REVIEW / ARTÍCULO DE REVISIÓN

The problem associated with tuberculosis in cattle and how this is being addressed

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Abstract: Bovine tuberculosis (bTB) is a zoonotic disease caused by Mycobacterium bovis that primarily infects cattle but has a wide range of hosts. It represents a global health problem affecting the livestock industry tremendously, with economic losses of about 3 billion annually. Dairy cattle produce a decline of 10% in terms of liters of milk produced and 5% in the meat industry because of live weight loss and seized carcasses in abattoirs. The core of the current control measures implemented in most countries against bTB is based on the diagnosis with tuberculin skin test (TST) and culling of infected animals. Unfortunately, control programs have failed to eradicate the disease since no vaccine protects cattle from infection. Moreover, the strain of M. bovis Bacillus Calmette Guerin, BCG used as a vaccine for human tuberculosis, interferes with surveillance tools. Nowadays, most researchers have been working on improving the efficacy of BCG through a prime-boost strategy that involves a first immunization with BCG and a booster with different types of vaccines. A less explored approach by experts has been the development of a new vaccine that only includes some protective antigens of M. bovis that should be absent ot in low representation in TST. On the other hand, because TST precludes implementation of Bacille Calmette-Guérin (BCG) vaccine–based control programs, several investigations have been carried out to replace the TST with a DIVA test that allows to differentiation infected from vaccinated animals. In this review, most of the studies cited agree that without an effective vaccine and a compatible diagnosis, no program for eradication would be successful against tuberculosis in cattle.

Key words: Bovine tuberculosis, bTB, *Mycobacterium bovis*, diagnosis, tuberculin skin test, TST, bPPD, vaccines, DIVA.

Introduction

Bovine tuberculosis (bTB) is a chronic zoonotic pathology caused by Mycobacterium bovis that belongs to the reportable diseases of the World Organization for Animal Health WOAH (OIE). *Mycobacterium bovis* may mostly affect cattle, humans, and other domestic and wildlife animals[1-3]. It belongs to the phylum Actinobacteria, family Mycobacteriaceae and genus *Mycobacterium*[4,5]. In the *Mycobacterium* genus has been identified more than 120 different species, and some of them belong to the *Mycobacterium tuberculosis complex* (MTC). The most common members of the MTC are *M. bovis, M. tuberculosis, M. canettii* (oldest strain), *M. africanum* (responsible for human tuberculosis in Africa), *M. pinnipedi* (infect seals), *M. microti* (rodents), and *M. caprae* (goats and other mammals). *M. bovis* and *M. tuberculosis* have 99.95% of genomic homology in their shared genes[6].

Bovine tuberculosis has a worldwide prevalence of about 13% and can be found in 82 countries around the world[7]. The biggest challenge in those countries is the control of *M. bovis* in wildlife animals as they act as a reservoir of the pathogen[8]. In all these countries, the disease is associated to tremendous economic losses for the livestock industry, which according to a study performed in USA were estimated in 3 billion dollars per year[9]. Research have shown that bTB generates a fertility loss in females and a decrease in milk production per lactation of 10%. On the

other side, the disease also generates losses of 5% in the meat industry since carcasses are seized in slaughterhouses, and there is a live weight loss of animals[10]. It has also been observed that close to 1% of positive bTB cows can develop mastitis[11]. On the other hand, the disease produces high costs in terms of international cattle trading restrictions and dairy and meat by-products to OTF countries[12]. Other costs associated with bTB are control and eradication programs that have as central measures the diagnosis through TST and culling of infected animals to avoid spreading the disease in the herd.

Furthermore, control and eradication programs require removing and replacing infected animals from the pen. Therefore, it causes economic losses not only to farmers but also to the government in terms of indemnity expenses and replacement cost of cattle which can vary from around $500 to 900 USD[13]. The measures included in the control and eradication programs implemented by governments in the countries where bovine tuberculosis is present have shown to be non-enough to prevent infection and eliminate the disease. The need for an effective vaccine that can be applied in cattle and would be authorized by the OIE is still a challenge. Moreover, it is imperative to have a diagnostic tool that can be used with an effective vaccine to support the surveillance strategies. An effective vaccine that strengthens and complements those programs is pivotal in contro-

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lling the pathology. Indeed, it has been demonstrated that a strong immunity triggered by a vaccine may defeat *M. bovis*. The vaccine BCG (Bacillus Calmette-Guérin) used against human tuberculosis but made of Mycobacterium bovis has been trialed before in cattle; however, it has demonstrated a variable range of protection[14]. Currently, most studies are led to improve the effectiveness of BCG in cattle, and in this context, it has been carrying out assays in cows and other animal models. The primary strategy implemented in those studies is based on the prime boost with BCG as a prime and a boost with different types of vaccines, such as subunit recombinant and adenovirus vaccines. Another less explored approach is the development of a new protective vaccine that could include only some immunogenic proteins of *M. bovis*. These proteins must be included in a low proportion of bPPD of tuberculin so they may not interfere with the TST. On the other side, researchers have also been working on differentiating infected and vaccinated animals and DIVA tests to replace TST because it is incompatible with BCG. The diagnosis of cattle with TST does not allow to discrimination between infected and vaccinated animals since it causes a cross-reaction with the BCG vaccine.

