

Animal tuberculosis: impact of disease heterogeneity in transmission, diagnosis, and control

Short Title

Animal tuberculosis: impact of disease heterogeneity

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Abstract

Animal tuberculosis (TB) in terrestrial mammals is mainly caused by *Mycobacterium bovis*. This pathogen is adapted to a wide range of host species, representing a threat to livestock, wildlife, and human health. Disease heterogeneity is a hallmark of multi-host TB and a challenge for control. Drivers of animal TB heterogeneity are very diverse and may act at the level of the causative agent, the host species, the interface between mycobacteria and the host, community of hosts, the environment, and even policy behind control programs. In this paper, we examine the drivers that seem to contribute to this phenomenon. We begin by reviewing evidence accumulated to date supporting the consensus that a complex range of genetic, biological, and socio-environmental factors contribute to the establishment and maintenance of animal TB, setting the grounds for heterogeneity. We then highlight the complex interplay between individual, species-specific and community protective factors with risk/maintenance variables that include animal movements and densities, co-infection and super-shedders. We finally consider how current interventions should seek to consider and explore heterogeneity in order to tackle potential limitations for diagnosis and control programs, simultaneously increasing their efficacy.

Keywords: Animal tuberculosis; *Mycobacterium bovis*; *Mycobacterium caprae*; Heterogeneity; TB control

1. Introduction

Animal tuberculosis (TB) is mainly caused by *Mycobacterium bovis* and, to a lesser extent, *Mycobacterium caprae* (Aranaz, Cousins, Mateos, & Dominguez, 2003; Palmer & Waters, 2006). It is a chronic disease, classically characterized by the formation of nodular granulomas, most frequently found in lymph nodes and lungs (Palmer & Waters, 2006), although lesion location may vary according to host species and primary infection route. Affecting a high range of mammal species, animal TB represents a threat to biodiversity, to public health due to the zoonotic potential of *M. bovis*, and to countries' economies and farmer livelihoods due to huge losses in animal production, trade, disease surveillance and control plans (Rodriguez-Campos, Smith, Boniotti, & Aranaz, 2014). Several efforts to eradicate TB have been made over the years in many of the affected nations, with estimations showing a gradual decline in the global burden (World Health Organization, 2019). However, the disease is still a significant problem in developing countries, but also in the United States of America (USA), Australia, and several European countries that have regional foci and are yet not considered animal TB-free (Murai, 2019). Several reports suggest that heterogeneity increases when TB burden declines and the disease becomes more unevenly distributed (Cohen, Colijn, Finklea, & Murray, 2007; Trauer et al., 2018). Several drivers reviewed here seem to contribute to this phenomenon. Heterogeneity in disease phenotypes and distribution are limiting the efficacy of management and control strategies and should thus be taken into account when delineating eradication programs (Zimpel et al., 2019).

2. Drivers of animal TB heterogeneity

2.1 The heterogeneity within the agents of animal TB

M. bovis is the major causative agent of TB in cattle and other species of the *Bovidae* family (Rodriguez-Campos et al., 2014). Also responsible for animal TB, *M. caprae* preferably infects goats, sheep and occasionally cattle (Aranaz et al., 2003). It is almost confined to Europe, while *M. bovis* is spread worldwide (N. H. Smith, 2012; Zimpel et al., 2019). Both pathogens have zoonotic potential. Information concerning human TB due to *M. bovis* is limited, even more, when developing countries are considered, however, zoonotic TB in humans has been confirmed in

African countries, India and China (Grange, 2001). The growing populations of cattle in India, Ethiopia, South Africa, and China could represent a serious animal and human health issue due to the circulation of *M. bovis* and also *M. tuberculosis* (the etiological agent of human TB) (Ameni et al., 2011; Chen et al., 2009; Hlokwe, Said, & Gcebe, 2017; Sweetline Anne, Ronald, Kumar, Kannan, & Thangavelu, 2017). When considering the Americas, both Mexico and USA reported details of TB epidemiology of zoonotic origin in specific areas, showing an increasing percentage of infections by *M. bovis* (Mark, Neha, & Jennifer, 2015; Torres-Gonzalez et al., 2016). In Europe, several countries, namely Austria, Germany and Spain, have reported cases of zoonotic TB due to *M. bovis* and also *M. caprae* (Borna et al., 2013; Kubica, Rusch-Gerdes, & Niemann, 2003; Proding, Eigntler, Allerberger, Schönbauer, & Glawischnig, 2002).

Several intrinsic characteristics of the causative agents of animal TB represent sources of heterogeneity, such as genetic variability that is reflected in virulence, host preference and/or geographic adaptation.

Virulence of pathogenic mycobacteria appears to be widely dependent on its genetic background. A clear example is the RD1 region, which is known to be absent in attenuated *M. bovis* vaccine strain Bacillus Calmette–Guérin (BCG), encoding *EsxA* gene in the ESX1 *locus* that results in the loss of virulence (Brodin et al., 2006; Pym, Brodin, Brosch, Huerre, & Cole, 2002). A recent report based on comparative genomics shows that *M. bovis* from wild boar and cattle may lead to different disease severities (de la Fuente et al., 2015), which was associated with specific alterations in the ESX *locus* that could relate with host tropism (de la Fuente et al., 2015).

The sources of natural genetic variability in pathogenic mycobacteria are diverse, one of which is the presence of transposable insertion sequences (IS) that represent major drivers of gene loss and pseudogenization (Moran & Plague, 2004; Toft & Andersson, 2010). Many IS contain promoter sequences that may regulate genes nearby the site they transpose into (Moran & Plague, 2004). For example, *M. bovis* shows mutations in *phoPR* virulence operon, which have been associated with reduced virulence in humans or, in contrast, with a reported case of increased virulence when the *IS6110* element is translocated into the upstream region of this operon, enabling human-to-

human transmission (Gonzalo-Asensio et al., 2014). Furthermore, the number of IS elements is positively correlated with host specialization, leading to the hypothesis that the reduced number of IS elements in *M. bovis*, in parallel with genome reduction and pseudogenization promote a possible adaptation to multi-host, contrasting with *M. tuberculosis* with a superior number of IS and tropism for one host (Humans) (Allen, 2017). Additionally, the existence of specific polymorphisms throughout the *M. bovis* genome can favor specific host adaptations, leading to host preference of specific genotypes. In agreement, several single nucleotide polymorphisms (SNPs), namely in genes encoding proteins linking stress response with lipid metabolism, have been associated to host preference and disease outcomes (de la Fuente et al., 2015).

Previous studies described the region of difference (RD) 4 as a marker for *M. bovis* (Warren et al., 2006). This region comprises genes encoding for the biosynthesis of glycolipids containing trehalose (Ru et al., 2017). However, a study by Rodriguez and co-workers described some RD4-specific sequences to be present in Spanish *M. caprae* isolates (Rodriguez et al., 2011). Also, another study reported that RD4 is not uniformly present in *M. caprae* isolates from Germany, confirming the heterogeneity within this genomic region and questioning its discriminative power, particularly when used for diagnostic purposes (Domogalla et al., 2013), since the detection of RD4 deletion is used in several molecular assays to differentiate *M. bovis* from other *Mycobacterium tuberculosis* complex (MTC) members (Costa et al., 2014; Halse, Escuyer, & Musser, 2011).

