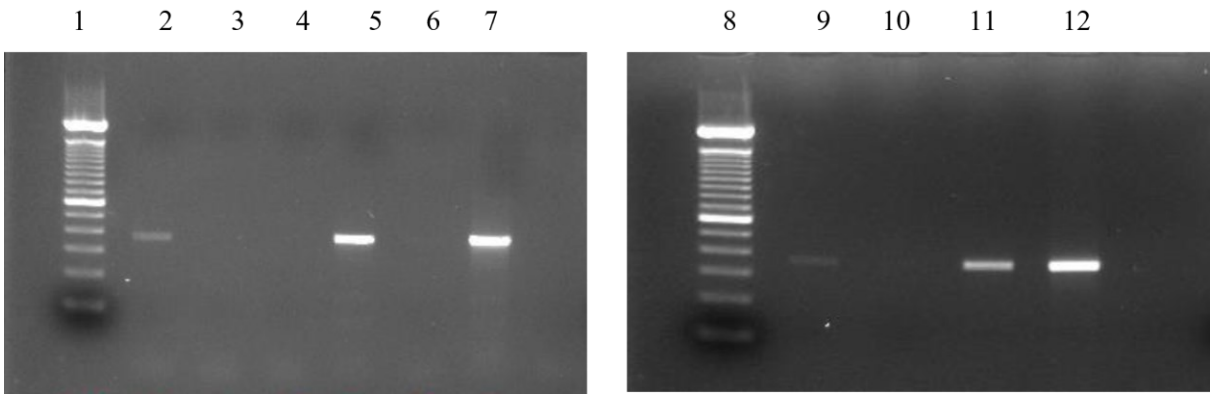


Fig.10 *Agarose gel**:** amplification of MAGP48 sequence (375bp).**1**: 1kb plus DNA ladder;**2-7**: BTM samples;**8**:1kb plus DNA ladder;**9-11**: BTM samples; **12**: positive control.*

Samples were sent for sequencing to BMR Genomics s.r.l. (Padova). The analysis of sequence (375bp nucleotides length) with BLASTN showed a 100% similarity with *Mycoplasma agalactiae*, confirming the specificity of the PCR tool.

4.3 Real-time PCR (Maedi-visna virus)

A total of 1000 samples of BTM have been examined in qPCR for detection Maedi-visna virus DNA. Bulk tank milk samples representing 55 herds were positive for the presence of MVV DNA. Cycle threshold vs copy number it was obtained with scalar dilutions ranging from 10 to 10000 copies/µl of pCR2.1/gag-pol plasmid. Samples with a cycle threshold (CT) value less than or equal to 38.8 were considered positive (Fig 11).

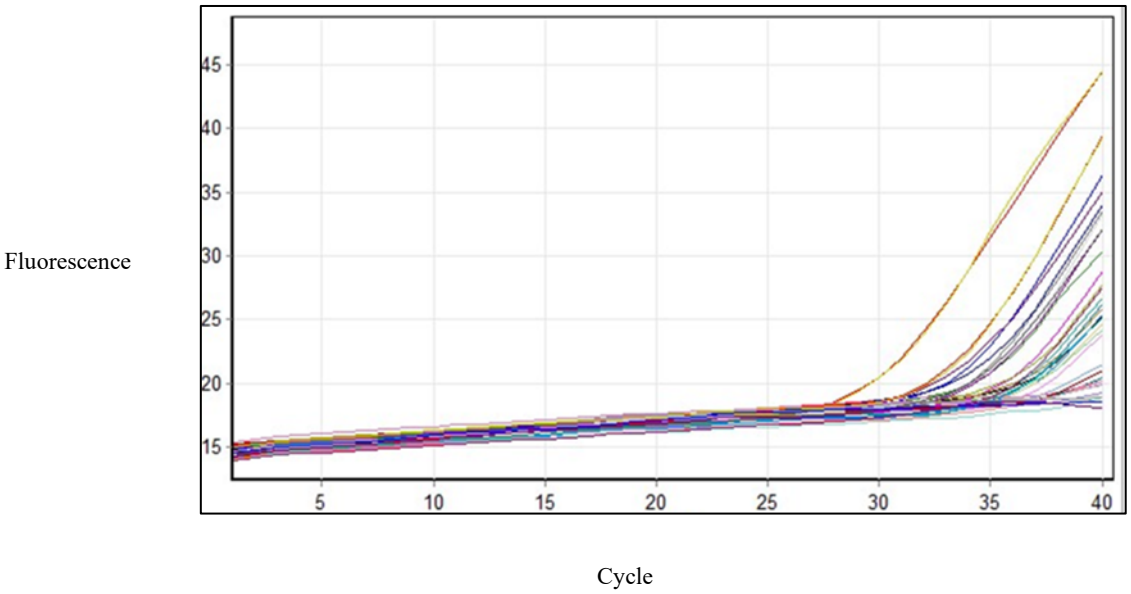


Fig.11 *Standard curve obtained by amplifying scalar dilutions of pCR2.1 / gag-pol plasmid with qGAG_fw_Monastir_Biorad primers with Monastir_Biorad probe*

4.3.1 Interpretation of the data

The sample is defined as compliant if an amplification curve is found similar to that observed in the positive control. Samples are tested in duplicate. Each sample was evaluated individually in the replicates following the scheme following: two positivity = positive sample; two negativities = negative sample; one positivity and one negativity = doubtful sample and repetition of the amplification.

4.3.2 Geographic distribution of positive herds

Herds positive for shedding of MVV DNA were identified in different areas of Sardinia. The number of positive samples was highest in the center of Sardinia (57.4%) than in North (46.2%) and South (21.8%). Out of 1000 tested samples, the total number of MVV positives in all the geographical areas considered was 366 samples (36.6%). While the number of positive samples out of the 1000 tested divided by geographical area is equal to: Center Sardinia (9.9%) North Sardinia (15.4%), and South Sardinia (11.3%) (Tab.3)

	Positive samples / n° geogr. area	Positive samples/ n° tot. Samples
North	99/214 (46.2%)	99/1000 (9.9%)
Center	154/268 (57.4%)	154/1000 (15.4%)
South	113/518 (21.8%)	113/1000 (11.3%)
Total	**366/1000 (36.6%)**	

Tab.3 *Geographic distribution of positive samples a MVV in dairy herds of Sardinia*

Some of the positive samples were tested in PCR have confirmed results obtained in qPCR. The gag/pol gene appareance was confirmed by its amplification from samples (Fig 12).

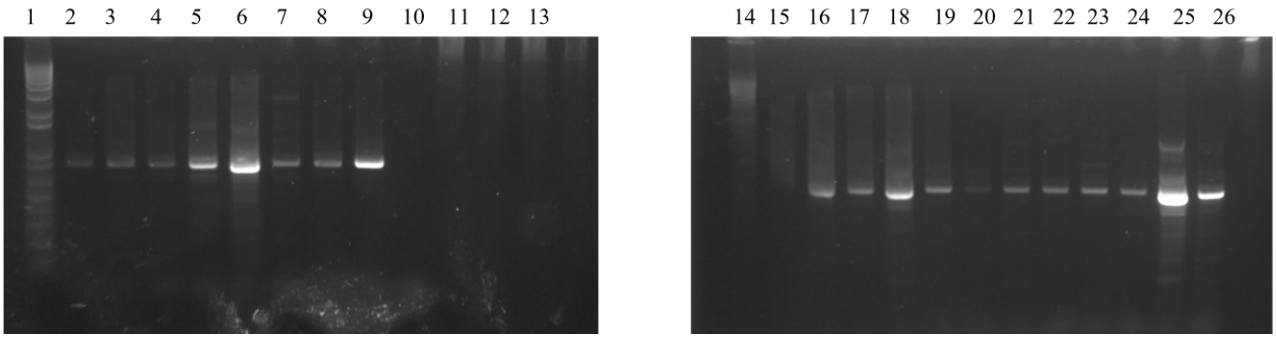


Fig.12 *Agarose gel**:** amplification of gag/pol gene (816bp).**1**:1kb plus DNA ladder;**2-13**: BTM sample;**14**:1kb plus DNA ladder;**15-25**: BTM samples; **26**: positive control.*

Samples were sent for sequencing to BMR Genomics s.r.l. (Padova). The analysis of sequence (816bp nucleotides length) with BLASTN showed a 100% similarity with MVV, confirming the specificity of the PCR tool.

4.3.3 Real-time PCR (MAP)

A total of 1000 samples of BTM have been examined in qPCR for detection of MAP DNA. Bulk tank milk samples representing 2 herds were positive for the presence of MAP DNA. Cycle threshold vs copy number it was obtained with scalar dilutions ranging from 10 to 1.000.000 copies/µl of pCR2.1/IS900 plasmid. Samples with a cycle threshold (CT) value less than or equal to 38.96 were considered positive.

4.3.4 Interpretation of the data

The sample is defined as compliant if an amplification curve is found similar to that observed in the positive control. Samples are tested in duplicate. Each sample was evaluated individually in the replicates following the scheme following: two positivity = positive sample; two negativities = negative sample; one positivity and one negativity = doubtful sample and repetition of the amplification. Some of

the positive samples were also tested in PCR. The IS900 sequence appareance was confirmed by its amplification from samples (Fig 13).

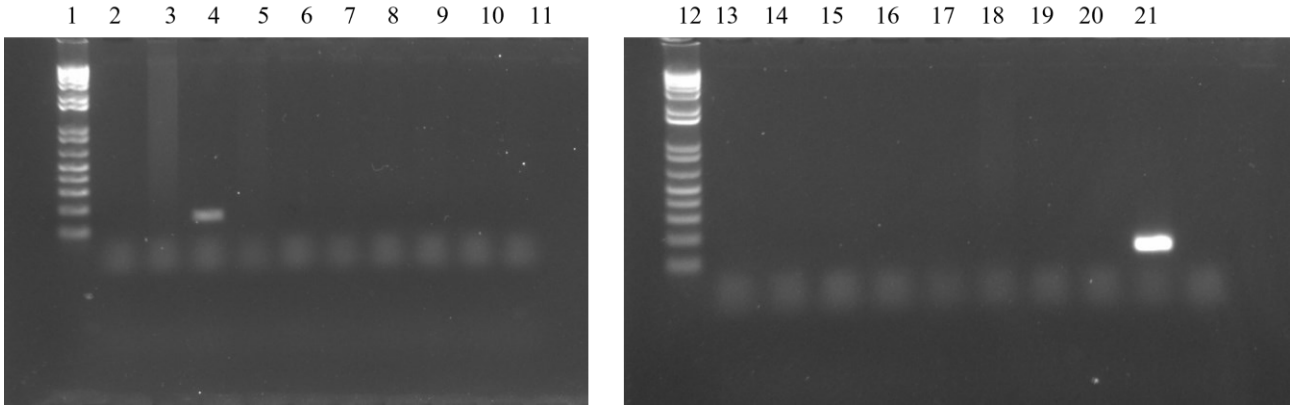


Fig.13 *Agarose gel**:** amplification of IS900 sequence (108bp).**1**: 1kb plus DNA ladder;**2-11**: BTM sample;**12**:1kb plus DNA ladder;**13-20**: BTM samples; **21**: positive control.*

Sample was sent for sequencing to BMR Genomics s.r.l. (Padova). The analysis of sequence (108 nucleotides length) with BLASTN showed a 100% similarity with MAP, confirming the specificity of the PCR tool.

