Veterinary Medicine

Chapter 1

Bovine Paratuberculosis

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1. General Information: Pathogen and Disease Characteristics

Johne's disease or paratuberculosis (PTB) is an intestinal infection caused by *Mycobacterium avium subsp. paratuberculosis* (MAP) that affects wild and domestic ruminant species, including sheep, goats, deer, camelids and primates [1]. The clinical manifestation of PTB may include diarrhea, decreased milk production, edema, infertility and weight loss and the control of the disease requires animal culling to prevent disease transmission [2].

In addition, MAP is associated with Crohn's disease (CD) in humans, which is an autoimmune disease related to chronic intestinal depletion in humans suggesting a zoonotic relevance [3].

MAP is a Gram-positive acid-fast bacterium from the *Mycobacterium avium* complex (MAC) [4,5]. In addition, MAC is a group of related species of non-tuberculous mycobacteria with members that are the most isolated species in human infections, such as lung and dermal diseases as well as soft tissue and systemic infections [6].

MAP, which has a slow reproduction rate (between 22 and 26 h), is an obligate intracellular pathogen characterized by lack of mycobactin (an iron chelating compound) and subsequently needs a constant source of iron [7]. As a Gram-positive, acid-fast bacillus, MAP has a cell wall consisting of mycolates as well as slayers of peptidoglycans and arabinogalactans [10]. According to the environmental conditions, the MAP cell wall can be conditioned as a vegetative dormant state or as a spore-like form [9]. This cell wall confers the ability to survive outside the host in environments such as feces and animal food products [10]. In addition, MAP can

modify its phenotype depending on the stage of the disease and, therefore, depending on the environment and the host cells it infect [11]. This environmental adaptation demonstrates an amazing genetic plasticity. The pathogen entries into the systemic circulation of the ruminant through the mucosa and in this layer remains as asymptomatic; afterwards, it enters into the macrophages [1].

The relationship between MAP and the environment depends on multiple factors such as physical characteristics and spatial location as well as competition with other microorganisms [12]. The pathogen is eliminated through the feces of infected cows in clinical and subclinical stages, and can survive outside the host for 12 to 120 weeks in water and soil [13].

Like for other infectious diseases, the structure and management of herd housing is important to the transmission of this pathogen. Calf-to-calf transmission occurs by contact with contaminated surfaces, whereas adult-to-calf transmission depends on exposure of calves to feces on housing fomites [14]. Another possible form of transmission is by aerosols, as supported by MAP isolations from air samples and water droplets [15]. These aerosols can travel long distances and deposit in soil and water and in this way, if contaminated, the pathogen may enter into grazing animals via the digestive or inhalation paths [16]. Another form of environmental persistence of mycobacteria is by forming pellicle biofilms, which can stick and grow on different surfaces allowing the bacteria to maintain their infective capacity [17].

The economic impact of paratuberculosis is associated with the high prevalence of this disease in Latin America, different European countries and Australia and depends on herd size, infection rate, bacterial excretion, occurrence of clinical manifestations and cases of clinical disease [18].

As was mentioned before, PTB infected animals presents long subclinic periods [20]; which makes culling and replacement less effective in controlling the disease. Actually, clinical cases occurred, and are detectable, in the final stages of the disease. However, the estimated global economic losses because of endemic cases of PTB exceed US$ 1.5 billion annually [19] and this number increases together with the prevalence of infected animals [20].

The reduction in milk yield is 15% and 19.5% in subclinically and clinically infected animals, respectively [21]. The estimated cost caused by PTB is usually lower than that caused by mastitis in dairy production. However, this is associated with a lack of monitoring of economic costs, because this disease does not represent a financial concern of equal magnitude for producers [22].

Because of the zoonotic potential of MAP and its importance in Public as well as Animal Health, the control of PTB requires prompt improvement. Herd management programs consist

of the diagnosis and subsequent elimination of positive animals. This has been partially effective, owing to the low sensitivity of the available diagnostic tests to detect positive animals in the early stages of the disease [23]. Active surveillance, farming education programs and improved diagnostic tests, along with epidemiology, are critical areas to promote.

The difficulty of controlling PTB worldwide exposes the urgent need to improve diagnostic methods, as well as to find new effective vaccines against this disease. Vaccines applied in conjunction with diagnosis and management would contribute to the success of control and eradication programs.

Although the World Organization for Animal Health (OIE) does not offer any guidelines for controlling paratuberculosis [18], recommendations for livestock trade created by the International Association for Paratuberculosis can be found in combination with the Sanitary and Phytosanitary WTO agreement. In addition, the European Union Parliament implemented a law that will be in use from 2021 in all member countries of the European Union with specific rules for each disease, which includes paratuberculosis [24].

2. Pathogenesis and Immune Response of Paratuberculosis

PTB may be described as an iceberg phenomenon according to mathematical models **(Figure 1)** [4]. The base of the iceberg is a silent stage, i. e. the pathogen is undetectable, in young calves and heifers. The second phase is the subclinical infection in adults and, in this case, the pathogen is typically detectable by antibody tests. The next stage is the clinical manifestation of the disease, with clinical signs such as diarrhea, decreased milk production, weight lossand and histopathological lesions. The last part of the iceberg is the advanced-stage disease, which is associated with disseminated infection, edema, infertility, severe dehydration, cachexia and death [25].

A triad of factors determines the infectious disease (paratuberculosis, PTB): the agent (MAP), the host (mainly, cow) and the environment (farm). The interplay between the host and pathogen, which involves host defense mechanisms and the efficiency of the pathogen to evade the host immune response, is pivotal to the resulting infectious disease [7].