In *M. bovis* and *M. caprae*, spoligotyping is still considered the gold standard for strain discrimination, however, in developed countries, whole-genome sequencing (WGS) techniques are being increasingly used on a routine basis. Also, Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR) allows to understand more deeply the genomic variability within *M. bovis* and *M. caprae* strains and has the resolution to detect mixed infections (Reis, Albuquerque, Botelho, & Cunha, 2020). Based on spoligotyping, RD characterization and SNPs in specific genes, clonal complexes of *M. bovis* can be defined (N. H. Smith, 2012; Zimpel et al., 2019). Up until now, *M. bovis* strains have been mainly distributed

into one of four clonal complexes: African 1 (Af1) (Müller et al., 2009), African 2 (Af2) (Berg et al., 2011), European 1 (Eu1) (N. H. Smith et al., 2011), and European 2 (Eu2) (Rodriguez-Campos et al., 2012). Another group of *M. bovis* isolates characterized by a BCG-like spoligotype signature (SB0120) is also hypothesized to constitute a fifth clonal complex (Boniotti et al., 2009). Each clonal complex is predominant in a specific geographic region: Af1 in West-Central Africa, Af2 in East Africa, Eu1 in the British Isles and other countries worldwide, possibly reflecting historical and commercial relationships, and Eu2 in the Iberian Peninsula (Rodriguez-Campos et al., 2014). The Eu1 lineage is typically found in geographic regions that suffer from a maintenance host problem in wildlife, which may indicate that this *M. bovis* lineage is more suited to infect and disseminate in multiple hosts when compared to other animal-adapted lineages (N. H. Smith et al., 2011). However, these assumptions need to be considered with caution, since it is known that this lineage distribution is also highly influenced by human demography (N. H. Smith et al., 2011). Besides this aspect, Eu1 lineage has been reported to exhibit high diversity that is reflected in phenotype, namely differential virulence in bovine hosts (Allen et al., 2013; Wright et al., 2013). Additionally, a recent publication by Ghavidel *et al.* (2018) combined spoligotyping data of bovine isolates from different geographic locations and evaluated the most common spoligotype signature in each continent (Ghavidel, Mansury, Nourian, & Ghazvini, 2018). The most predominant were spoligotype SB0120, found in Asia (Iran), Europe (France and Italy) and Africa (Zambia and Algeria); spoligotype SB0121 in Europe (Spain, Portugal), Africa (Algeria) and America (Mexico and Brazil); and SB0140, in Asia (China), Europe (Ireland) and America (Mexico). Spoligotype SB0121 isolates belong to Eu2 clonal complex, SB0140 to Eu1, and SB0120 to BCG-like (Ghavidel et al., 2018). Also, a group of strains enclosed in the F4-family, defined by a specific spoligotype and MIRU-VNTR profile, has been found mainly in southern France (Hauer et al., 2015). Garbaccio and co-workers also managed to relate different spoligotype signatures with differential virulence in Argentina (Garbaccio et al., 2014). Recently, Hauer *et al.* (2016) evaluated if MIRU-VNTR allelic variability in French isolates is related to clonal group identity, showing that each spoligo signature presents a different MIRU

locus as the most discriminatory (Hauer et al., 2016). In contrast with *M. bovis*, *M. caprae* isolates reported by large surveys in Europe (Bonioti et al., 2009; Duarte, Domingos, Amado, & Botelho, 2008; Haddad et al., 2001; Rodriguez et al., 2011) seem to be considerably less diverse, with only two main clusters, the Iberian and the Central and Eastern European, identified by differences in spoligotyping profiles (Aranaz et al., 2003; Duarte et al., 2008; Erler et al., 2004; Kubica et al., 2003; Pavlik, 2002; Prodinger et al., 2002; Rodriguez et al., 2011).

Several WGS studies of clinical isolates of *M. bovis* worldwide have been performed in the last years. These studies have been proving that, besides the high nucleotidic similarity between *M. bovis* strains, several SNPs can be found that help to differentiate clinical isolates. The analysis of 186 whole-genome sequences of MTC strains isolated worldwide revealed a highly clustered population in agreement with clustering based on clonal complexes (Lasserre et al., 2018). However, another study that analyzed 823 genomes of *M. bovis* strains from different countries and hosts, suggested the existence of three lineages (Lb1, Lb2, and Lb3) that do not fully represent the clonal complexes and are mainly clustered based on geographic location (Zimpel et al., 2019). In a recent work conducted in France, SNPs-based phylogenetic analysis of 87 *M. bovis* isolates was congruent with clusters previously defined by spoligotyping and MIRU-VNTR, however, some SNPs were specific of particular genotypic groups (Hauer et al., 2019). WGS is a higher resolution genotyping method and the information generated is starting to be used to trace infection sources, to refine transmission networks, and to improve knowledge concerning wildlife in TB transmission cycle, as well as in epidemiological scenarios with differential levels of complexity, from herd to multi-host environment frameworks (Crispell et al., 2017; Price-Carter et al., 2018).

Among the techniques mentioned above for strain differentiation, WGS are best suited for virulence assessment. WGS enables the statistic association of newly reported SNPs, insertions and deletions that may explain certain virulence phenotypes, but also the calculation of indicators of selective pressure exerted on specific virulence genes (Abdelaal et al., 2019; Cheng et al., 2018; Hauer et al., 2019). Besides,, virulence phenotype- genotype associations can also be inferred by

transcriptomic profiling and differential expression analysis in different strains (Malone et al., 2018).

As described up above, the heterogeneity of the causative agents of animal TB is highly dependent on their genetic heterogeneity, introduced by SNPs, IS regions, and RDs. These, along with clustering by WGS, spoligotyping, and MIRU-VNTR, are now starting to be related to host range, geographical distribution, and differential virulence (Garbaccio et al., 2014; Hauer et al., 2015, 2019; Ghavidel et al., 2018; Zimpel et al., 2019). So, although many descriptive molecular epidemiology studies have been performed so far, a more complex understanding of the connection between genetic markers and their phenotypical consequence needs to be developed, as it may greatly help epidemiological tracking, the prognosis of disease outcome and transmission risk prediction.

2.2 Host Range: the heterogeneity within animal species

Cattle are considered the main host of *M. bovis*, however, some wildlife species are described to act as reservoirs, namely the African buffalo (*Syncerus caffer*) in South Africa, badger (*Meles meles*) in Britain, brushtail possums (*Trichosurus vulpecula*) in New Zealand, Canadian bison (*Bison bison*) in Canada, lechwe antelope (*Kobus leche*) in Zambia, white-tailed deer (*Odocoileus virginianus*) in USA, and wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) in the Iberian Peninsula (Corner, 2006; Fitzgerald & Kaneene, 2013; Naranjo, Gortazar, Vicente, & de la Fuente, 2008; Santos et al., 2009). Furthermore, *M. bovis* was already reported in many other domestic and wild animal species, including diverse ungulates, carnivores, and marsupials (Bruning-Fann et al., 2001; Pesciaroli et al., 2014). They all have in common the fact that they are mammal species however host singularities, life-history traits, and the ecological context are expected to greatly influence disease establishment and maintenance.

In certain regions, TB can be maintained for as many as eight different animal species. It is the case of Iberia (encompassing Portugal and Spain), where cattle, sheep, goat, pig breeds that are managed in extensive husbandry, wild boar, red deer, fallow deer, and badger can support *M. bovis* maintenance in the ecosystem, adding several layers of heterogeneity in a disease system .-

2.2.1 Variation of immune responses to mycobacterial infection

It is well accepted that infection by *M. bovis* and *M. caprae* triggers an immune response in the host that can lead to the elimination of the agent, development of granuloma-like lesions in several organs, lymph nodes and cavities, or generalization throughout the organism, depending on the pathogen's virulence and the host's capacity to activate an effective immune response (Wangoo et al., 2005).

TB has a long, poorly quantified stage of disease progression and latent period estimates (O'Hare, Orton, Bessell, & Kao, 2014). Granuloma development in experimentally *M. bovis*-infected cattle can be divided into four different stages (stage I to IV) (Wangoo et al., 2005). In an initial phase (stage I), granulomas are formed by irregular unencapsulated clusters of epithelioid macrophages and lymphocytes of the B and T type; then, evolving to clusters with a thin capsule and necrotic areas (stage II); further to encapsulated granulomas with central necrosis and mineralized areas (stage III); and, finally, to thickly encapsulated multicentric granulomas, with prominent caseous necrosis and extensive islands of mineralization (stage IV) (Wangoo et al., 2005). However, in a work performed by Carrisoza-Urbina and collaborators (2019) with *M. bovis* naturally infected cattle, the granulomatous lesions of calves revealed an atypical pattern, not fitting into the previously established classification defined by Wangoo and collaborators (2005). These granulomas lacked a fibrous capsule and the classification of their formation was adapted to stages I, II, II-III and III-IV (Carrisoza-Urbina, Morales-Salinas, Bedolla-Alva, Hernández-Pando, & Gutiérrez-Pabello, 2019).

The degree of peripheral fibrosis is variable and hosts that are more resistant to the disease tend to develop more exuberant fibrosis followed by central necrosis and mineralization, a phenomenon already described for cattle and cervids (Canal et al., 2017).

The cellular immune response impacts granuloma's development and evolution to disease confinement or dissemination. Observation of granulomas in different developed stages within the same organ of *M. bovis* naturally infected cattle has been reported (Carrisoza-Urbina et al., 2019), which reveals the presence of different immune microenvironments.