5. Discussion and conclusion

The obtained results show that qPCR is a really sensitive diagnostic assay suggesting that it could be the "gold standard" for the detection of pathogens from biological samples. qPCR has high sensitivity, specificity and reproducibility and permit to analyze a large number of samples in a short time (Bottero and Dalmasso, 2011). Furthermore, this molecular assay allows timely diagnosis of infection by detection of very low quantity of the microorganism's DNA (Mackay, 2004). The methodology used in this project showed: i) the use of nucleic acid automatic extractor that has a sensitivity comparable to other methods; ii) the possibility to test more samples at a time, with less costs and in a short time. The use of Taq Man MGB (Minor groove binder) probe, FAM labeled (6-carboxy-fluorescein), allowed sensitive and specific detection of selected microorganisms. The detection of *C. burnetii* DNA in sheep BTM showed the presence of this important zoonotic pathogen in different areas of Sardinia. Positive samples number were 79/806 (9.8%). Etiological and pathogentic characteristics of coxiella, such as the ease of transmission through aerosol and food (raw milk), may constitute a risk for public health, as suggested by the increment of epidemic cases between 2009 and 2010 in livestock and in human in Netherlands. Seventyfive sheep and goat herds tested positive and there were over 3000 human cases of Q fever. People that are most exposed to coxiella infection are Veterinarians, laboratory staff, breeders, and slaughterers. For this reason, it would be important to establish a monitoring plan, control programs and prophylaxis activities aimed at limiting economic and health impact in the livestock and humans (Roest et al., 2011). In this project we developed qPCR assay for detecting and quantifying *M. agalactiae* DNA in BTM samples. For *M. agalactiae*, qPCR is more sensitive than traditional PCR. This assay is reproducible and linear in range of scalar orders of magnitude between 10^1 and 10^6 copies of plasmid DNA, ensuring a precise estimation of quantity of M. *agalactiae* DNA in milk samples. When compared to traditional PCR assay, qPCR takes less time for analysis of samples, shows minor contamination hazards, and has higher specificity because of the use of hybridization probes. Direct analysis of BTM samples by qPCR allows us to replace microbial cultures of Mag. Subclinical infected animals can shed the microorganism, therefore the use of this rapid, sensitive, and specific method is important for early

diagnosis. Rapid disease spread, the presence of different sources of infection, the vertical and horizontal transmission of the pathogen, are the principal causes of economy demage. For this reason, it would be necessary to implement research on microrganism epidemiology in domestic and wild animals, in order to manage and protect sheep populations from infection. This project allowed to confirm that a timely and accurate diagnosis of MV is also required to control and eradicate the disease in ovine flocks. Usually, for diagnoses Serological tests (enzyme-linked immunosorbent assays (ELISAs) were used to detect antibodies against the virus (Kaba et al., 2013). An important problem in disease eradication is the immune response to the disease beacause it takes a long time before clinical signs development, and the affected animals remain carriers of virus (Hüttner et al., 2010; Azkur and Aslan, 2011). The qPCR system developed in this project for MVV was used to evaluate the positivity of the BTM samples. This allowed us to confirm the development of a rapid and economical protocol based on proviral load of infected animals. The number of positive samples was 366/1000 (36.6%). The obtained results show the presence of this microorganism in several farms in Sardinia with high prevalence of positive samples in the central area of the island (57.4%) on the total number of farms sampled in this region. The obtained data are probably understimated because it has been shown in a previous study that aproximately 90% of sheep livestock presented at least one infected animal (Puggioni et al., 2007). Furthermore, it has been discovered that majority of sheep are genetically resistent to lentivirus infection. Transmembrane protein gene (TMEM154) has been identified as the responsible for this resistance in sheep (Heaton et al., 2012). For this reason, it would be appropiate to select genetically resistent animals in order to eradicate the disease. For this reason, it would be necessary to implement the number of samples in order to have a more suitable analysis. Finally, it would be important to increase appropriate monitoring and control plan with prophylaxis programmes, carryin out further research to verify the effects of the virus on public health. Among 1000 samples tested for MAP, only 2 samples resulted positive. This result is in contrast with the high positivity rate described in Sardinia before and this could be due to the different matrice used in this study. In fact, MAP is mainly present in fat milk whereas in this work the DNA was extracted from somatic cells. Early detection of microorganisms is of primary importance to allow prophylactic measures aimed to contain diseases diffusion in animals, and to identify the presence of zoonotic agents

that could represent a serious danger for human health (Rahman et al., 2020). The detection of microorganisms is extremely complex and articulated due to the multitude of bacteria genera and species, virus groups and fungi that can be found in milk (Tonamo et al., 2020).

References

Alba, A.; Allepuz, A.; Serrano, E.; Casal, J. Seroprevalence and spatial distribution of Maedi–Visna virus and pestiviruses in Catalonia (Spain). Small Rumin. Res. 2008, 78, 80–86

Alberti A, Robino P, Chessa B, Rosati S, Addis MF, Mercier P, Mannelli A, Cubeddu T, Profiti M, Bandino E, Thiery R, Pittau M. Characterisation of Mycoplasma capricolum P60 surface lipoprotein and its evaluation in a recombinant ELISA. Vet Microbiol. 2008 Apr 1;128(1-2):81-9. doi: 10.1016/j.vetmic.2007.09.020. Epub 2007 Oct 2. PMID: 17981406.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 Oct 5;215(3):403-10. doi: 10.1016/S0022-2836(05)80360-2. PMID: 2231712.

Amano K, Williams JC. Chemical and immunological characterization of lipopolysaccharides from phase I and phase II Coxiella burnetii. J Bacteriol. 1984 Dec;160(3):994-1002. doi: 10.1128/jb.160.3.994-1002.1984. PMID: 6438066; PMCID: PMC215808.

Angelakis E, Raoult D. Q Fever. Vet Microbiol. 2010 Jan 27;140(3-4):297-309. doi: 10.1016/j.vetmic.2009.07.016. Epub 2009 Aug 8. PMID: 19875249.

Angelopoulou, K.; Brellou, G.D.; Vlemmas, I. Detection of Maedi-Visna Virus in the Kidneys of Naturally Infected Sheep. J. Comp. Path. 2006, 134, 329–335.

Ariza-Miguel J, Rodríguez-Lázaro D, Hernández M. Molecular characterization of Mycoplasma agalactiae reveals the presence of an endemic clone in Spain. J Clin Microbiol. 2013 Feb;51(2):656-60. doi: 10.1128/JCM.02835-12. Epub 2012 Dec 5. PMID: 23224102; PMCID: PMC3553876.

Arricau Bouvery N, Souriau A, Lechopier P, Rodolakis A. Experimental Coxiella burnetii infection in pregnant goats: excretion routes. Vet Res. 2003 Jul-Aug;34(4):423-33. doi: 10.1051/vetres:2003017. PMID: 12911859.

Astruc, J. M., F. Barillet, A. Carta, M. Fioretti, E. Gootwine, D. Kompan, F. J. Romberg, and E. Ugarte. 2008. Report of the working group on milk recording of sheep. Pages 275‑282 in Proc. 36th Biennial Session of the International Committee for Animal Recording (ICAR), Niagara Falls, NY. ICAR technical series no. 13. Identification, breeding, production, health and recording on farm animals. J. D. Slatter, ed. ICAR, Rome, Italy.

Autschbach F, Eisold S, Hinz U, Zinser S, Linnebacher M, Giese T, Löffler T, Büchler MW, Schmidt J. High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. Gut. 2005 Jul;54(7):944-9. doi: 10.1136/gut.2004.045526. PMID: 15951539; PMCID: PMC1774626.

Ayele WY, Bartos M, Svastova P, Pavlik I. Distribution of Mycobacterium avium subsp. paratuberculosis in organs of naturally infected bull-calves and breeding bulls. Vet Microbiol. 2004 Nov 15;103(3-4):209-17. doi: 10.1016/j.vetmic.2004.07.011. PMID: 15504592.

Azkur, A.K.; Aslan, M.E. Serological and Epidemiological Investigation of Bluetongue, Maedi-Visna and Caprine Arthritis-Encephalitis Viruses in Small Ruminant in Kirikkale District in Turkey. Kafkas Univ. Vetr. Fak. Derg. 2011, 17, 803–808.

Babudieri B, Moscovici C. experimental and natural infection of birds by Coxiella burneti. Nature. 1952 Feb 2;169(4292):195-6. doi: 10.1038/169195a0. PMID: 14910721.

Bashiruddin JB, Frey J, Königsson MH, Johansson KE, Hotzel H, Diller R, de Santis P, Botelho A, Ayling RD, Nicholas RA, Thiaucourt F, Sachse K. Evaluation of PCR systems for the identification and differentiation of Mycoplasma agalactiae and Mycoplasma bovis: a collaborative trial. Vet J. 2005 Mar;169(2):268-75. doi: 10.1016/j.tvjl.2004.01.018. PMID: 15727920.

Bastida F, Juste RA. Paratuberculosis control: a review with a focus on vaccination. J Immune Based Ther Vaccines. 2011 Oct 31;9:8. doi: 10.1186/1476-8518-9-8. PMID: 22035107; PMCID: PMC3222599.

Beare PA, Samuel JE, Howe D, Virtaneva K, Porcella SF, Heinzen RA. Genetic diversity of the Q fever agent, Coxiella burnetii, assessed by microarray-based whole-genome comparisons. J Bacteriol. 2006 Apr;188(7):2309-24. doi: 10.1128/JB.188.7.2309-2324.2006. PMID: 16547017; PMCID: PMC1428397.

Benedictus A, Mitchell RM, Linde-Widmann M, Sweeney R, Fyock T, Schukken YH, Whitlock RH. Transmission parameters of Mycobacterium avium subspecies paratuberculosis infections in a dairy herd going through a control program. Prev Vet Med. 2008 Mar 17;83(3-4):215-27. doi: 10.1016/j.prevetmed.2007.07.008. Epub 2007 Sep 14. PMID: 17868937.

Bengis RG, Kock RA, Fischer J. Infectious animal diseases: the wildlife/livestock interface. Rev Sci Tech. 2002 Apr;21(1):53-65. doi: 10.20506/rst.21.1.1322. PMID: 11974630.

Bergonier D, De Simone F, Russo P, Solsona M, Lambert M, Poumarat F. Variable expression and geographic distribution of Mycoplasma agalactiae surface epitopes demonstrated with monoclonal antibodies. FEMS Microbiol Lett. 1996 Oct 1;143(2-3):159-65. doi: 10.1111/j.1574-6968.1996.tb08475.x. PMID: 8837468.

Bergonier D, Berthelot X, Poumarat F. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. *OIE Review in Scienific Techique.* 1997;16(3):848–873.

Berri M, Souriau A, Crosby M, Rodolakis A. Shedding of Coxiella burnetii in ewes in two pregnancies following an episode of Coxiella abortion in a sheep flock. Vet Microbiol. 2002 Feb 26;85(1):55-60. doi: 10.1016/s0378-1135(01)00480-1. PMID: 11792492.

Bertolotti, L., Reina, R., Mazzei, M., Preziuso, S., Camero, M., Carrozza, M.L., Cavalli, A., Juganaru, M., Profiti, M., De Meneghi, D., Perona, G., Renzoni, G., Tursi, M., Bertoni, G., Rosati, S., 2013. Small ruminant lentivirus genotype B and E interaction: evidences on the role of Roccaverano strain on reducing proviral load of the challenging CAEV strain. Vet. Microbiol. 163, 33–41.