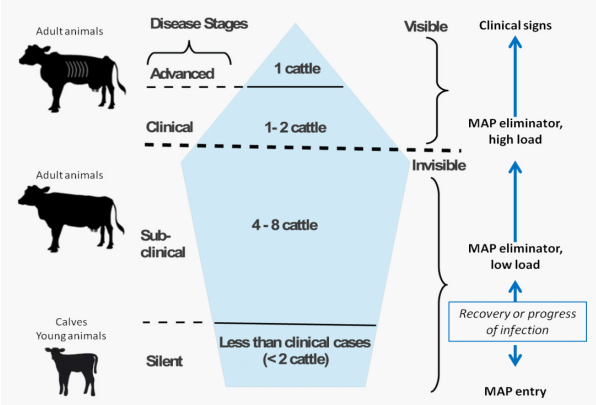

Figure 1: Iceberg phenomenon of Johne's Disease, adapted from Magombedze et al., 2013 (25).

Although newborn calves may be infected, paratuberculosis infection occurs primary from the first months of life and the likelihood of infection decreases with age. MAP is mainly spread horizontally through the fecal-oral route, but also through milk or colostrum to newborn calves and vertically in the uterus. [26–28] **(Figure 2)**. Indeed, even maternal MAP antibodies given to calves through the colostrum may enhance uptake by means of opsonization of the microorganisms [1]. MAP enters through the lymphoid tissue of the small intestine. The bacterium is first opsonized by fibronectin in the intestinal lumen and then binds to α5β1 integrins, which are mainly present in M cells (Peyer's plaques)In addition, MAP can weaken the junctions between epithelial cells that provide a mechanical barrier to the entry of infectious agents [29,30] and even enter by endocytosis [31] hematogenous route [32].

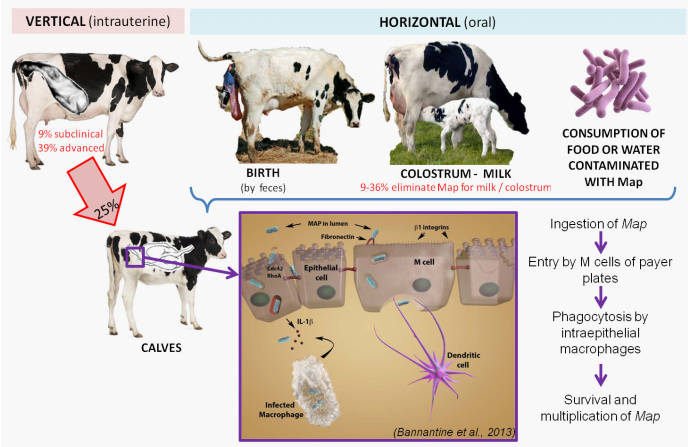

Figure 2: Transmission routes of PTB and entry through intestinal epithelium (adapted from Bannantine et al., 2013)

After crossing the intestinal epithelium, the bacterium is phagocytosed by submucosal macrophages and dentritic cells, where it replicates and persists **(Figure 2)**. MAP wall glycolipids such as lipoarabinomannans (LAM), phosphatidylinositolmannose and lipoproteins, which are pathogen-associated molecular patterns (PAMPs) in MAP, are recognized by cellular Toll-like receptors (TLRs), mainly TLR1, TLR2 and TLR4. TLR2/TLR6 heterodimers are engaged with the recognition of mycobacterial antigens. In addition, endosomal TLR9 recognizes dinucleotides (CpG) of bacterial DNA (33,34). The TLR2 receptor is one of the most important receptors since it induces macrophage activation. Some polymorphisms of this receptor in cattle are associated with increased susceptibility to MAP infection [35], the same is true for TLR4 [35–40]. The mannose receptor (C-type lecithin-like receptor) mainly recognizes LAM with mannose residue (Man-LAM) and trehalosedimycolate, which are present in pathogenic mycobacteria [41,42]. C3b of the complement system acts as an opsonin and interacts with CR1, CR3 and CR4 receptors on immune system cells, and is important for macrophage invasion [43].

Several bacilli degrade within the phagosome and stimulate CD4+ immune responses through antigen presentation via the MHC class II pathway. Alternatively, through inhibition of common destruction mechanisms, MAP can evade lysis within the phagosome, and proliferate. Secreted MAP proteins can pass from the phagosome into the cytosol and subsequently become

available for presentation to LTCD8+ through the MHC class I pathway or the non-classical CD1b pathway [44–46]. Survival and proliferation in macrophages are important virulence factors for PTB. NRAMP1 expression in cattle is associated with infection control, as it limits iron entry (essential for MAP multiplication) and stimulates the enzyme inducible nitric oxide synthase, which increases nitric oxide production [47].

These bacteria can inhibit phagosome maturation [48] and IL-12 production, which is important for Th1 and cytotoxic responses [49]. MAP can also suppress macrophage activation and enhance IL-10 and TGFb production [50]. On the other hand, MAP can inhibit the presentation of antigens to T lymphocytes by decreasing the expression of antigens of the major histocompatibility complex type II (MHC-II) and LFA-1 integrins, which could be related to the persistence of the infection [51]. Furthermore, the bacterium can inhibit macrophage apoptosis and thus maintain its proliferation niche and avoid stimulation of the adaptive immune response [52–54].

PTB subclinical stage may progress to clinical stage through macrophage polarization from a M1 pro-inflammatory phenotype, which actively participate in host defense, to a M2 phenotype that regulates the response to help control inflammation [55].

The adaptive immune response does not participate independently of the innate response, but rather both responses act together against infection. Dendritic cells (DCs) are involved in the immune response against mycobacterial infections and play a fundamental role as a key link between the innate and adaptive immune response. Indeed, upon activation they transmigrate to the mesenteric lymph nodes, where they process and present MAP antigens to virgin T cells; that interaction induces T cell polarization into various T cell subsets, including activation of γδ T cells [56]. Polarization into T cell phenotypes varies according to the type of cytokine released by different types of immune cells that are specifically activated. These mechanisms are critical at this stage to develop an effective immune response against MAP. However, MAP can interfere with these mechanisms.

