UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

Molecular diagnosis of *Mycoplasma bovis*

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Molecular diagnosis of *Mycoplasma bovis*

António Maria Nolasco Cordeiro Gomes Gonçalves

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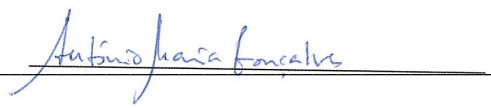
Assinatura: 

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Diagnóstico Molecular de *M. bovis*

Resumo

Mycoplasma bovis é uma bactéria cuja infecção pode ter diferentes apresentações tais como pneumonia, mastite, otite, afecções genitais, queratoconjuntivite e artrites. É um dos agentes considerados emergentes e que afecta a produção agropecuária, sendo responsável por perdas na ordem dos 150 milhões de euros na Europa. Tem uma patogénese multifactorial e as proteínas membranares à sua superfície conferem uma variabilidade que permite uma rápida e eficiente disseminação no hospedeiro, e no rebanho. Esta variabilidade e capacidade de resistir à imunidade do hospedeiro, assim como as suas interacções sinérgicas com outros agentes patogénicos, tornam as infecções por *M. bovis* um obstáculo difícil de conter e ultrapassar na Medicina Veterinária, isto porque quer a antibioterapia, quer a vacina, não são eficientes.

Este estudo procura desenvolver e contribuir para o estabelecimento de um protocolo de diagnóstico para a detecção de *M. bovis*. Vindas de 5 produções portuguesas diferentes, 93 amostras foram processadas e analisadas através de um qPCR, com os genes *uvrC* e *uvrC2024* como genes alvo. Dados os resultados, com uma positividade significativa pode-se considerar que ainda há trabalho pela frente em termos de estabelecer e uniformizar uma prática para combater a larga presença de *M. bovis* nas explorações.

Palavras-chave: *Mycoplasma bovis*; qPCR; diagnóstico; mastite; leite

Abstract

Mycoplasma bovis is a bacteria responsible for different disease presentations in cattle, such as pneumonia, mastitis, otitis, genital disorders, keratoconjuntivitis and arthritis, presently considered as one of the major emerging pathogens affecting cattle. Until this day, it is responsible for losses in animal production of over 150 million euros across Europe. The pathogenesis of *Mycoplasma*-associated diseases is multifactorial and the highly variable surface lipoproteins allows a fast and efficient dissemination of *M. bovis* within the host and the herd. Due to its high antigenic plasticity, its ability to survive within multiple host cells and the capacity to establish multiple synergistic interactions with other pathogens, makes *M. bovis* and associated infections are a major challenge in Veterinary Medicine, since the vaccine is not efficient and antibiotics are almost inefficient.

This study aims at developing and validating a quantitative PCR protocol for the diagnosis of *M. bovis*. 93 milk samples, from 5 different Portuguese farms, were collected, processed and each one's DNA extracted to be analyzed through a qPCR method targeting the *uvrC* and *uvrC2024* genes. Given the percentage of positivity, which was high, the conclusion we can take from the study is that there is still work to do, in terms of establishing a uniformed practice to tackle the wide presence of *M. bovis* in farms.

Keywords: *Mycoplasma bovis*, qPCR, diagnosis, mastitis, milk.

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MSC project final report

The project aimed to develop and validate a quantitative PCR (qPCR) protocol for the molecular diagnostic of *M. bovis* to be applied directly to clinical or biological samples (milk), allowing the prompt identification of the organism and establishment of therapeutics contributing to the decrease of the dissemination and economic impact of the disease. It may also allow a deeper knowledge on the approach to diagnosis of *M. bovis* that can be of universal use for future projects or assays. DNA extraction from milk was validated using *E. coli* contaminated milk tested with a conventional PCR system targeting the 16SRNA of *E. coli*. Further two qPCR protocols for *M. bovis* were established and developed according to Naikare et al, 2015; a qPCR using a hydrolysis probe with higher sensibility but with a higher cost, and a SYBR Green based qPCR with slightly less sensibility but at a more affordable price. Both qPCR targeted two distinct regions included within *M. bovis* specific *uvrC* gene.

Two qPCR systems were performed. The PCR was set in a 20µL final reaction volume, composed by either 10µL of SYBR Green Mix or Sensifast Mix, the first one with 0,3µM uvrC2024/2135 primer forward and reverse, the second with 0,5µM uvrC primer forward and reverse. On the Sensifast uvrC system, 0,3µM of hydrolysis probe was added. On both H2O was added to complete the 17µL of reaction mix on which was added 3µL of DNA template.

For assessing the specificity of the system both qPCR were performed using 1 yeast species and 11 different bacteria isolates. All isolates corresponded to field strains except for *Staphylococcus aureus* ATCC 25923. Amplification was detected on *Streptococcus agalactiae* and coagulase negative *Staphylococcus* when testing the hydrolysis probe uvrC qPCR system. To fully characterize this specificity leak, the amplicons were sequenced and subjected to Blast analysis confirming the amplified nucleotide sequence as *Mycoplasma bovis*.

Total DNA extraction from the milk matrix was performed with the commercial extraction kit Qiamp Blood and tissue kit, using three different protocols (Gram positive bacteria, Gram negative bacteria, culture cells). Contamination of the milk with *E. coli* suspension before extraction, followed by specific amplification of *E. coli* 16SrRNA gene, was completed to evaluate the extraction efficiency.

The qPCR systems were tested with raw milk samples in order to evaluate its robustness and to provide an insight about *M. bovis* distribution in dairy farms. A total of 93 samples from 75 animals divided by 5 dairy farms, which we named from A to D were processed and analyzed. Whenever available, samples from the different quarters of the udder were used.

Objectives

The project aims to develop and validate a quantitative PCR (qPCR) protocol for the molecular diagnostic of *M. bovis* to be applied directly to clinical or biological samples, allowing the prompt identification of the organism, facilitating therapeutics establishment and the control of *M. bovis* dissemination, allowing a significant decrease of the economic impact in the animal production industry. Also, the use of a fast and reliable molecular assay for the diagnostic of *M. bovis* will provide the means to rethink *M. bovis* diagnostic approach, useful in future projects or assays.

Introduction

Mycoplasma bovis is included in the Mycoplasmataceae family and *Mollicutes* class. *Mycoplasma bovis* is the most prevalent of the Mycoplasmataceae family and was first isolated from mastitic cows in the USA in 1961. It was originally named *Mycoplasma agalactiae* variety *bovis* because of the similarity with the small ruminant pathogen, *M. agalactiae*, with which shares biochemical, immunological and genetic features. The members of this bacterial species do not present a cell wall, have a small genome of 1,003,404bp (strain PG45), are slow growers and require complex growth media (sterols and enriched medium) for *in vitro* multiplication (Burki, Frey & Pilo, 2015; Calcutt et al., 2018). Their colonies have a “fried-egg” like appearance (Calcutt et al., 2018) and may take up to 10 days to grow at 37 °C in an atmosphere of 5%–10% CO_2 (Burki et al., 2015). *Mycoplasma* infections can affect directly or indirectly different organs, including the lungs, the udder, and even the eye. Frequently, this bacterium causes a chronic disease mainly identified through serological surveys. Organisms can be disseminated via the respiratory tract for long periods of time, acting the affected animals as infection reservoirs (Maunsell et al., 2011).

Different *Mycoplasma* species have a severe pathogenic impact in the bovine population: *Mycoplasma mycoides* subsp. *mycoides* is implicated in bovine severe respiratory diseases; *Mycoplasma bovigenitalium* is generally associated with bovine reproductive disorders, while *Mycoplasma bovoculi* has been isolated from infectious keratoconjunctivitis in cattle. *M. bovis* infections are non-zoonotic; however, the worldwide subclinical infection induces a substantial economic impact and severe consequences on cattle health and welfare. *M. bovis* affects animals from several age groups (prewean, postwean, neonate and adult), including beef and dairy cattle (Maunsell et al., 2011).

M. bovis can persist in the herd for long periods, with the possibility of pathogen shedding by the infected animals for periods ranging from a few weeks to several months

(Maunsell et al., 2011). It has the capacity to adhere to host cells, through protein interactions between the pathogen and the host cell proteins, and to invade and modulate the host immune system, having been found in macrophages, neutrophils and also in lymphocytes and monocytes (Van der Merwe J, Prysliak T, Perez-Casal J., 2016). It has the ability to induce or delay apoptosis and induce or suppress cytokines and leukines expression patterns. The persistence of *M. bovis* is facilitated by biofilm formation and the production of metabolites such as hydrogen peroxide (Duarte et al., 2012). Although the gold standard technique for *M. bovis* detection requires the bacteria isolation through conventional bacteriological methods, this approach does not offer the required sensitivity and specificity, with the intermittent and low-level shedding, uneven distribution of *M. bovis* throughout diseased tissue, suboptimal sample handling or culture conditions, and the presence of mycoplasma inhibitors in samples likely contribute to the low sensitivity of the isolation protocols (Naikare et al., 2015; Clothier et al., 2010). *Mycoplasma* laboratory diagnostic can also be based on serological assays, such as immunofluorescence and immunocytochemistry techniques and complement fixation tests, available exclusively for the major mycoplasma diseases and for international trading purposes. ELISA tests with whole cell or treated antigen are also available. Serology has its utility to detect mycoplasma negative populations, but the high sero-prevalence in many herds curbs the utility of serological tests in general (Burki et al, 2015; Calcutt et al., 2018). Several molecular tests have also been reported aiming at *M. bovis* identification (Naikare et al., 2015), including conventional Polymerase Chain Reaction (PCR), SYBR quantitative PCR (qPCR), hydrolysis probe qPCR or loop-mediated isothermal amplification (LAMP), with different sensitivity and specificity rates. The complete genome sequence of the international reference strain *M. bovis* PG45 (ATCC 25523) was reported by Wise K.S. (2011) allowing the choice of *16S rRNA* and *uvrC* genes as genomic targets used for PCR amplification (Naikare et al., 2015; McAuliffe, Ellis, Lawes, Ayling & Nicholas, 2005, Clothier et al., 2010).

Infections by *Mycoplasma bovis*

M. bovis can cause severe pneumonia, especially in calves, (Doherty, McElroy, Markey, Carter & Ball, 1994) and it can exacerbate respiratory infections by *Pasteurella* and *Mannheimia* species (Gourlay, Thomas & Wyld, 1989). In the lungs, this bacterium promotes the degeneration of the alveolar epithelium caused by the presence of a purulent interstitial exudate, which is followed by epithelial hyperplasia, ending with fibrosis and atrophy; as such, lesions of caseonecrotic bronchopneumonia are frequently observed in *M. bovis* outbreaks (Caswell & Archambault, 2007). As mentioned, *M. bovis* has also been associated

with mastitis. Polyarthritis can also occur but in association with respiratory disease or mastitis. Systemic involvement may follow these events. Mastitis caused by *M. bovis* may range from subclinical to severe with a dramatic loss of milk production (Maunsell et al., 2011), as the serous or purulent mastitic exudate has a high leukocyte count. In the etiological diagnosis of mastitis, *M. bovis* should be considered after discarding the hypothesis of the presence of other bacterial species (Maunsell et al., 2011).

Mycoplasma's virulence, persistence and dissemination (pathogenesis and pathogenicity)

Mycoplasma virulence is mainly linked to the mechanisms of host cell adherence and immunological evasion rather than the production of toxins or other virulence factors, although H_2O_2 production appears to be damaging for the colonized cells (Quinn et al., 2011), acting as a pathogenicity feature. European strains are less pathogenic than African strains, producing significantly less H_20_2 (Quinn et al., 2011).

Cell adhesion seems essential for *Mycoplasma* pathogenicity (Quinn et al., 2011). Some pathogenic species have peripheral structures formed by unique adhesion proteins, allowing the attachment to mammalian cells. Mycoplasmas can adhere to neutrophils and macrophages and can also impair phagocytic functions.

Variation in the surface proteins is an essential virulence factor of *Mycoplasma* species, allowing the organism to quickly adapt to the host environment and evade the immune response. Also, the similarity between some *Mycoplasma* antigens and host tissue proteins may interfere with *Mycoplasma* antigen recognition by the host immune system during invasion. Furthermore, it might predispose to the development of autoimmune disease, due to the potential damage of host tissues during the immune response towards *Mycoplasma* antigens (Quinn et al., 2011). Modulation or activation of the host immune system is critical for the pathogenesis of *Mycoplasma* diseases. Some pathogenic mycoplasmas, including those involved in pulmonary disease, are mitogenic for B and T lymphocytes. Mycoplasmas induce pneumonia by adhesion to the ciliated respiratory epithelium, leading to ciliostasis, loss of cilia and cytopathic change causing the release of proinflammatory cytokines that act during acute or chronic processes.

Modulation of the host's immune system (pathogenesis and pathogenicity)

Mycoplasma membrane proteins are important immunological targets. However, interactions are dependent on the cell type present in the peripheral blood. Studies showed induction of interferon-γ (IFN-γ) in T helper, cytotoxic T, natural killer cells (NK) and γδ T cells, while no IFN-γ induction was detected in monocytes, dendritic cells and B cells. In opposition, some available controversial studies point to PBMCs' apoptosis by *M. bovis*. Vanden Bush, Ricardo and Rosenbuch (2002) showed induction of apoptosis in lymphocytes by *M. bovis,* but Mulongo, Prysliak, Scruten, Napper and Perez-Casal (2014) detected a delay in the apoptosis of *M. bovis* infected monocytes. Various reports (Burki et al., 2015; Vanden Bush et al., 2002; Razin, Yogev & Naot 1998, Mulongo et al., 2014) also imply a stimulation or a suppression of the host's immune system. Stimulation occurs through macrophages, T cells, complement activation and cytokines' expression. Immune suppression is caused by the expression of anti-inflammatory cytokines and chemokines such as IL-10, and suppression of other anti-inflammatory cytokines like IFN-γ and TNF-α. IL-10 shifts the adaptive immune response to express T helper cells type 2 (Th2). In another perspective, suppression of the host immune system could be accomplished by the down-regulation of lymphocyte proliferation, by a putative inhibitory protein, or through interference with the lympho-proliferative response to phytoagglutinin. Consequently, the proliferation of lymphocytes is decreased, although their cytokine expression is not altered. *M. bovis* seems to suppress the lymphocyte-mediated immune response *via* a decrease of their population. Another strategy of *M. bovis* to restrain the host immune response is to bind neutrophils and thereby inhibit their oxidative burst. The modulation of the host's immune system leads to *M. bovis* higher capacity and ability to systemically survive and proliferate throughout long periods of time, creating one of the main challenges to outcome when it comes to manage and treat outbursts of *M. bovis* disease in animal production (Burki et al., 2015; Thomas et al., 2003; Maunsell et al., 2011).

Antigenic variation (pathogenesis and pathogenicity)

M. bovis have a highly variable, strain independent, antigenic profile (Burki et al., 2015). A number of mechanisms are responsible for this pattern, providing an extraordinary advantage when it comes to outmaneuver the immune system.

