Mycoplasma bovis Infections

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Mycoplasma bovis is a cause of bronchopneumonia, mastitis and arthritis but may also affect other main organs in cattle such us the eye, ear or brain. *M. bovis* infections are responsible for substantial economic health and welfare problems worldwide. *M. bovis* has spread worldwide, including to countries for a long time considered free of the pathogen.

Keywords: Mycoplasma bovis ; cattle ; disease ; prevalence ; control

1. Introduction

In 2017, New Zealand became the last of the major cattle-rearing countries to be infected with *Mycoplasma bovis* [1]. Finland had also remained free until relatively recently but became infected via imported cattle in 2012 [2]. Undoubtedly, *M. bovis* is now the most important mycoplasma of livestock being a primary cause of mastitis, arthritis, keratoconjunctivitis and other disorders as well as a major player in the bovine respiratory disease complex (BRD) [3]. Previously *Mycoplasma mycoides* subsp. *mycoides,* the aetiological agent of the World Organisation for Animal Health (OIE)-listed contagious bovine pleuropneumonia, had this dubious distinction but this mycoplasma is now confined to countries in sub Saharan Africa.

Mycoplasma bovis was first reported in the USA in 1961 from a case of bovine mastitis then was probably exported in cattle of high genetic quality to Israel [3]. It then spread around the world, reaching the UK and the rest of Europe in the mid1970s (**Figure 1**). International trade in cattle and cattle products like semen has enabled its silent spread to all continents where cattle are kept. The date of isolation in a particular country, of course, is not necessarily the date of introduction even in the USA as mycoplasmas were very much an unknown quantity and their fastidious nature made isolation and detection an extremely difficult task. Indeed, it has only been in the last two decades with the introduction of DNA amplification techniques that detection and identification have become routine in many parts of the world. However, not all countries have veterinary diagnostic laboratories which can identify these organisms.

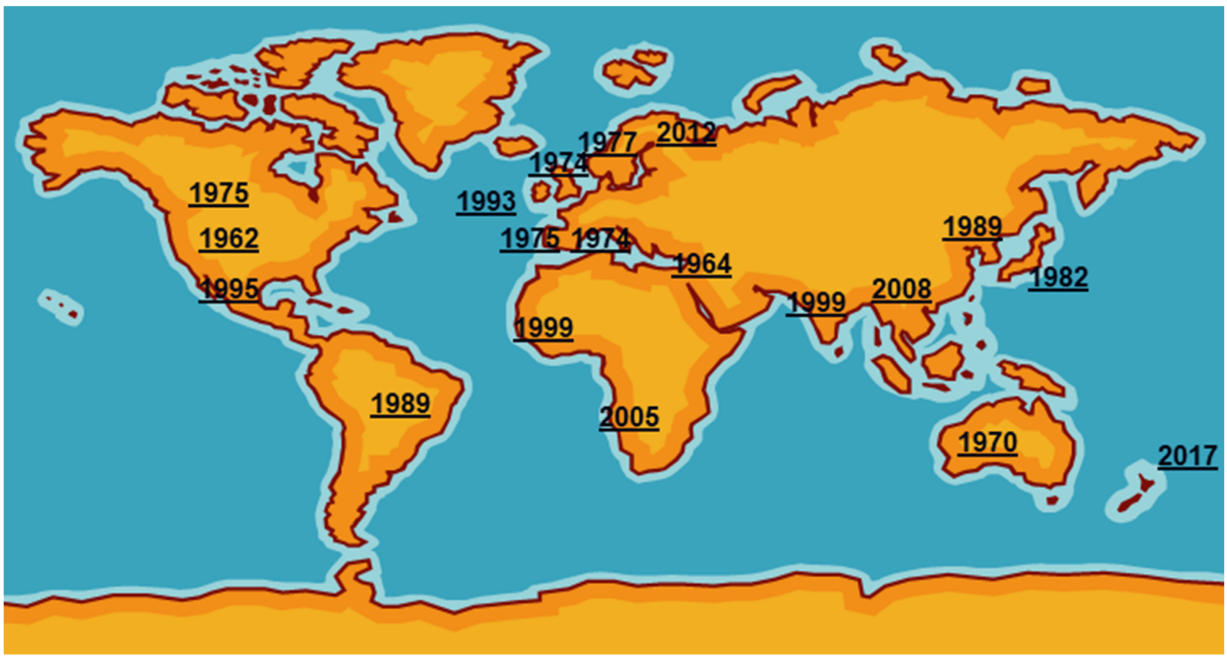

Figure 1. First detections of *Mycoplasma bovis* around the world.

