EXPERT REVIEWS

Vaccination against paratuberculosis

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Johne's disease, or paratuberculosis, is a chronic granulomatous enteritis in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) affecting principally cattle, sheep and goats. Primarily, there are two clinical signs: cachexia and chronic diarrhea (less common in goats and sheep). This disease results in considerable economic losses in livestock industry, particularly the dairy sector. The route of transmission is mostly by the fecal–oral route, but hygienic measures and culling of shedding animals are not sufficient to eradicate this disease. Moreover, diagnostic tools available at this moment are not powerful enough to perform early and specific diagnosis. Existing vaccines, based on whole killed or live-attenuated bacteria, can delay the unset of clinical symptoms but do not protect against infection. Moreover, vaccinated animals develop antibodies that interfere with existing serodiagnostic tests for paratuberculosis and they become reactive in the tuberculin skin test, used for the control of bovine tuberculosis. This review summarizes the current knowledge of the immune responses induced by MAP infection, with focus on cattle studies. It provides an overview of the existing MAP vaccines and comments on the development of second-generation subunit vaccines based on new technologies.

KEYWORDS: Johne's disease • *Mycobacterium avium* subsp. *paratuberculosis* • paratuberculosis • vaccine

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, or Johne's disease, a progressive, chronic and incurable enteritis affecting principally domestic ruminants, such as cattle, sheep and goats, and wild ruminants, such as deer, bison and elk [1]. Johne's disease causes considerable economic losses to affected farms and livestock industries resulting from premature culling or death, reduced milk production and decreasing fertility [2].

M. avium subsp. *paratuberculosis* infection is prevalent worldwide, principally in tempered and humid areas [3]. Actual herd and animal prevalence are unknown in most countries owing to the lack of performing diagnostic tests and to the lack of their standardization. Indeed, different tests are used for prevalence estimation such as fecal culture, tissue culture, or antibody ELISA and no standardization of these protocols has been performed. Moreover, estimation of exact prevalence is hampered by the lack of specificity and sensitivity of these diagnostic tests. Based on serological assays, Tiwari *et al.* have estimated the herd prevalence (at least one animal testing positive in a farm) in dairy herds in the USA and Europe to be between 7 and 66% [4].

Mycobacterium avium subsp. *paratuberculosis* is transmitted horizontally via the fecal–oral route [1]. Moreover, cows with clinical symptoms can transmit the infection vertically through the utero–placental route [5]. MAP has also been recovered from semen of infected bulls [6].

M. subsp. *paratuberculosis* can survive in the environment (e.g., soiled fields) for periods of more than 1 year and it has been suggested that MAP can enter into a stage of *in vitro* dormancy [7]. Owing to this capacity to persist in the environment, hygienic measures are an important (but not sufficient) containment tool.

Finally, the association between MAP and Crohn's disease has been controversial for a long time [8], but recent improvements in isolation and genomic techniques have provided evidence that MAP may be at least one of the triggers in the development of Crohn's disease through a complex interplay between genetic, infectious and immunologic factors [8–11].

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Immune response induced by MAP infection

As summarized in Figure 1, infection of cattle with MAP can be divided into four stages, according to the severity of the symptoms, the immune response induced and the potential to detect the infection by the available diagnostic tools [4,12]. In sheep and goats, ovine and caprine Johne's disease causes similar symptoms as in cattle, with the exception that diarrhea is less frequent and the onset of the clinical phase occurs in younger animals [13].

Silent, preclinical infection

In a proportion of (resistant?) animals, bacteria are cleared by the innate immune system and infection does not become established [14]. Infected susceptible animals will remain asymptomatic carriers during the first 2–4 years after contact, while intermittently shedding very low numbers of MAP in their feces. Mycobacteria-specific cell-mediated immune responses (CMIs; e.g., IFN-γ production, lymphoproliferation and DTH reactions) are readily detected, but existing tests, using purified protein derivative (PPD; basically crude bacterial lysate and filtrate), lack specificity because of interference with environmental *Mycobacterium* spp.

Subclinical infection

At this time, the animal presents no clinical signs of Johne's disease, but infection by MAP can be detected by fecal culture owing to the intermittent MAP shedding in the feces. A strong mycobacteria-specific cell-mediated immune response is developed during this stage while overall antibody levels are low [1].

Clinical infection

Often after a first or second calving (possibly owing to changes in hormonal balance [15]), infection is no longer controlled and animals develop progressive disease characterized by intermittent or persistent diarrhea, gradual weight loss despite normal food intake (caused by deficient nutrient absorption by the inflamed intestine), reduced milk production and decreased fertility [16]. At this stage, most animals present antibodies against MAP and show persistent bacterial shedding in feces. Mycobacteria-specific Th1-type immune responses are weak at this clinical stage.