In *M. bovis,* the genes coding for the integral proteins of the membrane belong to a family of *vsps* (phase and size variant variable membrane surface lipoproteins). For example, *M. bovis* PG45 strain contains 13 different, single-copy *vsp* genes, distributed in a chromosomal cluster, the *vsp*-locus, with approximately 23 kb. This locus also comprises 2 additional open reading frames (ORFs), rendering a total of 15 peptides coding ORFs already identified (vspA, B, C, D, E, F, G, H, I, J, K, L, M, N and O). These proteins are putative lipoproteins, amphiphilic, with fatty acids and cysteine-residues. The *vsp* genes have a conserved 5' noncoding sequence, split into two cassettes, with the first cassette (I) being 99% homologous in all *vsp* genes, and encodes a putative ribosomal binding site; the second cassette (II), located upstream of cassette I, displays a higher genetic diversity. The co-expression of these Vsps provide the assembly of protein mosaics on the surface of *M. bovis*, attributing specific structural and antigenic features to this species. Gene expression is limited to two genes per isolate, with the remainder of the *vsp* genes remaining transcriptionally silent (Burki et al., 2015; Razin et al., 1998).

Adhesion and Cell Invasion (pathogenesis and pathogenicity)

Adhesion is one of the first steps of *Mycoplasma* infection, enhancing the importance of the adhesins expressed on the membrane due to their role in this process.

Due to their small genome, mycoplasmas lack the battery of genes involved in essential biosynthetic pathways and have to acquire essential compounds such as amino acids, nucleotides and lipids from the host cells. A fusion between the *Mycoplasma* and the host membranes was proposed as a mechanism for exchanging membrane and intracellular components.

Furthermore, adhesion of *M. bovis* strain PG45 to embryonic lung cells (EBL) has been proven to be temperature dependent, with maximal adhesion at 37ºC. The binding capacity of the cell receptor reached saturation at a MOI (multiplicity of infection) of 225:1 in EBL cells and 100:1 in bovine bronchial epithelial (BBE) cells (Burki et al., 2015; Thomas et al., 2003).

A large variation in cyto-adherence rates (3.4%-19.1%) among various *M. bovis* strains was recorded for different host cells (EBL cells, embryonic bovine trachea cells, Madin Darby bovine kidney (MDBK) and rabbit kidney (EBTr) cells) in a study made by Thomas et al., (2003). A lower adherence rate was observed in less or non-pathogenic strains when compared to more virulent strains. A lower cyto-adherence was recorded in a fibroblast cell line (EBTr) and primary BBE cells than with epithelial cell lines. In addition, *M. bovis* seem to lose the ability to adhere after successive *in vitro* passages. *M. bovis* proteins and their interaction mediate this adhesion, and trypsin treatment will lead to a decrease in adherence. Sialic acid residues were shown to be involved in cytoadhesion (Burki et al., 2015; Thomas et al., 2003).

The glycolytic enzyme alpha-enolase, associated to the membrane, is also involved in the adhesion process, and by binding plasminogen induces adherence of *M. bovis* to EBL cells (plasminogen treatment of EBL cells increased adherence by 11.9%). The same study revealed that a pre-treatment with low concentrations of trypsin increased the proteolytic activity and adhesion rate to EBL cells, pointing that other proteolytic enzymes activated by partial digestion, due to the low trypsin concentration, are also involved in adhesion (Burki et al., 2015; Thomas et al., 2003).

As pointed above, in infected calves Mycoplasmas seem to be present in a variety of cells, such as macrophages, neutrophils (inhibiting their oxidative burst), hepatocytes, bile duct epithelium cells, renal tubular cells or even axons of facial nerves. *Mycoplasma* specific antigens were also found in monocytes, and often in bronchiolar epithelial cells. The survival of *M. bovis* inside the phagocytes is presumably possible by altering at least one of the steps of the phagocytosis, after the engulfment (Burki et al., 2015; Thomas et al., 2003).

An *in vitro* assay showed that *M. bovis* strain Mb1 persisted in a variety of peripheral blood mononuclear cells (PBMC) including subpopulations of T cells (T helper cells, γδ T cells, cytotoxic T cells) and B cells, natural killer (NK) cells, dendritic cells and even bovine erythrocytes (Vanden Bush et al., 2002). The same study showed that intracellular location of *M. bovis* were dependent on the cell type used and time of infection, either associated with the cytosolic side of the cell membrane, in vacuole-like structures, or as a diffuse distribution. This may happen due to different receptors required for adhesion and invasion. In general, *M. bovis* invasion of epithelial and immune cells might contribute to the dissemination of the pathogen to distinct infection sites and weakens the efficiency of the treatment.

Biofilm formation and secondary metabolites (pathogenicity)

The formation of biofilms is another factor contributing for bacteria persistence in the host, as is in the environment, leading to chronic disease. Biofilms can also add damage to the host tissue, attracting phagocytes, which in turn release lysosomal enzymes, reactive oxygen and nitrogen species (ROS and RNS), creating specific conditions that impair phagocytosis efficiency (Burki et al., 2015). The adhesion capacity of Mycoplasma species seems to be a very important starting point to biofilm development (Burki et al., 2015).

The extent of biofilm formation depends on Vsps patterns and molecular types involved in adherence and therefore varies with the *Mycoplasma* strain. The biofilm enhances the survival of the bacteria, protecting them against environmental stressors and the host's immune system (Burki et al., 2015).

Synergistic Interactions

Synergistic interactions were identified, namely, between *M. bovis* and Bovine Herpes Virus (BHV), Bovine Respiratory Syncytial Virus (BRSV), Bovine Viral Diarrhea Virus (BVDV), *Pasteurella multocida*, *Histophillus somnii* and *Mannheimia haemolytica* (Burki et al., 2015).

Mastitis

In dairy cattle, the mammary gland is the main reservoir of bacterial species potentially involved in contagious mastitis, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis* and *Corynebacterium bovis*. Transmission and control of the disease depends on the ability of the pathogen to colonize the host cells. *Mycoplasma* and streptococci can survive in the environment for shorter periods of time than staphylococci, being more susceptible to external conditions. The pathogenicity and virulence of the pathogen will modulate the severity of local and systemic immune responses (Quinn et al., 2011).

Within *Mycoplasma* species, *M. bovis* is highly relevant considering bovine mastitis, with mycoplasma mastitis being particularly prevalent in large dairy herds. The most probable reservoir of mycoplasmas are clinically healthy calves and young cattle, which harbor *M. bovis* in the respiratory tract. Sachse et al. (2010) used a real time PCR technique to monitor and quantify *M. bovis* in cattle herds with mastitis and respiratory disease,

revealing that one single cow with active mastitis can shed up to 10^5 to 10^8 Colony Forming Units (CFU)/ml of milk, potentially contributing for the contamination of milking machines, hands and cloths and disease transmission through milking. Haematogenous spread of the infection between quarters may occur as fetal infection during pregnancy, contributing for *M. bovis* maintenance in the herd (Nicholas, Fox & Lysnyansky, 2016; Aebi et al., 2015, Byrne et al., 2005; Timonen et al., 2020).

Though mastitis pathogenesis by *Mycoplasma* species is still unclear, surface proteins play a vital role in the colonization process, allowing cellular adherence and immune invasion, extrapolating this mechanism to the infection of other tissues and organs (Burki et al., 2015; Quinn et al., 2011).

Not all affected animals develop clinical signs, and subclinical infection allows the establishment of carrier animals as a source of infection for the remaining herd. When present, clinical signs include alterations in the milk consistency and a rapid decrease in milk yield. Infection can also result in agalactia (Quinn et al., 2011).

M. bovis laboratory diagnosis

Conventional culture protocols

A while back, diagnosis of *M. bovis* infection would be performed through conventional culture methods after mycoplasma isolation. However these methods required several days (5-10) and are laborious. Nevertheless, these culture methods will remain somehow important because of their high specificity and sensitivity, being a reliable test. Isolation can be of use when for diagnosis of individual animals (Sachse et al., 1993).

Indirect ELISA

As an alternative, *Mycoplasma* specific antibodies can be detected through an enzyme-linked immunosorbent assay (ELISA) technique. ELISA-based protocols have the advantage of allowing the screening of a larger number of samples. However, they may be limited by the fact that antibody titers only emerge ten to fourteen days after the onset of disease (time needed to mount a humoral immunological response). Consequently, the pathogen cannot be detected during the incubation period. The attainable sensitivity is insufficient for reliable identification of all animals shedding *M. bovis*. ELISA detection of

antibodies can be useful for regular screening of *M. bovis*-free herds for trade purposes and for routine monitoring.

There are a variety of rapid tests available on the market. These include the commercially available indirect ELISA kits for anti-*M. bovis* antibody detection by Bio-X Diagnostics (Rochefort, Belgium) for use on serum and milk samples and the Bovichek *M. bovis* antibody ELISA test kit by Biovet Inc (Quebec, Canada). Western blot is often used in concurrence with sequence alignment to provide both an *in vitro* analysis and theoretical analysis of the potential for cross-reactivity of an ELISA. However, cross-reactivity experienced in-field although difficult to determine, must be taken into account when interpreting results. (Calcutt et al., 2018; Parker, Sheehy; Hazelton, Bosward, House, 2018; McAuliffe et al., 2005)

An antigen ELISA method can also be used for targeting a particular protein rather than the whole protein complex. A surface protein, p26, involved in the adherence process, is often used as a target to capture *M. bovis,* having been proved to be successfully applied to the detection of this bacterial species in milk samples. It showed high specificity and presented no cross-reactions other than with *M. agalactiae* (Parker et al., 2018; McAuliffe et al., 2005).

Polymerase Chain Reaction (PCR) and qPCR

PCR is a revolutionary method developed by Kary Mullis in the 1980s, based on the ability of DNA polymerase to synthesize new strands of DNA complementary to the template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to target a specific region of template sequence to amplify. At the end of the PCR reaction, the specific sequence will be exponentially amplified in billions of copies (amplicons) (20).

The dynamics of a PCR reaction include the initial exponential generation of copies of the target sequence but due to reaction inhibitors found in the sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs.

In comparison with conventional culture based diagnosis, PCR-based methods present higher efficiency, specificity and sensitivity . As previously explained, analysis via PCR involves the amplification of the DNA (non degraded) of the target microorganism. Opposed to culture, the PCR does not need a viable organism to be detected (Naikare et al., 2015; Sachse et al., 2010).

The development of *Mycoplasma* diagnostic methods based on conventional PCR began with the 16S rRNA gene as a target. This gene is one of the most common targeted genes for bacterial identification due to its presence in all bacteria and its function remaining unchanged over time. The 16S rRNA gene is a small subunit within prokaryotic ribosomes, containing highly conserved regions and variable regions, which can be species specific, making it useful for bacterial identification. However, while the specificity of these PCR assays targeting the 16S rRNA gene of *M. bovis* appeared to be adequate against most *Mycoplasma* species, less specific amplification was seen with *M. agalactiae* (Parker et al., 2018). For the detection of multiple species, a PCR targeting the 16S-23S rRNA spacer region was developed for the detection of *Mycoplasma* spp. and *Acholeplasma* spp. contaminants (Parker et al., 2018). This intergenic region is a structural region situated between 2 ribosomal RNAs, essential for protein synthesis. After DNA amplification, the resulting product is digested and run on an agarose gel; the resulting banding pattern allows the differentiation between *Mycoplasma* spp. from *Acholeplasma* spp. (Parker et al., 2018) A similar approach was developed by McAuliffe et al. (2005), using *Mycoplasma*-16S rRNA specific primers followed by resolution of the PCR products using denaturing gradient gel electrophoresis (DGGE). This approach enabled the identification and differentiation of 67 *Mycoplasma* species of veterinary and human significance, and was useful in detecting this bacterial species in mixed cultures. Finally, a multiplex cPCR using a specific set of primers represents another approach, which can be applied to identify several *Mycoplasma* species (Parker et al., 2018).

Real Time PCR

Despite the above-discussed PCR techniques discussed have proved to be effective, they require additional labor and time, as they need to be visualized and analyzed by horizontal electrophoresis of the amplified fragments. In a conventional PCR, the amplicon is visualized by horizontal electrophoresis in an agarose gel, at the end of the amplification reaction in the plateau phase. In the real time PCR, also called quantitative PCR, the amplification is displayed in real time, allowing the quantitation of the initial quantity of the target DNA during the exponential phase of the reaction (due to the quantitation unreliability during the plateau phase), making the qPCR an unique tool for molecular diagnosis, due to its sensitivity, specificity and quantitation feature (Naikare et al., 2015).

Therefore the real time PCR technology came as an upgrade of great utility and rapidly applied to *Mycoplasma* diagnosis. This technique can be based in two different approaches, SYBR green dye intercalation and fluorescent reporter probes.

Because SYBR green binds to all double stranded DNA, it can create an increase in background signal and reduced specificity compared with probe-based real time PCR methodologies (Parker et al., 2018). This method has not been frequently used to detect *Mycoplasma* in cattle, but it was previously applied to detect *Mycoplasma* spp. in bulk tank samples by using a qPCR protocol targeting the 16S-23S intergenic spacer region. This method is not significantly more sensitive than culture methods but it allows the identification of several *Mycoplasma* species in the tested samples (Parker et al., 2018).

Fluorescent reporter probe qPCR methods, commonly using a hydrolysis probe, present higher specificity. In addition to primer hybridization, the probe binds to a targeted region internal to the primer binding sites. Because this probe is specific for the targeted sequence, it can greatly reduce the background signal and increase the specificity. Different probes can be conjugated to different dyes and quencher molecules, multiplexing assays in a single reaction, saving time and reagents (Parker et al., 2018).

Several PCR probe assays have been developed for the diagnosis of *M. bovis*. Since probe based PCR methods targeting the 16S rRNA gene of *M. bovis* may reveal cross amplification with *M. agalactiae*, other genes can be used as targets, including the *M. bovis uvrC* gene, which does not promote cross amplification with non-*M. bovis* species. The *uvrC* gene encodes for a deoxyribodipyrimidine photolyase, an enzyme essential for replication, involved with DNA repair, making it a highly stable gene. Being significantly different in *M. bovis* and *M. agalactiae* species, the *uvrC* gene has been validated as a qPCR target using several samples obtained from cattle, like milk, joint fluid, nasal swabs, bronchoalveolar lavage fluid, tracheal wash fluid, and ear swabs (Clothier et al., 2010).

Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is a useful tool for investigating bacterial genome sequences. It involves sequencing the entire genome of selected isolates that can be used for clinical diagnosis, disease outbreak investigation and assessing antimicrobial resistance. This technique has provided some insight into the content and dynamics of the *Mycoplasma* organism, uncovering putative virulent genes. It makes it possible to assess differences between the genomes of different strains (Clothier et al., 2010).