Initially the importance of *M. bovis*, particularly in BRD, was underestimated because of the promotion of more established and easier detectable organisms like the bacteria *Mannheimia haemolytica*, *Histophilus somni* and *Pasteurella*

multocida and viruses, namely bovine respiratory syncytial disease, parainfluenza-3 virus, bovine herpesviruses, coronaviruses and bovine viral diarrhoea virus. The presence of *M. bovis* in healthy cattle, although at a much lower levels than infected ones, delayed recognition of its pathogenicity. Once the importance of environmental factors such as weather, variation in strain virulence and its interaction with the BRD pathogens were known, studies quickly demonstrated its widespread prevalence in pneumonic calves and, later, older cattle.

Despite attempts going back nearly half a century, control of *M. bovis* diseases is still problematic because of a lack of an effective commercial vaccine. Many have been marketed, particularly in the USA, but little data exist to assess their immunogenicity and protective properties [4]. To be valuable they are required to be part of multivalent vaccines incorporating the causative bacteria and viruses currently available for BRD. Presently, no vaccine is available for mycoplasma mastitis, a major problem in large dairy herds of North America where they are often untreatable. Indeed, the major trend in the last two decades has been the alarming decrease in susceptibility of *M. bovis* to the commonly used antimicrobials including the fluoroquinolones [5].

2. Currently Used Diagnostic Methods

The clinical signs of infections in cattle associated with *M. bovis* are non-specific; for that reason, sensitive, accurate and rapid testing of animals is needed for reliable diagnosis. Culturing of *M. bovis* is a gold standard method but is time-consuming and requires specific conditions. Different kinds of media are widely used in experimental studies and in confirmation of infection caused by *M. bovis*, and include Hayflick's [6], modified PPLO [7] and Eaton's [8]. Mycoplasmas are fastidious, slow growing and can be easily overgrown by other bacteria. During the last few years various tests have been used for the detection of *M. bovis* infections in cattle.

2.1. Real-Time PCR Assays for M. bovis Detection

Detection of *M. bovis* by real-time PCR preceded by culture enrichment of the samples improves detection when DNA is present at low concentrations. Furthermore, a selective broth-enrichment step increases the probability of *Mycoplasma* recovery when compared to direct plating on agar [9]. In the real-time PCR assay [10], milk samples from dairies and lung tissue samples were culture-enriched in PPLO broth for 24 h before analysis. In another qPCR for *M. bovis* testing [11], the nasopharyngeal swabs were cultured for 3–5 days before the analysis. The molecular methods are optimized for the detection of *M. bovis* in nasopharyngeal swabs and milk samples, but they can be optimized to be used for the detection of *M. bovis* in different specimens [2][12][13][14][15]. In 2020, a qPCR was developed for the detection of *M. bovis* in tracheal aspirate samples derived from calves [15]. In research on *M. bovis* intramammary infection, the presence of this pathogen in colostrum and additionally in milk from clinical cases was assessed with qPCR [12]. It is also possible to detect *M. bovis* in processed semen [2][14]. The real-time PCR assays are characterised often by a low limit of detection (LOD) and specificity near to 100% [10][11][14][16]. Taking into consideration that the number of mycoplasmas that are shed during the infection is about >1 × 10^6 CFU/mL in milk [4] and the LOD for real-time PCR for *M. bovis* detection in milk is 1.3 × 10^2 CFU/mL [14], the probability of the detection of infected cow in a herd is high. To assess the best sensitivity, the real-time PCR assays for *M. bovis* detection are usually used after an enrichment procedure of the samples. Additionally, centrifugation of the milk and plating the resuspended pellet of bacteria improves detection of mycoplasmas with culture. After such treatment, it was four times more likely to detect of a positive sample when compared to traditional culture regarding very small concentrations [17]. The combination of culture of viable bacteria and qPCR results enables the most accurate confirmation of active infection in animals.