Bittante G, Cipolat-Gotet C, Pazzola M, Dettori ML, Vacca GM, Cecchinato A. Genetic analysis of coagulation properties, curd firming modeling, milk yield, composition, and acidity in Sarda dairy sheep. J Dairy Sci. 2017 Jan;100(1):385-394. doi: 10.3168/jds.2016-11212. Epub 2016 Oct 27. PMID: 28341048.

Blacklaws BA, Berriatua E, Torsteinsdottir S, Watt NJ, de Andres D, Klein D, Harkiss GD. Transmission of small ruminant lentiviruses. Vet Microbiol. 2004 Jul 14;101(3):199-208. doi: 10.1016/j.vetmic.2004.04.006. PMID: 15223124.

Blacklaws BA. Small ruminant lentiviruses: immunopathogenesis of visna-maedi and caprine arthritis and encephalitis virus. Comp Immunol Microbiol Infect Dis. 2012 May;35(3):259-69

Bohach DM, Stegniy BT, Bohach MV, Pavlov SL, Bolotin VI. Age and Seasonal Pattern of Contagious Agalactia in Small Ruminants in Ukraine. J Vet Res. 2021 Mar 9;65(1):67-72. doi: 10.2478/jvetres-2021-0014. PMID: 33817397; PMCID: PMC8009590.

Boor KJ. Fluid dairy product quality and safety: looking to the future. J Dairy Sci. 2001 Jan;84(1):1-11. doi: 10.3168/jds.s0022-0302(01)74445-1. PMID: 11210019.

Boor KJ, Wiedmann M, Murphy S, Alcaine S. A 100-Year Review: Microbiology and safety of milk handling. J Dairy Sci. 2017 Dec;100(12):9933-9951. doi: 10.3168/jds.2017-12969. PMID: 29153181.

Bosshard C, Stephan R, Tasara T. Application of an F57 sequence-based real-time PCR assay for Mycobacterium paratuberculosis detection in bulk tank raw milk and slaughtered healthy dairy cows. J Food Prot. 2006 Jul;69(7):1662-7. doi: 10.4315/0362-028x-69.7.1662. PMID: 16865901.

Bottero MT, Dalmasso A. Animal species identification in food products: evolution of biomolecular methods. Vet J. 2011 Oct;190(1):34-8. doi: 10.1016/j.tvjl.2010.09.024. Epub 2010 Oct 30. PMID: 21041103.

Bower K, Begg DJ, Whittington RJ. Optimisation of culture of Mycobacterium avium subspecies paratuberculosis from blood samples. J Microbiol Methods. 2010 Jan;80(1):93-9. doi: 10.1016/j.mimet.2009.11.005. Epub 2009 Nov 22. PMID: 19932719.

Brellou, G.D.; Angelopoulou, K.; Poutahidis, T.; Vlemmas, I. Detection of Maedi-Visna Virus in the Liver and Heart of Naturally Infected Sheep. J. Comp. Path. 2007, 136, 27–35 López, A.; Martinson,

S.A. Respiratory System, Mediastinum, and Pleurae, In Pathologic Basis of Veterinary Disease, 6th ed.; Zachary, J.F., Ed.; Mosby: St. Louis, MO, USA, 2017; pp. 471–560.

Bridre J, Donatien A. Le microbe de l'agalaxie contagieuse et sa culture *in vitro* . *Comptes Rendus de l'Académie des Sciences.* 1923;177:841–843.

Bridre J, Donatien A. Le microbe de l'agalaxie du mouton et de la chèvre. *Annals of Institute of Pasteur.* 1925;39:925–951.

Broughton-Neiswanger, L.E.; White, S.N.; Knowles, D.P.; Mousel, M.R.; Lewis, G.S.; Herndon, D.R.; Herrmann-Hoesing, L.M. Non-maternal transmission is the major mode of ovine lentivirus transmission in a ewe flock: A molecular epidemiology study. Infect. Genet. Evol. 2010, 10, 998–1007

Buhariwalla F, Cann B, Marrie TJ. A dog-related outbreak of Q fever. Clin Infect Dis. 1996 Oct;23(4):753-5. doi: 10.1093/clinids/23.4.753. PMID: 8909839.

Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J. Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. J Clin Microbiol. 2003 Jul;41(7):2915-23. doi: 10.1128/JCM.41.7.2915-2923.2003. PMID: 12843021; PMCID: PMC165291.

Cacciotto C, Addis MF, Pagnozzi D, Chessa B, Coradduzza E, Carcangiu L, Uzzau S, Alberti A, Pittau M. The liposoluble proteome of Mycoplasma agalactiae: an insight into the minimal protein complement of a bacterial membrane. BMC Microbiol. 2010 Aug 25;10:225. doi: 10.1186/1471-2180-10-225. PMID: 20738845; PMCID: PMC2941501.

Caporale VP, Foglini A, Lelli R, Mantovani A, Nannini D, Simoni P. Preliminary observations on the presence of visna-maedi in Italy. Vet Res Commun. 1983 Jan;6(1):31-5. doi: 10.1007/BF02214893. PMID: 6868345.

Carrozza, M.L.; Mazzei, M.; Bandecchi, P.; Fraisier, C.; Pérez, M.; Suzan-Monti, M.; de Andrés, D.; Amorena, B.; Rosati, S.; Andrésdottir, V.; et al. Development and comparison of strain specific gag and pol real-time PCR assays for the detection of Visna/maedi virus. J. Virol. Methods. 2010, 165, 161–167

Carta A, Casu S, Salaris S. Invited review: Current state of genetic improvement in dairy sheep. J Dairy Sci. 2009 Dec;92(12):5814-33. doi: 10.3168/jds.2009-2479. PMID: 19923587.

Casu, Sara & Marie-Etancelin, Christel & Robert-Granié, Christèle & Barillet, Francis & Carta, Antonello. (2008). Evolution during the productive life and individual variability of milk emission at machine milking in Sardinian× Lacaune back-cross ewes. Small Ruminant Research. 75. 7-16. 10.1016/j.smallrumres.2007.07.003.

Chessa B, Pittau M, Puricelli M, Zobba R, Coradduzza E, Dall'ara P, Rosati S, Poli G, Alberti A. Genetic immunization with the immunodominant antigen P48 of Mycoplasma agalactiae stimulates a mixed adaptive immune response in BALBc mice. Res Vet Sci. 2009 Jun;86(3):414-20. doi: 10.1016/j.rvsc.2008.09.010. Epub 2008 Nov 13. PMID: 19007952.

Chisu V, Mura L, Foxi C, Masala G. Coxiellaceae in Ticks from Human, Domestic and Wild Hosts from Sardinia, Italy: High Diversity of Coxiella-like Endosymbionts. Acta Parasitol. 2021 Jun;66(2):654-663. doi: 10.1007/s11686-020-00324-w. Epub 2021 Jan 25. PMID: 33492605.

Cirone F, Maggiolino A, Cirilli M, Sposato A, De Palo P, Ciappetta G, Pratelli A. Small ruminant lentiviruses in goats in southern Italy: Serological evidence, risk factors and implementation of control programs. Vet Microbiol. 2019 Jan;228:143-146. doi: 10.1016/j.vetmic.2018.11.023. Epub 2018 Nov 23. PMID: 30593360.

COTTEREAU, Ph; LAVAL, Arlette; JEANPERT, A. Identification du Mœdi dans l'Est de la France. *Bulletin de l'Académie Vétérinaire de France*, 1977.

Cottew GS, Watson WA, Erdag O, Arisoy F. Mycoplasmas of caprine pleuropneumonia in turkey and their relationship to other mycoplasmas of goats and M. mycoides var. mycoides. J Comp Pathol. 1969 Oct;79(4):541-51. doi: 10.1016/0021-9975(69)90075-9. PMID: 5351418.

Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Kluver P. Mycobacteria distenct from Mycobacterium avium subsp. paratuberculosis isolated from the faeces of ruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis. Mol Cell Probes. 1999 Dec;13(6):431-42. doi: 10.1006/mcpr.1999.0275. PMID: 10657148.

Christodoulopoulos G, Maedi–Visna: Clinical review and short reference on the disease status in Mediterranean countries, Small Ruminant Research,Volume 62, Issues 1–2, 2006, Pages 47-53, ISSN 0921-4488,

Cross AR, Baldwin VM, Roy S, Essex-Lopresti AE, Prior JL, Harmer NJ. Zoonoses under our noses. Microbes Infect. 2019 Jan-Feb;21(1):10-19. doi: 10.1016/j.micinf.2018.06.001. Epub 2018 Jun 18. PMID: 29913297; PMCID: PMC6386771.

da Massa AJ, Brooks DL. The external ear canal of goats and other animals as a mycoplasma habitat. *Small Ruminant Research.* 1991;4(1):85–93.

Dawson, M., 1980. Maedi/Visna: a review. Vet. Rec. 106, 212–216

Denison AM, Massung RF, Thompson HA. Analysis of the O-antigen biosynthesis regions of phase II isolates of Coxiella burnetii. FEMS Microbiol Lett. 2007 Feb;267(1):102-7. doi: 10.1111/j.1574-6968.2006.00544.x. Epub 2006 Nov 29. PMID: 17156123.

Desrosiers RC, Nonhuman lentiviruses. In: Knipe DM, Howley PM, editors. Fields virology. 5th ed. Philadelphia: Lippincott, Williams & Wilkins; 2007. p. 2215–43

Domingue GJ Sr, Woody HB. Bacterial persistence and expression of disease. Clin Microbiol Rev. 1997 Apr;10(2):320-44. doi: 10.1128/CMR.10.2.320. PMID: 9105757; PMCID: PMC172922.

Dukes TW, Greig AS, Corner AH. Maedi-visna in Canadian sheep. Can J Comp Med. 1979 Jul;43(3):313-20. PMID: 226248; PMCID: PMC1319895.

EDWARD DG. A difference in growth requirements between bacteria in the L-phase and organisms of the pleuropneumonia group. J Gen Microbiol. 1953 Apr;8(2):256-62. doi: 10.1099/00221287-8-2-256. PMID: 13044906.

EFSA BIOHAZ Panel Scientific Opinion on the public health risks related to the consumption of raw drinking milk. EFSA J. 2015;13(1):3940.

EFSA Scientific Opinion on Q fever. EFSA J., 8 (2010), p. 1595

Eldin C, Mélenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, Mege JL, Maurin M, Raoult D. From Q Fever to Coxiella burnetii Infection: a Paradigm Change. Clin Microbiol Rev. 2017 Jan;30(1):115-190. doi: 10.1128/CMR.00045-16. PMID: 27856520; PMCID: PMC5217791.

Ellingson JL, Bolin CA, Stabel JR. Identification of a gene unique to Mycobacterium avium subspecies paratuberculosis and application to diagnosis of paratuberculosis. Mol Cell Probes. 1998 Jun;12(3):133-42. doi: 10.1006/mcpr.1998.0167. PMID: 9664574.

Ellingson JL, Anderson JL, Koziczkowski JJ, Radcliff RP, Sloan SJ, Allen SE, Sullivan NM. Detection of viable Mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR. J Food Prot. 2005 May;68(5):966-72. doi: 10.4315/0362-028x-68.5.966. PMID: 15895728.