CD4+ T cells are essential for disease control, especially the T-helper 1 (Th1) subclass. These cells produce cytokines such as interferon gamma (IFNγ) that promote antimicrobial activity in the macrophage by increasing its phagocytic capacity as well as mycobacterial degradation. In turn, the response stimulates the recruitment of blood monocytes and therefore increases the number of phagocytes at the site of infection [57].

Epithelioid cells (which are activated macrophages) have increased secretory capacity and, participate in the inflammatory response by releasing cytokines and other regulatory molecules (TNFa, IL-1, TRAF-1, IL-6, IL-8, IL-12). Another consequence of macrophage activation is the formation of Langhans-type multinucleated giant cells from the fusion of several macrophages. These have high phagocytic power, and degrade the ingested material

with high efficiency [58]. As a consequence of the developed Th1 profile, numerous lymphocytes accompany the macrophages in the formation of the granuloma, among which are the aforementioned CD4+, together with CD8+ T lymphocytes and Tγδ lymphocytes. Particularly, Tγδ lymphocytes are a subtype of CD4- and CD8- lymphocyte with cytotoxic effect that are important in the control of infection at the local level [59].

The development of this Th1 response is related to the release of the proinflammatory cytokines TNFa, IL-1, IL-6, IL-2 as well as other lymphokines released by Th lymphocytes and other cells related to the response against the pathogen. Another important cytokine is IL-8, which is chemotactic and therefore attracts cells to the site of infection. All these cytokines, together with IFNγ (which is secreted mainly by TCD4+ of the Th1 profile), collaborate in the elimination of the microorganism [60,61].

LTγδ also seem to contribute in the innate immune response during an infections by agents circulating in the intestine of young ruminants, as is the case of MAP [62]. Specifically, the γδ WC1+ subtype can recognize antigens without being bound to MHC II and therefore play a very important role in the early stages of infection [63]. These cells, together with CD8+ LTs, are present in higher proportion in the intestine and associate with lymph nodes of animals in the early stages of PTB [64].

A third type of CD4+ T cells, related to PTB infection but independent of Th1 and Th2, are the so-called Th17 cells. These are a type of lymphocytes involved in host protective immunity against intracellular pathogens that, produce cytokines such as IL-17a, IL-17F, IL-23 and IL-22 [65,66].

Regarding the natural killer (NK) cytotoxic population, although these cells are mainly present in young animals, their role in infection and protection against PTB is not yet clear. NK appear early in lesions, blood circulation as well as milk of MAP-infected cattle, can produce IFNγ and may be a marker of early infection [62,67,68]. These cells in turn have been associated with protection against mycobacterial infection [69].

Finally, the subpopulation of T cells with immunosuppressive functions called regulatory T cells (Treg) also participate in the immunological response against PTB. These cells express the surface markers CD4+ and CD25+, in addition to the transcription factor "forkead box P3" (FoxP3) [70]. The CD4+ cells that after exposure to antigens can differentiate to Treg and secrete IL-10 are called Tr1 cells (mainly in peripheral blood), and if they secrete TGFb they become Th3 cells.[71–73]. **(Figure 3)** summarizes some of the main immune cells and their function in the different stages of the disease.

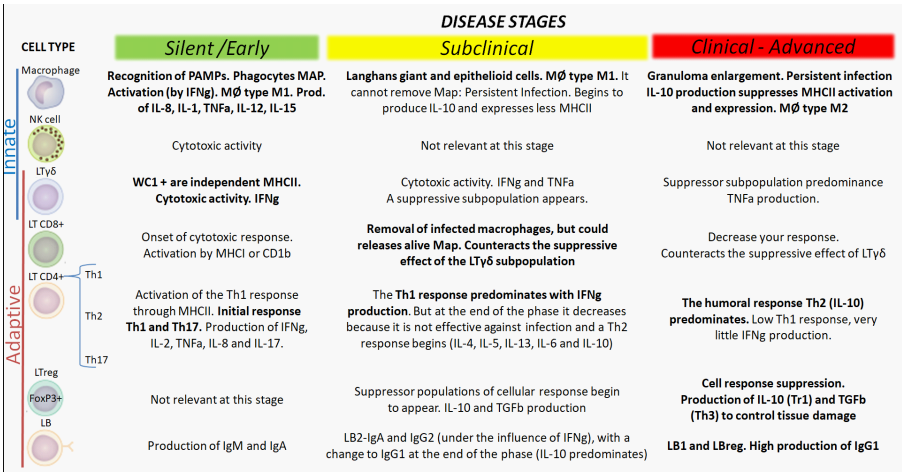

Figure 3: Immune cells and their function in the different stages of the disease.

Although the production of proinflammatory cytokines is necessary to control the initial PTB infection, their excessive or too prolonged secretion would be locally detrimental owing to the likelihood of tissue damage [74,75]. This is why Th2 immunity plays a crucial role in controlling excessive Th1 responses, mainly through IL-4 and IL-10. Both cytokines have an immunosuppressive effect against IFNγ. Without stimulation by IFNγ, macrophages revert to the M2 phenotype, unable to contain the invasion of the bacilli. That alteration of the host immune response itself would be detrimental and would allow the spread of mycobacteria in towards advanced stages. This inhibition of a Th1 cell-mediated immunity and the shift to a Th2 cell-mediated humoral immunity is related to the clinical stage of PTB and to a differentiation of B lymphocytes to produce IgG isotype 1 antibodies [60,75].

3. Paratuberculosis Vaccines

Members of the Johne's Disease Integrated Program (JDIP) have concluded that the best way of evaluating and developing paratuberculosis vaccines are a three-phase evaluation strategy: evaluation in an in-vitro macrophage model, followed by virulence and protection assays in the murine model, to finally evaluate in the native host (bovine, ovine, caprine) [76,77] **(Figure 4)**.