In this article, we seek to review and discuss different studies related to the global problem caused by tuberculosis in cattle. Here it has not only been described the current strategies used in the control of the disease and their drawbacks, but most importantly, we propose an approach that has not been deeply explored as an alternative in developing a new protective vaccine. This could be the study of the most immunogenic proteins of *M. bovis* that are less representative in bPPD to be included in producing a new vaccine that should not cross-react with the tuberculin skin test.

The Disease In Cattle

In natural conditions, airways are the most frequent route of infection of *M. bovis*. Using inhalation of the pathogen that is suspended in aerosols, bovines may get infected. Nevertheless, the oral route is the second most common infection in bovines, and they can ingest the bacterium from contaminated water and food[15]. When the microorganism comes from the upper airways, it moves to the pulmonary alveoli. In the alveoli, molecular patterns associated with pathogens are recognized by receptors of innate immune cells, such as macrophages[16]. These cells engulf *M. bovis* and release chemokines that attract neutrophils and other cells to the infection site, leading to edema with the accumulation of fibrin (inflammatory exudate). Macrophages display the antigen to lymphocytes which then are activated, and as a result, more cells from the immune system reach the site of infection[17]. When immune cells migrate to the site of infection, they accumulate and form tuberculous, which are granulomatous lesions pathognomonic of the disease. In some animals, the immune system can restrain the initial infection by eliminating the bacterium or inhibiting its multiplication into the tissue[18]. Thus, the microorganism can get into a latency phase in which *M. bovis* decreases its metabolism to basal levels and remain in a non-replicative state to survive. Nevertheless, the bacterium can reactivate, and the disease will progress, causing a transformation of the granuloma structural organization that in chronic cases, have a necrotic center surrounded by a thick fibrotic tissue layer[19]. The microorganism contained in the granuloma of the lower airways can be transported by phagocytic cells through the lymphatic system until a regional lymph node where a second infection occurs. The primary infection in the lungs, plus the new in the lymph node, integrates the "primary complex" of the disease. When the immune system cannot hold the infection in the primary complex, the pathogen disseminates through the blood (bacteremia) toward other tissues and organs, triggering the generalization of the bTb[20]. Owing to bTB being a chronic disease with slow progress, most infected animals do not manifest clinical signs in the short term but in months or years after infection. Most animals show unspecific symptoms at the onset of the disease, for example, fluctuating fever, cough, diarrhea, anorexia, weakness, and lymph node inflammation. While acute presentation of bTb is not frequent, it has been observed that the pathology evolves very fast in non-immunocompetent animals, causing death. On the other hand, in animals that overcome the initial infection, the pathology becomes chronic. Some of the clinical signs that infected animals exhibit in advanced stages of the disease are dyspnea, pneumonia, weight loss and emaciation, granulomatous lesions in different organs, and finally ending with the, death[21]. Granulomas can be encountered most frequently in lungs and lymph nodes if *M. bovis* has come in throughout the respiratory system. Otherwise, granulomas may be in the stomach, spleen, liver or mesenteric lymph nodes if the pathogen penetrates an oral or digestive route. In the inspection, post-mortem is very frequent to find granulomatous lesions in retropharyngeal, mediastinal, and tracheobronchial lymph nodes. Those lesions also commonly affect the lungs (mainly apex zone), stomach, liver, spleen, kidneys, and mammary gland[21]. In severe cases, there is an extrapulmonary presentation of the disease because of the dissemination of *M. bovis* to the rest of the organism.

Control and prevention of bovine tuberculosis

Despite several countries from European Union, North America, Asia, and Oceania being OTF, the disease has not been fully eradicated. In these countries, bTB is remitted to certain areas, and control and eradication programs against the pathogen's spread are still in force. These programs require huge economic investment because of animal screening and indemnity for culling infected individuals besides other measures such as restrictions in animal movement and surveillance strategies in farms and slaughterhouses.

Detection of *M. bovis*-infected cattle.

One of the central control measures achieved in the programs against bTB is the identification of infected cattle in the field through the tuberculin skin test TST[22]. The single intradermal tuberculin SIT consists of injecting 0.1 mL of 3000 IU of a bPPD (bovine purified protein derivative), eliciting delayed hypersensitivity response (type IV) mediated by cells when the animal has been previously exposed to the pathogen[23]. This cell immune response is triggered by chemokines and cytokines recruited in the PPD injection site due to previous contact with the microorganism. It has been reported that TST sensitivity range from 57 to 95%, whereas its specificity ranges from 55 to 100%[24]. There are three types of SIT: i) the single caudal fold test (SCFT) which a bPPD is administrated in the tail base, ii) the single intradermal cervical tuberculin (SICT) which a bPPD is injected in the middle of the neck of the animal, iii) the intradermal comparative cervical tuberculin (SICCT) which a bPPD and aPPD (avian purified protein derivative) are applied in two central points of the neck of cattle[25]. In this last, both PPDs are administrated since they allow to discriminate between infections produced by *M. bovis* and other environmental

mycobacteria such as *Mycobacterium avium spp* paratuberculosis. The tuberculin test results are read 72 hours post-administration and are interpreted according to the skin fold thickness of the inflammatory lesion generated at the injection site. Following the interpretation of the TST, it can be obtained two results: i) negative animals, when was neither observed nor touched any thickness changed at the inoculated site ii) reactor animals, when can be observed and touched a type of edema that maybe go along with blush, warm, and pain at the inoculated site. The intradermal test cannot be performed in animals with an interval of fewer than two months between each application to avoid false positive cases because of the previous sensitization with the PPD[26].