Englund S, Ballagi-Pordány A, Bölske G, Johansson KE. Single PCR and nested PCR with a mimic molecule for detection of Mycobacterium avium subsp. paratuberculosis. Diagn Microbiol Infect Dis. 1999 Mar;33(3):163-71. doi: 10.1016/s0732-8893(98)00098-4. PMID: 10092965.

Englund S, Bölske G, Johansson KE. An IS900-like sequence found in a Mycobacterium sp. other than Mycobacterium avium subsp. paratuberculosis. FEMS Microbiol Lett. 2002 Apr 9;209(2):267-71. doi: 10.1111/j.1574-6968.2002.tb11142.x. PMID: 12007816.

Fang F, Ge Q, Li R, Lv J, Zhang Y, Feng A, Kelly GT, Wang H, Wang X, Song C, Wang T, Qian Z. LPS restores protective immunity in macrophages against Mycobacterium tuberculosis via autophagy. Mol Immunol. 2020 Aug;124:18-24. doi: 10.1016/j.molimm.2020.05.001. Epub 2020 May 30. PMID: 32485435.

Field P R, Mitchell J L, Santiago A, Dickeson D J, Chan S W, Ho D W T, Murphy A M, Cuzzubbo A J, Devine P L. Comparison of a commercial enzyme-linked immunosorbent assay with immunofluorescence and complement fixation tests for detection of *Coxiella burnetii* (Q fever) immunoglobulin M. J. Clin. Microbiol., 38 (2000), pp. 1645-1647

Fishbein DB, Raoult D. A cluster of Coxiella burnetii infections associated with exposure to vaccinated goats and their unpasteurized dairy products. Am J Trop Med Hyg. 1992 Jul;47(1):35-40. doi: 10.4269/ajtmh.1992.47.35. PMID: 1636881.

Flannagan RS, Jaumouillé V, Grinstein S. The cell biology of phagocytosis. Annu Rev Pathol. 2012;7:61-98. doi: 10.1146/annurev-pathol-011811-132445. Epub 2011 Sep 9. PMID: 21910624.

Fleury B, Bergonier D, Berthelot X, Schlatter Y, Frey J, Vilei EM. Characterization and analysis of a stable serotype-associated membrane protein (P30) of Mycoplasma agalactiae. J Clin Microbiol. 2001 Aug;39(8):2814-22. doi: 10.1128/JCM.39.8.2814-2822.2001. PMID: 11473997; PMCID: PMC88244.

Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM. Characterization of P40, a cytadhesin of Mycoplasma agalactiae. Infect Immun. 2002 Oct;70(10):5612-21. doi: 10.1128/IAI.70.10.5612-5621.2002. PMID: 12228289; PMCID: PMC128363.

Gayo, E.; Polledo, L.; Balseiro, A.; Martinez, C.P.; Garcia Iglesias, M.J.; Preziuso, S.; Rossi, G.; García Marín, J.F. Inflammatory Lesion Patterns in Target Organs of Visna/Maedi in Sheep and their Significance in the Pathogenesis and Diagnosis of the Infection. J. Comp. Pathol. 2018, 159, 49–56.

Gendelman HE, Narayan O, Molineaux S, Clements JE, Ghotbi Z. Slow, persistent replication of lentiviruses: role of tissue macrophages and macrophage precursors in bone marrow. Proc Natl Acad Sci USA 1985;82:7086–90.

Gilardoni LR, Paolicchi FA, Mundo SL. Bovine paratuberculosis: a review of the advantages and disadvantages of different diagnostic tests. Rev Argent Microbiol. 2012 Jul-Sep;44(3):201-15. PMID: 23102470.

GIMENEZ DF. Staining rickettsiae in yolk-sac cultures. stain Technol. 1964 May;39:135-40. doi: 10.3109/10520296409061219. PMID: 14157454.

Gislason, G. (1947). Thattir um innflutning bufjarsjukd6ma. Iceland Department of AgriculturePublication, pp. 235-257. Reykjavik.

Gjerset, B., Storset, A.K., Rimstad, E., 2006. Genetic diversity of small-ruminant lentiviruses: characterization of Norwegian isolates of caprine arthritis encephalitis virus. J. Gen. Virol. 87, 573–580.

Glaria, I., Reina, R., Crespo, H., de Andres, X., Ramirez, H., Biescas, E., Perez, M.M., Badiola, J., Lujan, L., Amorena, B., de Andres, D., 2009. Phylogenetic analysis of SRLV sequences from an arthritic sheep outbreak demonstrates the introduction of CAEV-like viruses among Spanish sheep. Vet. Microbiol. 138, 156–162.

Glazunova O, Roux V, Freylikman O, Sekeyova Z, Fournous G, Tyczka J, Tokarevich N, Kovacava E, Marrie TJ, Raoult D. Coxiella burnetii genotyping. Emerg Infect Dis. 2005 Aug;11(8):1211-7. doi: 10.3201/eid1108.041354. PMID: 16102309; PMCID: PMC3320512.

Gomez-Lucia, E.; Barquero, N.; Domenech, A. Maedi-Visna virus: Current perspectives. Vet. Med. 2018, 9, 11–21.

Gómez-Martín A, De la Fe C, Amores J, Sánchez A, Contreras A, Paterna A, Buendía AJ, Corrales JC. Anatomic location of Mycoplasma mycoides subsp. capri and Mycoplasma agalactiae in naturally

infected goat male auricular carriers. Vet Microbiol. 2012 Jun 15;157(3-4):355-62. doi: 10.1016/j.vetmic.2012.01.004. Epub 2012 Jan 11. PMID: 22284907.

Gómez-Martín A, Amores J, Paterna A, De la Fe C. Contagious agalactia due to Mycoplasma spp. in small dairy ruminants: epidemiology and prospects for diagnosis and control. Vet J. 2013 Oct;198(1):48-56. doi: 10.1016/j.tvjl.2013.04.015. Epub 2013 Jun 10. PMID: 23759248.

Gonzales-Barron U, Gonçalves-Tenório A, Rodrigues V, Cadavez V. Foodborne pathogens in raw milk and cheese of sheep and goat origin: a meta-analysis approach, Current Opinion in Food Science,Volume 18,2017,Pages 7-13,ISSN 2214-7993.

Grant IR, Ball HJ, Rowe MT. Effect of high-temperature, short-time (HTST) pasteurization on milk containing low numbers of Mycobacterium paratuberculosis. Lett Appl Microbiol. 1998 Feb;26(2):166-70. doi: 10.1046/j.1472-765x.1998.00309.x. PMID: 9569703.

Grant IR, O'Riordan LM, Ball HJ, Rowe MT. Incidence of Mycobacterium paratuberculosis in raw sheep and goats' milk in England, Wales and Northern Ireland. Vet Microbiol. 2001 Mar 20;79(2):123-31. doi: 10.1016/s0378-1135(00)00344-8. PMID: 11230934.

Grant IR, Kirk RB, Hitchings E, Rowe MT. Comparative evaluation of the MGIT and BACTEC culture systems for the recovery of Mycobacterium avium subsp. paratuberculosis from milk. J Appl Microbiol. 2003;95(1):196-201. doi: 10.1046/j.1365-2672.2003.02003.x. PMID: 12807471.

Grant IR. Zoonotic potential of Mycobacterium avium ssp. paratuberculosis: the current position. J Appl Microbiol. 2005;98(6):1282-93. doi: 10.1111/j.1365-2672.2005.02598.x. PMID: 15916642.

Greco G, Corrente M, Martella V, Pratelli A, Buonavoglia D. A multiplex-PCR for the diagnosis of contagious agalactia of sheep and goats. Mol Cell Probes. 2001 Feb;15(1):21-5. doi: 10.1006/mcpr.2000.0337. PMID: 11162077.

Grego E, Bertolotti L, Quasso A, Profiti M, Lacerenza D, Muz D, Rosati S. Genetic characterization of small ruminant lentivirus in Italian mixed flocks: evidence for a novel genotype circulating in a local goat population. J Gen Virol. 2007 Dec;88(Pt 12):3423-3427. doi: 10.1099/vir.0.83292-0. PMID: 18024912.

Grego, E., Lacerenza, D., Arias, R.R., Profiti, M., Rosati, S., 2009. Serological characterization of the new genotype E of small ruminant lentivirus in Roccaverano goat flocks. Vet. Res. Commun. 33, 137–140.

Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of Mycobacterium paratuberculosis. Nucleic Acids Res. 1989 Nov 25;17(22):9063-73. doi: 10.1093/nar/17.22.9063. PMID: 2555783; PMCID: PMC335114.

Grossi P, Giudice C, Bertoletti I, Cioccarelli G, Brocchi E, Cammarata G, et al. Immunohistochemical detection of the p27 capsid protein of caprine arthritis–encephalitis virus (CAEV) in bone-marrow cells of seropositive goats. J Comp Pathol 2005;133:197–200.

Haase AT, Stowring L, Narayan P, Griffin D, Price D. Slow persistent infection caused by visna virus: role of host restriction. Science 1977;195:175–7.

Hailat, N. Q., Hananeh, W., Metekia, A. S., Stabel, J. R., Al-Majali, A., & Lafi, S. (2010). Pathology of subclinical paratuberculosis (Johne's Disease) in Awassi sheep with reference to its occurrence in Jordan.

Hamilton KA, Ahmed W, Toze S, Haas CN. Human health risks for Legionella and Mycobacterium avium complex (MAC) from potable and non-potable uses of roof-harvested rainwater. Water Res. 2017 Aug 1;119:288-303. doi: 10.1016/j.watres.2017.04.004. Epub 2017 Apr 5. PMID: 28500949.

Harmache A, Russo P, Guiguen F, Vitu C, Vignoni M, Bouyac M, et al. Requirement of caprine arthritis encephalitis virus vif gene for in vivo replication. Virology 1996a;224:246–55;

Harmache A, Russo P, Vitu C, Guiguen F, Mornex JF, Pepin M, et al. Replication in goats in vivo of caprine arthritis–encephalitis virus deleted in vif or tat genes: possible use of these deletion mutants as live vaccines. AIDS Res Hum Retroviruses 1996b;12:409–11;

Herrmann-Hoesing, L.M. Diagnostic assays used to control small ruminant lentiviruses. J. Vet. Diagn. Investig. **2010**, 22, 843–855.

Hatchette TF, Hudson RC, Schlech WF, Campbell NA, Hatchette JE, Ratnam S, Raoult D, Donovan C, Marrie TJ. Goat-associated Q fever: a new disease in Newfoundland. Emerg Infect Dis. 2001 May-Jun;7(3):413-9. doi: 10.3201/eid0703.010308. PMID: 11384518; PMCID: PMC2631794.

Heaton MP, Clawson ML, Chitko-Mckown CG, Leymaster KA, Smith TP, Harhay GP, White SN, Herrmann-Hoesing LM, Mousel MR, Lewis GS, Kalbfleisch TS, Keen JE, Laegreid WW. Reduced lentivirus susceptibility in sheep with TMEM154 mutations. PLoS Genet. 2012 Jan;8(1):e1002467. doi: 10.1371/journal.pgen.1002467. Epub 2012 Jan 26. PMID: 22291605; PMCID: PMC3266874.

Heinzen RA, Hackstadt T, Samuel JE. Developmental biology of Coxiella burnettii. Trends Microbiol. 1999 Apr;7(4):149-54. doi: 10.1016/s0966-842x(99)01475-4. PMID: 10217829.