Epithelial cells of the respiratory tract and immune cells both contribute for the establishment of the immune microenvironment around and inside granulomas, with the balance between pro- and anti-inflammatory cytokines, in parallel with the trade-off of control of bacterial spread and tissue damage, being the major biological challenges (Miranda, 2012). The cytokines, tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ) are particularly important in promoting the formation and function of the granuloma (Miranda, 2012).

In a work by Thacker and collaborators (2006), the experimental infected group of white-tailed deer expressed higher levels of cytokines (IFN- γ , interleukin [IL]-12p40, IL-4 and granulocyte-monocyte colony-stimulating factor) than the uninfected group. Furthermore, animals classified in high-pathology group express higher levels of IFN- γ in early infection, when compared to low-pathology group, being this cytokine associated with increased pathogenesis (Thacker, Palmer, & Waters, 2006).

Other recent studies assessed granuloma-level immune responses showing that granuloma lesions of naturally infected fallow deer had higher levels of IFN- γ in initial stages than in late granuloma development stages (Garcia-Jimenez et al., 2012), which is in agreement with the previously mentioned study. The same work assessed the expression levels of another cytokine associated with granuloma development and revealed small differences in TNF- α expression along the evolution process (Garcia-Jimenez et al., 2012). Differential cytokine [IFN- γ , TNF- α , IL-1 β , IL-10 and transforming growth factor beta (TGF- β)] expression between the initial and late stages of granuloma development, was also studied in naturally infected cattle using immunohistochemistry (Canal et al., 2017). Globally, cytokines expression in late stages (stage III and IV) was significant, predominantly IFN- γ , once more associated with disease severity (Canal et al., 2017). Moreover, higher expression of IFN- γ and TGF- β was observed in lymphocytes, macrophages and necrotic areas of late stage granulomas, whereas IL-10 immunoreactivity was absent from necrotic areas (Canal et al., 2017). TGF- β is associated with fibrosis processes and so higher levels of this cytokine could indicate the establishment of the chronic process (Canal et al., 2017).

The humoral immune response also contributes to the host's arms race against *M. bovis* infection. The production of antibodies against *M. bovis* antigens increases with time after infection, being dependent on individual characteristics as suggested by others (Infantes-Lorenzo et al., 2019; Thacker et al., 2006). Furthermore, in a recent study performed in a population of experimentally infected badgers, the proportion of animals with circulating antibodies differs significantly through time (Infantes-Lorenzo et al., 2019).

The understanding of immune response in different host species, including the assessment of cytokines and antibodies in the regulation of immune response dynamics and disease progression, is critical for the development of a protective and high coverage vaccine strategy.

Although there are differences across host species that are related to bioecological characters, the anatomical localization, and features of animal TB lesions can provide information on the primary infection route and on the excretion pathways. Lesions in the thoracic cavity may arise from inhalation of aerosols, while lesions in abdominal organs are thought to be related with the ingestion of infected animals or contaminated pastures/water (Cunha et al., 2011; Palmer, Thacker, Waters, Gortazar, & Corner, 2012; Phillips, Foster, Morris, & Teverson, 2003).

A recent work from Palmer and collaborators (2016) revealed a differential gene expression of cytokines in lung and lymph nodes granulomas in cattle experimentally infected with *M. bovis* (Palmer, Thacker, & Waters, 2016). Moreover, when comparing cytokines (IFN- γ , TNF- α , IL-10, IL-17A, IL-22 and TGF- β) expression between two lymph nodes with a distinct anatomical localization, tracheobronchial and mediastinal, the expression was also differential (Palmer et al., 2016). This work highlighted the importance of considering the anatomical location of lesions when assessing host immune response and that cytokine expression might be related to the nature of specific granuloma microenvironments.

Also, vitamin D has been shown to be an important stimulator of mammalian immunity (Waters et al., 2001). The mycobacterial killing ability is enhanced, in response to IFN- γ , by the increased production of nitric oxide, unleashed by fully activated macrophages (Waters et al., 2001).

The immunological capacity of an individual can be improved by vaccination. Cattle vaccination with attenuated *M. bovis* BCG can negatively interfere with the intradermal tuberculin test applied by routine for diagnosis, so it is forbidden in several countries, including the European Union (EU) member-states. However, several experimental trials are currently being conducted in wildlife hosts, such as badger (Aznar et al., 2018; Gormley et al., 2017) and wild boar (Díez-Delgado et al., 2018; Garrido et al., 2011) in Europe or brushtail possum (Nugent et al., 2016) in New Zealand. The specific characteristics of the vaccine (*M. bovis* BCG or heat-inactivated *M. bovis*; with or without adjuvants); the administration method (oral, parenteral); the characteristics of oral baits; and, in field trials, the unknown immune condition of the host undergoing vaccination together with the number of times the same individual could be in contact with the vaccine, all contribute to shaping the (variable) immune response (Aznar et al., 2018; Chambers et al., 2017; Díez-Delgado et al., 2018; Gormley et al., 2017; Nugent et al., 2016).

The oral and parenteral delivery of vaccines containing inactivated *M. bovis* or *M. bovis* BCG to wild boar, in experimentally controlled conditions in Spain, allowed the reduction of the number and severity of lesions, as well as the infection burden when compared with unvaccinated individuals (Garrido et al., 2011). However, subsequent field trials using bait oral vaccination, performed in free-ranging wild boar populations, showed no significant differences in lesion scores between vaccinated and unvaccinated groups (Díez-Delgado et al., 2018). Even in controlled experimental conditions, the protection conferred by vaccination suffers an intra-group variability, as for example in trials conducted on badgers (Chambers et al., 2017) and wild boars (Gortazar et al., 2014), where differences in the total number of lesions and granuloma evolution between vaccinated individuals were registered. Thus, wildlife vaccination is an emergent area, with a series of challenging questions to address, namely the specific characteristics of the vaccine, the delivery system, the geographic area of application, and the efficacy of the strategy. To date, the majority of works were performed in experimentally controlled conditions, so it is still very difficult to assess and quantify the economic and health impacts of this measure.

When considering transmission models to predict the effectiveness of vaccination in a community, the differential protective effect promoted on each individual by the vaccine is a factor that needs to be included and quantified or estimated. The success of any vaccination strategy is dependent on the efficacy of the vaccine and on the delivery to a sufficient proportion of susceptible animals, protecting the unvaccinated proportion of the population by reducing the quantity and frequency of mycobacterial excretion in the vaccinated population, consequently reducing transmission burden of the disease.

Thus, the most considerable limitation of modeling animal TB transmission is the lack of knowledge relating to key epidemiological aspects, such as infectiousness, basic reproduction number, the performance of diagnostic tests, host community and power of infection. The quantification of these dynamics within host species and within populations is of extreme importance to forecast animal TB transmission and to inform the development and management of vaccines.

2.2.2 Detection of infected hosts: heterogeneity within intradermal tuberculin testing and the IFN- γ assay

Depending on the infection phase, the IFN- γ assay and the intradermal tuberculin skin test may fail to detect infected animals (Rodriguez-Campos et al., 2014), perpetuating transmission to other animals and enabling animal movement to other herds, meaning an increase in animal and environmental heterogeneity once an external source of infection is introduced into a naïve ecosystem.

A fast and accurate way to detect infected animals is a key component of any disease control and eradication program. In TB, the immune response is connected to disease progression, from a latent state to rapid disease progression and organ dissemination.

The intradermal tuberculin test is recognized by the World Organization of Animal Health (OIE) and the European Commission as a primary screening test for *antemortem* identification of TB in cattle (Council Directive 64/432/ECC) (World Organisation for Animal Health, 2015). The result

of the intradermal tuberculin can be influenced by several factors, namely the immunological state of the host, the purified-protein derivatives (PPDs) used as antigens, the methodology, and rigor of test application and interpretation. The “pre-allergic” period, i.e. the time between when the host is infected and the development of an immune response that can be detected by the skin test; TB terminal stages that can cause a depression in immune response, also known as anergic state, preventing a reaction to the skin test; desensitization to bovine tuberculin, which may happen when the tuberculin skin test is administered too soon after a previous one; states of immunosuppression, as in *postpartum*; and/or co-infection; can each, or altogether, affect the result of the test (Bezoz et al., 2014; de la Rua-Domenech et al., 2006).