2.2. Fast and Cost-Effective Assays for M. bovis Detection

Another approach for *M. bovis* detection is to design a simple and cost-effective assay run at a single temperature without the need of using specific equipment, which will be useful to process in developing countries. LAMP (loop-mediated Isothermal amplification) is recently of interest because it enables results to be received quickly, and the reaction is normally completed in less than 2 h; furthermore, there is no need to have expensive laboratory equipment, as it is performed at a single temperature [18]. LAMP gives better results than qPCR when performed on purified DNA but is susceptible to contamination. Two assays, namely LAMP and qPCR developed for *M. bovis* detection in milk samples from individual cow quarters and bulk tank milk samples, accurately detected *M. bovis* isolates but gave false positive results for one *Mycoplasma bovigenitalium* isolate [16]. Another method called isothermal DNA amplification assay, a technique based on recombinase polymerase amplification (RPA) with lateral flow dipstick (LFD), allows one to obtain the result in 30 min and is dedicated for *M. bovis* DNA extracted directly from clinical samples i.e., nasal swabs, lungs tissue samples, joint fluids and bulk tank milk samples; no cross-reactions were observed with other *Mycoplasma* species [19]. Usually, LAMP assays are more sensitive than end-point PCRs, for example high sensitivity and specificity for all milk

sample types was obtained with the use of LAMP combined with a procedure for ultra-rapid extraction (PURE-LAMP), in which various sample types i.e., bulk tank milk, mature milk, colostrum/transitional milk and mastitis milk were examined [20]. Similar parameters were obtained in LAMP for the examination of *M. bovis* in milk from mastitis cases [21].

2.3. Immunohistochemistry and In-Situ Hybridization

Although molecular methods are advantageous, they can only provide the data on *M. bovis* DNA, and there is lacking information about the presence of viable bacteria and their associaation with the lesion. Immunohistochemistry (IHC) and in-situ hybridization (ISH) are types of techniques which have the advantage that they are able to detect the localization of *M. bovis* antigen or DNA, respectively, in the examined tissue of the infected animals [22][23][24][25][26]. The IHC used in the study on calves experimentally infected with *M. bovis* allows one to detect *M. bovis* antigen in the bronchiolar epithelial cells in the lung tissue with histopathological changes that are characteristic for bronchiolitis [23]. Results of another experiment proved that *M. bovis* antigen was detected on the surface and inside the cytoplasm of bronchiolar epithelial cells in the pneumonic foci and in the cytoplasm of phagocytes at the margin of bronchiolar exudates [25]. In the study on aborted foetus and neonatal calf that were infected with *M. bovis*, its antigen was found with the use of IHC in the brain, liver, lungs and placenta of aborted foetus, and ISH showed the presence of its DNA i.e., in lungs and placenta of the examined animals [24]. The research on long-term survival of *M. bovis* in tissues of infected calves showed the persistence of this pathogen in necrotic lung lesions several weeks after the infection with the use of both methods [26]. It is also possible to examine the pulmonary samples of calves with BRD. IHC was used to detect the *M. bovis* antigen intralesional in different areas of the lungs [22]. However, while these techniques allow one to obtain significant information, they are also expensive, labour intensive and require trained staff.

2.4. A Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for M. bovis Detection

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) procedure has been applied to *M. bovis* detection. It was optimised for the detection of *M. bovis* isolates and found to be a suitable test for routine diagnostics in cattle, especially those from BRD cases. The protocol enables the identification of *M. bovis* from bronchoalveolar lavage fluid (BALF) after enrichment in culture. The higher number of positive samples was obtained after 72 h of enrichment. The main advantage of MALDI-TOF MS is that it only detects viable bacteria, which indicates that cattle have active rather than historic infections [27].