The first report of vaccination against MAP consisted of a live non-virulent strain of MAP adjuvanted in a mix of olive oil, liquid paraffin and pumice powder (Vallée and Rinjard, 1926). Today, several types of vaccines are under development: inactivated (killed whole-cell bacteria) or live-attenuated, DNA or viral vectored, recombinant protein-based and subunit vaccines(78). However, the commercial MAP vaccines currently available are based on heat-inactivated MAP formulated with an oil adjuvant **(Table 1)**. These commercial vaccines are applied in young animals (4-16 weeks of age), preferably before the first month of age. Actually, vaccination does not prevent the infection of animals [79,80], but reduces the number of clinically and subclinically infected animals [81]. Thus, there is a reduction in the presentation of clinical signs and in the excretion of mycobacteria in feces [23]. On the other hand, the

vaccine could serve as a "treatment", because in some cases it can reverse the disease process, with animals returning to a healthy state and normal milk production; it may even decree as the lesions caused by other mycobacteria such as *M. bovis* or *M. caprae* [81–84].Thus, vaccination has provided satisfactory results in relation to the maintenance of production and reduction of economic losses attributable to the disease.

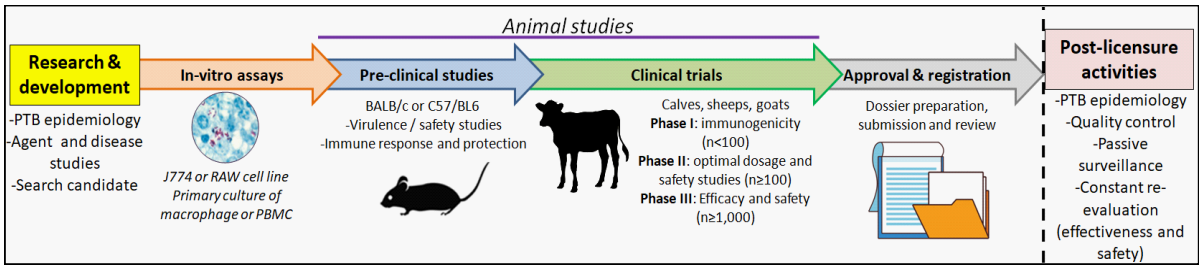

Figure 4: Stages in PTB vaccine development (adapted from [85]).

Sheep vaccination has been an important strategy to significantly reduce PTB prevalence in Spain, Australian and Icelandic. In other countries such as Greece, The Netherlands, New Zealand, UK and India MAP, vaccination is also implementedfor small ruminants [18].

The control of PTB in cattle, however, usually do not include vaccination with the available vaccine, because of possible interference with bovine tuberculosis diagnosis [18]. This problem can be solved with the use of the Comparative Cervical Tuberculin Test (CCTT) [86,87]. In addition, some studies suggest doing the first Simple Cervical Tuberculin Test (SCTT) one year after PTB vaccination, since interference tends to decrease over time [87–90]. Another possibility would be to use other antigens for the intradermal reaction, which have a greater specificity in the diagnosis of bovine tuberculosis [83,91]. Moreover, it is important to mention that MAP infection causes more false positives to SCTT (8.3-26.8%) than vaccination itself (6.55%) [88]. Actually, this response can also be reduced with the use of CCTT (0.15% of false positives in vaccinated animals). On the other hand, the sensitivity of the diagnostic techniques for bovine tuberculosis (bTB) is reduced in animals infected with *M. bovis* and co-infected with MAP. In these co-infected animals, the occurrence of false negatives increases, thus making the eradication of bTB more difficult [92–98]

The indirect costs of vaccination would be low, since vaccination itself would reduce MAP prevalence as well as false positives in bTB-free herds [99] and false negatives in bTB-herds. However, further research is required to determine the effect of PTB vaccination and co-infection on the diagnosis of bovine tuberculosis.

Research efforts have focused on the development of a more efficient vaccine and DIVA diagnostics in order to differentiate infected from vaccinated animals. An effective response against MAP would require a vaccine that generates a cellular immune response. This is because this vaccination, among other things, would guarantee the participation of activated macrophages capable of destroying the bacillus at the focus of infection [100]. Ideally, the

vaccines hould produce a Th1/Th17 response with production of IFNγ, TNFα, IL-2 IL-17, IL-22 and IL-27 as well as cytokines related to protection against mycobacterial infection [101–103]. In turn, it should induce an effector memory T response (Tem), together with a central memory T response (Tcm), including both CD4+ and CD8+ [104].

Inactivated vaccines are safe, but compromise the microbial antigens integrity, and possibly its antigenicity, due to the heat-inactivation and, furthermore, usually require the addition of an adjuvant. This kind of vaccine primarily develops a humoral immune response generally accompanied by a cellular response [105]. Live attenuated vaccines, on the other hand, generate a greater cellular response, but may face the risk of possible residual virulence or reversal pathogenicity, with unknown consequences if ingested by non-target species, even humans that consume meat from that vaccinated animals [85]. Tables 1 and 2 summarize the different types of vaccine.

Table 1: Summary of commercial and experimental killed whole-cell- based vaccines against paratuberculosis.