M. bovis prevalence in Portugal

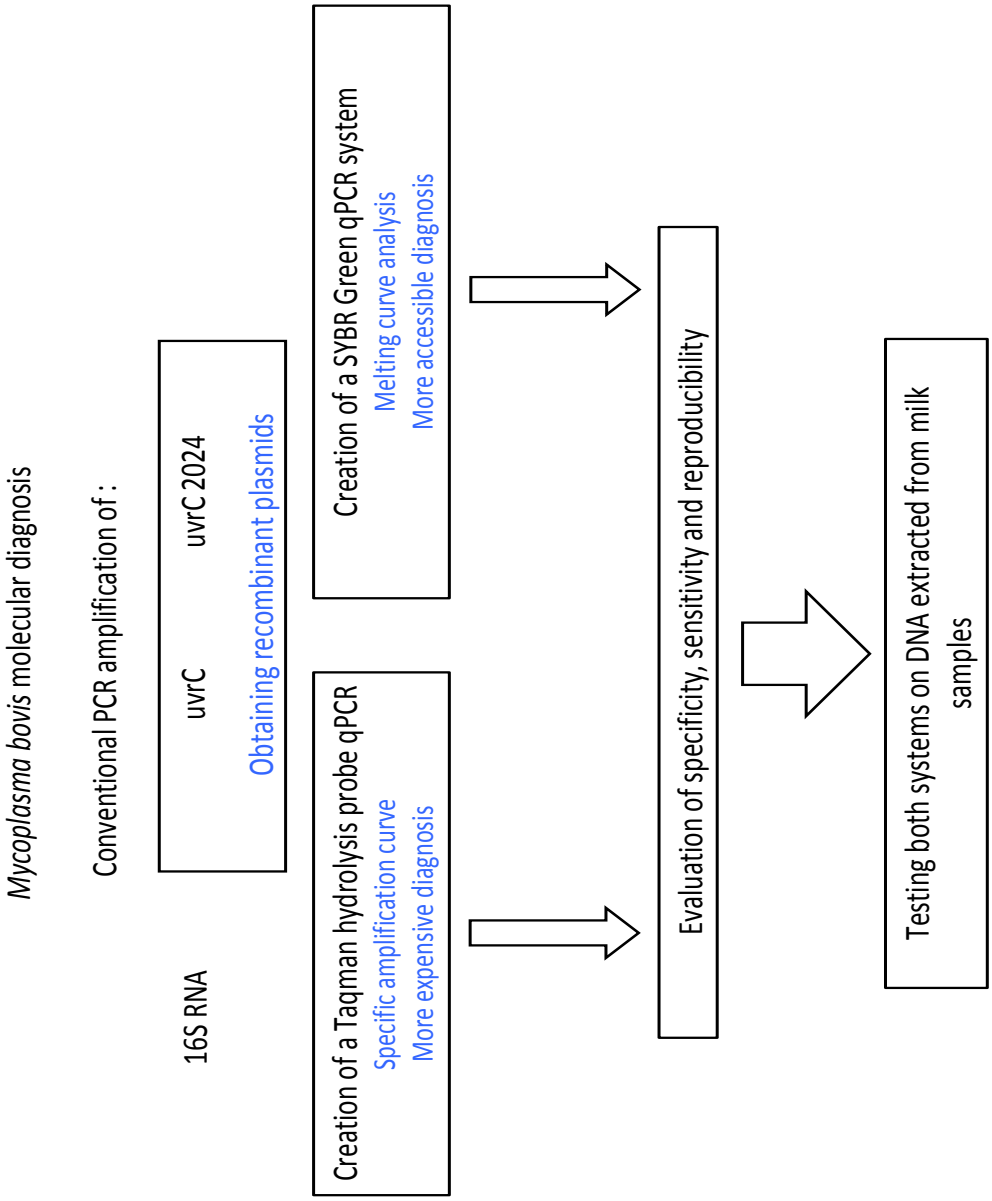
In 2002, the results of a survey including 871 dairies in the United States claimed that 6.8% were *M. bovis* positive on a bulk tank milk culture. In other studies, mycoplasma was identified in bulk tank milk samples in 7–20% of dairies sampled. Because mycoplasmas are shed intermittently and mastitic milk is withheld from the bulk tank, these values likely underestimate the true prevalence (Maunsell et al., 2011). In Central and South America, Mexico and Paraná, Brazil, respectively, the values can reach maximums of 55% in México (Miranda-Morales et al., 2008), and 35% in Paraná (Junqueira et al., 2020).

In Europe, *M. bovis* is the dominant species, with other mycoplasmas being rarely detected, although this may reflect a lack of thorough investigation (Nicholas et al., 2016). Until recently, outbreaks of *Mycoplasma* mastitis in Europe and Israel were rare, although this was possibly due to under-reporting. Routine *Mycoplasma* investigations were rarely conducted on undiagnosed cases of mastitis, being estimated to be associated with over a quarter of undiagnosed clinical and nearly 40% of subclinical cases (Bradley et al., 2007). The prevalence of *Mycoplasma* mastitis may be somewhat higher in other European countries based on sampling bulk milk tanks, with reports of a prevalence of 1.5% in Belgium (Passchyn et al., 2012) and 5.4% in Greece (Filioussis, Christodoulopoulos, Thatcher, Petridou & Bourtzi-Chatzopoulos, 2007). Outbreaks of *Mycoplasma* mastitis have been reported in Denmark (Nielsen et al., 2015), Austria (Spergser et al., 2013), The Netherlands (van Engelen et al., 2015), Switzerland (Aebi et al., 2015).

Only a few studies have been developed in order to uncover the presence and prevalence of this bacterium and its impact on Portuguese dairy farms (Pinho, Thompson, Machado & Carvalheira, 2013; Gonçalves, Regalla, Ayling & Nicholas, 2008). Moreover, they were performed in 2002, 2006 and 2008, pointing out for the urgent need of setting an efficient method of diagnosis for regular surveillance of *M. bovis* in Portugal. Gonçalves, R et al. studied the impact of *Mycoplasma bovis* infection under a serological surveillance study for contagious bovine pleuropneumonia in Portugal stating that the percentage of *M. bovis* seropositive samples on the Portuguese mainland was just under 23%, and on the Islands was 3.2%, with 6.3% and 3.2%, respectively, being classified as suspect. The regions with the highest percentage of seropositive samples were Algarve, with 33% of samples testing positive (although the total number of samples was small), and Entre Douro e Minho, with 26% of samples testing positive. In another study, Pinho, L. et al. (2013) evaluated the effect of some management practices on the prevalence of *Mycoplasma* spp. in Northwestern Portuguese dairy farms from bulk tank milk (BTM) samples. Between 2007 and 2008, 492 BTM samples from 164 dairy farms were analyzed. Five herds were positive in the bulk tank

milk to *Mycoplasma* spp., and four of them yielded *M. bovis*. Being *M. bovis* a bacterial species that impacts both the welfare of the animals and their production yields, the development of specific and sensitive diagnostic methods (Figure 1) is required for the establishment of appropriate control measures, aiming to control the negative impact of *M. bovis* in animal production

Figure 1 - Schematics of the procedures to develop the two qPCR systems, hydrolysis probe system and SYBR Green system, targeting the uvrC and uvrC2024, respectively



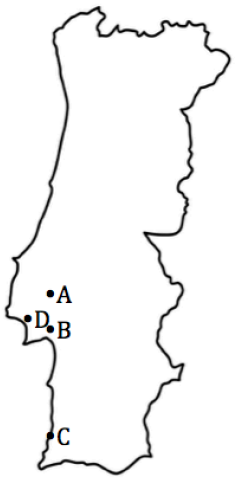
Material and Methods

As it is shown in the figure 1, the protocols and methods used throughout the project had the objective of either obtaining the products and the material to be used in the development and validation of both qPCR assays, or to apply these same assays to the samples collected.

Samples origin

Milk Samples

Figure 2- Geographical location of the 4 farms present in the study, on mainland Portugal.



In this study, 93 samples from 75 animals from four dairy farms, with suspicions of masititis, (A - Benavente, B – Palmela, C – Zambujeira do Mar and D – Moita) were processed and analyzed. Samples with the same numeric code correspond to the same animal. All samples are listed in the annexes.

Previous to total DNA extraction, as a pre treatment of the samples, 1mL of milk was centrifuged at 4.000xg for 20 minutes at room temperature and the pellet ressuspended in 0.4mL of Phosphate Buffer Solution (PBS). After an additional centrifugation step at 4.000 xg for 10 minutes at room temperature the pellets were stored at -80ºC until total DNA extraction.

Total DNA extraction

Total DNA extraction from milk samples and pellet cells was performed with the commercial extraction kit Qiamp Blood and Tissue Kit (Qiagen®), using three different protocols, established by the manufacturer: a) gram negative bacteria, b) gram positive bacteria, c) cultured cells (Volk et al., 2014).

The workflow of this extraction kit includes a chemical and enzymatic treatment of the sample, followed by nucleic acid precipitation. Samples are then loaded on silica columns with high affinity to dehydrated nucleic acids. Each column is attached to a collection tube. After two consecutive washes with buffers with different composition and ethanol concentration, the nucleic acid is eluted from the silica membrane with an aqueous buffer. Each described step is accomplished by serially apply the sample to the column, the washing buffers and the elution buffer, followed by a centrifugation step at 16.000 xg for 1 minute switching the collection tubes between each step.

a) Gram negative bacteria total DNA extraction

The sample was ressuspended in 180µl of ATL buffer with 20µl of Proteinase K and incubated at 56ºC for at least 2h for chemical and enzymatic lysis. To complete sample deproteinization and DNA precipitation, 200µL of AL buffer were added followed by 200µL of 96% ethanol. The sample was transferred to a silica column and centrifuged at 16000xg/1 min, for DNA attachment on the silica membrane. The columns were washed twice with 500µL of washing buffer AW1 and AW2. The DNA was eluted with 100 µL of elution buffer AE.

b) Gram positive bacteria total DNA extraction

The bacteria pellets were lysed with 200µL of ELB buffer with 20 mg/mL of Lysozyme for 30 minutes at 56ºC. Bacteria RNA was degraded by adding 4µL of RNAse A 5 minutes at room temperature. The sample was deproteinized with 25µL of Proteinase K and 200µL of AL at 56ºC for 30 minutes. After DNA precipitation with 200µL of ethanol, the suspension was loaded onto the silica column, washed twice with AW1 and AW2 buffers and eluted with 100 µ buffer AE.

c) DNA plasmid extraction (cultured cells)

For plasmid extraction, the ThermoScientific® Miniprep Kit was used, according to the manufacters' instruction. *E. coli* strain DH5α pelleted cells, obtained from 5 ml overnight culture in Luria-Bertani liquid medium (LB - 1% Tryptone; 1% NaCl; 0,5% Yeast extract), were ressuspended in 250 µL of Resuspension Solution. The cells were lysed with Lysis Solution (250 µL) and precipitation of bacterial genomic DNA was induced by adding 350 µL of Neutralization Solution. The solution was centrifuged at 12.000xg for 5 minutes and the supernatant was transferred to a Thermo Scientific GeneJET Spin Column and centrifuged for 1 minute at 14000xg. After two consecutive washes with 500µL of washing buffer, under the same centrifugation conditions, the plasmid DNA was eluted in 50µL of Elution Buffer.

Hydrolysis (Taqman®) probe q-PCR

The qPCR assay with Taqman® probe includes two primers (forward and reverse and a Taqman® probe, a primer-like sequence, complementary to an inner region, within the amplicon, labelled with a reporter (fluophore) in the 5' end, and a quencher in the 3' end). Due to the physical distance between both, the quencher blocks the fluorescence emission by the reporter. The probe only binds to a specific DNA region, within the forward and reverse primers targeted region. Once the binding occurs, the probe is hydrolyzed by the Taq polymerase during the primer extension, releasing the reporter from the blocking action of the quencher and allowing the emission of fluorescence, which is detected by the thermocycler. This fluorescence is proportional to the quantity of target sequence amplified (VanGuilder et al., 2008).

The qPCR reaction of this system was set in a reaction volume of 20µL including 10µL of Sensifast Mix (Citomed®) 0.5µM of uvrC primer forward and reverse each, 0.075µM of uvrC hydrolysis probe and 20-50ng of template DNA. The PCR cycling conditions included an initial denaturation step at 95ºC/10minutes, followed by 45 amplification cycles at 90ºC/15seconds and 60ºC/1minute. Fluorescence was read at the end of each round during the annealing step (Naikare et al., 2015). Primer and probes sequences are presented in the table 1.

SYBR Green q-PCR

With the SYBR Green assay, SYBR Green mix promotes the emission of the fluorescence when in the presence of double stranded DNA. Its dissociation temperature, specific of its nucleotide sequence, allows the evaluation of the specificity of the amplicon.
The qPCR in this assay was based on the study of Naikare et al. (2015) and Clothier et al. (2010) and was set in a reaction volume of 20µL including 10µL of SYBR Green Mix (Thermo Scientific®), 0.3µM of uvrC2024/2135 primer forward and reverse each and 20-50ng of template DNA (table 1). PCR cycling conditions were the same as described for the Hydrolysis probe qPCR, followed by a melting curve (95ºC/15seconds; 60ºC/1minute) to assess the dissociation temperature of the amplicons.

To evaluate the best amplification conditions, for both qPCR systems, different primer concentrations, 0,3 and 0,5µM for each system, and probe concentrations were assessed (0,15µM/0,075µM). Different cycles, and corresponding temperatures and durations, were also attempted, in order to define the ideal cycling conditions.

On both assays, reaction mixtures containing water and no DNA template were used as negative controls. Recombinant plasmids of each qPCR amplicon were used as positive controls.

Both PCR were run in the Applied Biosystems StepOne Plus thermal cycler instrument (Applied Biosystems®, Foster City, CA, USA).

Table 1 - Primer and probe sequences, target product size used in this study.

Gene Target	Oligo	Sequence (5'→3')	Product Size	References
uvrC	Primer Forward	**GAGAATGCTTCA GTATTTTGACGG**	170 bp	Naikare et al. (2015)
	Primer Reverse	**CAAAAGCAAAAT GTTAAATTCAGG**		Naikare et al. (2015)
	Probe	**(6-FAM)CATATA TAAGTGAGACTA ACTTATT(MGB)**		Lee K.H. et al. (2008)
uvrC	F2024	**TCTAATTTTTTCATC ATCGCTAATGC**	112 bp	Clothier et al. (2010)
	R2135	**TCAGGCCTTTGC TACAATGAAC**		
16S rRNA	Primer Forward	**CCTTTTAGATTG GGATAGCGGATG**	360 bp	Gonzalez et al. (1995)
	Primer Reverse	**CCGTCAAGGTAG CATCATTTCCTAT**		

Standard curve construction

The recombinant DNA pJETMbovis uvrC and the recombinant DNA pJETMbovis 2024-uvrC were diluted in 10-fold series from 10^{-1} to 10^{-8}; then, each set of recombinant DNA dilution was subjected to the Hydrolysis probe qPCR and SYBR green qPCR, with the described amplification conditions.

To quantify the target detection for each qPCR system, a standard curve was constructed, using ten-fold serial dilutions of each quantified recombinant plasmid. The Ct values of each dilution were used to calculate the standard curve, considering the number of target copies of each plasmid dilution (Fig.3).

Molecules:

$$1{,}04 \times 10^{11}(\#\ \text{molecules}) = \frac{\text{Weight (g)} = 0{,}000000312}{660\ (\text{MW bp}) \times \text{bp recombinant} = 1806565{,}5} \times 6{,}022\times10^{23}\ (\text{Avogadro number})$$

Figure 3– Equation used to calculate the number of molecules present in each dilution.

Recovery and purification of PCR product

For the recovery and purification of PCR products, the Zymoresearch® Clean and Concentrator Kit was used. The PCR reaction with the amplified amplicon, confirmed by horizontal electrophoresis, was added to Binding Buffer (5:1 proportion), transferred to the Zymo-Spin™ Column and centrifuged at 16.000xg for 30 seconds. After two washes with 200µL of the Washing Buffer provided by the kit, the amplicon DNA was eluted with 6µL of Elution Buffer.