Hendrix LR, Samuel JE, Mallavia LP. Differentiation of Coxiella burnetii isolates by analysis of restriction-endonuclease-digested DNA separated by SDS-PAGE. J Gen Microbiol. 1991 Feb;137(2):269-76. doi: 10.1099/00221287-137-2-269. PMID: 1673152.

Hoff-Jørgensen, R. Slow virus infections with particular reference to maedi-visna and enzootic bovine leukaemia. *Vet Res Commun* **1,** 251–263 (1977).

Hogerwerf L, van den Brom R, Roest HI, Bouma A, Vellema P, Pieterse M, Dercksen D, Nielen M. Reduction of Coxiella burnetii prevalence by vaccination of goats and sheep, The Netherlands. Emerg Infect Dis. 2011 Mar;17(3):379-86. doi: 10.3201/eid1703.101157. PMID: 21392427; PMCID: PMC3166012.

Huntley JF, Stabel JR, Paustian ML, Reinhardt TA, Bannantine JP. Expression library immunization confers protection against Mycobacterium avium subsp. paratuberculosis infection. Infect Immun. 2005 Oct;73(10):6877-84. doi: 10.1128/IAI.73.10.6877-6884.2005. PMID: 16177367; PMCID: PMC1230947.

Hüttner, K.; Seelmann, M.; Feldhusen, F. Prevalence and risk factors for Maedi-Visna in sheep farms in Mecklenburg-Western-Pomerania. Berl. Münch. Tierärztl. 2010, 123, 463–467.,14

Ibrahim A, Norlander L, Macellaro A, Sjöstedt A. Specific detection of Coxiella burnetii through partial amplification of 23S rDNA. Eur J Epidemiol. 1997 Apr;13(3):329-34. doi: 10.1023/a:1007385104687. PMID: 9258533.

Jarand J, Davis JP, Cowie RL, Field SK, Fisher DA. Long-term Follow-up of Mycobacterium avium Complex Lung Disease in Patients Treated With Regimens Including Clofazimine and/or Rifampin. Chest. 2016 May;149(5):1285-93. doi: 10.1378/chest.15-0543. Epub 2016 Jan 12. PMID: 26513209.

Jarvis, Lovell S., and Pablo Valdes-Donoso. A selective review of the economic analysis of animal health management. Journal of agricultural economics 69.1 (2018): 201-225.

Junkuszewa, A.; Dudko, P.; Bojar, W.; Olech, M.; Osinski, Z.; Gruszecki, T.M.; Kania, M.G.; Ku´zmak, J.; Czerski, G. Risk factors associated with small ruminant lentivirus infection in eastern Poland sheep flocks. Prev. Vet. Med. 2016, 127, 44–49.

Kaba, J.; Czopowicz, M.; Ganter, M.; Nowicki, M.; Witkowski, L.; Nowicka, D.; Szalu´s-Jordanow, O. Risk factors associated with seropositivity to small ruminant lentiviruses in goat herds. Res. Vet. Sci. 2013, 94, 225–227

Karesh WB, Dobson A, Lloyd-Smith JO, Lubroth J, Dixon MA, Bennett M, Aldrich S, Harrington T, Formenty P, Loh EH, Machalaba CC, Thomas MJ, Heymann DL. Ecology of zoonoses: natural and unnatural histories. Lancet. 2012 Dec 1;380(9857):1936-45. doi: 10.1016/S0140-6736(12)61678-X. PMID: 23200502; PMCID: PMC7138068.

Kinde H, da Massa AJ, Wakenell PS, Petty R. Mycoplasma infection in a commercial goat dairy caused by *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *mycoides* (caprine biotype) *Journal of Veterinary Diagnostic Investigation.* 1994;6(4):423–427.

Kizil O, Ozdemir H. With mycoplasma agalactiae. *Bull Vet Inst Pulawy*, 2006, 50: 325-328.

Klee SR, Tyczka J, Ellerbrok H, Franz T, Linke S, Baljer G, Appel B. Highly sensitive real-time PCR for specific detection and quantification of Coxiella burnetii. BMC Microbiol. 2006 Jan 19;6:2. doi: 10.1186/1471-2180-6-2. PMID: 16423303; PMCID: PMC1360083.

Klempner MS, Shapiro DS. Crossing the species barrier--one small step to man, one giant leap to mankind. N Engl J Med. 2004 Mar 18;350(12):1171-2. doi: 10.1056/NEJMp048039. Epub 2004 Feb 25. PMID: 14985471.

Klinkenberg D, Koets A. The long subclinical phase of Mycobacterium avium ssp. paratuberculosis infections explained without adaptive immunity. Vet Res. 2015 Jun 19;46(1):63. doi: 10.1186/s13567-015-0202-3. PMID: 26092036; PMCID: PMC4473850.

Koets AP, Eda S, Sreevatsan S. The within host dynamics of Mycobacterium avium ssp. paratuberculosis infection in cattle: where time and place matter. Vet Res. 2015 Jun 19;46(1):61. doi: 10.1186/s13567-015-0185-0. PMID: 26092382; PMCID: PMC4473847.

Kristbjornsdottir HB, Andresdottir V, Svansson V, Torsteinsdottir S, Matthiasdottir S, Andresson OS. The vif gene of maedi-visna virus is essential for infectivity in vivo and in vitro. Virology 2004;318:350–9.

Kumar A, Verma AK, Gangwar N, Rahal A. Isolation, characterization and antibiogram of *Mycoplasma bovis* in sheep pneumonia. *Asian Journal of Animal and Veterinary Advances.* 2012;7(2):149–157.

Kumar A, Rahal A, Chakraborty S, Verma AK, Dhama K. Mycoplasma agalactiae, an Etiological Agent of Contagious Agalactia in Small Ruminants: A Review. Vet Med Int. 2014;2014:286752. doi: 10.1155/2014/286752. Epub 2014 Jul 3. PMID: 25097796; PMCID: PMC4109668.

Lambert M. Contagious agalactia of sheep and goats. *OIE Review in Scienific Techique.* 1987;6(3):699–711.

Lago, N.; López, C.; Panadero, R.; Cienfuegos, J.; Pato, A.; Prieto, P.; Díaz, P.; Mourazos, N.; Fernández, G.Seroprevalence and risk factors associated with Visna/Maedi virus in semi-intensive lamb-producing flocksin northwestern Spain. Prev. Vet. Med. 2012, 103, 163–169.

Langer AJ, Ayers T, Grass J, Lynch M, Angulo FJ, Mahon BE. Nonpasteurized dairy products, disease outbreaks, and state laws-United States, 1993-2006. Emerg Infect Dis. 2012 Mar;18(3):385-91. doi: 10.3201/eid1803.111370. PMID: 22377202; PMCID: PMC3309640

Leedom JM. Milk of nonhuman origin and infectious diseases in humans. Clin Infect Dis. 2006 Sep 1;43(5):610-5. doi: 10.1086/507035. Epub 2006 Jul 31. PMID: 16886155.

Lerondelle C, Godet M, Mornex JF. Infection of primary cultures of mammary epithelial cells by small ruminant lentiviruses. Vet Res 1999;30:467–74.

Leroux, C., Cruz, J.C. & Mornex, J.F. (2010). SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. Current HIV Research, 8, 94-100

Levisohn S, Davidson I, Caro Vergara MR, Rapoport E. Use of an ELISA for differential diagnosis of Mycoplasma agalactiae and M mycoides subspecies mycoides (LC) in naturally infected goat herds. Res Vet Sci. 1991 Jul;51(1):66-71. doi: 10.1016/0034-5288(91)90033-k. PMID: 1716774.

L'Homme, Y.; Leboeuf, A.; Arsenault, J.; Fras, M. Identification and characterization of an emerging small ruminant lentivirus circulating recombinant form (CRF). Virology 2015, 475, 159–171

Li L, Mahan CS, Palaci M, Horter L, Loeffelholz L, Johnson JL, Dietze R, Debanne SM, Joloba ML, Okwera A, Boom WH, Eisenach KD. Sputum Mycobacterium tuberculosis mRNA as a marker of bacteriologic clearance in response to antituberculosis therapy. J Clin Microbiol. 2010 Jan;48(1):46-51. doi: 10.1128/JCM.01526-09. Epub 2009 Nov 18. PMID: 19923475; PMCID: PMC2812283.

Lloyd-Smith JO, George D, Pepin KM. Epidemic dynamics at the human-animal interface. Science. 2009;326:1362–1367.

López, A.; Martinson, S.A. Respiratory System, Mediastinum, and Pleurae, In Pathologic Basis of Veterinary Disease, 6th ed.; Zachary, J.F., Ed.; Mosby: St. Louis, MO, USA, 2017; pp. 471–560

Lund BM, Gould GW, Rampling AM. Pasteurization of milk and the heat resistance of Mycobacterium avium subsp. paratuberculosis: a critical review of the data. Int J Food Microbiol. 2002 Jul 25;77(1-2):135-45. doi: 10.1016/s0168-1605(02)00057-0. Erratum in: Int J Food Microbiol 2002 Aug 25;77(3):245. PMID: 12076031.

Macciotta N.P.P, Mele M, Cappio-Borlino A, Secchiari P. Issues and perspectives in dairy sheep breeding. *Ital. J. Anim. Sci.* 2005; 4: 5-23.

Mackay IM. Real-time PCR in the microbiology laboratory. Clin Microbiol Infect. 2004 Mar;10(3):190-212. doi: 10.1111/j.1198-743x.2004.00722.x. PMID: 15008940.

Mallavia LP, Paretsky D. Physiology of rickettsiae. VII. Amino acid incorporation by Coxiella burnetii and by infected hosts. J Bacteriol. 1967 May;93(5):1479-83. doi: 10.1128/jb.93.5.1479-1483.1967. PMID: 6025436; PMCID: PMC276636.

McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to Mycobacterium paratuberculosis, as determined by DNA probes that distinguish between mycobacterial species. J Clin Microbiol. 1987 May;25(5):796-801. doi: 10.1128/jcm.25.5.796-801.1987. PMID: 2884232; PMCID: PMC266091.

Maltezou H C, Constantopoulou I, Kallergi C, Vlahou V, Georgakopoulos D, Kafetzis D A *et al.* Q fever in children in Greece. American Journal of Tropical Medicine and Hygiene, 70 (2004), pp. 540-544

Marrie TJ, Durant H, Williams JC, Mintz E, Waag DM. Exposure to parturient cats: a risk factor for acquisition of Q fever in Maritime Canada. J Infect Dis. 1988 Jul;158(1):101-8. doi: 10.1093/infdis/158.1.101. PMID: 3392409.

Masala G, Porcu R, Sanna G, Chessa G, Cillara G, Chisu V, Tola S. Occurrence, distribution, and role in abortion of Coxiella burnetii in sheep and goats in Sardinia, Italy. Vet Microbiol. 2004 Apr 19;99(3-4):301-5. doi: 10.1016/j.vetmic.2004.01.006. PMID: 15066733.

Masala G, Porcu R, Daga C, Denti S, Canu G, Patta C, Tola S. Detection of pathogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR. J Vet Diagn Invest. 2007 Jan;19(1):96-8. doi: 10.1177/104063870701900116. PMID: 17459840.