Killed whole-cell-based vaccines	Strain	Description	Reference
Mycopar (BoehringerIngelheim Corp.)	Strain 18 of *Mycobacterium avium* subsp. *Avium*	Oil adjuvant. Used in cattle(USA) Combined with rIL-12 failed to improve protection	
Gudair (CZ Veterinaria)	Attenuated 316F Weybridge MAP strain	Oil adjuvant. Used in small ruminants (Australia and Europe)	[80,106,107]
Silirum (CZ Veterinaria)		Oil adjuvant (W/O/W) Used mainly in cattle, but also in small ruminants (New Zealand, Australia)	[82,108,109]
Bio-JD Oil & Gel (Biovet Pvt. Ltd.)	Native MAP strain 'S 5' 'Indian Bison type'	Aluminium hydroxide gel and oil adjuvant. Used mainly in small ruminants, but also in cattleand buffaloes. (India)	[110–113]
ParaVax -6611 strain (experimental)	Argentinian virulent MAP strain C-type	Oil adjuvant. Used in cattle. (Argentina) Comparisons with the Silirum vaccine in the murine and bovine model revealed better results, with humoral an cellular immune responses. Current evaluations are being performedin a dairy herd with high PTB prevalence.	[114]
E	5889 Bergey MAP strain	Oil adjuvant. Used in cattle (Hungary). Partial protection with a predominant humoral response.	[23,115,116]
Leystad (experimental)	*ID-Leystad MAP strain*	Oil adjuvant.	[117,118]
CWD-QS21 (experimental)	*Cell wall deficient inactivated MAP strains (spheroplasts)*	QS21 adjuvant. These vaccines failed to protect vaccinated and challenged goats.	[119]

Table 2: Summary of commercial and experimental live-attenuated-based vaccines against paratuberculosis.

Live-attenuated-based vaccines	Strain	Description	Reference
Neoparasec (Merial Corp.):	316F MAP strain	Oil adjuvant. Used in cattle and sheep (France, until 2002). Similar protection to inactivated vaccines, with cellular and humoral immune responses.	[120–123]
AquaVax MAP (experimental)		Aqueous solution. Used in sheep. (New Zealand). Similarprotectiontothat of Neoparasec.	[122]
Paratuberkulosevaksine – OSLO (experimental)	Two British strains of MAP: 316F and 2E.	In conjuction with an adjuvant consisting of a mix of olive oil, liquid paraffin and pumice powder. Used in goats. (Norway)	[124,125]
Mutants K-10 MAP strains *(experimental)*	*WAg906 (MAP1566)* *WAg913 (MAP0053c)* *WAg915 (ppiA)*	Used in a mice model. These vaccines failed to protect against the challenge	[126]
	Five strains obtained by mutagenesis with transposons	Used in a mice model and goats. Similar response to that of the commercial Silirum vaccine. None of the mutant strains managed to protect the goats oral vaccinated.	[76,127]
	*ΔrelA** *ΔpknG*	Only the *ΔrelA* was selected as a vaccine candidate. Used in cattle and goats. CD4 + and CD8 + cellular immune response.	[128–130]
	*ΔleuD** *Δmpt64* *ΔsecA2*	Only the *ΔleuD* was selected as a vaccine candidate in a murine model and goats. These vaccines improved the protection in relation to the Mycopar vaccine.	[131,132]
	*ΔsigH** *ΔsigL*	*ΔsigH* showedbetter proteccion than that of the Mycopar vaccine in goats. *ΔsigL* displayed significant protection in mice. No available data regarding comparisons with commercial vaccines.	[133–135]
	*ΔumaA1** *ΔimpA* *ΔfabG2*	*ΔumaA1* was selected as a vaccine candidate in murine model. Th1/Th17 response. No comparisons were made with the commercial vaccine.	[136,137]
	ΔlipN	Used in cattle with or without QuilA adjuvant. These vaccines displayed slightly better results than Mycopar	[138]

On the other hand, the recombinant-protein subunit vaccines, with the proper potent adjuvant, are designed to induce mainly a Th1 cell type response with the production of IFN□. These vaccines are safe and would avoid the misdiagnosis of false reactors. They can be applied with adjuvants (such as dimethyldioctadecylammonium (DDA) [139] or Monophosphoryl Lipid A (MPLA), combined or not with IL-12) that exacerbate this response [140]. These vaccines, however, activate a restricted number of T-cell clones and consequently require the addition of an adjuvant to obtain activation of an extended T-cell repertoire [141]. Nevertheless, vaccines

elaborated with subunits have a lower degree of protection [139,142]. Delivery of expressed MAP antigens through attenuated strains like *Salmonella* and *Lactobacillus salivarius* is an alternative way to stimulate protective mucosal immune responses [45,132,143].

The selected antigens for heterologous vaccines include Ag85 complex (A, B and C), superoxide dismutase (SOD), heat shock proteins (Hsp70), PPE/PEE family (MAP1518 and MAP3184), lipoprotein P22 (LprG), polyprotein 74F and Alkyl-hydroperoxide reductase (AhpC and AhpD), among others. Most immune response studies and some cases of protection assays with these vaccines displayed results similar to those of the live attenuated or inactivated vaccines, but without the interference with bTB diagnosis[139,140,142,144–154].

DNA subunit vaccines consist of genes encoding antigens cloned into mammalian expression vectors (plasmids) that elicit a response modulated by CD4+ and CD8+ lymphocytes and generate protective immunity in animal models. Researchers have evaluated this strategy in sheep, cattle and in the murine model with partial protection to PTB infection. Plasmids expressing MAP immunogenic proteins such as Ag85 complex, Hsp65, SOD, MAP0586c, MAP2121c, Ag 16.8 kDa, among others, were used to evaluate this strategy [155–160].

Among the viral vector vaccines are human adenovirus serotype 5 (Ad5), deficient simian adenovirus-vectored vaccine (ChAdOx2) and modified vaccinia Ankara virus (MVA), among others. The advantages of these vaccines are that they provide high antigen delivery to antigen presenting cells [68,161], induce an increased antigen-specific response to antigens by CD4+ and CD8+ T cells [162,163] and maintain IFN-γ release with a consequent increased macrophage activation in order to increase the ability to eliminate the bacteria. For instance, *Bull et al. (2014)* used a prime-boost strategy with Ad5-MVA expressing a pool of MAP antigens in mice and cattle. The vaccination produced a reduction in lesions and decreased CFU counts in tissues after the challenge; no adverse effects were detected upon vaccination [164]. Recently, in a study on Crohn`s Disease, *Folegatti et al.* (2019) used ChAdOx2 expressing 4 genes of MAP and demonstrated that the vaccine was immunogenic and safe for humans [161].