Features related to the PPDs, such as manufacturing errors or low potency, might cause false-negative results (Bezoz et al., 2014). In a trial conducted in Ireland, cattle population tested with low and high potency tuberculin’s led to significant differences in the number of reactors detected

The single intradermal comparative cervical tuberculin (SICCT) test measures the immune response of the animal to the injection of both bovine and avian antigen-purified protein derivatives, with the aim to increase specificity, and discard false-positive reactions that result from exposure to environmental mycobacteria, when compared to the single test. The application procedures of the test, as for doses, site of injection and interpretation criteria of the results, are standardized for cattle, so the extrapolation to other animal species, domestic or wild, might be prone to variabilities of immune reaction and to difficulties in the interpretation of test result (World Organisation for Animal Health, 2015).

The reaction size to tuberculin inoculation was suggested to be significantly positively associated with the *postmortem* number of lesions and their size (Byrne et al., 2018). Therefore, the reaction size has been suggested to be used as a predictor of both the extent and the progression of infection and a useful measure to identify super-shedders in a community herd (Byrne et al., 2018).

The IFN- γ assay is a complimentary blood test used alongside the SICCT, to maximize the probability of detecting TB-infected animals in cattle herds affected by TB breakdowns. This test was approved by OIE in 2015 and is the only blood test approved in the EU to supplement the

intradermal tuberculin skin test for TB in cattle (World Organisation for Animal Health, 2015). This assay is robust and relatively easy to standardize across different laboratories. Plus, the definition of interpretation criteria based on quantitative values allows for a more objective interpretation of the results, when comparing with the more subjective interpretation of skin test results (European Food Safety Authority, 2012).

The outcome of the IFN- γ assay can be influenced by the characteristics of the cattle populations in which the test is applied, the previous intradermal tuberculin test, sample handling and storage and methodology used. Sample handling is a critical aspect, with a decrease in the IFN- γ response associated with the increasing time between blood collection and sample processing (de la Rua-Domenech et al., 2006).

The IFN- γ assay shares some disadvantages with SICCT, namely: the pre-allergic period; the anergic state; the interference by co-infection with environmental mycobacteria; and the vaccination against *M. avium* subsp. *paratuberculosis* that can cause false-negative results (de la Rua-Domenech et al., 2006). Published data along the years on the influence exerted by prior application of SICCT on IFN- γ assay outcomes are contradictory. The type of skin test applied, the PPDs used, the time mediating the two tests, and the cattle population examined (experimental or naturally infected) can boost the IFN- γ result (de la Rua-Domenech et al., 2006; Schiller et al., 2010).

Novel diagnostic strategies are now being developed to distinguish infected from vaccinated animals (DIVA). The DIVA test takes advantage of antigens uniquely present in *M. bovis* but whose coding genes are deleted in BCG strains. The mostly used antigens are ESAT-6 and CFP-10, encoded by the RD1 region (Vordermeier, Jones, Buddle, Hewinson, & Villarreal-Ramos, 2016). DIVA tests were initially blood tests, being laborious and time-consuming and showing lower sensitivity in relation to SICCT (82% vs. 96%) (Vordermeier et al., 2016). In an attempt to increase sensitivity, the Rv3615c antigen, which is the ESX-1 secretion-associated protein EspC that is not secreted in BCG, was joined to the DIVA cocktail, with a marked increase in sensitivity (Vordermeier et al., 2016). With the transition to DIVA skin tests, previous disadvantages were

overcome and a sensitivity similar to SICCT (78% vs 76%) was achieved (Vordermeier et al., 2016). Furthermore, the addition of Rv3020c antigen (ESAT-6 like protein EsxS encoded in BCG genome) increased the sensitivity of the DIVA skin test to 90% (Vordermeier et al., 2016). The cornerstone of TB control is the rapid, accurate identification and removal of infected animals before they can spread the disease to others, therefore any improvements on the accuracy of the diagnostic strategy in place can positively influence this purpose. The opposite is also true.

2.2.3 The confounding effect of the co-infection state of the host

Alongside infection with *M. bovis* or *M. caprae*, co-infection with other bacterial, parasite or viral pathogens may weaken body condition and the capacity of the individual to mount an effective immune response, facilitating disease establishment and progression, impacting on transmissibility and on the ability to detect infection (Acevedo-Whitehouse et al., 2005; Byrne et al., 2018; Garza-Cuartero et al., 2016).

Co-morbidity with other infectious diseases, of bacterial or other etiology, has been associated with impacts on the performance of tuberculin skin test. Recently published work conducted in cattle suggested that liver infection by the parasite *Fasciola hepatica* can decrease the size of intradermal tuberculin reaction in animals experimentally infected with *M. bovis* (Claridge et al., 2012) and that the presence of the parasite can reduce infection progression, leading to slower lesion development (Byrne et al., 2018; Garza-Cuartero et al., 2016; Risco et al., 2014). Moreover, in livestock animals infected with *Mycobacterium avium* subsp. *paratuberculosis* or vaccinated against paratuberculosis, cross-reactivity may be registered, interfering with the interpretation of the skin test in the same way as colonization or previous contact with non-tuberculous mycobacteria do (de la Rua-Domenech et al., 2006). Furthermore, co-infection might also be related to disease progression outcomes. Recent work conducted in wild boar in Spain revealed a positive correlation between TB severity and the co-infection with porcine circovirus type 2, Aujeszky's disease virus or with *Metastrongylus* spp. (Risco et al., 2014).

These studies support the notion that the life history of the host, which includes exposure to different pathogens at different timepoints and scales, is a major driver of heterogeneity, with impacts in diagnosis and disease outcome.

2.2.4 Influence of host genetics

The host genetic variability has been suggested as an important predictor of disease outcome following exposure to *M. bovis*, producing effects on both susceptibilities to infection and/or greater risk of disease progression.

Work conducted on wild boar populations in Spain suggested that genetic heterozygosity in nuclear markers confers significant resistance to *M. bovis* infection and that it modulates disease progression in infected animals (Acevedo-Whitehouse et al., 2005). Specifically, a significant association between TB occurrence and the KS604 and S0101 markers, as well as between disease progression and SW208, S0025, S0068, and SW2021 markers was revealed (Acevedo-Whitehouse et al., 2005). The individuals with higher heterozygosity presented fewer disseminated lesions in comparison with homozygous ones, suggesting that host genetic heterozygosity may confer a significant advantage to confine the pathogen once the infection is acquired (Acevedo-Whitehouse et al., 2005). More recent studies in Iberian red deer populations also positively correlate population genetic diversity with the host's ability to control disease progression, indicating that inbred populations might present a higher risk for developing severe animal TB (Queirós, Vicente, Alves, de la Fuente, & Gortazar, 2016). Furthermore, studies conducted in Iberian wild boar populations identified SNPs and genes as candidates for biomarkers associated with wild boar genetic susceptibility to animal TB (Queirós, Alves, Vicente, Gortázar, & de la Fuente, 2018).

Polymorphisms in genes directly related to immune response have also been associated with TB outcomes. The allelic diversity of the major histocompatibility complex class II was assessed in a population of Iberian red deer (Fernandez-de-Mera, Vicente, Perez de la lastra, et al., 2009). The deer population presented high levels of variation, with 17% of nucleotide and 25% of amino acid positions being polymorphic among the 18 alleles identified (Fernandez-de-Mera, Vicente,

Perez de la lastra, et al., 2009). In another work, also conducted in the red deer population, DRB-2 haplotypes were significantly associated with animal TB prevalence (Fernandez-de-Mera, Vicente, Naranjo, et al., 2009).

The genetic improvement of cattle can be explored with the purpose of reducing susceptibility to TB. Different cattle breeds exhibit different susceptibility to animal TB (Allen et al., 2010). Heritability is one of the most important parameters to address when applying a selective breeding approach, and this factor has been estimated in several works applied to cattle and red deer (Bermingham et al., 2009; Mackintosh et al., 2000). Work conducted with Holstein, Zebu and Zebu-Holstein crosses cattle revealed a higher prevalence and severity of animal TB in the Holstein breed (Ameni et al., 2011).

Data concerning over 650,000 Holstein cows with TB, recorded by the Animal and Plant Health Agency (APHA), UK, was modeled to identify genetic signatures associated with TB resistance (APHA, 2016). Following this work, *TB advantage*, a genetic traits index, was created, to help dairy farmers making informed decisions on to the selection of cattle breeds with improved resistance to animal TB and, also, to help creating breeding plans suitable for their herds (APHA, 2016). However, this information is still only available for Holstein.