2.5. Molecular Typing

The analysis of *M. bovis* isolates with typing and sequencing methods can give additional information about their relationships and evolution. The multilocus sequence typing (MLST) analysis was proved to be suitable for molecular typing of *M. bovis* and the assessment of geographical relatedness of isolates. The MLST scheme based on eleven housekeeping genes was evaluated. Three genes, *dnaN*, *metS* and *hsp70*, were taken for the sequence analysis and the remaining eight genes, i.e., *adk*, *efp*, *gmk*, *gyrB*, *polC*, *rpoB*, *tpiA* and *uvrC* were not chosen for the further analysis. It allows the acquiring of information on sequence variation, its type of distribution and disappearance of some sequence types [28]. A later study [29] assessed two MLST schemes for *M. bovis* isolate typing. The comparison of the performance of the two MLST schemes and additional identification of a new reference scheme capable of full typing of the examined isolates was made. The PubMLST reference method contains *adh-1*, *gltX*, *gspA*, *gyrA*, *gyrB*, *pta-2*, *tdk* and *tkt* locus; it is thought to be discriminatory and informative enough, but in this study, *adh-1*, one of the typing loci of *M. bovis* isolates, was missed. According to this reference scheme, the *adh-1* locus should be retired from the analysis. This approach was not beneficial for the study because the discrimination index received with the use of the six remaining PubMLST loci failed to reach the benchmark recommended for a reference method, and the addition of a seventh locus had to be made. The alternative scheme contains seven loci: *aptA*, *dnaA*, *metS*, *recA*, *rpoD*, *tkt* and *tufA*. The comparisons of examined *M. bovis* genome sequences identified the *dnaA* locus from the alternative scheme as the optimal replacement for *adh-1*.

Another approach for epidemiological studies is the use of whole genome sequencing (WGS) to evaluate the molecular epidemiology and genomic diversity of *M. bovis* isolates as well as their genetic relationship. The single nucleotide polymorphism (SNP) analysis can be used to assess the intraspecies relationship and the presence of a dominant genotype that can be associated with one type of disease. This study is relevant to better understand the global epidemiology of this important pathogen and to assess control strategies [30]. Comparison of the *M. bovis* sequences can be used in assessing the genetic diversity of the strains [31] or to get the information about gene virulence [32].

WGS was used in New Zealand to track the outbreaks first identified in 2017. In all, 171 isolates from 30 infected herds have so far been sequenced, and results indicate that the current outbreak was probably caused by recent entry of the

mycoplasma, perhaps 1–2 years before detection, from a single source either as a single border crossing of a single clone or, potentially, up to three border crossings of three very closely related clones from the same source (TAG 2019) probably in germplasm imported from Europe.

2.6. Serological Approaches

Serological diagnosis based on detection of specific antibodies to *M. bovis* is suitable and practical for the assessment of prevalence and epidemiological studies of herds [33]. Although serological testing is a reliable method for identification of infected animals, specific antibodies do not appear until 10 to 14 days after the infection but remain elevated for several months [34]. Various indirect ELISAs are used for anti-*M. bovis* antibody detection in cattle herds. The BIO K302 ELISA (BioX Diagnostics) was applied for evaluation of antibody response to *M. bovis* in serum and milk samples [35][36][37]. A study conducted in Belgium [37] showed that the ELISA is able to detect *M. bovis* specific antibodies in bulk tank milk up to 12 months after the outbreak of the disease. Researchers [36] examined bulk milk tank samples for all Danish herds with this ELISA and concluded that the cut-off value should be increased from 37%, as suggested for animal-level diagnosis, to 50%, to obtain more adequate sensitivity and specificity for bulk tank milk analysis. On the other hand, as a result of a European inter-laboratory comparison conducted on 180 serum samples, the sensitivity and specificity of BIO K302 ELISA was determined to be 49.1% and 89.6%, respectively [38]. However, in 2020 it was confirmed that this ELISA was suitable for the serological evaluation of anti-*M. bovis* antibodies in longitudinal studies. Despite the low number of apparent clinical mastitis cases, it was useful in evaluation of *M. bovis* seroprevalence in dairy herds, which was on average 38% (16–76%), as mentioned before [35].