4. Diagnosis of Paratuberculosis

The diagnosis of PTB is done by different diagnostic tests for the detection of MAP in clinical samples or evaluation of the immune response of the host against the bacterium. The assays to detect the pathogen include the polymerase chain reaction (PCR), bacterium selection and identification in cultures and Ziehl-Neelsen (ZN) staining. The faecal culture is the gold diagnosis of PTB but because of the slow growth rate of MAP (20 h generation time), the detection of visible colonies takes a long time. Another disadvantage of this diagnostic test is the contamination normally encountered when using faecal samples [13,121,165]. An alternative direct MAP detection technique is ZN staining. Although this assay is fast and inexpensive, it lacks specificity, as it detects all acid-fast bacteria. In addition, ZN staining

has low sensitivity, since it requires between 5,000 and 10,000 acid-fast bacilli(AFB) / mLto obtain a positive result [166]. By contrast, although PCR diagnosis is specific, it is not a common practice in veterinary laboratories [13].

Finally, the tests based on the immune response of the host are easy to perform and adapt and, furthermore, are routine procedures in all diagnostic laboratories. Particularly serological tests, and among these the ELISA, are low cost, fast and easy to perform [86,160]. However, adequate antigens are essential to develop a sensitive and specific test. In the case of PTB, the specificity of the test is compromised because of the very high sequence similarity between MAP and other mycobacteria. Other problem is the detection of animals in subclinical stages of infection, since at this stage the immune responses are not so evident **(Figure 5)**. Apart from the main MAP antigens, the potential diagnostic antigens for an ELISA test could be the secreted MAP proteins. These proteins are considered immunogenic owing to their interaction with host receptors and their availability to immune system cells [167]. Furthermore, these proteins play a vital role in macrophages regarding virulence, intracellular survival and proliferation [168]. A study carried out in our laboratory, in collaboration with a Brazilian laboratory, showed that a protein rich in alanine and proline (Apa-MAP1569) is a secretory protein present in cattle faeces with PTB positive results; which suggests that Apa is a potential biomarker for the detection of MAP infections [169].

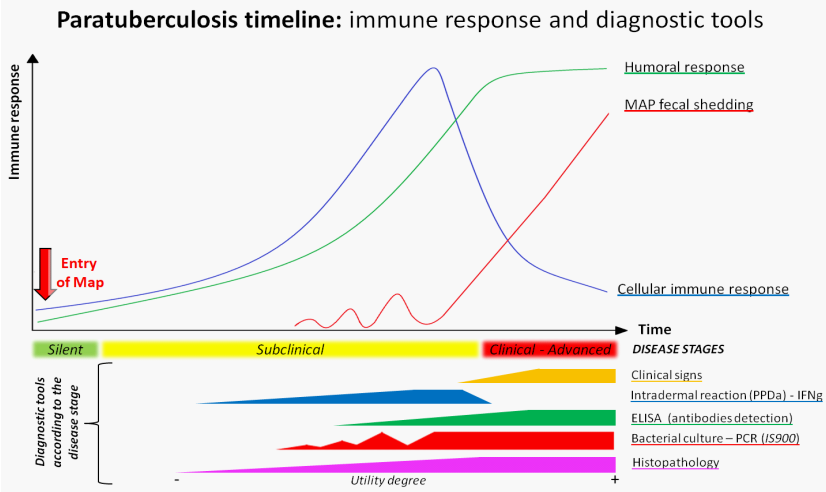

Figure 5: Immune response scheme and diagnostic tools according to progress of paratuberculosis.

On the other hand, MAP cell envelope proteins play a critical role in bacterial pathophysiology during attachment, colonization and infection of target tissues and cells. These cell envelope proteins are accessible to host immune system and apparently are highly immunogenic [4,7,170,171]. An analysis of MAP cell wall proteins performed in search of immunogenic proteins exposed on the surface of MAP consisted of analysing the proteins of a wall protein fraction (CW), which had been previously resolved by 2DSDS-PAGEand transferred to a nitrocellulose membrane, with sera from MAP-infected animals. The analysis revealed 18 proteins that were subsequently identified by MALDI-MS. Interestingly, one of the identified

proteins was the elongation factor Tu (EF-Tu). This protein functions as a fibronectin (FN)-binding protein, thus it would facilitate interactions between bacteria and extracellular matrix [172].

Regarding the humoral response, FN-binding proteins could play a role in adhesion to host cells and in immunomodulation. An evaluation of EF-Tu using a comprehensive set of sera, including sera from animals with bovine tuberculosis (TBB) and negative controls, showed that EF-Tu was recognizable by 64% of the sera from PTB-positive animals. However, this protein resulted non-especific, as some of the sera from healthy animals and with TBB also detected this antigen. This suggests the presence of conserved antigenic epitopes among mycobacterial species[172].

Other antigens extensively study are the lipoprotein antigens. Indeed, lipid-conjugated macromolecules are the main components of the mycobacterial cell wall and are accessible to the host immune system; thus several hosts produce antibodies against them. For example, Lipoarabinomannan is an immunogenic macromolecule present in the MAP cell wall, although non-specific, because it is widely conserved in mycobacteria [173]. An evaluation of different ELISAs, one of which included the specific lipoprotein of MAP lipopentapeptide (L5P) [174], revealed that the ELISA withPPA-3 (Paratuberculosis Protoplasmic Antigen 3) of MAP detected 31% of the positive sera, whereas, despite the high specificity of the lipopentapeptide, the ELISA with L5P detected fewer animals (17%) [175]. Different stages of MAP infection may require particular antigens. In this line, a study using a cocktail with seven antigens from different sources (cell wall, periplasm, etc) displayed high diagnostic sensitivity [176]. Therefore, multiplexing with antigens from different fractions would be more appropriate than a single type of antigen.