Recovering and purification of DNA from agarose

The recovery of the amplicons directly from the agarose, after horizontal electrophoresis was performed with the Zymoresearch® Gel DNA Recovery Kit. After the amplicon excision from the agarose, 3 volumes of buffer ADB were added and incubated at 55ºC until complete dissolution of the agarose. The volume was then applied to a Zymo-Spin™ column, centrifuged at 16.000xg for 30 seconds, washed twice with 200µL of washing buffer after which the amplicon DNA was eluted with 6µL of Elution Buffer.

DNA Cloning

In order to obtain the recombinant plasmids containing the insert of interest, PCR amplicons were directly cloned into pJET1.2 (Thermo Scientific®). pJET1.2 is a positive cloning vector, with a lethal gene included within the multiple cloning site of the plasmid vector. The pJET1.2 also includes an ampicillin resistance gene for positive selection of recombinant colonies. The lethal gene is disrupted if the ligation with the inserted fragment is successful. This approach implies that all bacterial colonies able to propagate in the LB agar medium supplemented with ampicillin (100 µg/ml) are recombinant colonies. DNA cloning into bacterial plasmid includes the following steps:

I) Ligation of the insert within the plasmid-cloning site

II) Transformation of competent bacterial cells

III) Recombinant screening

IV) Amplification and extraction of recombinant plasmid DNA

I) Ligation of the insert within the plasmid-cloning site

The ligation reaction was performed using the Clone Jet PCR cloning kit (Thermo Scientific®). To a final 18µL reaction volume, 10 µL of 2x Reaction Buffer, 5ng of PCR product and 1µL of blunting enzyme were added. The insert weight added to the reaction mix, report to its length (bp) and conditioned the water volume. After an incubation step at 70 º C for 5 minutes, the reaction was chilled on ice and 50ng (1µL) of pJet 1.2/blunt Cloning Vector and 1µL of the T4 DNA ligase were added. After an additional incubation step at 22ºC for 5 minutes and the reaction was stored at 4ºC until the transformation process.

II) Transformation of competent bacterial cells

The transformation step was performed according to Clone Jet PCR cloning kit (Thermo Scientific®). To 50µL of competent cells (*E. coli* DH5α), 2-5µL of ligation mix were added and followed by incubation on ice for 20 minutes. The cells were subjected to a heat shock at 42ºC for 45 seconds, followed by 2 minutes on ice. After 1-3h recovery in 950µL of SOC (Super Optimal broth with Catabolite repression - 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) medium, at 37ºC in an orbital incubator with agitation (200rpm/minute), the cells were collected by

centrifugation (1000 xg/ 5minutes), inoculated in Luria-Bertania solid medium with 100 µg/ml ampicillin and incubated at 37ºC overnight.

III) Recombinant screening

Selected isolated colonies were picked from the transformation LB (1% Tryptone; 1% NaCl; 0,5% Yeast extract; 2% agar) amp plates and streaked into LB amp plates. After overnight incubation at 37ºC, the streaks were ressuspended in PBS and submitted to the specific qPCR. Positive streaks were amplified by an overnight culture in LB liquid medium supplemented with 100µg/ml of Ampicillin and frozen at -80ºC in 30% glycerol. The remaining overnight culture was used for DNA extraction of the recombinant plasmid.

IV) Amplification and extraction of the recombinant plasmid DNA (as above – see DNA plasmid extraction)

Extraction efficiency

To evaluate the extraction efficiency, milk samples were spiked with *E. coli* suspension (OD=1.5x10^8 CFU/ml: 0.5 McFarland Scale. The McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension 1.5x10^8 colony forming units (CFU/ml)) before extraction. After total DNA extraction the samples were subjected to conventional PCR for the amplification of a fragment included in *E. coli 16S*rRNA gene according to (Gonzalez et al.). Conventional PCR (cPCR) was performed in a 25µl reaction volume, with 12,5µL of DreamTaq (ThermoScientific®), 1µM of primer forward and primer reverse and 50-100ng of template DNA. The amplification cycle included a denaturation step at 94°C for 45 seconds, primer annealing at 60°C for 1 minute and extension at 72°C for 2 minutes (40 cycles).

Horizontal Electrophoresis

Conventional PCR amplicons were submitted to horizontal electrophoresis, in a 1.5% agarose gel. After complete dissolution of the agarose, 5µl/100ml agarose of Gel Red 10.000x (Biotium®) was added and the mixture poured into a gel casket. The horizontal electrophoresis was run at 90V for 50 minutes. The NZYDNA Ladder V (Nzytech) was

included in the electrophoresis run. Once completed, the gel was photographed and scanned using Biorad®Chemidoc imaging and analysis system.

Results

Specificity

For assessing the specificity of the systems both qPCR were performed against a set of different microorganisms including 1 yeast species and 11 different bacteria isolates. *Candida spp.*, *Proteus mirabilis*, *Salmonella* spp., *Pasteurella multocida*, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* ATCC25923, *Streptococcus agalactiae,* coagulase negative *Staphylococcus* JB09PE1, *Serratia marcescens*, *Klebsiella pneumonia* and *Enterococcus faecalis*. All isolates corresponded to field strains except for *Staphylococcus aureus* ATCC25923. Amplification was detected on *Streptococcus agalactiae* and coagulase negative *Staphylococcus* JB09PE1 when testing the hydrolysis probe *uvrC* qPCR system.

To fully characterize this lack of specificity, the amplicons were cloned, sequenced and the nucleotide sequences subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) confirming the amplified nucleotide sequence as corresponding to *Mycoplasma bovis* with an e value of 2e-79, resulting in a similarity of 99.41% for the *Streptococcus agalactiae* Blast analysis, while for the coagulase negative *Staphylococcus* the e value was 9e-78, corresponding to a 98.82% similarity to *M. bovis*, as seen in the annexes nº 3 and 4.

Figure 4- Taqman hydrolysis probe system sensitivity and concentration of primers and probe used (0,5µM/0,5µM /0,075µM). Representation of the amplification curves for each recombinant plasmid dilution.

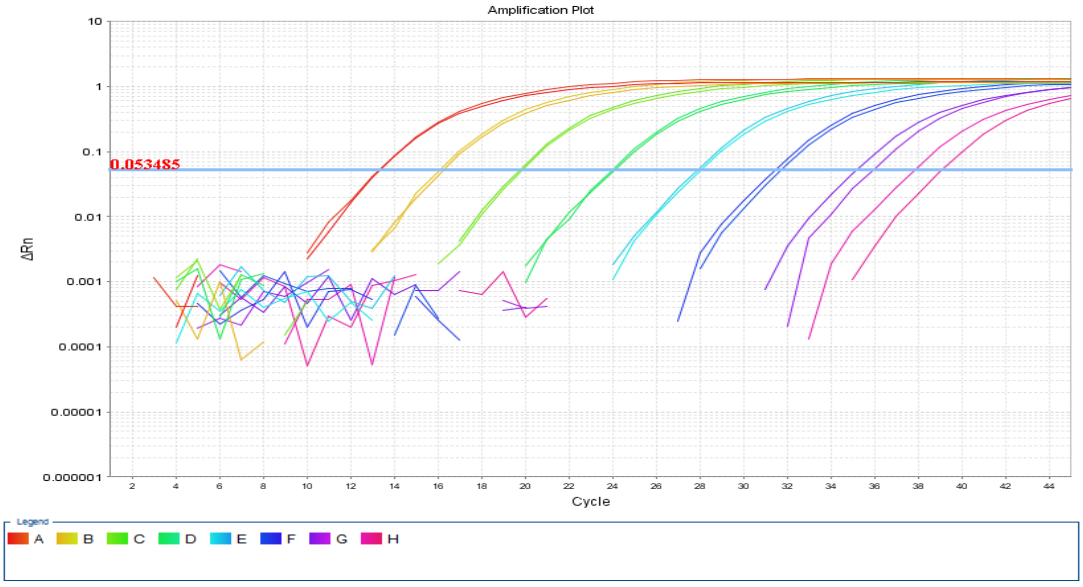


Figure 5 - SYBR Green system sensitivity and concentration of primers used in the mix (0,3µM/0,3µM). Representation of the amplification curves for each recombinant plasmid dilution.

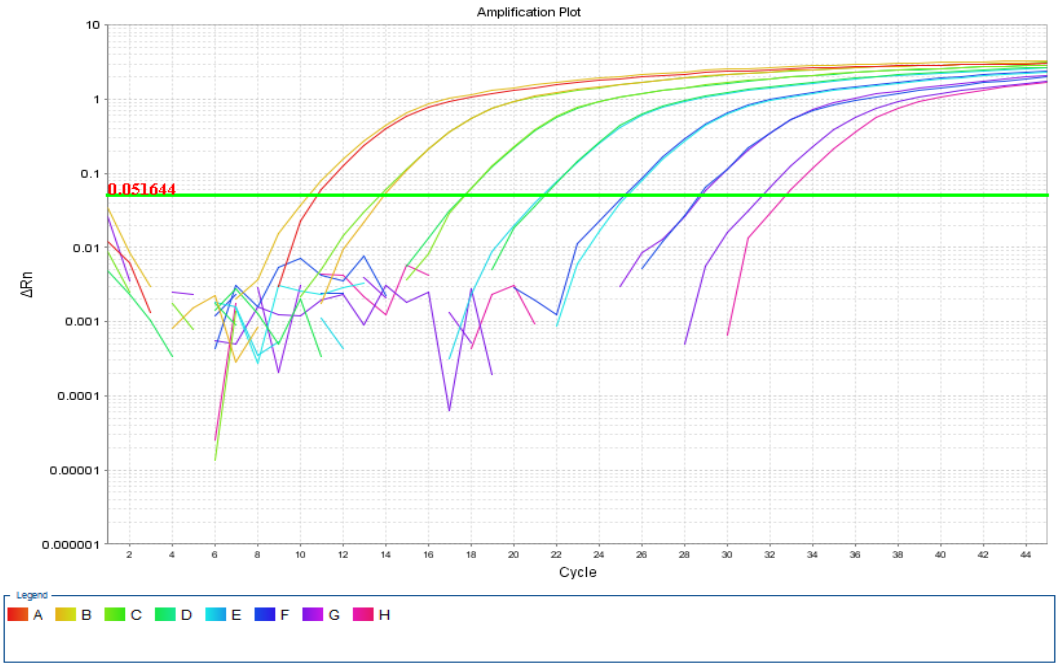
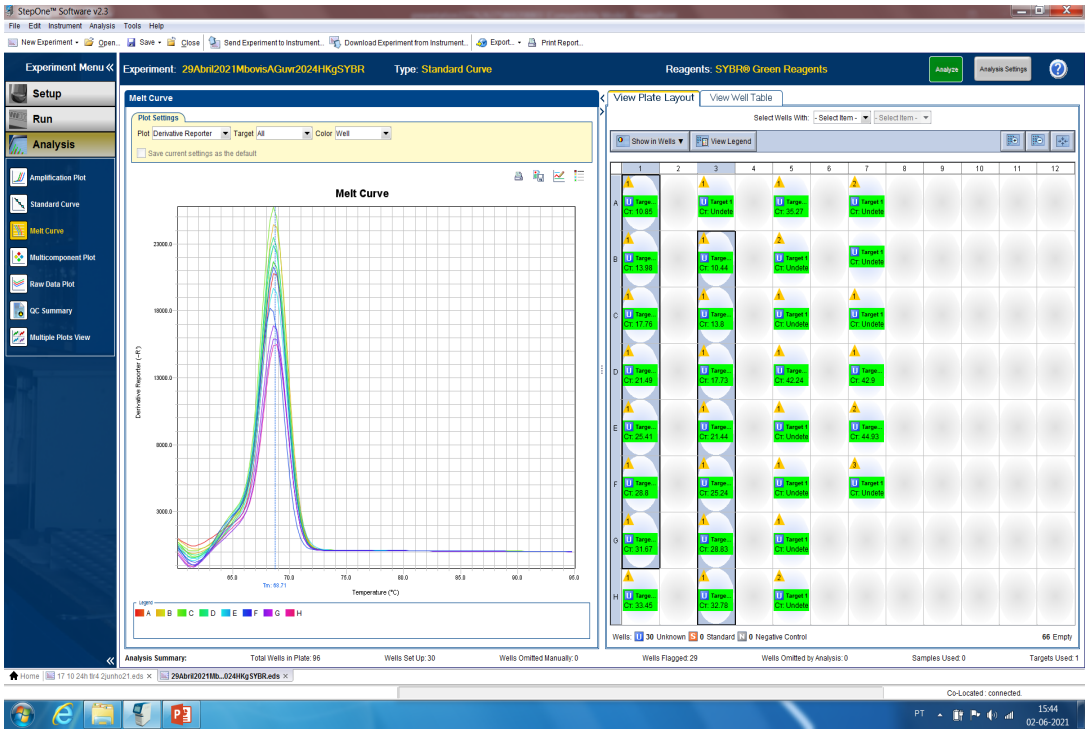


Figure 5a - SYBR Green system sensitivity and concentration of primers used in the mix (0,3µM/0,3µM). Representation of the melting curves for each recombinant plasmid dilution.



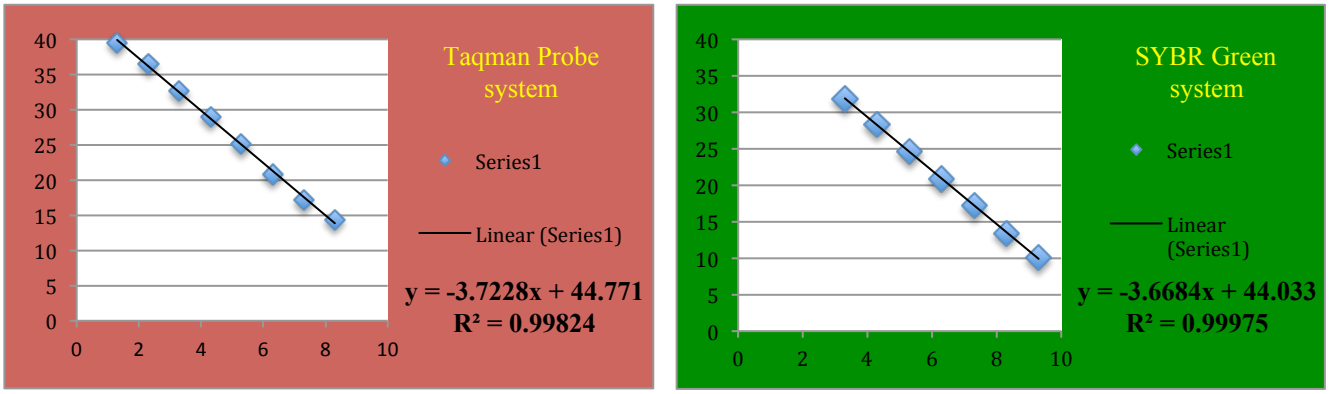
Sensitivity (limit of detection) and efficiency

The sensitivity of the q-PCR method determined as the limit of detection (LOD), was evaluated by using a set of serial dilutions of each pJET1.2 recombinant DNA (10^{-1} to 10^{-11}) ($2x10^{10}$ to 2 molecules), as can be seen next, in table 2. The highest dilution with positive and specific amplification was considered as the LOD.