All suspected or responder animals to the tuberculin test are confirmed through laboratory techniques such as interferon-gamma release assay (IGRAs) and enzyme-linked immunosorbent assay (ELISA).

The IGRA is an in vitro technique that measures a cell-mediated immunity stimulated in response to *M. bovis* infection. The cytokine IFNγ produced by T lymphocytes of an infected animal is detected in blood samples stimulated with PPD throughout an ELISA plate which is coated with a monoclonal antibody anti- IFNγ. With this assay, infected animals can be identified as soon as two weeks since they were in contact with the pathogen[27].

On the other side, the ELISA test kit IDDEX is frequently employed in diagnosing infected herds with tuberculosis. However, unlike IGRA, the ELISA is a serological technique that measures antibody immune response against two specific antigens of *M. bovis*, MPB70 and MPB83, which are immobilized in the plate. Antibodies in sera samples are detected using a secondary conjugated anti-bovine antibody[28].

Cattle culling and surveillance in slaughterhouses

When a cow is reactive to the tuberculin skin test or confirmed as positive through a laboratory diagnosis, the animal is first isolated and eventually removed from the pen and slaughtered, limiting the risk of continuing to transmit the disease toward the rest of the animals[29]. Culling is one of the control measures mostly used in programs against bovine tuberculosis worldwide. Veterinarians kill the animal in an official abattoir, where the individuals are subjected to an inspection for granulomatous lesions findings[30]. This procedure involves the inspection and incision of lymph nodes in the head (retropharyngeal and tracheobronchial), chest cavity (mediastinal), and organs such as lungs and liver[31]. Samples from these lymph nodes are then sent to an official accredited laboratory for further analysis, such as culture, PCR, and histopathology, to confirm the cases. Once reactive and suspect animal cases are confirmed as positive after organs and carcass inspection, they are included in an official register by the respective government organization[3]. Consequently, in infected farms is performed a sanitation process which provides for quarantine, restriction on animal movement, and the disinfection of premises to remove the disease. After the removal of infected animals and sanitization of the farms, whole herds must periodically be re-evaluated through a tuberculin skin test until the entire herd in negative. These tests should be applied in animals with at least 2 months between and complemented with laboratory analysis[33,34]. Besides the sanitation and removal strategies, in several countries, the governments provide financial compensation per culled animal to farmers[30]. In England, for example, per animal slaughtered, it

is paid about €1500, and in the USA, between $500-2500 US[35]. Because test-and-slaughter approaches are unfeasible, there is an imperative need to develop a new effective vaccine that can support control and eradication programs. Vaccines provide immunity that may protect individuals from pathogens; in reality, they are nearly the most cost-effective solution for controlling infectious diseases.

The vaccine against bTB must meet two main criteria: first, do not interfere with the intradermal tuberculin skin test performed in vivo on animals, and second provide full protection against the *Mycobacterium bovis*, which is responsible for causing the pathology.

Challenges of vaccination in cattle

Currently, no vaccine is approved by the OIE to be used in cattle against bTB. In the case of human tuberculosis (hTB) produced by *Mycobacterium tuberculosis*, the strain of *M. bovis Bacillus* Calmette-Guerin (BCG) is authorized by the World Health Organization (OMS) to be employed as an attenuated vaccine. Despite, both pathologies are closely related in terms of high homology between the microorganisms and similarity in the clinical manifestation of the disease, the use of BCG in cattle for immunization is not allowed[36]. There are two key reasons for this: firstly, BCG is a live attenuated vaccine that includes the whole pathogen; therefore, it shares several antigens with bovine PPD used in the tuberculin skin test or TST[37]. This means that TST failed to distinguish BCG-vaccinated cattle from infected ones with a live virulent strain of *M. bovis*. The interference between BCG and bPPD generates a cellular immune cross-response mediated by memory T cells. These cells generate a recall response when they have been previously exposed to the pathogen, and then animals become positive in TST[38]. Second, BCG has been successfully tested in cattle; however, it has been demonstrated to induce varying levels of protection against *M. bovis*[39]. The lack of an efficient vaccine against bovine tuberculosis during all these years has provoked the problem caused by the disease has increased due to it still spreading between the species. Hence, it is crucial to have a protective vaccine that complements control and eradication programs executed in most countries where the disease is present. Based on this, in the last 15 years have resurged the interest on the development of a vaccine against bTB that must fulfill all the criteria required to be used in the immunization of cattle.

Requirements must meet a suitable vaccine against bTB

First, an ideal vaccine to bTB must prevent the infection's transmission and establishment, offering whole protection in cattle. Secondly, it must not interfere with the disease's diagnosis and must be cost-effective[39].