Altogether, to date researchers have studied and identified several MAP antigens with varying degrees of success for their use in diagnosis. Despite the large number of MAP proteins tested for antigenicity, the evaluated proteins represent only 20% of the MAP proteome [177]. To date, none of the characterized MAP antigens is species-specific and sensitive for the detection of animals with PTB in the different phases of the disease, either through cellular response (IFN-γ) or immune response mediated by antibodies. This is because most MAP antigenic epitopes are shared with other closely related mycobacterial species. In addition, the disease presents an immunological and pathological spectrum and thus no single MAP specific antigen is effective to detect all infected animals at different stages of infection. This poses a considerable challenge regarding the selection of antigens suitable for diagnostic tests. Recent advances in computational biology, gene editing, and synthetic gene biology may help in the search of appropriate antigens to differentiate animals at early stages of MAP infection from uninfected animals. Upon identification, these antigens could be recombinant expressed in mycobacteria, or other suitable vector, to retain their antigenicity or biological activity. The

evaluation of multiplex containing many of these antigens, either in large longitudinal and large-scale studies, could lead to the development of next-generation diagnostics for PTB.

5. MAP Diversity

Phenotypic observations as well as epidemiological and molecular studies have shown heterogeneity among the different MAP isolates studied so far. The isolates have been classified into two main groups or type of strains, bovine and ovine. This differentiation was first performed by restriction fragment length polymorphism analysis (RFLP) in combination with hybridization with the IS900 sequence (IS900-RFLP). [178,179]. The designation was subsequently replaced by 3 types of MAP according to the species from which they were isolated: ovine strains or Type "S" (Type sheep), isolated from sheep; bovine strains or Type "C" (Type cattle), obtained mainly from cattle; and intermediate Type or "I", isolated from small ruminants [179]. However, researchers have reported that all of these MAP types can also be isolated from diverse species; the species of origin, consequently, is not necessarily an adequate indicator of strain type. For this reason, a most current classification suggest naming these strains as Type I (corresponding to Type S), Type II (corresponding to Type C) and Type III (or Intermediate Type) [167,168]. Advances in molecular typing techniques have allowed genetic differentiation between MAP isolates.

The use of IS1311 PCR-REA assay allowed the identification of a distinctive kind of strain obtained from bison samples. According to this analysis, the novel strain corresponded to a different type of strain that was called Type B (Type bison) [169,170].The characterization of the strain by using SNPs (single nucleotide polymorphism)revealed that Type-B strains presented only copies of IS1311 with a Thymine (T) at position 223, whereas Type S strains had only a Cytokine (C) at that position. In the case of Type C strains, some copies of the IS1311 element presented a C at position 223, whereas others had a T.

Further analysis of the B-type isolates revealed that the Indian B-type isolates differed from the US bison isolates and therefore the isolates from India ere reported as the "Indian bison type". [171]. More recently, Indian B-type isolates have been identified by a TG deletion at positions 64 and 65 of IS1311 at locus 2 [180]. In addition, are a sub-lineage of C-type strains. On the other hand, the strains isolated from camels in Arabia are considered a sub-lineage of the S-type strains.

At the epidemiological level, there seems to be some tendency for MAP types to be associated with aspects such as transmission, host preference and susceptibility to infection; however, the different methodologies employed in the different studies on isolation and typing have made the understanding of these associations difficult. Nevertheless, there is phenotypic and genotypic evidence that differentiates MAP strains.

Phenotypically, the distinct strains are distinguished by the difference in growth time, the type of culture medium in which they can be isolated, as well as their pigmentation, as some strains of ovine origin show a distinct pigmentation not seen in bovine strains [167]. With regard to host preferences, Type II strains show the widest host range, as they have been commonly isolated from different species of animals, both ruminants and non-ruminants [175], as well as from human samples from Crohn's disease patients [165,176,181,182]. By contrast, Type I and III strains are more host-restricted, as they have been most commonly isolated from sheep and goats [183]. According to other reports, sheep are also susceptible to Type II strains [7,184,185]and can manifest the disease if infected with this strain. The geographical distribution of the different types of MAP strains probably depends on factors such as animal circulation from one geographical region to another, virulence of the strains [185] and the management systems in the fields.

To date, the technique based on tandem repeats that can be characterized in 2 categories, ranging from 1 to 9 nucleotides called microsatellites, or short sequence repeats (SSR), is mostly used to characterize MAP, Long repeats (10-100 nt) called variable number tandem repeats (MIRU-VNTR). There are databases that use a pattern of 8 locus to characterize MAP, thus finding great variability in the genotypes. This allowed a worldwide comparison that revealed that some genotypes, such as INMV 1 and 2, are predominant (http://mac-inmv.tours.inra.fr/macinmv/index.php?p=fa_db). A recent analysis suggests that MAP genomes show no evidence of widespread horizontal gene transfer and that a single strain can embody the MAP subspecies, at least for type II strains. This would indicate that the MAP genome is stable [186].

Despite the great progress regarding the molecular epidemiology of MAP, the phenotypic, epidemiological and pathogenic traits of the different strains require further investigation. Complete genome sequencing will transform the capacity to characterize isolates and will provide greater resolution and epidemiological analysis at regional, national and international levels.

6. Relevance of the Study of *Mycobacterium Avium* Subsp *Paratuberculosis* Mutants

Over the last decade, researchers have made great progress in the development of Genetic Engineering strategies that allow the genetic manipulation of microorganisms of biotechnological interest.