Table 2 - pJET1.2 recombinant DNA dilutions and corresponding copy numbers.

10^{-1}	$2x10^{10}$
10^{-2}	$2x10^{9}$
10^{-3}	$2x10^{8}$
10^{-4}	$2x10^{7}$
10^{-5}	$2x10^{6}$
10^{-6}	$2x10^{5}$
10^{-7}	$2x10^{4}$
10^{-8}	$2x10^{3}$
10^{-9}	$2x10^{2}$
10^{-10}	2x10
10^{-11}	2

Figure 6- Sensitivity of the two qPCR systems.



The efficiency of reaction is calculated according to the following equation:

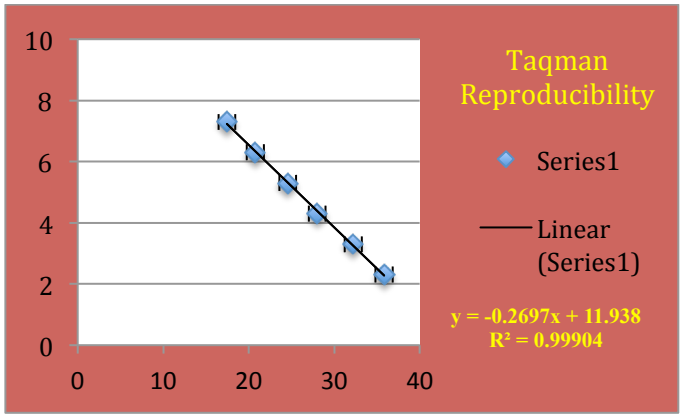
$$10^{(-1/slope)} - 1$$

The hydrolysis probe system revealed an efficiency of 85.6%, and a LOD of $2.00x10^{2}$ molecules, whereas the SYBR Green system showed an efficiency of 87.3% with a LOD of $2.00x10^{3}$ molecules (Fig. 8).

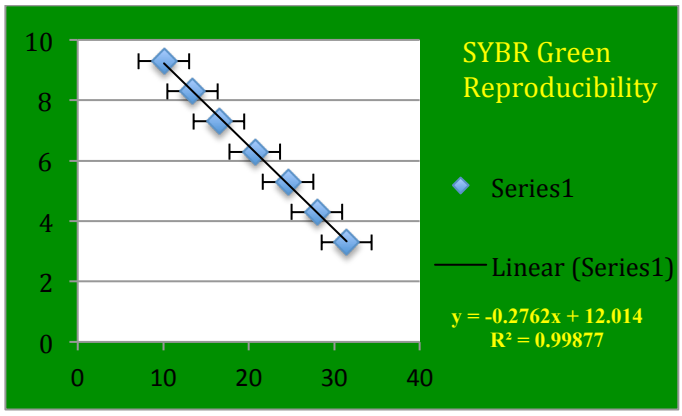
Reproducibility

To evaluate the inter-assay and intra-assay variability, each dilution series (10^{-4} - 10^{-9}) was repeated in three independent runs, performed in three different occasions. The variability between the hydrolysis probe qPCR runs was 1.2 Ct for the same dilution, and in the SYBR Green assay a maximum of 1.4 in Ct count for the same dilution, and is depicted below (Fig. 9).

Figure 7 - Reproducibility of the qPCR systems. Variation of Ct counts on different qPCR runs.



Log7	17.50	17.78	17.28	17.22
Log6	20.78	20.94	20.69	20.65
Log5	23.96	24.38	24.93	24.97
Log4	27.60	27.40	28.46	28.52
Log3	32.51	32.78	32.32	32.10
Log2	34.94	35.24	35.89	35.76



Log7	17.19	17.09	16.55	17.22
Log6	20.83	20.70	20.15	21.24
Log5	24.76	24.57	23.87	25.22
Log4	28.16	28.18	27.61	29.52
Log3	31.02	32.17	31.13	32,10

M. bovis molecular diagnosis in raw milk samples

Both qPCR systems were tested with raw milk samples in order to evaluate its robustness and to provide an initial insight about *M. bovis* distribution in dairy farms.

A total of 93 milk samples from 75 animals divided by 4 different milk production farms (A, n=8; B, n=5; C, n=45 e D, n=34) were processed and analyzed. Whenever available samples from the different quarters of the udder were used.

Table 3 - Results of milk sample testing for *M. bovis* in the 4 farms. Qualitative classification.

	A (n=8)	B (n=5)	C (n=45)	D (n=34)
POSITIVE	8	4	31	1
NEGATIVE	0	0	3	4
DISCORDANT	0	1	12	29

The samples were tested with the two systems, SYBR Green and the Taqman hydrolysis probe system. The Ct threshold value was set at 35.5. Samples with a Ct count inferior to 35.5, on both systems, were considered positive. Samples with a Ct count over 35.5 or undetermined, on both systems, were considered negative. Samples that differed in classification as positive or negative between the two systems were determined to be discordant. There was no discrepancies regarding the positive and negative classification of the samples, but the detection timing represented by the recorded Ct ranged between the Ct 22 to 38 with the SYB green system, whereas with the hydrolysis probe assay ranged between the Ct 28 to 40, according to the tables in the annexes. The qualitative results divided by each farm are represented above, in table 3.

In the end, and coming as a surprise, most of the analyzed samples tested positive to *M. bovis* DNA, although with low copy number (with a bacterial load per mL of milk of 5.33E+2 to 7.23E+5 in the probe system and 1.2E+2 to 1.8E+7 in the SYBR Green system) in both qPCR systems. In the qPCR system with the hydrolysis probe 44 out of 93 milk samples tested positive while in the SYBR Green qPCR system 87 out of 93 milk samples were positive to *M. bovis*. The tables in the annexes can show, more precisely, which samples turned out positive, negative or discordant, in each system, from the 4 farms.

Discussion

Data availability concerning the prevalence of *M. bovis* infection throughout the world is still scarce. Information regarding *M. bovis* infections is available in several countries. Considering this, a survey in the United States claimed that 6.8% of the farms were *M. bovis* positive on a bulk tank milk culture (Maunsell et al., 2011). In Central and South America, the prevalence values can be higher. Outbreaks of *Mycoplasma bovis* mastitis have also been reported in Europe. Though, a concerted approach towards diagnosis and treatment of *M. bovis* infections it is still of utmost importance to define. For this, several diagnosis assays have been suggested and it is under this context that this study was developed.

What are the risk factors?

Prophylaxis and other measures to adopt, in the survey and control of *M. bovis* infections

M. bovis infections of the udder have specific characteristics, including high contagiousness; affects more than one quarter; leads to a significant loss in milk production; are frequently refractory to antibiotic treatment; are clinically silent affecting cows without or few signs of disease (Nicholas et al., 2016), the infection through milk feeding in the farm and mastitis of pre-pubescent animals are also reoccurring during *M. bovis* infections. Therefore, it is essential to early recognize and tackle the problem, enabling the rapid removal or culling of the infected animals. Late diagnosis, which is often the case due to the diagnosis difficulties, is more problematic. Historically, it has been considered that mycoplasma mastitis might be best controlled by the introduction of a surveillance test and slaughter program (Pfutzner & Sachse, 1996). The diagnosis methods commonly used to test the animals in dairy farms are bacteriological culture from bulk tank milk (BTM), which provides a cost effective way of monitoring herd status, although negative cultures do not necessarily guarantee absence of disease (Fox, 2012). BTM ELISA is useful in determining the herd seroprevalence (Parker et al., 2018), particularly when correlating the results also with the optical density of the BTM (Petersen et al., 2019). The optical density is directly linked to somatic cell counts (SCC), which when high are useful to detect subclinical infections (Hazelton et al., 2020). However, low SCC are not uncommon in cows with subclinical

intramammary infection with *M. bovis* (Gonzalez & Wilson, 2003; Higuchi, Iwano, Gondaira, Kawai, Nagahata, 2011).

When *M. bovis* is detected in the bulk tank, and eradication is being attempted, Pinho et al. (2013) recommended sampling of individual animals in the herd rather than evaluating only animals with clinical signs, using measures such as SCC. Sampling and testing methods for detection of subclinical intramammary infections must be practical and cost-effective. The options are to use milk samples collected during commercial milk recordings or by pooling samples. In herds that already use routine milk recording, this option would allow collection of samples from each lactating cow at minimal marginal cost, with costs further reduced by pooling samples. This involves testing pools of samples from multiple cows, with subsequent individual testing of animals associated with positive pool tests. There is evidence that pooling reduces diagnostic sensitivity but only modestly (Hazelton et al., 2020). For example, Murai, Lehenbauer, Champagne, Glenn and Aly (2014) found that PCR on pools of samples from 50 cows followed by culture of individual cow samples from PCR-positive pools had a diagnostic sensitivity of 81.3%. Hazelton's study also suggests that exposure to and infection with *M. bovis* increases the risk that heifers will have to be removed from dairy herds before the first calving. A supplementary analysis suggested that the death/euthanasia among the young calves are affected by the disease among the lactating cows, rather than disease among the other youngstock. There are a number of risk factors associated to infection sources, transmission and spread of udder pathogens within and between dairy farms such as:

Herd size

Larger herd sizes provide greater opportunity for the organism to spread and therefore easier to maintain itself within the population. In opposition, smaller herds allow more frequent transmission breaks, probably as a result of lower stocking densities and fewer susceptible animals (Nicholas et al., 2016).

McCluskey, Lombard and Hirst (2003), demonstrated prevalence of mycoplasma in bulk milk tanks of 2.1%, 3.9% and 21.7% in herds with < 100, 100-499 and more than 500 cows, respectively, providing conclusive evidence of herd size risk.

Introduced cattle

The contagious nature of mycoplasma infections means that infected animals are the main source of infections for other livestock. Bovine mycoplasmosis is no exception and new infections invariably can be traced to introduction or contact with clinically or subclinically affected cattle (Punyapornwithaya, Fox, Hancock, Gay & Alldredge, 2010). Cows with previous contact with infected animals may harbor Mycoplasma until stressful conditions such as calving, results in the development of contagious disease. Once introduced into a herd, Mycoplasma can be transmitted rapidly to up to 40% of healthy cattle unless they are segregated (Punyapornwithaya et al., 2010). Consequently, tt is of major relevance to test the animals before introduction.

Other risk factors

Feeding waste milk or colostrum to livestock increases the risk of transmission to the rest of the herd (Foster et al., 2008); waste milk pasteurization is recommended in these cases. The lack of a well-separated sick or hospital pen has also been identified as a risk factor in the spread of the infection (Pfuzner & Sachse, 1996; Jensen, 2015). Return of hospitalized cattle to the healthy pen should be considered very carefully, since they may sporadically excrete Mycoplasma for over a year. As evidenced above the presence of calves in close contact with dairy cattle is a risk factor, since respiratory secretions via aerosols and nose-to-nose contact are important in the spread of respiratory disease (Maunsell et al., 2011; Lysnyansky et al., 2016). Prolonged colonization of the nasal cavity by *M. bovis* of young stock has been reported (Aebi et al., 2015); this could lead to the dissemination of the agent to the lungs of cows, followed by spread to the mammary glands via the blood stream. The discovery of Mycoplasma biofilms, in which there is differential gene expression, possibly leading to higher virulence, makes this route of infection increasingly more frequent (McAuliffe et al., 2006; Simmons & Dybvig, 2007).

Mycoplasma mastitis is considered untreatable and, consequently, culling remains the most common frequent recommendation aiming at its control. But some authors (Punyapornwithaya et al., 2010) suggest that due to the infection's largely self-limiting nature, that control can be achieved without culling, but with specific removal of infected cows into well separated hospital pens (testing and segregating) where full milking time hygiene practices can be applied. Transmission of mycoplasma mastitis in a hospital pen can be > 100 fold higher than in the healthy pen, often despite the use of excellent milking time hygiene. Quarantine before introducing animals into a new herd is, in theory, a good

management practice and can be considered as an effective control strategy (Fox, 2012), however it is rarely applied mostly because it requires considerable management and is not always cost effective. As such, biosecurity practices of isolation should be considered in areas of high risk or where mycoplasma free status needs to be maintained.

Control of Mycoplasma mastitis by antibiotic treatment is generally not effective for a number of reasons. Firstly, mycoplasmas are inherently resistant to penicillin and cephalosporins. Secondly, in *M. bovis* isolates over the last two decades, *in vitro* resistance has been detected to all the main antibiotic classes, including the fluoroquinolones, which probably explains their lack of impact *in vivo* (Waites, Lysnyansky & Bébéar, 2014). Thirdly, the ability of mycoplasmas to infect different body sites means that treatment is rarely sufficiently thorough, enabling their survival. Fourthly, mycoplasmas are able to invade host cells and form biofilms, which allows them to evade antibiotic therapy (McAuliffe et al., 2006). Most investigators have concluded that, based on their field experience, antibiotic therapy of *Mycoplasma* mastitis is not an economically viable control strategy.

Regarding vaccination, due to the *Mycoplasma* antigenic nature and adaptive strategies to outmaneuver the immune system (ability to evade the host by altering their surface proteins and inducing immunomodulatory effects), it is hardly believed that the existing vaccines, aimed mainly at the pneumonic form of the disease, are fully efficient. Also the development of successful *Mycoplasma* vaccines has presented serious difficulties.

Concluding, it is clear that *Mycoplasma* mastitis is an important and distinct problem in countries with large dairy herds and could be a growing risk for those countries where plans are to increase herd size. On the other hand, there are many documented reports of herds that are free from Mycoplasma mastitis, including some that have remained free of this microorganism for decades. In some regions of the world, Mycoplasma mastitis may affect up to half of all herds, whereas in other areas it has a very low to zero prevalence (Fox, 2012). The largest risk factor is importation of cattle into a herd. Such importation is becoming more common as increasing numbers of herds outsource their heifer rearing and expand their herd sizes through the purchase of replacements. Thus, control and biosecurity are of major importance in preventing the introduction of the agent into the herd and minimizing the impact of an outbreak. In the 1990s, following the restocking of herds in Ireland following the bovine spongiform encephalopathy cull, The Agriculture and Food Development Authority included *M. bovis* in its list of organisms to be avoided in its national herd (O'Farrell et al., 2001). Serological screening was adopted to sample donor herds, with a great deal of success, providing a future blueprint for *M. bovis* free herds. The development of molecular epidemiologic methods, as is genotyping the *M. bovis* isolates by Multi-locus sequence typing (MLST) (Lysnyansky et al., 2016) and core genome MLST (cg-MLST) (Haapala et al., 2018), enables a comparison between *M. bovis* strains at herd and animal

level and provides a useful tool to identify infection sources and different transmission routes of *M. bovis* infections.

For Mycoplasma control, Nicholas et al. (2016) recommended the following approach: [1] sample and test bulk tank milk weekly to rapidly identify infected cows and to monitor success of management changes; [2] clean and disinfect milking equipment between milking sessions; [3] test milk samples from all cows before they enter or re-enter the lactating herd; [4] if possible, rapidly segregate cows found to be mycoplasma positive or showing mycoplasma mastitis to hospital pens and carefully monitor contacts; [5] cull cows where welfare is compromised; [6] antibiotic treatment should be discouraged when *M. bovis* has been confirmed; [7] regarding infected cows, these should remain segregated for life unless milk is shown to be *M. bovis*-free over three successive monthly phases of testing; and [8] in farms where *M. bovis* is suspected or confirmed, waste milk should be discarded or pasteurized before feeding to calves.