Maurin M, Raoult D. Q fever. Clin. Microbiol. Rev., 12 (1999), pp. 518-553

Mazarin V, Gourdou I, Querat G, Sauze N, Audoly G, Vitu C, et al. Subcellular localization of rev-gene product in visna virus-infected cells. Virology 1990;178:305–10

McArthur DB. Emerging Infectious Diseases. Nurs Clin North Am. 2019 Jun;54(2):297-311. doi: 10.1016/j.cnur.2019.02.006. Epub 2019 Mar 27. PMID: 31027668; PMCID: PMC7096727.

McGregor H, Dhand NK, Dhungyel OP, Whittington RJ. Transmission of Mycobacterium avium subsp. paratuberculosis: dose-response and age-based susceptibility in a sheep model. Prev Vet Med. 2012 Nov 1;107(1-2):76-84. doi: 10.1016/j.prevetmed.2012.05.014. Epub 2012 Jul 2. PMID: 22762894.

McNeil MR, Brennan PJ. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. Res Microbiol. 1991 May;142(4):451-63. doi: 10.1016/0923-2508(91)90120-y. PMID: 1871433.

Mediannikov O, Fenollar F, Socolovschi C, Diatta G, Bassene H, Molez JF, Sokhna C, Trape JF, Raoult D. Coxiella burnetii in humans and ticks in rural Senegal. PLoS Negl Trop Dis. 2010 Apr 6;4(4):e654. doi: 10.1371/journal.pntd.0000654. PMID: 20386603; PMCID: PMC2850317.

Michiels, R.; van Mael, E.; Quinet, C.; Welby, S.; Cay, A.B.; de Regge, N. Seroprevalence and risk factors related to small ruminant lentivirus infections in Belgian sheep and goats. Prev. Vet. Med. 2018, 151, 13–20.

Millar D, Ford J, Sanderson J, Withey S, Tizard M, Doran T, Hermon-Taylor J. IS900 PCR to detect Mycobacterium paratuberculosis in retail supplies of whole pasteurized cows' milk in England and Wales. Appl Environ Microbiol. 1996 Sep;62(9):3446-52. doi: 10.1128/aem.62.9.3446-3452.1996. PMID: 8795236; PMCID: PMC168142.

Minguijon, E., Reina, R., Perez, M., Polledo, L., Villoria, M., Ramirez, H., Leginagoikoa, I., Badiola, J.J., Garcia-Marin, J.F., de Andres, D., Lujan, L., Amorena, B., Juste, R.A., 2015. Small ruminant lentivirus infections and diseases. Vet. Microbiol. 181, 75–89.

McNeilly TN, Tennant P, Lujan L, Perez M, Harkiss GD. Differential infection efficiencies of peripheral lung and tracheal tissues in sheep infected with Visna/maedi virus via the respiratory tract. J Gen Virol 2007;88:670–9.

McNeilly TN, Baker A, Brown JK, Collie D, Maclachlan G, Rhind SM, et al. Role of alveolar macrophages in respiratory transmission of visna/maedi virus. J Virol 2008;82:1526–36.

Möbius P, Hotzel H, Rassbach A, Köhler H. Comparison of 13 single-round and nested PCR assays targeting IS900, ISMav2, f57 and locus 255 for detection of Mycobacterium avium subsp. paratuberculosis. Vet Microbiol. 2008 Jan 25;126(4):324-33. doi: 10.1016/j.vetmic.2007.07.016. Epub 2007 Jul 25. PMID: 17889455.

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis. 1995;1:7–15.

Moss MT, Sanderson JD, Tizard ML, Hermon-Taylor J, el-Zaatari FA, Markesich DC, Graham DY. Polymerase chain reaction detection of Mycobacterium paratuberculosis and Mycobacterium avium

subsp silvaticum in long term cultures from Crohn's disease and control tissues. Gut. 1992 Sep;33(9):1209-13. doi: 10.1136/gut.33.9.1209. PMID: 1427373; PMCID: PMC1379488.

Narayan O, Wolinsky JS, Clements JE, Strandberg JD, Griffin DE, Cork LC. Slow virus-replication: the role of macrophages in the persistence and expression of visna viruses of sheep and goats. J Gen Virol 1982;59:345–56

Narayan O, Kennedy-Stoskopf S, Sheffer D, Griffin DE, Clements JE. Activation of caprine arthritis–encephalitis virus expression during maturation of monocytes to macrophages. Infect Immun 1983;41:67–73.

Naser SA, Sagramsingh SR, Naser AS, Thanigachalam S. Mycobacterium avium subspecies paratuberculosis causes Crohn's disease in some inflammatory bowel disease patients. World J Gastroenterol. 2014 Jun 21;20(23):7403-15. doi: 10.3748/wjg.v20.i23.7403. PMID: 24966610; PMCID: PMC4064085.

Nicholas RA, Ayling RD, Stipkovits LP. An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings. Vaccine. 2002 Oct 4;20(29-30):3569-75. doi: 10.1016/s0264-410x(02)00340-7. PMID: 12297403; PMCID: PMC7125750.

Niesalla H, McNeilly TN, Ross M, Rhind SM, Harkiss GD. Experimental infection of sheep with visna/maedi virus via the conjunctival space. J Gen Virol 2008;89:1329–37.

Odintsov Vaintrub M, Levit H, Chincarini M, Fusaro I, Giammarco M, Vignola G. Review: Precision livestock farming, automats and new technologies: possible applications in extensive dairy sheep farming. Animal. 2021 Mar;15(3):100143. doi: 10.1016/j.animal.2020.100143. Epub 2020 Dec 23. PMID: 33518488.

Olech, M., Rachid, A., Croise, B., Kuźmak, J., Valas, S., 2012. Genetic and antigenic characterization of small ruminant lentiviruses circulating in Poland. Virus Res. 163, 528–536

O'Mahony J, Hill C. A real time PCR assay for the detection and quantitation of Mycobacterium avium subsp. paratuberculosis using SYBR Green and the Light Cycler. J Microbiol Methods. 2002 Nov;51(3):283-93. doi: 10.1016/s0167-7012(02)00098-2. PMID: 12223288.

Oravcová K, López-Enríquez L, Rodríguez-Lázaro D, Hernández M. Mycoplasma agalactiae p40 Gene, a novel marker for diagnosis of contagious agalactia in sheep by real-time PCR: assessment of analytical performance and in-house validation using naturally contaminated milk samples. J Clin Microbiol. 2009 Feb;47(2):445-50. doi: 10.1128/JCM.01442-08. Epub 2008 Nov 19. PMID: 19020058; PMCID: PMC2643663.

Panning M, Kilwinski J, Greiner-Fischer S, Peters M, Kramme S, Frangoulidis D, Meyer H, Henning K, Drosten C. High throughput detection of Coxiella burnetii by real-time PCR with internal control system and automated DNA preparation. BMC Microbiol. 2008 May 19;8:77. doi: 10.1186/1471-2180-8-77. PMID: 18489780; PMCID: PMC2397412.

Pazzola M, Puggioni G, Ponti MN, Scivoli R, Dettori ML, Cecchinato A, Vacca GM. Test positivity for Maedi-Visna virus and Mycobacterium avium ssp. paratuberculosis in Sarda ewes: Effects on milk composition and coagulation traits and heritability estimates for susceptibility. J Dairy Sci. 2020 Oct;103(10):9213-9223. doi: 10.3168/jds.2019-18026. Epub 2020 Aug 20. PMID: 32828507.

Peluso R, Haase A, Stowring L, Edwards M, Ventura P. A Trojan Horse mechanism for the spread of visna virus in monocytes. Virology 1985;147:231–6.

Pérez, M.; Biescas, E.; de Andrés, X.; Leginagoikoa, I.; Salazar, E.; Berriatua, E.; Reina, R.; Bolea, R.; de Andrés, D.; Juste, R.A.; et al. Visna/Maedi virus

Pérez, M.; Biescas, E.; de Andrés, X.; Leginagoikoa, I.; Salazar, E.; Berriatua, E.; Reina, R.; Bolea, R.; de Andrés, D.; Juste, R.A.; et al. Visna/Maedi virus serology in sheep: Survey, risk factors and implementation of a successful control programme in Aragón (Spain). Vet. J. 2010, 186, 221–225.

Peterhans, E.; Greenland, T.; Badiola, J.; Harkiss, G.; Bertoni, G.; Amorena, B.; Eliaszewicz, M.; Juste, R.A.; Kra_nig, R.; Lafont, J.P.; et al. Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. Vet. Res. 2004, 35, 257–274.

Pexara A, Solomakos N, Govaris A. Q fever and seroprevalence of Coxiella burnetii in domestic ruminants. Vet Ital. 2018 Dec 31;54(4):265-279. doi: 10.12834/VetIt.1113.6046.3. PMID: 30681125.

Pickup RW, Rhodes G, Arnott S, Sidi-Boumedine K, Bull TJ, Weightman A, Hurley M, Hermon-Taylor J. Mycobacterium avium subsp. paratuberculosis in the catchment area and water of the River Taff in South Wales, United Kingdom, and its potential relationship to clustering of Crohn's disease cases in the city of Cardiff. Appl Environ Microbiol. 2005 Apr;71(4):2130-9. doi: 10.1128/AEM.71.4.2130-2139.2005. PMID: 15812047; PMCID: PMC1082532.

Pierce ES. Could *Mycobacterium avium* subspecies *paratuberculosis* cause Crohn's disease, ulcerative colitis…and colorectal cancer? Infect Agent Cancer. 2018 Jan 4;13:1. doi: 10.1186/s13027-017-0172-3. PMID: 29308085; PMCID: PMC5753485.

Pillai SR, Jayarao BM. Application of IS900 PCR for detection of Mycobacterium avium subsp. paratuberculosis directly from raw milk. J Dairy Sci. 2002 May;85(5):1052-7. doi: 10.3168/jds.S0022-0302(02)74165-9. PMID: 12086038.

Pistone D, Marone P, Pajoro M, Fabbi M, Vicari N, Daffara S, Dalla Valle C, Gabba S, Sassera D, Verri A, Montagna M, Epis S, Monti C, Strada EG, Grazioli V, Arrigoni N, Giacosa A, Bandi C. Mycobacterium avium paratuberculosis in Italy: commensal or emerging human pathogen? Dig Liver Dis. 2012 Jun;44(6):461-5. doi: 10.1016/j.dld.2011.12.022. Epub 2012 Jan 28. PMID: 22285147.

Poumarat F, Gautier-Bouchardon AV, Bergonier D, Gay E, Tardy F. Diversity and variation in antimicrobial susceptibility patterns over time in Mycoplasma agalactiae isolates collected from sheep

and goats in France. J Appl Microbiol. 2016 May;120(5):1208-18. doi: 10.1111/jam.13083. Epub 2016 Mar 11. PMID: 26835882.

Poupart P, Coene M, Van Heuverswyn H, Cocito C. Preparation of a specific RNA probe for detection of Mycobacterium paratuberculosis and diagnosis of Johne's disease. J Clin Microbiol. 1993 Jun;31(6):1601-5. doi: 10.1128/jcm.31.6.1601-1605.1993. PMID: 8315002; PMCID: PMC265585.

Primm TP, Lucero CA, Falkinham JO 3rd. Health impacts of environmental mycobacteria. Clin Microbiol Rev. 2004 Jan;17(1):98-106. doi: 10.1128/CMR.17.1.98-106.2004. PMID: 14726457; PMCID: PMC321467.