Intrinsic biological factors, such as age and gender, also need to be contemplated when considering the heterogeneity component of TB. Regarding age, cubs are more susceptible to infection than juveniles or adults, since their immune system is still under maturation. Recently published work conducted in groups of badgers demonstrated that cubs that were born into groups with reproductively active excretory females were the most likely to be detected as infected (Tomlinson, Chambers, Carter, et al., 2013). So, TB-infected females are still able to reproduce successfully and, thus, to transmit the infection to their offspring (Tomlinson, Chambers, Carter, et al., 2013). However, it also needs to be considered that the duration of exposure increases with age, meaning that older animals are more likely to have been exposed to pathogenic mycobacteria than the younger ones (Humblet, Boschirolì, & Saegerman, 2009). As for gender, female badgers seemed to be more resilient to infection than male (Tomlinson, Chambers, Wilson, McDonald, &

Delahay, 2013). This difference can be due to testosterone-induced immunosuppression in males, since sex hormones can influence the way the immunological response is triggered (Tomlinson, Chambers, Wilson, et al., 2013). In addition, females and males perform different functions in the social hierarchy, depending on the host species, thereby influencing transmission.

2.3 Environmental factors play a role in heterogeneity and persistence of multi-host TB

Environmental contamination with *M. bovis* remains poorly understood. The presence of *M. bovis* DNA, not necessarily viable, in environmental matrices, such as soil, sediments, water, grass, corn, and hay has been reported in several studies (Adams, Bolin, Fine, Bolin, & Kaneene, 2013; Barasona et al., 2017; Barbier, Boschioli, et al., 2016; Barbier, Rochelet, Gal, Boschioli, & Hartmann, 2017; Fine, O'Brien, Winterstein, & Kaneene, 2011; Santos, Santos, et al., 2015). Substrate contamination is believed to be the result of urine, feces, saliva, and/or nasal excretion by infected animals. The cultivation of *M. bovis* from the environment has been virtually impossible to accomplish due to difficulties in the culture procedure: sample over-contamination with other microorganisms leading to overgrowth of rapidly-dividing bacteria; decontamination process using bactericides that are harsh to cope with, even by mycobacteria; and the existence of viable but non-culturable bacteria (Adams et al., 2013; Fine et al., 2011). Additionally, the time lag between shedding and sample collection is a crucial factor to obtain viable bacteria and can compromise the reconstruction of eco-epidemiological transmission routes if the shedding individual cannot be identified (Fine et al., 2011). A previous study has demonstrated the existence of viable bacteria using an immunomagnetic capture technique (Sweeney et al., 2006). The scale-up of this methodology in order to test a larger number of environmental samples is difficult and has not yet been accomplished (Sweeney et al., 2007; Sweeney et al., 2006). Besides this technique, the experimental demonstration of viable and infectious *M. bovis* present in environmental substrates has not been proficient, although it is a hot topic among the scientific community working on TB.

The persistence of *M. bovis* in environmental matrices is thought to be associated with several environmental conditions that may facilitate bacteria survival (Rodríguez-Hernández et al., 2016).

DNA from MTC, namely *M. bovis*, has been found more frequently associated with sediments than with water, with more positive samples being detected in spring and major DNA concentrations being detected in autumn, while less concentrated samples are found in summer (Santos, Santos, et al., 2015). The temperature of air and soil, but also the evapotranspiration rate are amongst the most consistent positively associated variables with the probability of detecting MTC DNA in environmental samples. *M. bovis* has been found in higher concentration in moderate temperature and high water content, opposite to the characteristic high temperature and low water content of the dry season in summer (Adams et al., 2013; Barbier, Boschioli, et al., 2016; Barbier et al., 2017; Santos, Almeida, Gortázar, & Correia-Neves, 2015; Santos, Santos, et al., 2015; Young, Gormley, & Wellington, 2005). Besides, shade has been correlated with persistence of environmental *M. bovis* due to the maintenance of high-water content and moderate temperature, but also due to low sunlight association with low ultraviolet radiation, leading to less cell stress and mutations, improving bacterial survival (Rodríguez-Hernández et al., 2016). Moreover, the increase in exposure to sunlight allows the increase of vitamin D production in mammals, which is hypothesized to improve the host immune response, thereby counteracting transmission events enabled by increased contacts in water aggregation points during the summer (Nelson, Reinhardt, Thacker, Beitz, & Lippolis, 2010).

Besides abiotic factors that facilitate *M. bovis* survival and persistence in the environment, cell-intrinsic physiology also contributes to this phenomenon, since *M. bovis* possesses a specific cell wall composed of arabinogalactan, mycolic and glycolipid acids that act as a protective layer against several environmental factors usually considered detrimental to cell viability, such as pH, water content, oxidative stress, and temperature variations, allowing bacterial persistence for long periods of time (Chiaradia et al., 2017). In particular, the resistance to desiccation is thought to occur mainly due to the presence of endogenous synthesis of trehalose, a major constituent of the external cell wall (Harland, Rabuka, Bertozzi, & Parthasarathy, 2008).

M. bovis has been found in the free-living amoeba that may work as long-term reservoirs of these bacteria in environmental samples, particularly when present in cystic forms, retaining their

ability to transmit bacteria and cause disease in mice (Sanchez-Hidalgo, Obregon-Henao, Wheat, Jackson, & Gonzalez-Juarrero, 2017). Moreover, earthworms were found to be able to disseminate *M. bovis* from contaminated animal feces to the surrounding soil through casting egestion (Barbier, Chantemesse, et al., 2016). Besides, earthworms can also shed bacteria for four days after the initial ingestion of contaminated feces (Barbier, Chantemesse, et al., 2016). Both organisms act as environmental reservoirs of *M. bovis*, that could lead to cattle and wildlife infection (Barbier, Chantemesse, et al., 2016; Sanchez-Hidalgo et al., 2017), and maybe associated to the reemergence of animal TB in areas where no infected animal was present or no infected animal was introduced.

The high density of wildlife and cattle and habitat overlap usually promote aggregation at the wildlife-livestock interface. These convergence points include both feeding and drinking spots (i.e. pastures and waterholes, respectively) (Barasona et al., 2017; Santos, Santos, et al., 2015; Vicente et al., 2007), which can increase animal density, promoting animal contact and, subsequently, an increased probability of pathogen transmission, directly via secretions of infected animals, but also indirectly through the contamination of the environment. Waterhole size has been inversely related to the probability of positive detection of *M. bovis* since smaller waterholes have a higher probability of animal aggregation (Barasona et al., 2017). Besides, the detection of *M. bovis* in environmental spots is positively correlated with the observation of cachectic animals, related to advanced stages of animal TB and usually connected to increase excretion and infectiousness (Barasona et al., 2017).

Modelling TB in wildlife and at the livestock-wildlife interface requires information on spatial distribution, animal densities, landscape structure, climate, as well as animal management features, as these will impact on the model outcome (Gortazar et al., 2011; Madeira et al., 2017). So, the variability of abiotic factors also greatly conditions the heterogeneity of TB, both in disease burden and infection risk.

2.4 Heterogeneity of transmission within and amongst host populations and communities

Several studies report the influence of population and community factors in the transmission rate and infectivity of *M. bovis*. Animal movement appears to be one of the most important factors contributing to TB epidemiology, with cattle movements shown to be essential in badger disease dynamics in France (Palisson, Courcoul, & Durand, 2016), leading to an increased prevalence at a farm level in Spain (Pozo et al., 2019), and being the main cause of disease spreading in Great Britain (Brooks-Pollock, Roberts, & Keeling, 2014; Gilbert et al., 2005; Green, Kiss, Mitchell, & Kao, 2008). Animal movements were already used to develop epidemiological models to predict TB spread in United Kingdom (UK). These models are dependent on the spatial and temporal patterns of cattle movement, which need to have some consistency in order to improve model confidence. Movements of infected cattle were already associated with the introduction of the disease in TB-free herds, in England (Gopal, Goodchild, Hewinson, de la Rua Domenech, & Clifton-Hadley, 2006). The 2001 foot-and-mouth disease (FMD) epidemic was associated with disease dissemination since the reduction in bovine TB testing was followed by atypical movements of cattle to replenish FMD-affected farms (Gilbert et al., 2005).

Animal movement between herds and wildlife translocations associated with more frequent illegal commercial trade can alter *M. bovis* transmission dynamics, with the introduction of more virulent strains in susceptible communities (Avila, Gonçalves, & Perez, 2018).