Two main parameters support the effectiveness of a vaccine against bovine tuberculosis. Firstly, the level of lessons in pathognomonic organs and tissues directly relates to bacterial load after a challenge with a virulent strain of *M. bovis*. In the second place, the immune response triggered by the vaccine is determined by different subsets of T cells and antibodies[40].

It has been necessary to establish a set of immunological markers, predictors and correlatives of a protective response toward *M. bovis*. Thanks to different studies performed on human and bovine tuberculosis, it has been discovered essential attributes are involved in protective response against virulent strains of the pathogen[41]. A protective

vaccine must lead to a robust innate response, the first line of protection. Specialized antigen-presenting cells (APCs) like dendritic cells (CDs), macrophages, natural killer (NK) cells and gamma delta T (γδ T) cells play a crucial role then for stimulating proper adaptive immunity[42].

If the vaccine can induce a solid initial innate response, it can later trigger an early and potent adaptive immunity mediated by T CD4+ and CD8+ lymphocytes. The adaptive response through T CD4+ lymphocytes it should be Th1 type, characterized by the expression of proinflammatory cytokines such as IFNγ, TNFα, IL12 e IL2[43]. The vaccine must induce high levels of IFNγ produced by memory and effector T cells. In several studies, IFNγ has been associated with a protective immune response against *M. bovis* and its role in activating effector T lymphocytes and macrophages responsible for killing the mycobacteria. On the other side, it has also been described that IFNγ stimulates cytotoxic cells capable of destroying infected cells. An ideal vaccine must also induce a proper memory cell response that can answer faster and competently in reinfection cases[44].

Furthermore, the vaccine should stimulate polyfunctional cells that express the surface markers CD44hi, CD45RO+, and CD62Lo, and have the capacity of simultaneously secrete IFNγ, TNFα, and IL2[45]. On the other hand, it has been demonstrated that cell immune response type Th17 also plays a crucial role in protection against *M. bovis* during the initial establishment of the infection. This Th17 response is specific toward some antigens of the microorganism, and it is mediated by the cytokines IL17, IL21, e IL22. The action of Th17 cells and the respective cytokines have been negatively correlated with the development of the pathology and positively with protection against the disease in cattle[46]. The level of security a vaccine against tuberculosis provides is measured according to bacterial loading associated with the number of colony-forming units (CFU).

Consequently, in vaccinated groups of animals, an ideal vaccine must avoid or reduce the colonization of the microorganism in the different organs. This last means that the vaccine must inhibit the establishment of the infection in comparison with animal control groups that have not been received vaccinated. Figure 1 briefly displayed the main cell type, signaling pathways activated and cytokines pattern that is expected to be involved in the innate and adaptive immune response after vaccination of cattle with a protective vaccine.

The second requirement that a bTB-suitable vaccine candidate must meet is not to sensitize animals to react with the TST, which is used as the main tool for cattle diagnosis. This cross-reactivity is in response to immunogenic proteins of *Mycobacterium bovis*, which are components of bovine PPD (bPPD) of tuberculin skin test. Therefore, when an animal is immunized with a vaccine that shares the same antigens as the TST, as is the case of the live attenuated BCG when TST is performed, the animal will develop a local cellular response in the site of injection. This, hinder differentiating vaccinated from infected animals with a pathogenic strain[47]. One approximation for this problem may consider the development of new protective vaccines that only include some antigens from *M. bovis* that would be absent or in low proportion in the bPPD of the tuberculin. This list is based on the theory that as bPPD is a cocktail of numerous proteins, the immune response of the antigens that are in low amounts can be waned by the others that are in high doses. According to this, different studies have described the composition of the bPPD; however, none provide full knowledge of the protein conformation. A well-defined understanding of bPPD composition can contribute to identifying and selecting suitable candidate proteins for developing a new vaccine. It is important to note that has been observed some discrepancies in terms of protein components of different PPD preparations. Currently, there are different research that has studied the PPD composition. Among them stand out, a study performed by Rberto *et al.* 2017 compared the composition of four other preparations of bPPD from the AN5 strain. One of the bPPD was from Spain, two from Italy and the last one from the Netherlands. Proteins from each bPPD were precipitated by TCA and analyzed through bottom-up proteomics and then by mass spectrometry. In this work, 356 proteins were identified; 85% were found to be shared among the four bPPD preparations. From the total, 198 proteins were detected for the first time; 19 proteins were concordant with previously analyzed bPPD proteomes from the United Kingdom and Brazil, and 78 with bPPD from Korea. In addition, 78 proteins were found in common with the United Kingdom, Brazilian and Korean bPPDs. According to the same research, the most abundant proteins in all bPPD preparations are ten: 1) ESAT-6-like protein EsxB, 2) 6 kDa early secretory antigenic target, 3) Immunogenic protein MPB70, 4) ESAT-6-like protein EsxN, 5) 14 kDa antigen, 6) Meromycolate extension acyl carrier protein, 7)10 kDa chaperonin, 8) GroES protein, 9) Immunogenic protein MPB63, and 10) 50S ribosomal protein L7/L12.