Pritchard, G.C., Dawson, M., 2000. Maedi–Visna. In: Martin,W.B., Aitken, I.D. (Eds.), Diseases of Sheep, 3rd ed. Blackwell, pp. 187–191

Rahman MT, Sobur MA, Islam MS, Ievy S, Hossain MJ, El Zowalaty ME, Rahman AT, Ashour HM. Zoonotic Diseases: Etiology, Impact, and Control. Microorganisms. 2020 Sep 12;8(9):1405. doi: 10.3390/microorganisms8091405. PMID: 32932606; PMCID: PMC7563794.

Rathnaiah G, Zinniel DK, Bannantine JP, Stabel JR, Gröhn YT, Collins MT, Barletta RG. Pathogenesis, Molecular Genetics, and Genomics of *Mycobacterium avium* subsp. *paratuberculosis*, the Etiologic Agent of Johne's Disease. Front Vet Sci. 2017 Nov 6;4:187. doi: 10.3389/fvets.2017.00187. PMID: 29164142; PMCID: PMC5681481.

Kizil S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev. 1998 Dec;62(4):1094-156. doi: 10.1128/MMBR.62.4.1094-1156.1998. PMID: 9841667; PMCID: PMC98941.

Real F, Deniz S, Acosta B, Ferrer O, Poveda JB. Caprine contagious agalactia caused by *Mycoplasma agalactiae* in the Canary Islands. *Veterinary Record.* 1994;135(1):15–16.

Reina, R.; Berriatua, E.; Lujan, L.; Juste, R.; Sanchez, A.; de Andres, D.; Amorena, B. Prevention strategies against small ruminant lentiviruses: An update. Vet. J. 2009, 182, 31–37.

Reina, R., Bertolotti, L., Dei Giudici, S., Puggioni, G., Ponti, N., Profiti, M., Patta, C., Rosati, S., 2010. Small ruminant lentivirus genotype E is widespread in Sarda goat. Vet. Microbiol. 144, 24–31.

Reina, R.; de Andrés, D.; Amorena, B. Immunization against Small Ruminant Lentiviruses. Viruses 2013, 5, 1948–1963.

Ritter C, Wolf R, Adams CL, Kelton DF, Pickel C, Mason S, Orsel K, De Buck J, Barkema HW. Short communication: Herd-level prevalence of Mycobacterium avium ssp. paratuberculosis is not associated with participation in a voluntary Alberta Johne's disease control program. J Dairy Sci. 2016 Mar;99(3):2157-2160. doi: 10.3168/jds.2015-10356. Epub 2016 Jan 6. PMID: 26778309.

Robino P, Alberti A, Pittau M, Chessa B, Miciletta M, Nebbia P, Le Grand D, Rosati S. Genetic and antigenic characterization of the surface lipoprotein P48 of Mycoplasma bovis. Vet Microbiol. 2005 Aug 30;109(3-4):201-9. doi: 10.1016/j.vetmic.2005.05.007. PMID: 15985342.

Roest HI, Ruuls RC, Tilburg JJ, Nabuurs-Franssen MH, Klaassen CH, Vellema P, van den Brom R, Dercksen D, Wouda W, Spierenburg MA, van der Spek AN, Buijs R, de Boer AG, Willemsen PT, van Zijderveld FG. Molecular epidemiology of Coxiella burnetii from ruminants in Q fever outbreak, the Netherlands. Emerg Infect Dis. 2011 Apr;17(4):668-75. doi: 10.3201/eid1704.101562. PMID: 21470457; PMCID: PMC3377418.

Roger P A, The impact of disease and disease prevention on sheep welfare, Small Ruminant Research, Volume 76, Issues 1–2, 2008, Pages 104-111, ISSN 0921-4488.

Rosati S, Pozzi S, Robino P, Montinaro B, Conti A, Fadda M, Pittau M. P48 major surface antigen of Mycoplasma agalactiae is homologous to a malp product of Mycoplasma fermentans and belongs to a selected family of bacterial lipoproteins. Infect Immun. 1999 Nov;67(11):6213-6. doi: 10.1128/IAI.67.11.6213-6216.1999. PMID: 10531294; PMCID: PMC97020.

Rosati S, Robino P, Fadda M, Pozzi S, Mannelli A, Pittau M. Expression and antigenic characterization of recombinant Mycoplasma agalactiae P48 major surface protein. Vet Microbiol. 2000 Feb;71(3-4):201-10. doi: 10.1016/s0378-1135(99)00164-9. PMID: 10703704.

Ryan S, Tiley L, McConnell I, Blacklaws B. Infection of dendritic cells by the Maedi-Visna lentivirus. J Virol 2000;74:10096–103.

Samuel JE, Frazier ME, Kahn ML, Thomashow LS, Mallavia LP. Isolation and characterization of a plasmid from phase I Coxiella burnetii. Infect Immun. 1983 Aug;41(2):488-93. doi: 10.1128/iai.41.2.488-493.1983. PMID: 6307871; PMCID: PMC264667.

Sanderson JD, Moss MT, Tizard ML, Hermon-Taylor J. Mycobacterium paratuberculosis DNA in Crohn's disease tissue. Gut. 1992 Jul;33(7):890-6. doi: 10.1136/gut.33.7.890. PMID: 1644328; PMCID: PMC1379400.

Saxena, Shailendra K., and Sai V. Chitti. "Molecular Biology and Pathogenesis of Retroviruses." *Advances in Molecular Retrovirology*. IntechOpen, 2016.

Schairer DO, Chouake JS, Nosanchuk JD, Friedman AJ. The potential of nitric oxide releasing therapies as antimicrobial agents. Virulence. 2012 May 1;3(3):271-9. doi: 10.4161/viru.20328. Epub 2012 May 1. PMID: 22546899; PMCID: PMC3442839.

Schaller, P.; Vogt, H.R.; Strasser, M.; Nettleton, P.F.; Peterhans, E.; Zanoni, R. Seroprevalence of maedi-visna and border disease in Switzerland. Schweiz. Arch. Tierh. 2000, 142, 145–153

Schönenbrücher H, Abdulmawjood A, Failing K, Bülte M. New triplex real-time PCR assay for detection of Mycobacterium avium subsp. paratuberculosis in bovine feces. Appl Environ Microbiol. 2008 May;74(9):2751-8. doi: 10.1128/AEM.02534-07. Epub 2008 Mar 7. PMID: 18326682; PMCID: PMC2394907.

Serrano M, Elguezabal N, Sevilla IA, Geijo MV, Molina E, Juste RA, Garrido JM. Preliminary Results Indicate That Inactivated Vaccine against Paratuberculosis Could Modify the Course of

Experimental *Mycobacterium bovis* Infection in Calves. Front Vet Sci. 2017 Oct 18;4:175. doi: 10.3389/fvets.2017.00175. PMID: 29094040; PMCID: PMC5651274.

Seshadri R, Paulsen IT, Eisen JA, Read TD, Nelson KE, Nelson WC, Ward NL, Tettelin H, Davidsen TM, Beanan MJ, Deboy RT, Daugherty SC, Brinkac LM, Madupu R, Dodson RJ, Khouri HM, Lee KH, Carty HA, Scanlan D, Heinzen RA, Thompson HA, Samuel JE, Fraser CM, Heidelberg JF. Complete genome sequence of the Q-fever pathogen Coxiella burnetii. Proc Natl Acad Sci U S A. 2003 Apr 29;100(9):5455-60. doi: 10.1073/pnas.0931379100. Epub 2003 Apr 18. PMID: 12704232; PMCID: PMC154366.

Shuaib, M.; Green, C.; Rashid, M.; Duizer, G.; Whiting, T.L. Herd risk factors associated with sero-prevalence of Maedi-Visna in the Manitoba sheep population. Can. Vet. J. 2010, 51, 385–390

Sigurdsson, B. (1954). Maedi, a slow progressive pneumonia of sheep: an epizoological and pathological study. Brit. vet. J., 110, 255- 270.

Sihvonen L, Hirvelä-Koski V, Nuotio L, Kokkonen UM. Serological survey and epidemiological investigation of maedi-visna in sheep in Finland. Vet Microbiol. 1999 Mar 19;65(4):265-70. doi: 10.1016/s0378-1135(98)00312-5. PMID: 10223325.

Sihvonen L, Nuotio L, Rikula U, Hirvelä-Koski V, Kokkonen U. Preventing the spread of maedi-visna in sheep through a voluntary control programme in Finland. Prev Vet Med. 2000 Nov 16;47(3):213-20. doi: 10.1016/s0167-5877(00)00162-8. PMID: 11058781.

Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barré A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet. 2007 May 18;3(5):e75. doi: 10.1371/journal.pgen.0030075. PMID: 17511520; PMCID: PMC1868952.

Srivastava NC. *Studies on the mycoplasma and acholeplasma of respiratory tract of buffaloes [Ph.D. thesis]* Bareilly, India: Rohilkhand University; 1982.

Stabel JR, Lambertz A. Efficacy of pasteurization conditions for the inactivation of Mycobacterium avium subsp. paratuberculosis in milk. J Food Prot. 2004 Dec;67(12):2719-26. doi: 10.4315/0362-028x-67.12.2719. PMID: 15633677.

Stephan R, Schumacher S, Tasara T, Grant IR. Prevalence of Mycobacterium avium subspecies paratuberculosis in Swiss raw milk cheeses collected at the retail level. J Dairy Sci. 2007 Aug;90(8):3590-5. doi: 10.3168/jds.2007-0015. PMID: 17638968.

Stein A, Kruszewska D, Gouvernet J, Raoult D. Study of the 16S-23S ribosomal DNA internal spacer of Coxiella burnetii. Eur J Epidemiol. 1997 Jun;13(4):471-5. doi: 10.1023/a:1007389315808. PMID: 9258555.

Stoenner HG, Holdenried R, Lackman D, Orsborn JS., Jr The occurrence of *Coxiella burnetii*, *Brucella*, and other pathogens among fauna of the great salt lake desert in Utah. *Am J Trop Med Hyg.* 1959;8:590–596.

Stoenner HG, Lackman DB. The biologic properties of *Coxiella burnetii* isolated from rodents collected in Utah. *Am J Hyg.* 1960;71:45–51.

Stowring L, Haase AT, Petursson G, Georgsson G, Palsson P, Lutley R, et al. Detection of visna virus antigens and RNA in glial cells in foci of demyelination. Virology 1985;141:311–8.

Straub OC. Maedi-Visna virus infection in sheep. History and present knowledge. Comp Immunol Microbiol Infect Dis. 2004 Jan;27(1):1-5. doi: 10.1016/S0147-9571(02)00078-4. PMID: 14656537.

Strommenger B, Stevenson K, Gerlach GF. Isolation and diagnostic potential of ISMav2, a novel insertion sequence-like element from Mycobacterium avium subspecies paratuberculosis. FEMS Microbiol Lett. 2001 Mar 1;196(1):31-7. doi: 10.1111/j.1574-6968.2001.tb10536.x. PMID: 11257544.

Subramaniam S, Bergonier D, Poumarat F, Capaul S, Schlatter Y, Nicolet J, Frey J. Species identification of Mycoplasma bovis and Mycoplasma agalactiae based on the uvrC genes by PCR. Mol Cell Probes. 1998 Jun;12(3):161-9. doi: 10.1006/mcpr.1998.0160. PMID: 9664578.

Sweeney RW, Whitlock RH, Rosenberger AE. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J Clin Microbiol. 1992 Jan;30(1):166-71. doi: 10.1128/jcm.30.1.166-171.1992. PMID: 1734049; PMCID: PMC265014.