In the particular case of mycobacteria, gene manipulation of these microorganisms is quite complex, because conventional methods, such as those using thermal shock or electrical pulse, are not effective. This is due to certain particular characteristics of mycobacteria, such as the presence of an important and complex cell wall with high lipid content, which are essential components for the pathogenicity and survival of these bacteria in macrophages (Abra-

hams and Gurdyal, 2021; Alonso -Hearn et al., 2017).

Regarding the gene manipulation of MAP, the situation is even more complex owing to their extremely slow growth rate, low transformation efficiency, the occurrence of a high rate of illegitimate recombination, along to the limited availability of selection antibiotics for these bacteria (Borgers et al., 2019). For this reason, the information available on the virulence and pathogenesis factors of MAP is scarce, especially in relation to all the information reported on other pathogenic mycobacteria. This type of study is extremely important, since mutations in specific genes allow the identification of genes associated with the virulence of microorganisms and the development of strategies that contribute to control diseases, such as the generation of live vaccines. In general, the mutagenesis methods used in mycobacteria can be targeted towards specific genes or fragments directly by site-directed mutagenesis or can be generated randomly through the use of transposons. The selected strategy will depend on the type of mycobacterium, the available tools and the purpose of the study.

The first reports of site-directed mutagenesis in mycobacteria used negative selection vectors or suicide vectors. This allows, in one or two steps, the selection of bacteria that undergo homologous recombination, and therefore enables the editing of the gene, or fragment, of interest. In this line, one of the techniques most widely used in mycobacteria to delete genes has been the one described by Pelicic et al. (1997) [187,188] (2002; Duque-Villegas et al., 2020). Although still in use, these techniques are subject to the low transformation efficiency of mycobacteria, which is an extremely difficult procedure to perform in MAP.

Indeed, the most successful technique to obtain MAP mutants is specialized transduction, which was first described by Bardarov et al. (2002), [155,189,190] (Mukai et al., 2014;). Although this technique requires many steps and is somewhat laborious, it solves the problem of the low transformation efficiency of MAP. This is because the specialized transduction takes advantage of the natural infective capacities of the phage pHAE87, which infects and introduces its DNA inside the mycobacteria naturally. First, the wild type pHAE87 is modified to contain the flanking regions of the fragment of interest and the recombinant phage is subsequently introduced within the mycobacterium. As a result, the phage leads to the deletion and / or modification of the fragment of interest through homologous recombination.

In 2008, Park et al. obtained the first MAP mutants in the reference strain K10by specialized transduction. In their study, the authors selected 3 genes for mutagenesis: pknG and relA, because of their association with virulence in *M. tuberculosis* and *M. bovis*, and lsr2, for its association with the regulation of lipid biosynthesis and resistance to antibiotics [141]. From the evaluation of the resulting mutants, Park et al. proposed the live attenuated strain ΔrelA as a candidate for a vaccine strain against PTB (Park et al., 2011). More recently, Garber et al.(2018) reported that this mutant strain induced the development of cytotoxic CD8 T cells

(CTL) with the capacity of killing intracellular bacteria in an ex vivo model from peripheral blood mononuclear cells (PBMC).

On the other hand, in 2012 a study of 3 MAP mutant strains obtained by specialized transduction, ΔleuD, Δmpt64 and ΔsecA2, reported attenuation of the three mutant strains in a murine model [132]. Furthermore, all the mutants induced cytokine responses in J774 mouse cells, thus suggesting a role in MAP virulence for these selected genes. More recently, the ΔleuD strain was evaluated as a vaccine candidate in a goat model [133].

Another mutant obtained by specialized transduction in MAP is ΔmptD. The selection of the *mptD* gene was due to its exclusiveness inMAPand because it had not been previously characterized. The deletion of this gene caused the attenuation of the mutant in macrophages as well as in a murine model, and led to an important alteration of the metabolic profile of MAP, mainly of lipid metabolism [191].

In addition, several groups have obtained MAPmutants through the use of transposons. In a previous study [137], Shinand colleague's generated library of approximately 1500 mutants obtained by transposition, and then sequenced and analyzed the mutants with bioinformatic tools. The identification of genes potentially associated with MAP virulence led to the evaluation of two mutant strains displaying attenuation in a murine model (pgs1360 and pgs3965) as vaccine candidates against PTB. The candidate *pgs3965* has an alteration in the mycolic acid synthase enzyme (umaA1), whereas pgs 1360 has an alteration in the *fabG2 2* gene that codes for an oxide-reducatase [137]. These researchers not only suggested the use of these attenuated strains as live vaccine candidates, but also highlighted the importance of *pgs1360* and *pgs3965* in MAP virulence.

Moreover, Rathnaiah et al. (2014) have constructed and screened a comprehensive mutant bank of 13,536 K10 MAP mutants generated using the Tn5367 transposon in order to broaden the study of MAP infection mechanisms [192]. The screening of this mutant bank was performed in vitro and was focused on the search of specific phenotypes: virulence decrease, alterations of colony morphology, susceptibility to D cycloserine, reduction of cell association, and decrease of biofilm formation, among others. This type of approach consists of random insertions of the transposon and therefore allows researchers to obtain mutants at a large-scale.

It is important to highlight that other techniques have been implemented to obtain mutants in mycobacteria. Among these procedures are Recombineering, Recombination-mediated genetic engineering [49,193], ORBIT or oligonucleotide-mediated recombineering followed by Bxb1 integrase targeting (194). The use of CRISPR-Cas9 in the generation of MAP mutants [195,196], however, remains unexplored in this bacteria. Moreover, over the years, researchers have developed optimizations or combinations of the different methodologies to increase their efficiency. Finally, the complete genome sequencing (WGS) of mycobacteria

has allowed very important advances in the development of new tools, either for diagnostic techniques, epidemiological studies or mainly for the identification of new virulence factors and / or therapeutic targets, among others [186,197].

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