Another research carried out by Borsuk *et al.*, 2009, compared two bovine and two avium PPD preparation from Brazil and UK. This study used the microcolumn reversed-phase liquid chromatography-electrospray ionization tandem mass spectrometry (LC/MS/MS). This method allows the separation of proteins through HPLC and their detection by a mass spectrometer. In total, 171 proteins were discovered among the four bPPD. Among the Brazilian and UK bovine PPD was found 37 common proteins, 11 were unique in Brazilian bPPD and 52 to UK bPPD. On the other hand, 21 proteins were present exclusively in the bovine PPDs but were not identified in the avium PPDs. According to this work, in the Brazilian bPPD it was observed that the most abundant proteins were elongation factor EF-Tu – Rv0685 and Rv0865 (probable molyb- dopterin biosynthesis Mog protein), whereas in the UK bPPDs the most abundant proteins were EsxB protein (ESAT-6 like protein EsxB – Rv3874) and the 10 kDa chaperone protein (Rv3418c).

A second approach to the problem of the cross-reaction between bTB vaccine and TST is the development of differentiating infected from vaccinated animals (DIVA) tests. These assays may replace the tuberculin skin test, and heterologous prime boost strategies with BCG can be used as effective immunization. In order to develop a DIVA test to diagnose bovine tuberculosis in animals, it is necessary first to identify potential protein candidates. The two antigens well-characterized from *M. tuberculosis* and *M. bovis* ESAT-6 and CFP-10 have been tested as putative DIVA candidates. Both proteins are encoded in the RD1 region of the genome of the Mycobacterium, which is absent in BCG strains because of its attenuation process. As the RD1 locus is not in the genome of no BCG, neither ESAT-6 nor CFP-10 would be expressed in the strain. Thus, these antigens should not elicit a cross reactivity when animals are vaccinated with BCG. In fact, in a study, ESTA-6 and CFP-10 have been used as diagnostic proteins that can differentiate infected from vaccinated animals.
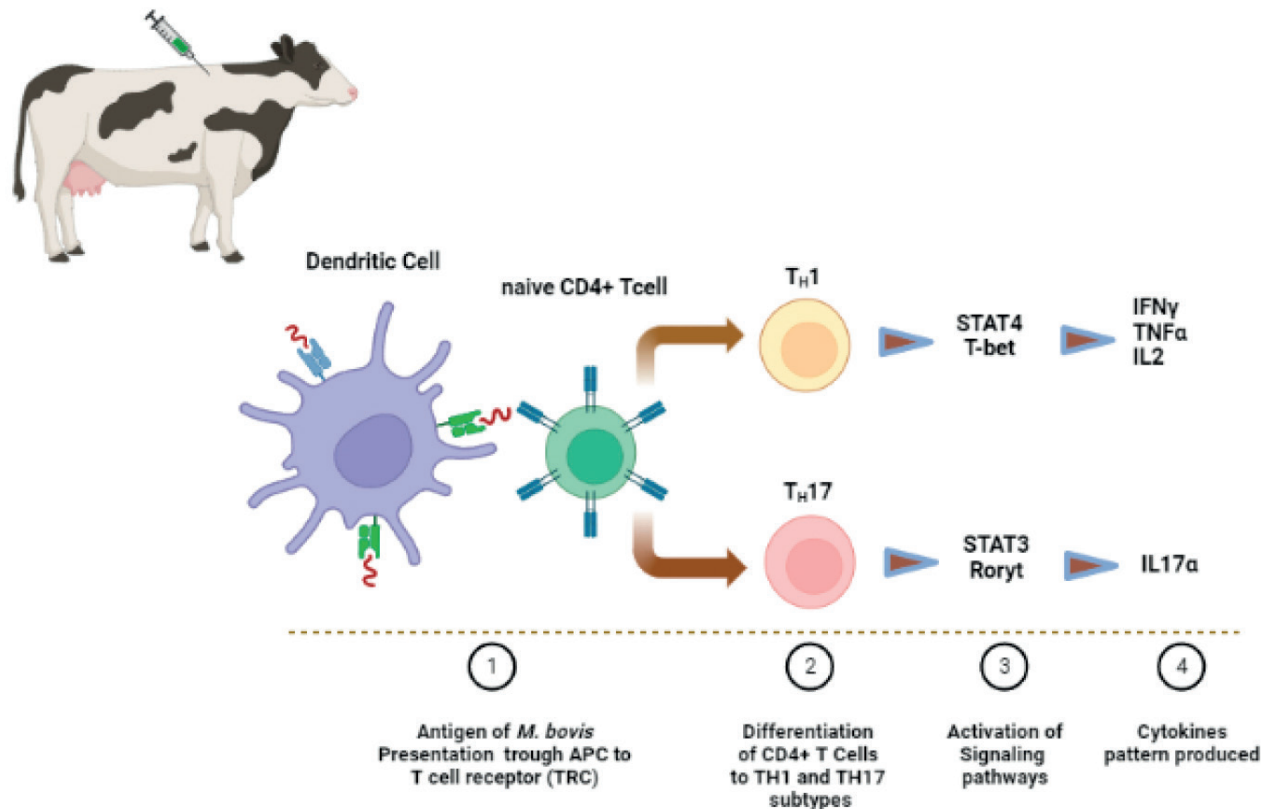
Figure 1. Expected immune response triggered by a protective vaccine against tuberculosis in cattle. The microorganism or antigens of *M. bovis* inoculated through a vaccine are first recognized by phagocytic cells that ingest the pathogen. This is processed inside of specialized vesicles containing different types of enzymes. Then, the antigens are exposed through histocompatibility molecules (MHC) located on the surface of antigen-presenting cells (APC) to naïve lymphocytes. A protective vaccine against *Mycobacterium bovis* should mainly activate CD4+ T cells and prompt its differentiation into T helper Th1 and Th17 subsets. Specific signaling pathways must be activated in both responses, the STAT4 and the STAT3, for the expression of the transcription factor T-bet and Roryt since they are responsible for the proinflammatory cytokines profile related to protective responses that involve INFγ, TNFα, IL2 e IL17, respectively.