Tasara T, Hoelzle LE, Stephan R. Development and evaluation of a Mycobacterium avium subspecies paratuberculosis (MAP) specific multiplex PCR assay. Int J Food Microbiol. 2005 Oct 25;104(3):279-87. doi: 10.1016/j.ijfoodmicro.2005.03.009. Epub 2005 Jun 27. PMID: 15982769.

Tasara T, Stephan R. Development of an F57 sequence-based real-time PCR assay for detection of Mycobacterium avium subsp. paratuberculosis in milk. Appl Environ Microbiol. 2005 Oct;71(10):5957-68. doi: 10.1128/AEM.71.10.5957-5968.2005. PMID: 16204510; PMCID: PMC1266021.

Thorel MF, Krichevsky M, Lévy-Frébault VV. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of Mycobacterium avium, and description of Mycobacterium avium subsp. avium subsp. nov., Mycobacterium avium subsp. paratuberculosis subsp. nov., and Mycobacterium avium subsp. silvaticum subsp. nov. Int J Syst Bacteriol. 1990 Jul;40(3):254-60. doi: 10.1099/00207713-40-3-254. PMID: 2397193.

Tiley LS, Brown PH, Le SY, Maizel JV, Clements JE, Cullen BR. Visna virus encodes a post-transcriptional regulator of viral structural gene expression. Proc Natl Acad Sci USA 1990;87:7497–501

Todaro M, Dattena M, Acciaioli A, Bonanno A, Bruni G, Caroprese M, Mele M, Sevi A, Trabalza Marinucci M. Aseasonal sheep and goat milk production in the Mediterranean area: Physiological and technical insights, Small Ruminant Research. Volume 126, Supplement 1, 2015, Pages 59-66, ISSN 0921-4488.

Tola S, Idini G, Manunta D, et al. Comparison of *Mycoplasma agalactiae* isolates by pulsed field gel eIectrophoresis, SDS-PAGE and immunoblotting. *FEMS Microbiology Letters.* 1996;143(2-3):259–265.

Tola S, Idini G, Rocchigiani AM, et al. Comparison of restriction pattern polymorphism of *Mycoplasma agalactiae* and *Mycoplasma bovis* by pulsed field gel electrophoresis. *Journal of Veterinary Medicine B.* 1999;46(3):199–206.

Tola S, Manunta D, Rocca S, Rocchigiani AM, Idini G, Angioi PP, Leori G. Experimental vaccination against Mycoplasma agalactiae using different inactivated vaccines. Vaccine. 1999 Jul 16;17(22):2764-8. doi: 10.1016/s0264-410x(99)00070-5. PMID: 10438045.

Tonamo A, Komlósi I, Varga L, Czeglédi L, Peles F. Bacteriological Quality of Raw Ovine Milk from Different Sheep Farms. Animals (Basel). 2020 Jul 9;10(7):1163. doi: 10.3390/ani10071163. PMID: 32660002; PMCID: PMC7401633.

Toohey KL, Haase AT. The rev gene of visna virus is required for productive infection. Virology 1994;200:276–80;

Torgerson PR, Macpherson CN. The socioeconomic burden of parasitic zoonoses: global trends. Vet Parasitol. 2011 Nov 24;182(1):79-95. doi: 10.1016/j.vetpar.2011.07.017. Epub 2011 Jul 14. PMID: 21862222.

Tissot-Dupont H, Raoult D. Q fever. Infect Dis Clin North Am. 2008 Sep;22(3):505-14, ix. doi: 10.1016/j.idc.2008.03.002. PMID: 18755387.

Yakes BJ, Lipert RJ, Bannantine JP, Porter MD. Detection of Mycobacterium avium subsp. paratuberculosis by a sonicate immunoassay based on surface-enhanced Raman scattering. Clin Vaccine Immunol. 2008 Feb;15(2):227-34. doi: 10.1128/CVI.00334-07. Epub 2007 Dec 12. PMID: 18077613; PMCID: PMC2238065.

Van den Brom R, Schimmer B, Schneeberger PM, Swart WA, van der Hoek W, Vellema P. Seroepidemiological survey for Coxiella burnetii antibodies and associated risk factors in Dutch livestock veterinarians. PLoS One. 2013;8(1):e54021. doi: 10.1371/journal.pone.0054021. Epub 2013 Jan 16. PMID: 23342063; PMCID: PMC3546960.

Van den Brom R, Santman-Berends I, Luttikholt S, Moll L, Van Engelen E, Vellema P. Bulk tank milk surveillance as a measure to detect Coxiella burnetii shedding dairy goat herds in the Netherlands between 2009 and 2014. J Dairy Sci. 2015 Jun;98(6):3814-25. doi: 10.3168/jds.2014-9029. Epub 2015 Mar 18. PMID: 25795490.

Van den Brom R, van Engelen E, Roest HI, van der Hoek W, Vellema P. Coxiella burnetii infections in sheep or goats: an opinionated review. Vet Microbiol. 2015 Dec 14;181(1-2):119-29. doi: 10.1016/j.vetmic.2015.07.011. Epub 2015 Jul 15. PMID: 26315774.

van den Brom R, de Jong A, van Engelen E, Heuvelink A, Vellema P. Zoonotic risks of pathogens from sheep and their milk borne transmission. Small Rumin Res. 2020 Aug;189:106123. doi: 10.1016/j.smallrumres.2020.106123. Epub 2020 May 15. PMID: 32427176; PMCID: PMC7227596.

Vanderburg S, Rubach MP, Halliday JE, Cleaveland S, Reddy EA, Crump JA. Epidemiology of Coxiella burnetii infection in Africa: a OneHealth systematic review. PLoS Negl Trop Dis. 2014 Apr 10;8(4):e2787. doi: 10.1371/journal.pntd.0002787. PMID: 24722554; PMCID: PMC3983093.

Vansnick E, De Rijk P, Vercammen F, Geysen D, Rigouts L, Portaels F. Newly developed primers for the detection of Mycobacterium avium subspecies paratuberculosis. Vet Microbiol. 2004 Jun 3;100(3-4):197-204. doi: 10.1016/j.vetmic.2004.02.006. PMID: 15145498

Vary PH, Andersen PR, Green E, Hermon-Taylor J, McFadden JJ. Use of highly specific DNA probes and the polymerase chain reaction to detect Mycobacterium paratuberculosis in Johne's disease. J Clin Microbiol. 1990 May;28(5):933-7. doi: 10.1128/jcm.28.5.933-937.1990. PMID: 2351737; PMCID: PMC267840.

Vigne R, Filippi P, Querat G, Sauze N, Vitu C, Russo P, et al. Precursorpolypeptides to structural proteins of visna virus. J Virol 1982;42:1046–56

Villet S, Bouzar BA, Morin T, Verdier G, Legras C, Chebloune Y. Maedi-visna virus and caprine arthritis encephalitis virus genomes encode a Vpr-like but no Tat protein. J Virol 2003;77:9632–8

Vogiatzis I, Dimoglou G, Sachpekidis V. Q fever myocarditis. Hippokratia. 2008 Jan;12(1):46-9. PMID: 18923753; PMCID: PMC2532961..

Waddell LA, Rajić A, Sargeant J, Harris J, Amezcua R, Downey L, Read S, McEwen SA. The zoonotic potential of Mycobacterium avium spp. paratuberculosis: a systematic review. Can J Public Health. 2008 Mar-Apr;99(2):145-55. doi: 10.1007/BF03405464. PMID: 18457292; PMCID: PMC6975835.

Wang Y, Kelly CG, Karttunen JT, Whittall T, Lehner PJ, Duncan L, MacAry P, Younson JS, Singh M, Oehlmann W, Cheng G, Bergmeier L, Lehner T. CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunity. 2001 Dec;15(6):971-83. doi: 10.1016/s1074-7613(01)00242-4. PMID: 11754818.

Waters WR, Miller JM, Palmer MV, Stabel JR, Jones DE, Koistinen KA, Steadham EM, Hamilton MJ, Davis WC, Bannantine JP. Early induction of humoral and cellular immune responses during experimental Mycobacterium avium subsp. paratuberculosis infection of calves. Infect Immun. 2003 Sep;71(9):5130-8. doi: 10.1128/IAI.71.9.5130-5138.2003. PMID: 12933856; PMCID: PMC187349.

Wells SJ, Collins MT, Faaberg KS, Wees C, Tavornpanich S, Petrini KR, Collins JE, Cernicchiaro N, Whitlock RH. Evaluation of a rapid fecal PCR test for detection of Mycobacterium avium subsp. paratuberculosis in dairy cattle. Clin Vaccine Immunol. 2006 Oct;13(10):1125-30. doi: 10.1128/CVI.00236-06. Epub 2006 Aug 23. PMID: 16928884; PMCID: PMC1595318.

Whelan J, Schimmer B, Schneeberger P, Meekelenkamp J, Ijff A, van der Hoek W, Robert-Du Ry van Beest Holle M. Q fever among culling workers, the Netherlands, 2009-2010. Emerg Infect Dis. 2011 Sep;17(9):1719-23. doi: 10.3201/eid1709.110051. PMID: 21888803; PMCID: PMC3322078.

Whittington RJ, Hope AF, Marshall DJ, Taragel CA, Marsh I. Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis: IS900 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. J Clin Microbiol. 2000 Sep;38(9):3240-8. doi: 10.1128/JCM.38.9.3240-3248.2000. PMID: 10970365; PMCID: PMC87366.

Whittington RJ, Windsor PA. In utero infection of cattle with Mycobacterium avium subsp. paratuberculosis: a critical review and meta-analysis. Vet J. 2009 Jan;179(1):60-9. doi: 10.1016/j.tvjl.2007.08.023. Epub 2007 Oct 24. PMID: 17928247.

Whittington, Richard. (2019). Opening Strategy: Professional Strategists and Practice Change, 1960 to Today, OUP 2019 ONLY FOR LIMITED SEMINAR CIRCULATION Chapter 1.

Windsor PA. Paratuberculosis in sheep and goats. Vet Microbiol. 2015 Dec 14;181(1-2):161-9. doi: 10.1016/j.vetmic.2015.07.019. Epub 2015 Jul 16. PMID: 26255556.

Wouda W, Dercksen DP. Abortus en doodgeboorte op melkgeitenbedrijven ten gevolge van Coxiella burnetii [Abortion and stillbirth among dairy goats as a consequence of Coxiella burnetii]. Tijdschr Diergeneeskd. 2007 Dec 1;132(23):908-11. Dutch. PMID: 18085173.

Woldehiwet Z. Q fever (coxiellosis): epidemiology and pathogenesis. Res Vet Sci. 2004 Oct;77(2):93-100. doi: 10.1016/j.rvsc.2003.09.001. Erratum in: Res Vet Sci. 2004 Dec;77(3):269. PMID: 15196898.

Wolfe ND, Dunavan CP, Diamond J. Origins of major human infectious diseases. Nature. 2007;447:279–283.

Woolhouse MEJ. Where do emerging pathogens come from? Microbe. 2006;1:511–515.

Zinsstag J, Schelling E, Wyss K, Mahamat MB. Potential of cooperation between human and animal health to strengthen health systems. Lancet. 2005 Dec 17;366(9503):2142-5. doi: 10.1016/S0140-6736(05)67731-8. PMID: 16360795.