The homology between *M. bovis* and *M. agalactiae*. The *uvrC*/*uvrC2024* as a target gene.

In Portugal, information regarding *M. bovis* frequency is limited and in an effort to contribute for a concerted approach a previously reported q-PCR (Nakare et al. and Clothier et al.) was evaluated as a diagnostic tool, to identify *M. bovis* infection/presence in Portuguese dairy farms.

Based on these previous developments, a q-PCR approach was developed, and two systems were considered in order to tackle the necessities referred above. For these two systems, two gene targets were established, the *uvrC* for the hydrolysis probe system, and the *uvrC2024* for the SYBR Green system as previously described by Nakare et al. (2015)
Nevertheless, when testing the two systems for its specificity we came across two cases of amplification and positivity for the presence of *M. bovis'* DNA when running the Taqman hydrolysis probe system with coagulase negative *Staphylococcus*' DNA and *Streptococcus agalactiae*'s DNA. Cloning and sequencing of each amplicon, revealed the amplification of *M. bovis* DNA excluding the lack of specificity of the qPCR assay. One possible explanation could be the biological matrix (raw milk) from which *Streptococcus agalactiae* was isolated, allowing the maintenance of Mycoplasma DNA within the bacterial biofilms.
Considering the already described homology between *M. bovis* and *M. agalactiae* genome (Gonzalez et al., 1995), and the potential risk of lack of specificity of molecular diagnostic assays, could the *uvrC* gene be paired with other target genes? Several authors have addressed this question. Marenda et al. (2005) identified two sets of *M. agalactiae* and *M.*

bovis species specific DNA sequences by PCR-based suppressive subtractive hybridization (SSH), a broad approach that allows the comparison of closely related bacterial genomes, even in the presence of limited sequence information and presented the possibility of adding the *polC* gene as a complimentary target gene to *uvrC*. Foddai. et al. (2005) also presented the possibility of using the *ma/mb-mp81* gene (membrane protein 81 gene) as a target to differentiate *M. bovis* from *M. agalactiae*. Sasche et al. (2010) targeted the *oppD* (from the *oppD/F* gene region of *M. bovis*, which encodes ATP-binding proteins of the ABC-transporter family) while S. Boonyayatra et al. (2012) used the *fusA* gene, a housekeeping gene, encoding for elongation factor G for such differentiation. Despite the validation of the *uvrC* gene as a suitable target gene to a qPCR diagnosis of *M. bovis* (Subramaniam et al., 1998) it is of interest to set a multi target qPCR technique to secure the specificity of the molecular test used for *M. bovis* and even *M. agalactiae* diagnosis. These bacteria are, phylogenetically close, although with different host species, bovine (*M. bovis*) and small ruminants (*M. agalactiae*). In this study, the processed milk samples had a mastitic appearance, and the choice of the dairy farms was biased. The main objective of this work was to evaluate a robust molecular assay for *M. bovis*; it was our interest to collect potential positive samples, justifying our positive results (44/93). Nevertheless the 47.3% of positivity recorded in our biased sample, suggests the need to implement control measures, which include the development of a surveillance net.

Molecular detection of *M. bovis* in the analyzed sample does necessarily mean sickness? Which Ct (cycle threshold) count or CFU/ml of milk leads to sickness or low performance?

Our samples were collected from dairy farms with previous and present events of mastitis or previous records of *M. bovis* infection. Despite the fact that the majority of the samples came from apparently healthy animals, Farm D presented a high number of discordant analysis (29/34) and a higher Ct count, which can possibly point to a subclinical condition. rather than an acute infection, raising the need to sample diseased but also healthy animals. Timmonen et al. (2020) studied the dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds. Four herds were analyzed. Many of these animals had no reports of disease, some with only a slight decrease of weight, and others showed signs of infection, as they can develop mastitis with intermittent or low shedding of the agent. *M. bovis* may cause subclinical or mild clinical mastitis, which can progress to chronic infection. However, severe clinical mastitis outbreaks may also occur. Intermittent mycoplasma shedding in cows with intra mammary infection

(IMI) could lead to differences in the shedding patterns and herd prevalence). Additionally, some cows with mycoplasma IMI excrete a low concentration of mycoplasma organisms (10^6 cells/ml) in composite milk samples (CMS) (Timmonen et al., 2020).

According to Castillo-Alcala et al. (2012), *M. bovis* can be detected through culture methods, serological testing and PCR analysis in several groups of animals with no clinical signs, including subclinical carriers and animals with chronic disease. In this study samples were collected in more than one moment; in the early sample collection period, collected at the time of arrival to the feedlot, *M. bovis* was isolated from 26 of 36 (72.2%) calves. Specifically, *M. bovis* was isolated in pure culture from 14 of 36 (38.9%) calves and in mixed bacterial infections with *M. bovirhinis*, *M. haemolytica*, *Pasteurella spp*, or *H. somni* in 12 of 36 (33.3%) calves. In the late sample collection period, 55 days later, *M. bovis* was isolated from 13 of 13 calves coinfected with Pasteurellaceae and from 23 of 29 (79.3%) calves from which Pasteurellaceae were not isolated. Mixed mycoplasmal and bacterial cultures were obtained from 39 of 42 (92.9%) calves.

As accounted previously, the presence of *M. bovis* in a herd does not necessarily mean that the animals developed disease, and when using a molecular diagnosis method such as the qPCR, a quantitative assay, the Ct count can provide an insight regarding positivity quantification. In a q-PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample; the lower the Ct value the higher the concentration of target nucleic acid in the sample). Sachse et al. (2010) monitored and quantified *M. bovis* infection in cattle herds with mastitis and respiratory disease, in Germany. Before implementation of sanitary measures, milk samples were collected from a herd with persistent clinical mastitis, recording *M. bovis* loads of 271 to 4.25 x 10^9 CFU/mL of milk. In an attempt to reduce the level of infection within this herd, diseased animals were repeatedly removed, resulting in the disappearance of clinical mastitis in the herd. After implementing the sanitary measures, a considerable number of the second batch samples collected from this heard (23/36), taken from apparently healthy and disease-free animals, were still positive. However, when the level of shedding in milk was compared, a significant decrease was detected (the first batch had a mean of $2.62x10^8$ CFU/mL, relative to 3.85 10^6 CFU/mL after the removal of the animals with clinical signs). A third batch was collected from the herd with mastitis suspicions and only from animals with clinical signs or altered milk consistency, registering a minimum copy number of $1.99x10^4$, a maximum of $4.42x10^8$, and a mean of $6.63x10^7$ (CFU/mL). Interpreting this information it is possible to associate the presence of clinical signs with higher bacterial load as it was already expected, on the other

side, lower bacterial loads may come from animals that show no signs, or only mild symptoms, of disease.

In this study, already considering the high amount of positive samples (44/93), there is still a significant number of discordant cases, whether positive in the hydrolysis probe system or positive in the SYBR Green system, differing in Ct count, due to the differences in the sensitivity of the assays. Most of these discordant samples showed a specific amplification curve, and in the SYBR Green system, a specific melting curve. Keeping this in mind, a reassessment of the positivity criteria led to a rise of the positive cases and a decrease in the discordant cases. Considering that positive samples yield specific amplification curves, the frequency of positive samples changes from 47.3% to 93.5% (87/93), with 4 samples still considered discordant, (annex nº 5).

There is still much work to develop on what could be a sensitive, specific and, overall, reliable diagnosis method to the presence of *M. bovis*. This includes not only the correct qPCR assay to be developed, but it also involves a standardization of the procedures, from a proper collection and handling of the collected samples, to the nucleic acid extraction and testing. The main goal of this study was to present another alternative to what could be an assay for *M. bovis* diagnosis, preferably, a fast and cheap method, justifying the two proposed assays.

Conclusion

The wide presence of *M. bovis* in dairy farms makes the challenge of tackling this issue urgent. This study was developed with the main goal of presenting another possibility to diagnose *M. bovis* in biological samples. Testing on milk samples can turn the samples' collection simpler and more efficient, benefiting not only the farm manager, but also the animals and even the veterinarian responsible. The qPCR assays developed also allow the diagnosis to be less laborious comparing to the gold standard method of bacteriological culture. Two assays were presented, with slight differences when it comes to the sensitivity of the method, but with the advantage of having a choice between a cheaper and less sensitive and a more expensive method but with lower LOD. In this study it was also discussed the probable risk factors to the presence of *M. bovis* infections in herds. Taking this in account, the objective of the study was reached and in here lays another possibility to help setting a needed standard procedure, from prophylaxis to diagnosis of *M. bovis*.

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Annexes

1. pJET uvrC *Mycoplasma bovis*

M. bovis uvrC sequence

AAAAGCAAAATGTTAAATTCAGGATTATATCTGTCAATCATTGCTTTTTCTAAAAGCAATGCCTCTT
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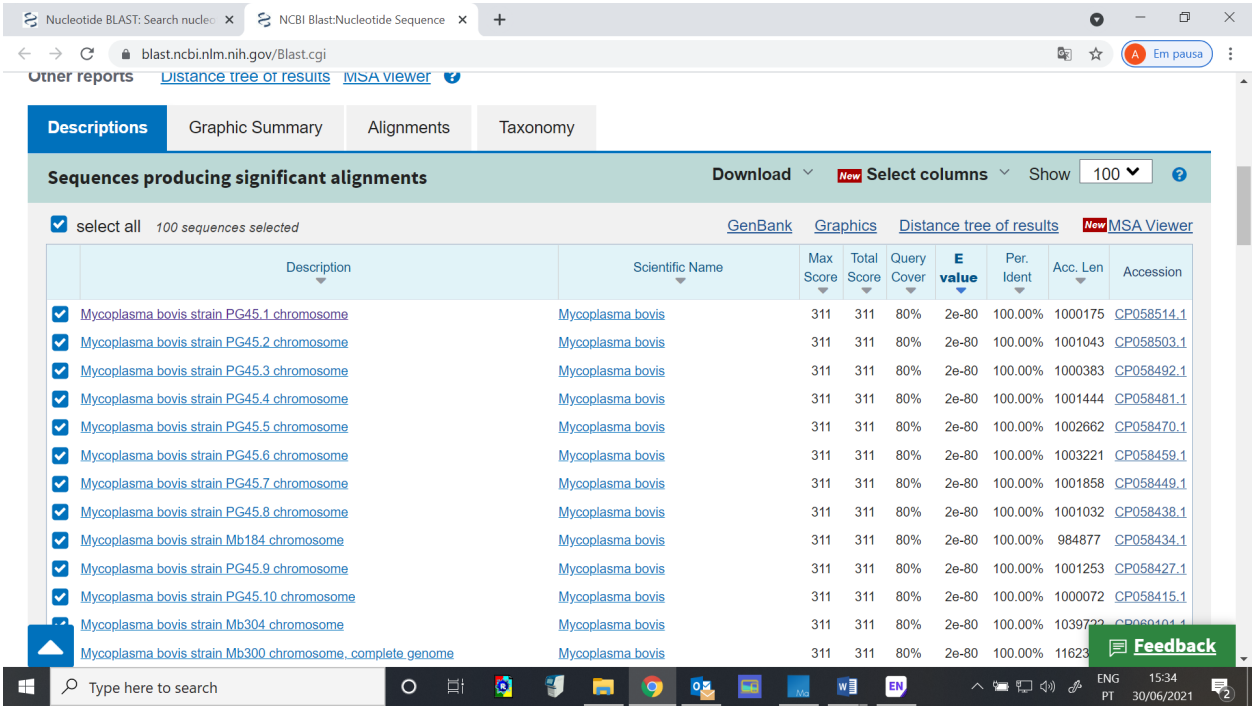

Blast Analysis results for the uvrC recombinant plasmid.

2. pJET uvrC2024 *Mycoplasma bovis*

M. bovis uvrC2024 sequence

TCAGGCCTTTGCTACAATGAACTTATTTTTAACTAACGCAAATAAAACATATAGTATGTGATATGAT
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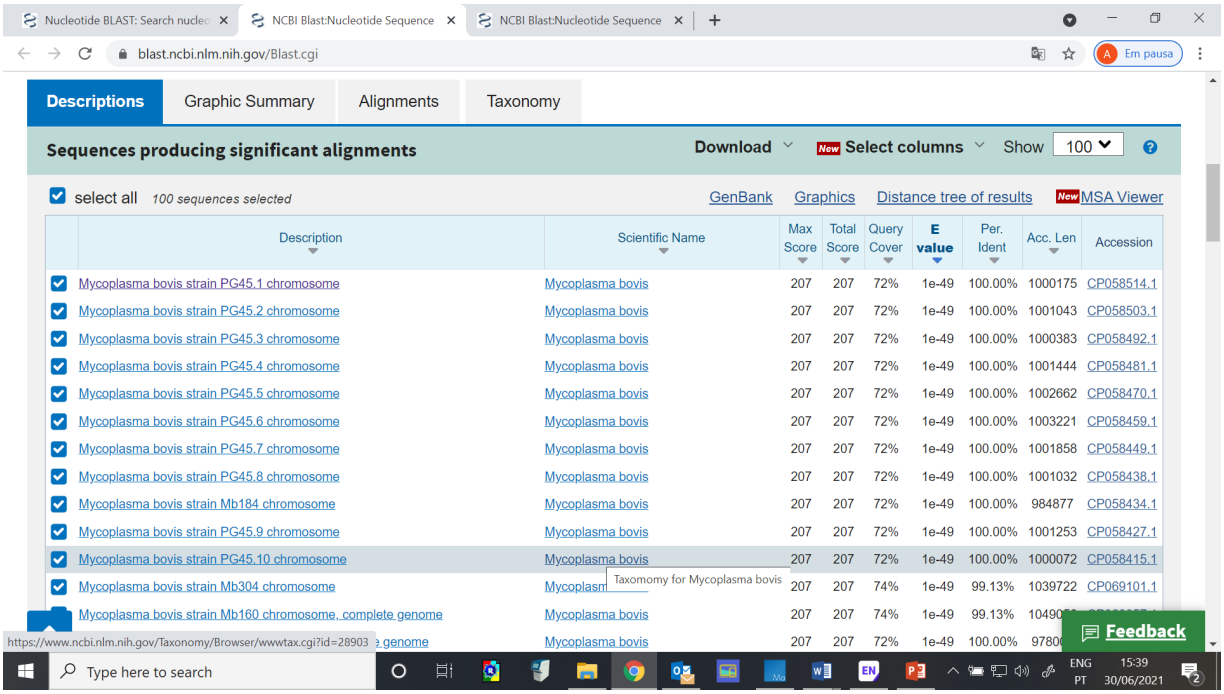

Blast analysis results for *uvrC* 2024 recombinant plasmid.