The comprehensive cattle tracing systems in place for the past two decades (in European countries, in particular) have revealed considerable heterogeneity in the trading patterns of herds relevant for disease transmission. There is a big bias in the data available on cattle compared to wildlife populations, which are central to the choices and focus of mathematical models design as well as to the epidemiological questions that are considered. Nevertheless, different models, with different degrees of complexity, have been formulated over the years to explore various aspects of animal TB, including within- and between-herd models (Ciaravino et al., 2018), diagnostic techniques reliability (Conlan et al., 2012), contribution of wildlife reservoirs to animal TB persistence (Gortazar et al., 2011; Madeira et al., 2017), and the application of alternative control measures (R. L. Smith, Tauer, Schukken, Lu, & Grohn, 2013). Simulation models in cattle

consider disease transmission to occur between compartmentalized states among which the individuals circulate with different probabilities (Álvarez et al., 2014). The community composition, TB prevalence rate, herd dimension, cattle movements, and interaction with wildlife reservoirs using as proxy the number of and distance to aggregation points (e.g. feeding and watering) are other variables included in the majority of within-herd transmission models (Álvarez et al., 2014; Guta et al., 2014).

The commercial trade of domestic animals, namely cattle, seems to also play a significant role in TB spreading between long-distance locations (Bouchez-Zacria, Courcoul, & Durand, 2018). One such example is the wide spreading of *M. bovis* lineage Eu1 promoted by animal trading (N. H. Smith et al., 2011).

Aspects of host social organization should be taken into account in the epidemiology of wildlife TB since, in different host species, individual and group behavioral differences that account for variability in sociability or gregariousness, as well as scavenging habits, may influence pathogen exposure and burden, thereby influencing the patterns of infection at different scales.

The study of free-ranging populations of wild boar, red deer, and fallow deer, in the same geographic area in Spain, suggested that red deer and wild boar individuals from infected social groups were more probably infected than those from non-infected groups, a situation that was not supported in fallow deer (Gortazar et al., 2011). Thus, animals that, by social or bioecological reasons, are more gregarious, are generally more exposed to infection. Badgers have been under the focus for years due to the high prevalence rate of cattle TB in the UK and Ireland. Several studies report that the social stability of badgers mitigates the spread of animal TB through the maintenance of distinct social groups with little interaction between them (Riordan, Delahay, Cheeseman, Johnson, & Macdonald, 2011; Weber et al., 2013). However, culling perturbs this social structure, increasing badgers' movement and increasing interactions between individuals of different social groups, leading to an increase in disease spreading (Riordan et al., 2011; Weber et al., 2013). Moreover, small badger groups with low badger densities register high animal TB prevalence, contrary to initially thought, probably due to more intensive and frequent interactions

with members from neighboring groups (Woodroffe et al., 2009). Furthermore, infected badgers are socially isolated from their own groups but have more interactions with other groups, an extremely important component in TB transmission dynamics between neighboring social groups (Pope et al., 2007). Also, terrain features and food availability may influence animal TB spread between badger and cattle populations, leading to concomitant infection (Bouchez-Zacria et al., 2018).

A study concerning the interaction between four different species (cattle, domestic pigs, red deer, and wild boar), in a complex wildlife/livestock disease community, revealed extremely low percentages of direct interactions between those different species, but high percentages of indirect interactions in a time frame of three days, increasing the potential for indirect transmission among the different species via *M. bovis*-contaminated environment (Cowie et al., 2016). This study exposed high spatial and temporal overlap and a high rate of cross-boundary between farms, leading to interactions between different farm animals and disease spreading (Cowie et al., 2016). A similar study also performed in France detected an equally low rate of direct contact between different animal populations but evidenced a high frequency of indirect interactions in waterholes and baited places (Payne, Philippon, Hars, Dufour, & Gilot-Fromont, 2017). In agreement with these notions, red deer density has been positively associated with *M. bovis* infection in both red deer and wild boar in TB hotspot areas in Portugal (Madeira et al., 2017), possibly as the result of interactions in an area where high densities of ungulates are maintained and artificially managed for hunting purposes.

More recently, the existence of super-shedders was reported. Super-shedders are individuals that excrete *M. bovis* consistently through time, space and several routes, and that promote large numbers of secondary cases (Delahay, Langton, Smith, Clifton-Hadley, & Cheeseman, 2000). The identification of these super-shedders is essential since they play a disproportionately important role in both transmission and maintenance of infection in a population. Super-shedders have been already reported in populations of cattle (O'Hare et al., 2014), badgers (Delahay et al., 2000), wild boar and red deer (Santos, Almeida, et al., 2015).

The high variability of social dynamics between wildlife reservoirs of animal TB, the host species-specific vital area, the probabilistic nature of animal movements, the heterogeneity of intraspecific and interspecific interactions, and the commercial trade of domestic animals, are all factors contributing to heterogeneity of *M. bovis* transmission, adding layers of complexity to animal TB and hampering disease management and control programs. However, these factors are still not well quantified.

2.5 Layers of heterogeneity in TB management, control, and eradication programs

Poor management and hygienic status of livestock herds and deficient game management policies increase the risk of acquiring infectious diseases. Several measures to enhance livestock biosecurity focus on prevention: regular testing of individuals and culling of test-positive; pre-movement testing; regular decontamination and cleaning procedures and application of specific measures to herd facilities, vehicles, and equipment when positive animals are detected (Layton, Choudhary, & Bean, 2017). In cases of extensive husbandry and/or existence of wildlife reservoirs, surveillance, and especially compartmentalization, artificial feeding and water spots, as well as physical limitation of contacts between domestic and wildlife animals, are recommended (Gortazar et al., 2015). In some ecosystems, wildlife animals are farmed, with artificial management, including fencing, feed supplementation, and population translocation, affecting population structure and dynamics, and promoting aggregation and animal contacts. This increased exploitation of farmed wildlife for hunting or game meat industry purposes increases the probability of transmission events among individuals. Disease monitoring and vaccination provide another layer of biosecurity for wildlife disease management, especially in highly manipulated ecosystems (Gortazar et al., 2015). Additionally, treatment of animal TB is usually not attempted, since administration of antibiotics would be necessary for a long period of time; it is uneconomical and safety questions arise concerning the consumption of animal products and animal derived products. Moreover, TB treatment in cattle is prohibited in many countries, including European Union members.

Some of the major challenges in quantifying heterogeneity are also related to the economic interest of each potential host species, particularly in the case of non-commercial animal productions and non-game animals. The legislation is strongly biased towards commercial animal productions and, in several countries, only deals with infection by *M. bovis*, excluding *M. caprae* as a causal agent of animal TB (Rodriguez-Campos et al., 2014). One of the biggest issues concerning heterogeneity quantification is non-commercial animal production, as testing is not obligatory (Gormley & Corner, 2018). Still, those animals remain a source of contamination and may interact with both wildlife and commercial production animals. Since those animals are not tested, they may represent a missing link in the epidemiological tracking further ahead. When it comes to non-game animals, the same kind of issue arises since most wildlife data is generated by testing game species, whose samples are more easily obtained and donated for scientific purposes. Neglecting non-game species in surveillance strategies due to logistic, operational, economic or legislation issues implies losing an important piece of the puzzle. Another source of heterogeneity is TB-infected animals that go undetected and perish in the fields. These end up contaminating the environment, infecting other sympatric animals and scavengers. Since *M. bovis* can infect a wide range of hosts, both wildlife and domestic animals may become in contact with infected animal leftovers, triggering a complex epidemiological web. In this aspect, intervention from people in the field like hunters, landowners, gamekeepers or shepherds is of utmost importance, since dead animal sighting and removal are crucial to interrupt transmission chains.

Another topic that influences the heterogeneity of available data is the fact that the delineation of animal TB control areas is frequently outdated and does not correspond to the current distribution of the disease, biasing surveillance, animal removal and further analyses for heterogeneity. Those areas retain the obligatory testing programs for years, frequently developed for a specific land area and epidemiological context, but are not adjusted to differences in community structure and composition, inter-species interactions, husbandry, management systems or environmental contamination.