On the other hand, the bPPD applied in animals during the same study showed that over 70% of BCG-vaccinated cattle reacted to the TST 50. In another work performed in Chile, researchers from University of Chile implemented and validated an ELISA DIVA assay using the proteins ESAT-6, CFP-10 and Rv3615. Based on this study, it was found that the test was effective in detecting infected animals with M. bovis, yet it is inefficient to test many samples because it is costly and slow[51]. More studies are required to discover new DIVA antigens that can be used in diagnosing bTB. Figure 2 summarizes two potential approaches mentioned in this article to develop a strategy that could allow the use of a compatible vaccine with a diagnosis in bovine tuberculosis.

New strategies of vaccination

Thus far, there is not an approved vaccine by the OIE against bovine tuberculosis. On the contrary, for human tuberculosis caused by *Mycobacterium tuberculosis*, the Bacillus Calmette-Guérin, BCG is allowed, which is a live attenuated vaccine produced from *M. bovis*. BCG is administrated in infants all around the world as an immunization routine for *M. tuberculosis*[52]. Although *M. tuberculosis* and *M. bovis* are closely related regarding genomic homology and disease presentation, BCG can not be used to prevent bTB since it has low effectiveness and interferes with the major surveillance tool, the tuberculin skin test[53].

Because bTB tremendously impacts the livestock industry and public health, in the last decades have emerged high interest in developing new effective vaccines against the disease. Researchers have been studying different type of vaccines in animal models to assess their ability to protect against a challenge with *M. tuberculosis* and *M. bovis*. The approaches in the development of human tuberculosis vaccines could also be applied for bovine tuberculosis as *M. bovis* and *M. tuberculosis* share high genomic homology, and thus, the mechanism of both pathogens in causing the disease is very alike. Therefore, it is important to consider human tuberculosis advances in terms of studying how to face the problem of tuberculosis in cattle caused by *M. bovis*.

Nowadays, the efforts are mostly focused on vaccination strategies that increase BCG efficacy. These involve a heterologous prime boost with BCG as a prime and recombinant BCG (rBCG)[54,55], subunit proteins[56], DNA vaccines[57], or viral vector vaccines[55] as a boost. Next, we described some of these vaccines and those currently under clinical trials (Table 1).

Recombinant BCG includes strains producing Th1 cytokines to augment the efficacy of wild-type BCG. One type of this rBCG is rBCG::IL-2, that shown to enhance the T-cell and antibody immune response in mice after immunization, proving its greater immunomodulatory effects compared with regular BCG[58]. There are rBCG strains producing non-bacterial immunomodulatory proteins, such as rBCG::-Flt3L. The Fms-like tyrosine kinase 3 ligand (Ftl3L) promoted APCs and was over-produced in an rBCG strain. Mice
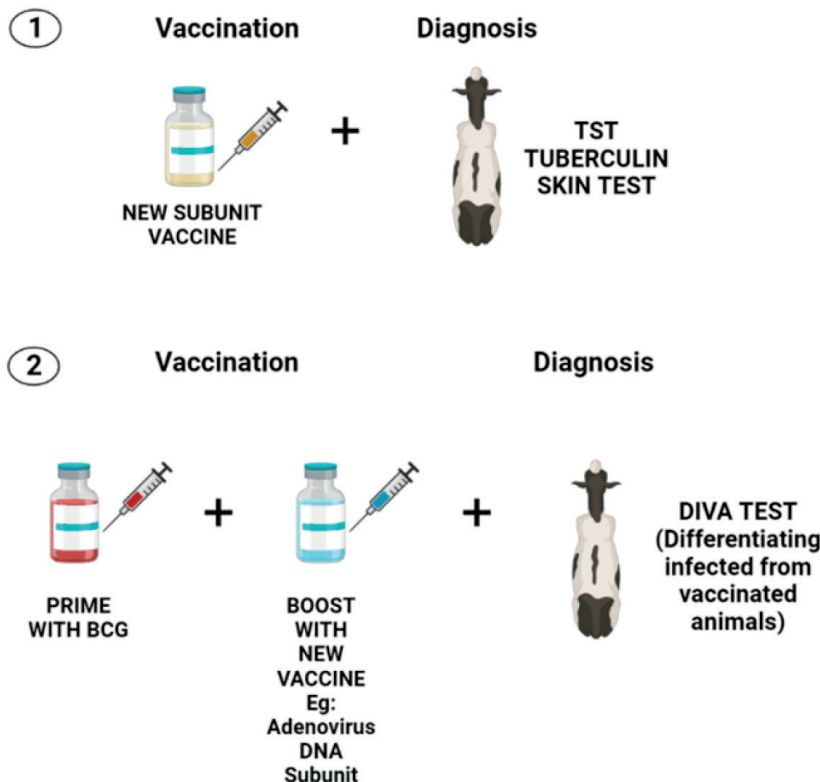
Figure 2. Two potential approaches for consistent vaccination and diagnosis in bovine tuberculosis. The first approach (1) involves the development of a new subunit vaccine that should include the most immunogenic proteins of *M. bovis*. Those proteins must be absent or in a lower proportion in bPPD to diminished cross-reactivity with TST. This first approach seems to be less studied because only a few reports describe the protein composition of bPPD. In addition, it entails the development of a new vaccine that must demonstrate to elicit a better immune response than BCG. The second approach (2) requires a heterologous prime boost using BCG and a new vaccine that must improve the immunity offered by BCG. Furthermore, this vaccination strategy needs the development of a DIVA diagnosis that only includes antigens that are missing in BCG to avoid cross-reactivity with the vaccine.