Another indirect ELISA, made in-house and based on a fragment of a recombinant mycoplasma immunogenic lipase A (MilA), was developed [39]. This assay can be also useful for bulk tank milk sample analysis. The results of the presence of anti-*M. bovis* antibodies in bulk tank milk were positively correlated with the antibody detection in sera of the examined animals. Additionally, there was made a comparison between BIO K 260 (BioX Diagnostics) and the MilA ELISA [40], and the latter test gave a higher number of positive samples for *M. bovis*, and they were more convergent with those obtained with culture or real-time PCR. The obtained sensitivity and specificity for this test was 94.3% and 94.4%, respectively. Additionally, it was shown that the MilA ELISA is also suitable for testing the presence of anti-*M. bovis* antibodies in the early stages of calf life (from the 3rd week of life) [41].

2.7. Interlaboratory Trials of Diagnostic Tests

M. bovis causes serious health problems in cattle herds almost all over the world, but its detection is not harmonised as yet and relies on different diagnostic methods, often in-house molecular techniques based on a variety of target genes and various different DNA extraction methods. There was conducted a European interlaboratory comparison of the diagnostic utility of the molecular tests for *M. bovis* detection [42]. Six laboratories from different countries were included in the study. Five different DNA extraction methods from bacterial culture and BALF samples were used. The molecular tests were made with the use of seven different PCR assays based on *polC*, *oppD*, *uvrC* and V4-V4 16S rRNA target genes. The comparison revealed that although the research used various assays, they had comparable diagnostic utility for *M. bovis* detection in cattle. The analytical specificity of the different PCR methods was comparable for all of the laboratories, except one, where *M. agalactiae* was detected because of the use of 16S rRNA target gene. The LOD was from 10 to 10^3 for the real-time, and from 10^3 to 10^6 CFU/mL for the end-point assays. According to the authors, this difference was acceptable. Cultures correctly detected the presence of *M. bovis* in bronchoalveolar lavage fluid samples and were consistent with PCR results. The recent comparison of diagnostic methods used in the different veterinary laboratories fortunately showed consensus.

2.8. Mixed Infections

Other *Mycoplasma* spp. can also be associated with *M. bovis* infections in cattle. In BRD cases, most often *M. dispar*, *M. canis* and *M. arginini* are implicated [3][43]. In mastitis and reproductive disorders, *M. bovigenitalium*, *M. californicum* and *M. alkalescens* can also participate [44][45]. A test based on PCR with the 16SrRNA target gene and separation of the PCR products using denaturing gradient gel electrophoresis (PCR–DGGE) enabled the differentiation of 13 *Mycoplasma* spp. of bovine origin in mixed infections [46]. Traditionally, culture is used for the confirmation of BRD infections, but the incubation period for each examined bacterial pathogen is different and samples inoculated onto agar plates are often overgrown with other fast-growing bacteria. For that reason, the multiplex real-time PCRs used by some laboratories [15][47][48] are the most suitable for simultaneous direct detection of *M. bovis* and other pathogens involved in BRD, such as *P. multocida*, *M. haemolytica* and *H. somni*, in contrast to methods not dedicated for different pathogen identification in mixed infections such as one-target PCR, traditional culture or MALDI-TOF MS [49]. When using one target PCR, there is no information about the involvement of other pathogens in the disease, different bacteria have various

growth requirements and slow growing bacteria can be easily overgrown by others, and MALDI-TOF MS is not able properly detect all organisms from polymicrobial samples.

Various diagnostics methods for fast and accurate detection of *M. bovis* in various sample types and typing methods for identification and analysis of its strains in the last few years have been developed for evaluation of the disease course. Methods should be chosen according to the purpose of the survey, for herd-level testing or for individuals, or should be considered in terms of its usage for the specimen. The use of a combination of molecular, serological and culture-based methods is necessary for reliable diagnosis of diseases caused by this pathogen in cattle.

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