Moreover, the difference between disease reservoir and dead-end spillover host is important when developing management and control programs and defining the host targets to be monitored. Additionally, this distinction impacts the stakeholders' perspective and level of engagement with the disease problem, with stakeholders normally agreeing with a radical response to deal with spillover hosts (Gormley & Corner, 2018). However, the problem complexity increases when a reservoir host is the target of intervention, with an increase on the number of stakeholders being involved and more complex ethical issues arise that need to be solved, together with the economic cost-benefit effectiveness of dealing with the disease in a multi-host and long time-spatial system (Gormley & Corner, 2018). Stakeholders are also strongly influenced by different perspectives on the economic, technical or scientific knowledge, ethics, social and cultural background, ecological concerns, and political will (Chardonnet et al., 2002; Ryser-Degiorgis, Pewsner, & Angst, 2015). All these factors add conflicting perspectives when designing management and control programs which may compromise effective implementation and observance to guidelines and rules. Heterogeneity in adherence to herd biosecurity measures and compliance with surveillance and slaughter of the reactor animal also increases the heterogeneity of control performance worldwide. So, in a management and control program, not all species are of equal significance in the epidemiology of the disease or are subjected to uniform disease management (Gormley & Corner, 2018), leaving room for individual interpretations and, once again, increasing heterogeneity in an already complex epidemiological scenario.

Several studies on the economic side of animal TB have been conducted in recent years, however, but the real cost-benefit of the different control and eradication programs are still poorly known and communicated, leading to the controversy on the taxpayers' money allocation for this purpose (Caminiti et al., 2016). Most of such studies were conducted in Europe, predominantly in the UK, as for example the analysis of cost-benefit of seven different control strategies in cattle (Bennett, Cooke, & Ijpelaar, 2005), the evaluation of costs borne by farmers for pre-movement testing of cattle (Bennett et al., 2005), and the assessment of the cost-benefit of different badger culling strategies for TB control in cattle (G. C. Smith, Bennett, Wilkinson, & Cooke, 2007; Wilkinson

et al., 2009). The first two studies were inconclusive due to the lack of information on several parameters, such as expenditure or indirect costs. The last couple of studies were more conclusive, with badger culling being effective to control animal TB in cattle, namely gassing, but all strategies showed costs that outweighed the benefits. However, these models are only predictive and, when put into practice, do not show the expected results, due to confounding effects.

Moreover, a study in Spain reported the cost-benefit of both animal TB and brucellosis eradication programs in cattle but did not take into consideration the community benefits, concluding on the economic inefficiency of the programs (Bernues, Manrique, & Maza, 1997). The evaluation of a total of 35 animal TB surveillance programs was also performed in the Netherlands, reporting Enzyme-Linked Immunosorbent Assay (ELISA) testing of bulk-tank milk in combination with the visceral inspection at the slaughterhouse as the best surveillance program (van Asseldonk, van Roermund, Fischer, de Jong, & Huirne, 2005). However, the Netherlands is an officially-free TB country, so the conclusion of efficiency is not directly transposable to countries with different epidemiological scenarios in which animal TB has a considerable prevalence. In Italy, the cost of TB, brucellosis, and leukosis in cattle has also been evaluated, with authors reporting an average of 50% reduction in costs associated with the eradication program when the certification of disease freedom is accomplished (Caminiti et al., 2017).

Regarding non-European countries, an evaluation of the probability of success of an eradication program for animal TB in Argentina, if costs were to be borne by farmers, was performed with a positive cost-benefit being achieved if caudal-fold tuberculin test would be used, but only as a long-term investment program, with inherent possible loss of will by the farmers (Perez, Ward, & Ritacco, 2011). Moreover, the control program applied in Zambia at the interface between wildlife and livestock, together with an awareness campaign aimed at the local population, was economically evaluated and the cost seemed to exceed the benefits, however the zoonotic impact of animal TB was not fully taken into consideration in this study, which could have led to an outweighed of benefits over costs (Mwacalimba, Mumba, & Munyeme, 2013). Furthermore, a study performed in India highlighted: 1) the economic constraints disparities between developed

and developing countries raised by the loss of capital when an infected animal needs to be culled, or when its derivate-products cannot be commercialized, due to lack of financial compensation; 2) economic power constraint differences regarding the ability to pay for better feeding, infrastructures, and veterinary services, together with the lower financial capacity to implement efficient biosecurity, control and eradication programs; and 3) the social and religious singularities of India and Nepal since cattle in these countries are considered sacred and slaughter is constitutionally banned, leading to alternative less efficient strategies to control animal TB (Chauhan, George, Lindahl, Grace, & Kakkar, 2019).

These studies show that financial, social, and religious heterogeneity that exist in particular between developed and developing countries do contribute to the heterogeneity of success and options in the control and eradication of TB worldwide.

3. Implications of animal TB heterogeneity in management, control, and eradication programs

The drivers for heterogeneity can be divided into three main groups: protective factors that help to prevent the establishment and spreading of animal TB; risk factors that contribute to easier and/or faster spreading of animal TB; and maintenance factors that influence animal TB infection persistence and burden on the community (Figure 1).

The main protective factors identified to date are biosecurity measures, accurate diagnosis, and vaccination. These factors are of extreme importance to be considered in management and control programs. The implementation of biosecurity measures such as fencing of cattle and waterholes, avoiding wildlife-livestock contact, the correct design of artificial feeding supplementation devices that avoid wildlife interference, and animal movement restrictions, all are important to prevent disease transmission between wildlife-livestock and within-herd transmission. Diagnosis performance within SICCT and IFN- γ tests are essential to detect infected animals and to avoid disease spreading across the herd since reactor animals are separated and culled. Broadening TB diagnosis to a larger panel of host species, for instance, sheep, goat, and pig in extensive management regime would enhance the protective role of diagnosis. Vaccination of cattle and,

eventually, of wildlife reservoirs has not been allowed in most countries due to the absence of regulated DIVA tests. However, vaccination, by increasing herd immunity, should be considered to augment the success of control programs, preventing the establishment and spreading of TB.

In contrast, several risk factors contribute to increasing animal TB heterogeneity, with super-shedder hosts, co-infected hosts, host age and sex, aggregation points, and animal density and movements being the most relevant. The detection and removal of super-shedders should be a priority since these animals are foci of direct and indirect contamination. The improvement in animal healthcare and welfare are of critical importance to maintain good body condition, immunity, and avoid infections. The co-infection status of the animals should also be evaluated on a regular basis, particularly in cattle, to prevent immune imbalance and susceptibility to disease. Moreover, cubs and badger males tend to be more prone to infection, so the biosecurity and diagnosis measures should focus on these risk groups. Aggregation points (e.g. waterholes and pastures) where animal density is higher should also be controlled to avoid animal accumulation by increasing waterhole sizes, the number of feeding points and the separating distance. Control of animal movement, either cattle or wildlife (for repopulation/reintroduction purposes) should be stricter and more surveilled to avoid transmission among farms and wildlife-livestock transmission. Furthermore, pre-movement testing of TB-susceptible species before trading should be reinforced and/or carried out once again to avoid disease spreading to a free-TB farm/area.

Maintenance factors are often neglected in management and control programs. These factors contribute to the indirect maintenance of animal TB in the community. One of the most important and neglected factors of animal TB maintenance is the environmental persistence of TB causative agents. The existence of high-water contents, moderate air, and soil temperature and invertebrate environmental reservoirs (e.g. free-living amoeba and earthworms) contribute to the persistence of environmental *M. bovis*. Besides, host social behavior and genetics are also related to susceptibility to disease, and advances in disease control can be achieved by selective breeding programs. In a work performed by Raphaka and collaborators (2018), the contribution of genetic

selection for TB eradication was assessed in the UK, and genetic selection was associated with a reduction of TB breakdowns severity over generations (Raphaka et al., 2018). It is important to note that this option is a long-term control measure that should be complemented with other management and surveillance approaches.

Given the heterogeneity of animal TB emphasized in this work, the key challenge for mathematical modelling as a tool for informing the best intervention strategy is the definition of the appropriate level of model complexity required to understand the dynamics of transmission in each context. Many aspects of animal TB epidemiology are yet to be elucidated, which limits the estimation of transmission model parameters. Disease modelling approaches could be helpful in decision-making, by evaluating potential alternative diagnostic and control measures in high prevalence scenarios (Guta et al., 2014); and to improve surveillance and streamline the eradication strategies in low prevalence scenarios, when a disease outbreak occurs (Salvador et al., 2019), never losing the heterogeneity parameter from the equation. The way these knowledge gaps are considered within the mathematical models, and the different considered assumptions, may have a critical impact on the model outcomes and robustness, so extrapolation of results to a certain scenario and the underlying definition for interventions should be done with caution (Álvarez et al., 2014; Conlan et al., 2012).