vaccinated with rBCG::Flt3L exhibited more robust IFN-γ responses in the spleen and draining lymph nodes contrasted to wild-type BCG. The recombinant strain was also safer than the parental strain in immune-deficient mice[59]. Other rBCG are strained, over-producing high immunogenic *M. bovis* proteins like Ag85b. Vaccination with rBCG::Ag85b has shown in mice a lessing of the lessons in lungs compared to immunized groups with the wild-type BCG[54]. Another example of recombinant BCG is the one that lacks of zmp-1gene, associated with hindering phagolysosome fusion in infected macrophages. It was reported that this vaccine induce a more robust T-cell response characterized by a rise in the secretion of IFNγ compared with the wild-type BCG[60].

Several protein subunit vaccines have been tested alone or co-administrated with BCG in animals under experimental conditions. These vaccines have demonstrated its capacity to induce a protective response by itself or boost BCG immunity against virulent *M. bovis* and *M. tuberculosis* challenge. In a study, culture filtrate proteins or CFP from M. bovis in combination with bovine IL-2 were used to immunize calves of 6 months old to assesses the effectiveness of the vaccine for stimulating a protective immune response against an intratracheal challenge. The results showed that the vaccine prompted a strong cellular and antibody response significantly reducing the degree of lung lesions in comparison with the control group[61].

In another investigation, a protein subunit vaccine known as H107 that included antigens from *M. tuberculosis* (PPE68, ESAT-6, EspI, EspC, and EspA, MPT64, MPT70, and MPT83) was tried. The analysis performed with the vaccine in a murine model disclosed that all chosen antigens induced substantial protection except for MPT64. Protein ESAT-6 was the most protective antigen in mice, followed by EspI, EspA, and EspC. Furthermore, it was found that combining BCG with H107 increases the immunity triggered by BCG alone, characterized by an expansion of the CD4 T cells and a Th17 cell response[62]. Recently, a group

of researchers have been developing a new subunit vaccine based on highly antigenic fusion proteins from *M. bovis* and *M. tuberculosis*. This is H65, composed of six ESX-secreted antigens EsxD, EsxC, ExsG, EsxH, ExsW and EsxV formulated in CAF01 liposomal adjuvant. According to this, it was shown that in the *M. bovis* mouse model of infection the administration of BCG plus H65 was much more protective than H65 alone[63]. In addition to protein subunit vaccines, DNA vaccines have been developed that encode specific antigens of M. bovis. An example is a DNA-E6 that encodes the protein ESAT-6, which is absent in BCG strains. Researchers immunized groups of BALB/c mice with DNA-E6 alone and co-administrated with BCG, and afterward, the animals were challenged with low-dose virulent M. *tuberculosis* H37Rv. This study found that the administration of DNA-E6 and BCG induced more secretion of IFNγ and less pathological changes in lung and spleen than the DNA-E6 alone[64]. In another study, a DNA vaccine that encodes for three mycobacterial antigens Rv3407, Ag85A, and HspX was tested in a murine model. The vaccine triggered an antigen-specific cellular and humoral immune response as well as conferred protection against a challenge performed with *M. Tuberculosis* aerosol[65].

Moreover, adenoviruses vaccines boosting BCG have been tried. Among these is MVA85A, which corresponds to a modified vaccine from a recombinant attenuated Ankara virus engineered to express Ag85A from *M. tuberculosis*. Individuals immunized with a prime-boost BCG- MVA85A showed high antigen 85A-specific CD8+ T cells after being boosted with MVA85A[66]. Another adenovirus vaccine used to boost BCG is Ad85A, an attenuated recombinant vaccine based on the human adenovirus type 5 (AdHu5), which was also developed to express Ag85A. It was found that Ad85A stimulates polyfunctional CD4+ and CD8+ T cell immunity in previously BCG-vaccinated individuals[67].