3. pJET uvrC *Streptococcus agalactiae*

Streptococcus agalactiae (Taqman system) sequence

GAGAATGCTTCAGTATTTTGACGGCGCTATCAATTCATATAAAACTAATAAGTTAGTCTCACTTATA
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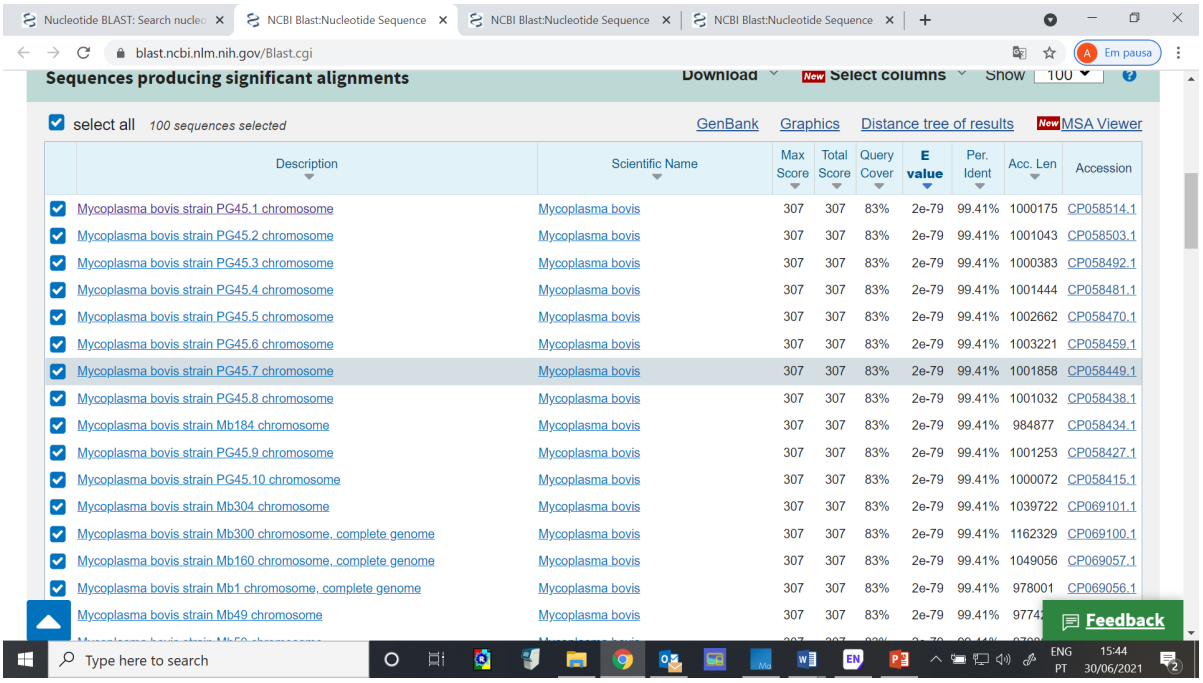
TGACAGATATAATCCTGAATTTAACATTTTGCTTTTG



Blast analysis result for *Streptococcus agalactiae* positivity, when assessing the *uvrC* Taqman probe system specificity.

4. pJET uvrC coagulase negative *Staphylococcus*

Coagulase negative *Staphylococcus* (Taqman system) sequence

CAAAAGCAAAATGTTAAATTCAGGATTATATCTGTCGATCATTGCTTTTTCTAAAAGCAATGCCTCT
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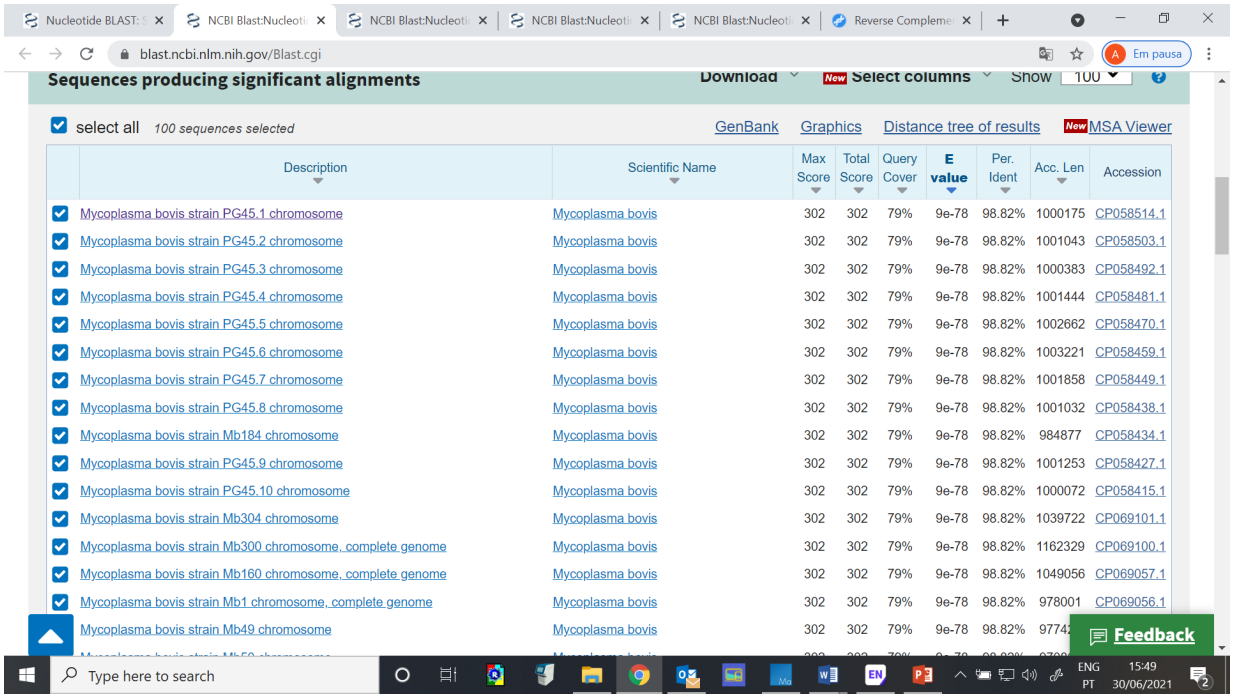


Blast analysis result for Coagulase Negative *Staphylococcus* positivity when assessing the *uvrC* taqman probe system specificity.

5. qPCR analysis results per sample

(plate 1 including samples from: C)

Sample nº	Ct value (SYBR)	Ct value (HP)	Result
9521D	33,595	31,90	+
9521E	Und.	36,63	-
9494TD+FE	26,92	31,98	+
9494TE	33,81	35,17	+
4838T	33,90	39,15	+/-
4838FD	31,96	34,64	+
2694D	28,53	33,43	+
2694E	25,76	31,40	+
4433D	30,46	35,33	+
4433F	32,99	36,58	+/-
2532TD	Und.	Und.	-
620FD	Und.	36,73	-
720FD	29,07	35,54	+
27TE	28,82	34,40	+
110TE	26,18	31,16	+
5655TD	26,34	32,12	+
3779TD	27,05	32,02	+
9233TD	28,10	31,33	+
1406TD	32,68	Und.	+/-
4306T	34,32	35,61	+/-
5736TD	26,55	31,63	+
9168TD	25,29	32,82	+
5749PE+TD	29,11	32,88	+
4808TD	27,62	33,24	+
9451TE	26,97	Und.	+/-
5775FD	30,76	Und.	+/-
9155TD	30,00	34,62	+
9422TE+FD	32,94	33,77	+
BTM1	31,71	36,73	+/-
BTM2	23,15	28,71	+

(plate 2 including samples from: A, B, C)

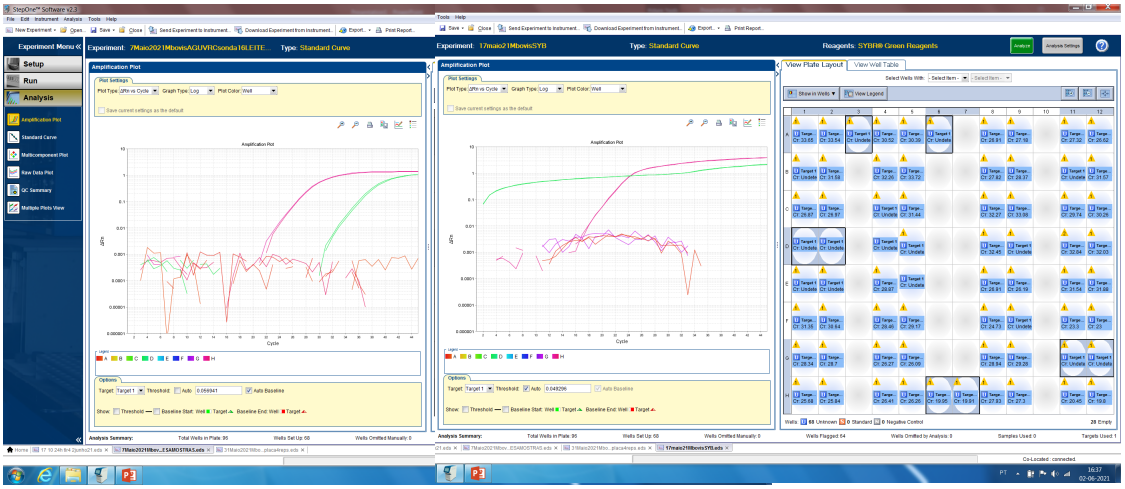
Sample nº	Ct value (SYBR)	Ct value (HP)	Result
9080D	27,68	33,32	+
9080TE	27,52	34,00	+
5690TD	27,07	33,31	+
5690F	27,37	34,33	+
5690E	27,50	34,22	+
5746TD	28,00	34,41	+
5746FD	26,74	33,70	+
8175TD	26,74	33,45	+
7107D	32,75	36,18	+/-
7107TE	27,89	33,87	+
610D	27,89	34,39	+
849E	27,64	34,29	+
853T	27,51	33,86	+
42TD	24,59	Und.	+/-
31TE	28,51	34,08	+
38T	29,68	35,70	+/-
60D	31,32	37,44	+/-
94D	27,67	35,13	+
253FD	27,99	33,60	+
2515TD	28,87	34,48	+
3749D	28,26	34,56	+
3749E	27,30	34,20	+
5775FE	26,95	33,53	+
5839FD	27,23	33,74	+
7953TE	32,53	36,87	+/-
9235D	27,32	33,98	+
9410TE	33,52	35,64	+/-
4271FD	27,18	31,87	+
5709TE	30,51	32,65	+

(plate 3 including samples from: D)

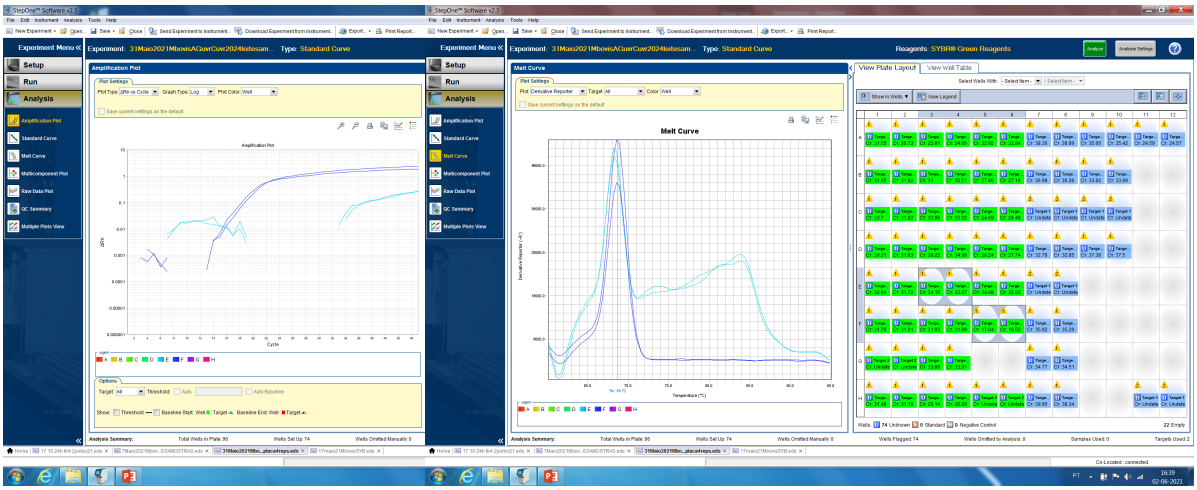
Sample nº	Ct value (SYBR)	Ct value (HP)	Result
8181D	32,30	37,27	+/-
8181E	30,94	36,87	+/-
7190FD	31,84	38,54	+/-
7190E	31,98	36,18	+/-
726D	33,20	28,89	+
726E	32,42	38,43	+/-
5153D	32,23	37,25	+/-
5153E	32,59	36,41	+/-
906FE+TD	33,59	36,99	+/-
906TE	32,10	37,60	+/-
882TE	31,14	38,57	+/-
882FD	33,13	37,52	+/-
884E	32,70	36,94	+/-
845TD	33,27	38,19	+/-
670E	33,62	37,79	+/-
720TE	32,66	36,04	+/-
711T	31,92	36,55	+/-
748D	31,92	37,11	+/-
849FE	34,04	36,23	+/-
8211FD	32,62	37,77	+/-
8216TD	35,11	37,70	+/-
8226TE	31,64	37,65	+/-
6214FE+TD	32,81	37,85	+/-
7187FD+TE	34,32	36,81	+/-
8133TD	35,98	36,54	-
6112TD	35,76	37,28	-
6126FE	31,02	36,30	+/-
7108TD	32,18	38,68	+/-
7109T	31,84	37,56	+/-
7122TE	Und.	36,94	-
8191TE	33,56	36,97	+/-
9246TE	31,33	39,85	+/-
6184F	33,79	36,90	+/-
6241TE	33,21	37,50	+/-

SAMPLES – AN INSIDE LOOK

9494TE



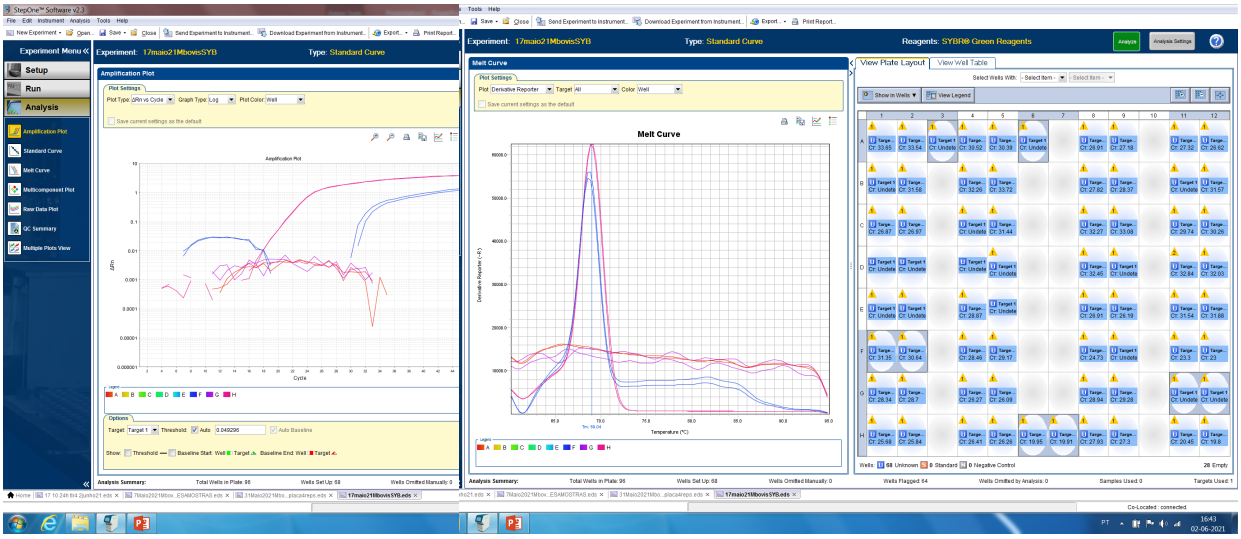
Taqman Hydrolysis Probe System (Ct = 35.17)