A consequence of heterogeneity in animal TB epidemiology is that the outcome of intervention strategies will vary greatly depending on the target group, with most interventions being focused on high-risk infection groups, such as cattle ("Regulation (EU) 2016/429 - "Animal Health Law", 2016). Intervention targeting wildlife reservoirs is often underperformed, with these hosts being mostly screened *postmortem* and not removed earlier to prevent transmission cascades, due to high operational costs. The focus on high-risk groups can be efficient, but the success of the control programs is highly dependent on the mono-host or multi-host transmission system existing in the region and on the ongoing environmental cycle (Allen, Skuce, & Byrne, 2018; Gormley & Corner, 2018; Trauer et al., 2018).

Regular screening and general health check-ups are performed in livestock animals in developed countries and also in some developing countries ("Regulation (EU) 2016/429 - "Animal Health Law", 2016). These screenings look to ensure animal health and exclude infection caused by specific microorganisms. However, the screening for animal TB in other susceptible livestock animals, such as goats, sheep or pigs does not usually occur by routine. The regular screening for animal TB in these species (or certain breeds) that usually are managed outdoors, and thus may also constitute sources of direct and indirect contamination, could help to spot unknown TB-infected areas. Furthermore, the *antemortem* screening of free-ranging game species known to act as animal TB reservoirs should be performed on a regular basis to prevent disease spreading and outbreaks. Moreover, random screening of other wildlife species should also be performed when ecological scientific studies are performed to identify possible dead-end spillover hosts or even new unknown reservoirs. Additionally, companion animals, in particular, those that usually interact with cattle or wildlife animals, such as hunting and shepherd dogs, should be regularly screened in veterinarian services to avoid TB spreading and zoonotic transmission. Besides hunting and shepherd dogs, which are commonly related with bovine TB due to their interactions with cattle, there is also the presence of cats. Those are reported to have large hunting territories by which they become in contact with cattle and wildlife animals as well (Pesciaroli et al., 2014). Most infections in cats are reported to be caused by *Mycobacterium microti* due to direct contact with small rodents or *M. bovis* by direct contact with infected cattle or consumption of contaminated by-products (Pesciaroli et al., 2014). Cases of cat-to-human transmission are supposed to represent a limited risk, but were reported by the British Government in 2014 (British Government, 2014). More recently, in 2019, a study by O'Halloran and coworkers, discussed the impacts of cat-to-human transmission due to a case of consumption of commercial raw food by pet cats (O'Halloran et al., 2019).

The incidence of animal TB shows geographical clustering, so spatial targeting of interventions should be considered (Humblet et al., 2009). The geographical targeting of management and control programs focusing on hotspot areas has been shown to help to accomplish a reduction of

general disease burden. However, the interaction between individuals, and their broader populations, at hotspot areas are important factors to be considered for optimized regional interventions. In several countries, namely Portugal, the epidemiological risk areas for animal TB that are under the focus of specific measures, should be revised and geographically extended to guarantee the implementation of current, evidence-based interventions. Buffer areas should also be included. Besides the focus on hotspot areas, sporadic screening of sentinels in non-risk areas should be guaranteed for early warning and preparedness.

The intervention on risk groups can decrease the general burden of animal TB but increase heterogeneity in non-surveilled populations (Trauer et al., 2018). When control programs are focused in high disease burden populations, the interventions can reduce heterogeneity even if the overall burden of the disease does not decrease significantly (Allen et al., 2018; Trauer et al., 2018). However, the focus on the overall community can decrease disease burden but increase the existing heterogeneity (Allen et al., 2018; Trauer et al., 2018). So, it is plausible that the slaughter of an infected animal is more important in controlling disease spreading in small and/or low disease burden populations than in large and/or higher disease burden populations.

4. Conclusions

Causes of heterogeneity in animal TB are multiple and include features of (i) the causative agent – genetic diversity, host and geographical range, virulence; (ii) the host species – species diversity, genetic variability, immune response, disease progression, co-infection, sensitivity to testing; (iii) the environmental conditions – environmental matrices diversity, environmental reservoirs, host density, abiotic factors such as climate, temperature, humidity, ultraviolet radiation; (iv) the population and community factors - social dynamics, vital area, animal movements, population interactions, community composition and structure, commercial trade, translocations; (v) management, control and eradication programs – economic, social, political, cultural and ethical factors (Figure 1). All these factors share intricate and complex interactions with each other, influencing and compounding disease burden. Knowledge of these accumulated heterogeneity

layers and the cross-interaction between them is necessary to understand the persistence and transmission dynamics of *M. bovis* or *M. caprae* in each ecosystem.

Several challenges can arise in assessing animal TB heterogeneity, with observed disease heterogeneity not reflecting epidemiological reality. These can be highly problematic depending on the economic interest or ecological value of the host, the areas undergoing control, and the monitoring system.

Animal TB heterogeneity has several implications on the effectiveness and efficiency of control. The outcome of intervention strategies will vary greatly depending on the target group, geographical and spatial locations, operational parameters and burden of the disease. The designed strategies to deal with animal TB should take heterogeneity into consideration and apply the available resources to ensure the efficiency of programs. The flexibility of national programs needs to be urgently considered, creating space for the implementation of specific measures at least to resolve regional and local epidemics.

5. Ethics approval and consent to participate

The authors confirm to adhere to the ethical policies of the journal. No ethical approval was required as this is a review article with no original research data.

6. Conflict of interest statement

The authors declare that they have no conflict of interests.

7. Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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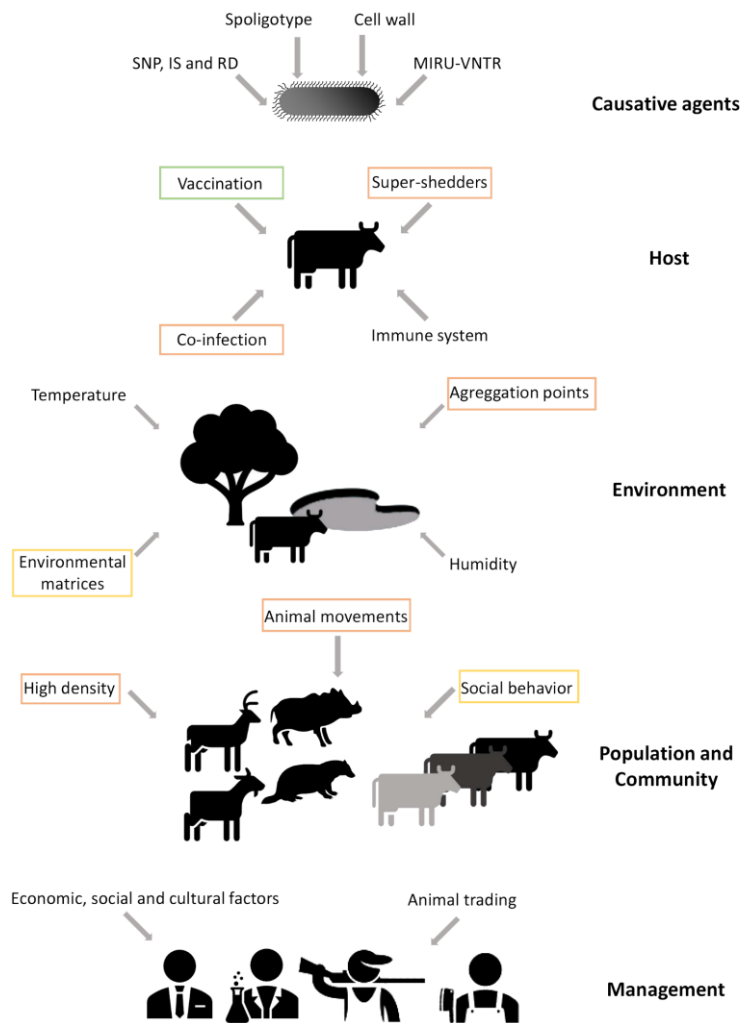
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1472 **Figure caption**



1473

1474 **Figure 1 – Framework for the understanding of animal TB heterogeneity.** Pinpoint of
1475 heterogeneity drivers associated with their agents, including causative agents, host, environment,
1476 population and community, and management entities. Protective, maintenance and risk factors
1477 are coded in dashed, dotted and full lines, respectively. IS – insertion sequence; SNP – single
1478 nucleotide polymorphism; RD – region of difference; MIRU-VNTR - Mycobacterial Interspersed
1479 Repetitive Units-Variable Number Tandem Repeats.