Viral Vector	Subunit Recombinant	Whole Cell/Extract	Attenuated/Live Recombinant
MVA85A **Composition:** Vaccinia Virus Ankara (MVA) expressing antigen 85 A (MVA85A). **Use:** Preventive **Clinical Trial phase:** I **Sponsoring:** University of Oxford.	**H56:IC31** **Composition:** Fusion protein antigens Ag85B, ESAT-6 and Rv2660c formulated in IC31. **Use:** Preventive **Clinical Trial phase:** IIb **Sponsoring:** Aeras.	**Vaccae™** **Composition:** Whole cell from *M. vaccae*. **Use:** Therapeutic **Clinical Trial phase:** III **Sponsoring:** AnHui Longcom.	**MTBVAC** **Composition:** Live attenuated *M. tuberculosis* from a human isolate MT103. **Use:** Preventive **Clinical Trial phase:** IIa **Sponsoring:** Biofabri,SL; University of Saragoza; Centre Hospitalier Universitaire Vaudois; TuBerculosis Vaccine Initiative.
Ad5Ag85A **Composition:** Adenovirus serotype 5 vaccine vector expressing antigen 85A. **Use:** Preventive **Clinical Trial phase:** I **Sponsoring:** McMaster University, CanSino.	**H4:IC31** **Composition:** Fusion protein antigens Ag85B and TB10.4 formulated in IC31. **Use:** Preventive **Clinical Trial phase:** IIa **Sponsoring:** HIV Vaccines Trials Network; Sanofi Pasteur; Statens Serum Institute.	**RUTI®** **Composition:** Whole cell extract from *M. tuberculosis* delivered in liposomes. **Use:** Therapeutic **Clinical Trial phase:** III **Sponsoring:** Archivel Farma, SL.	**VPM1002** **Composition:** Live recombinant BCG that uses the Lysteriolysin O (LLO)- encoding gene from *Listeria. monocytogenes* (Hly) to replace the urease C encoding gene (UreC). **Use:** Preventive **Clinical Trial phase:** III **Sponsoring:** University Health Network, Toronto; Serum Institute of India Pvt. Ltd; Max Planck Institute for Infection Biology; Verity Pharmaceutycals.
TB/FLU-04L **Composition:** Influenza virus mucosal vector expressing antigens Ag85A and ESAT-6 **Use:** Preventive **Clinical Trial phase:** IIa **Sponsoring:** Research Institute for Biological Safety Problems.	**M72+AS01E** **Composition:** Fusion protein antigens MTB32A and MTB39A formulated in AS01E. **Use:** Preventive **Clinical Trial phase:** IIb **Sponsoring:** GlaxoSmithKline; Aeras.	**DAR-901** **Composition:** Whole-cell, heated-killed NTM vaccine. **Use:** Preventive **Clinical Trial phase:** IIb **Sponsoring:** Darmouth University; Aeras.	
	ID93+GLA-SE **Composition:** Fusion protein antigens Rv2608, Rv3619, Rv3620 and Rv1813 formulated in GLA-SE. **Use:** Preventive **Clinical Trial Phase:** IIa **Sponsoring:** Quratis Inc; IDRI.		

Table 1. Shows several vaccine candidates currently under clinical trials and were classified according to its composition. The information for all these vaccines was retrieved from the following website https://clinicaltrials.gov/.

Conclusions

This article briefly describes the enormous damage caused by *Mycobacterium bovis* in the livestock industry and gives an approach to its possible solutions. According to the studies reviewed, all of them agree that without an effective vaccine and a compatible diagnosis, no program for eradication would be victorious against tuberculosis in cattle. In these terms, based on the scientific information gathered, two approaches could offer a true solution for bovine tuberculosis. The first one is the development of a new protective vaccine that precludes in the near future the use of BCG in animals, so it may be used with TST. This vaccine should comprise some of the most immunogenic proteins from M. bovis to protect against de pathogens.

Moreover, those selected proteins should be less representative in bPPD of tuberculin, hence may not cause a cross-reaction with the diagnosis. However, we noticed that only few studies describe the protein composition of bPPD. For this purpose, it is essential a deep knowledge of all protein components of bPPD. The second one is the replacement of TST with a DIVA test which also involves a strategy of vaccination that allows to the improvement of the effectiveness of BCG. Then finally, BCG would be used in cattle. The DIVA tests consider *M. bovis* proteins that are not expressed in BCG (e.g.: ESAT6/CFP10), so unlikely could occur a cross-reaction between the vaccine and the surveillance tool.

On the other hand, BCG enhancement consists of a prime-boost where the immune system is first primed with a dose of BCG, and later it is boosted with a subunit vaccine. This last approach seems to be more explored than the first one because more studies about it are available, and it was easier to incorporate them in our review. In brief, even though there is a lot of evidence in the literature that prime-boost with BCG and other types of vaccines gives better results in terms of protection than administrating BCG by itself, it needs to be compatible with an efficient diagnosis of the disease. Therefore, a definitive solution for tuberculosis in cattle would not be accomplished in the short term.

Conflicts of Interest

The authors declare no conflict of interest.

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