SYBR Green System (Ct = 33.81)

4838FD

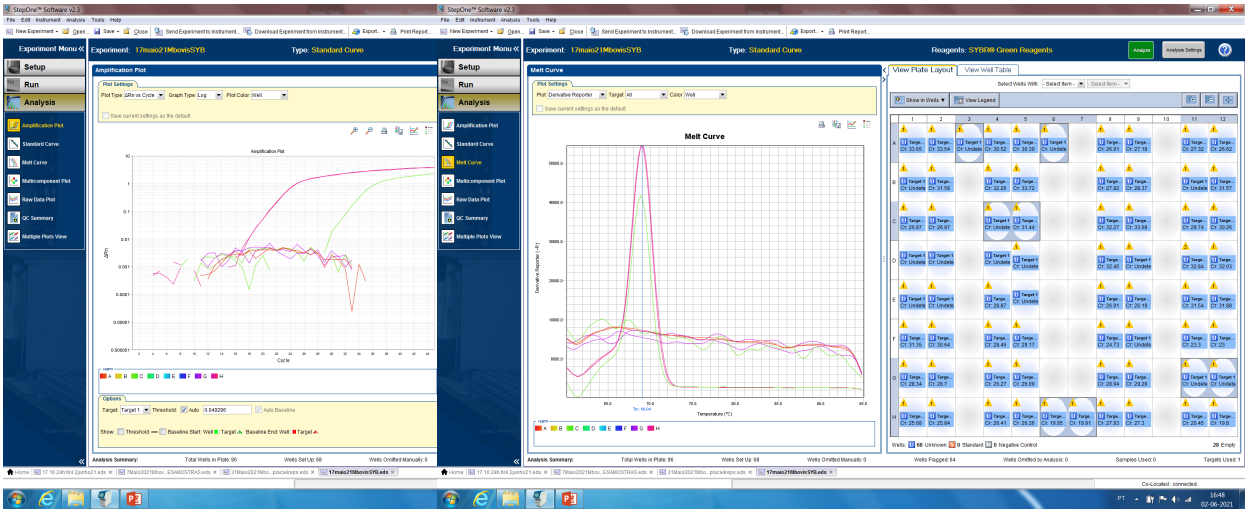
Taqman Hydrolysis Probe System (Ct = 34.64)



SYBR Green System (Ct = 31.96)

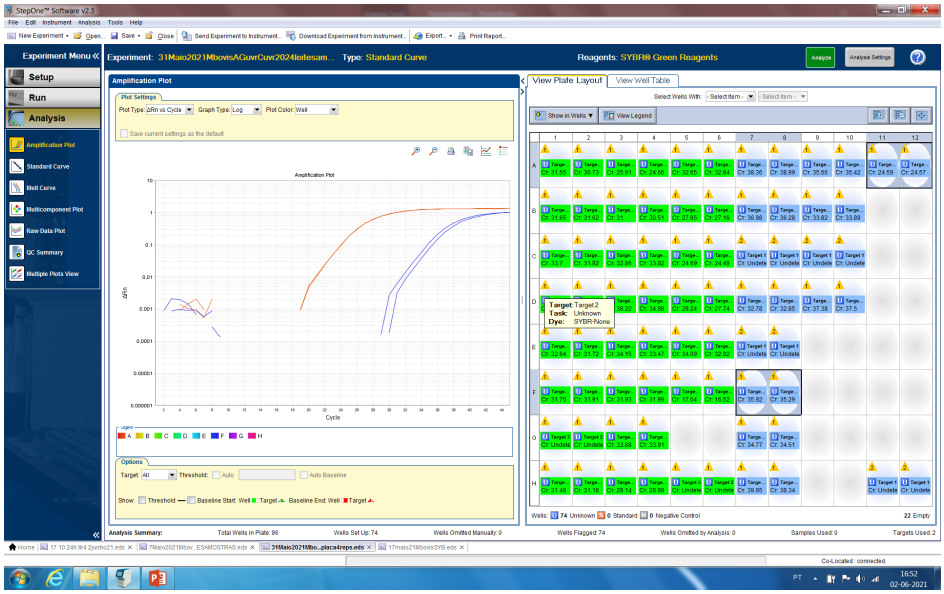
2532TD

Taqman Hydrolysis Probe System (Undetermined Ct value)



SYBR Green System (Undetermined Ct value)

4306T



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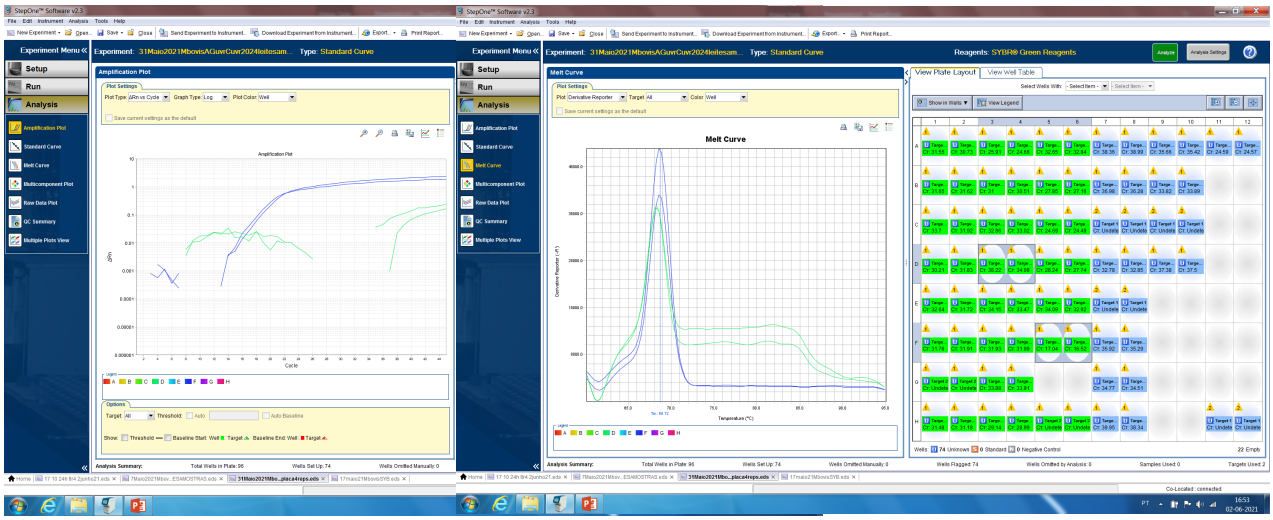
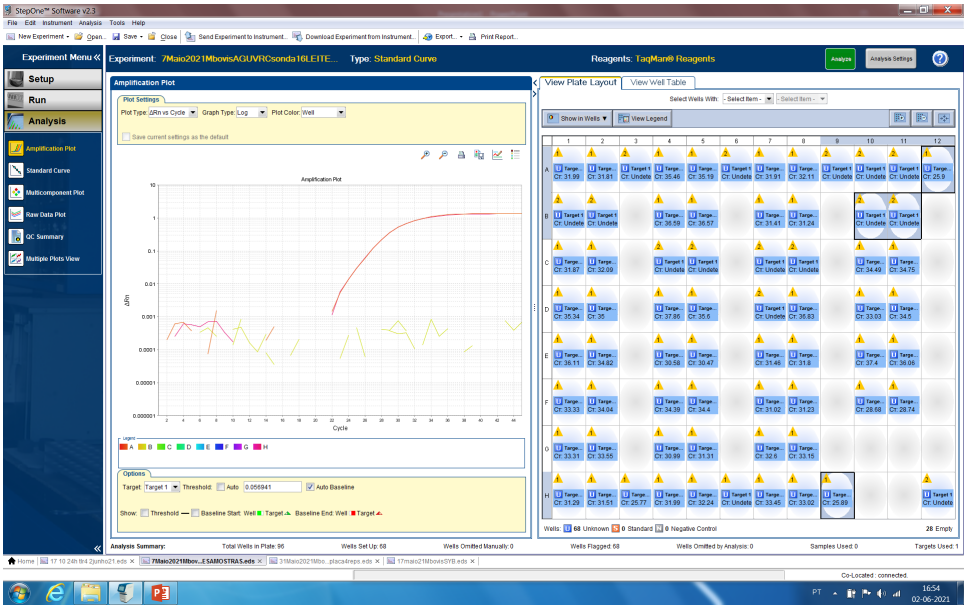
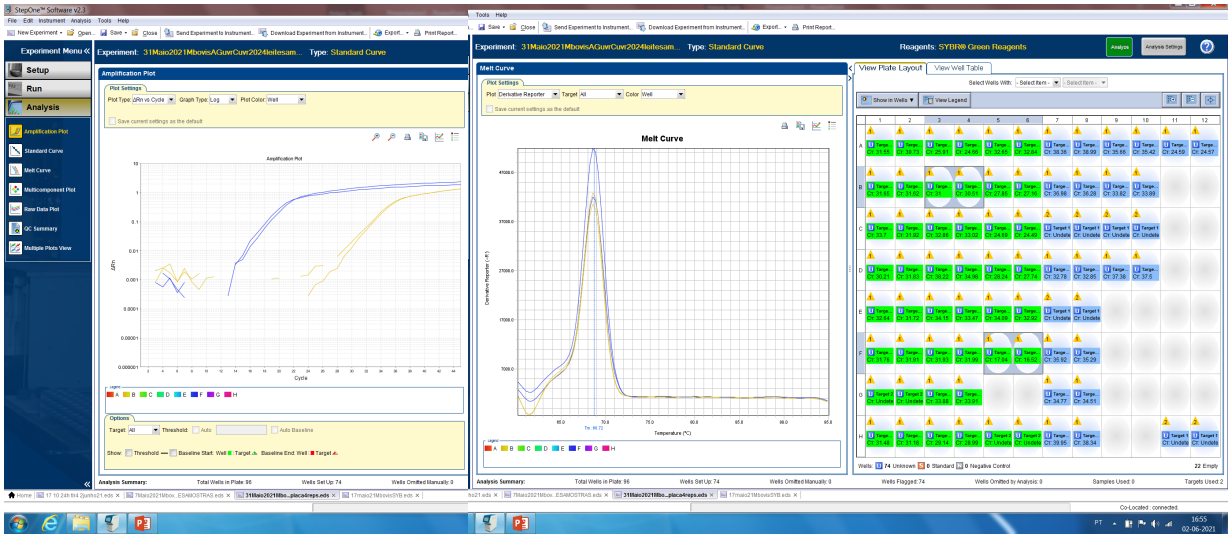


SYBR Green System (Ct = 34.32)

5775FD

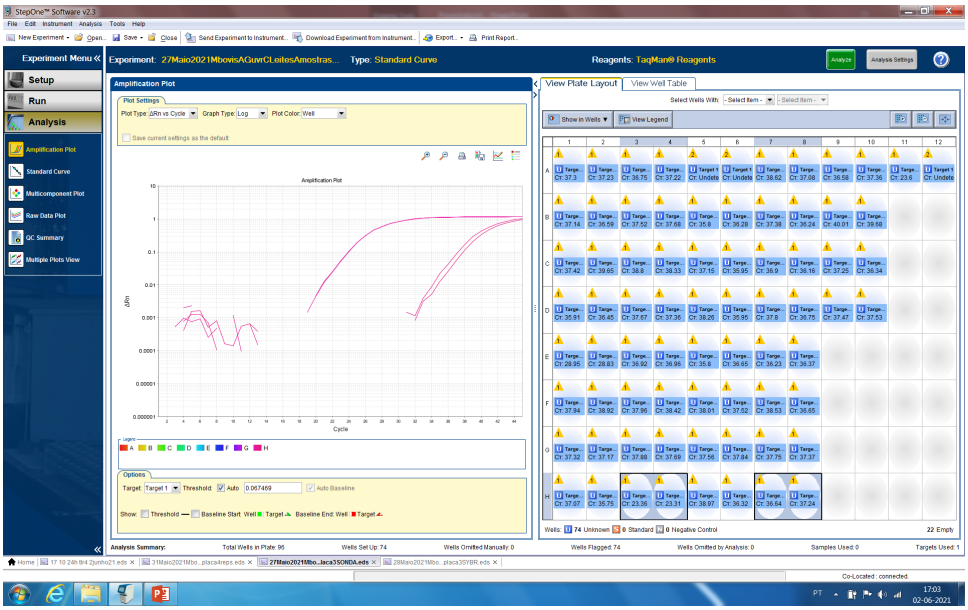


Taqman Hydrolysis Probe System (Undetermined Ct value)

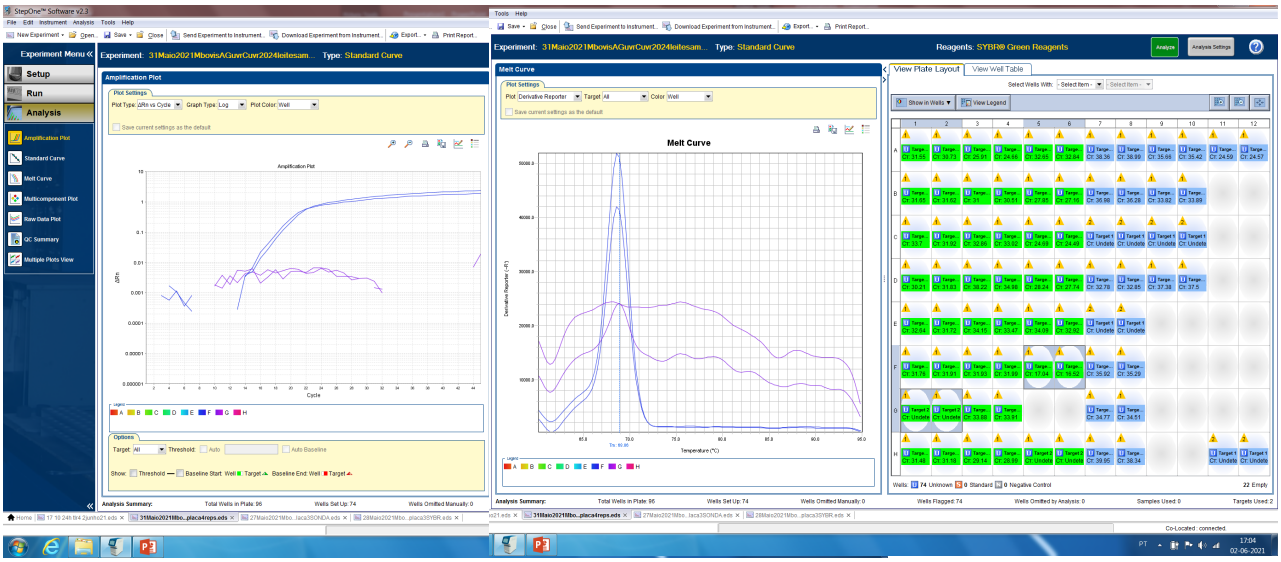


SYBR Green System (Ct = 30.76)

7122TE



Taqman Hydrolysis Probe System (Ct = 36.94)



SYBR Green System (Undetermined Ct value)