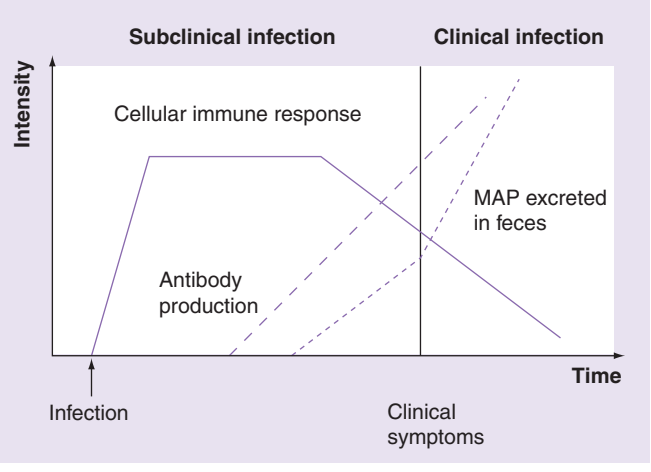

Figure 1. Stages of MAP infection in cattle.
MAP: *Mycobacterium avium* subsp. *paratuberculosis*.

Advanced clinical infection

Eventually, infection can lead to an advanced form of disease, characterized by persistent diarrhea, emaciation, debilitation and eventual death [4]. Most animals are culled, however, before reaching this stage.

Young animals are particularly susceptible to MAP

In endemic areas, animals are exposed daily to MAP from the contaminated environment, but animals younger than 6 months, with a functionally immature immune system, are particularly susceptible to MAP [17] and will become infected during the first months of life by ingestion of contaminated colostrum, milk (not yet reported in sheep and goats [18]), water and feed [3]. During gestation, the maternal immune system is regulated to suppress the rejection of the developing fetus. This imbalance of the maternal immune system leads to a regulation of the developing immune system of the fetus itself and this regulation may be extended into the early neonatal period [19,20]. Moreover, the immune system of the newborn has a strong tendency to adopt a Th2-type profile, whereas a Th1-type profile is thought to be essential for protection against intracellular pathogens, such as mycobacteria.

Neonates receive maternal antibodies, immune cells and various cytokines through ingestion of colostrum [21], and these molecules are able to cross the neonatal intestinal barrier during the first few hours following birth [22,23]. The half-life of colostrum-derived antibodies in the neonate is approximately 15 days. It has not been determined how long colostrum-derived cells can persist. In this context, vaccination of dams may represent an alternative approach in the control of infection of newborns. As mentioned previously, in addition to antibodies, numerous others constituents in colostrums, such as cytokines, chemokines and leukocytes, may potentially confer a passive immunity to newborn animals and function as a first line of defense against infection [24,25]. It is clear that maternal vaccination strategies for MAP are an interesting – so far theoretical – avenue, but more research is needed to understand the mechanisms involved in the transfer of immunity from the dam to her newborn calf, kid or lamb.

A number of genetic factors influence innate & acquired immunity to MAP infection

A number of genetic factors influence innate defense and acquired immunity after an initial exposure to MAP. In sheep, a large study of two Merino flocks highly infected with MAP demonstrated associations of particular polymorphisms in the

Slc11a1 (formerly called *Nramp1*) gene and in MHC loci with susceptibility or resistance to infection [26]. Resistance and susceptibility of mice to MAP is also controlled by the *Slc11a1* gene at early but not late stages of infection [27]. Other genetic factors influence MAP-specific antibody responses, as detected by ELISA in milk [28] and, recently, a potential quantitative trait locus was identified on BTA20 (bovine chromosome 20) affecting susceptibility to MAP in US Holsteins, but the precise mechanisms involved have not yet been elucidated [29].

Intestinal infection

Following oral ingestion, MAP passes through the epithelial barrier of the intestine. Experimental infection models performed in goat kids and calves have been reviewed by Sigurethardottir *et al.* [30]. In the ileum principally, intact and degraded MAP can pass mainly through M cells to the underlying Peyer's patches but MAP can also enter through 'enterocyte-like' cells [31]. After crossing the epithelial layer of the intestinal mucosa, MAP is phagocytosed by subepithelial macrophages. Once inside the macrophage, mycobacteria can evade immune elimination and modulate immune response (this event can be considered as an establishment of a persistent intracellular infection) but, at the same time, some bacilli can be ingested and processed by professional antigen-presenting cells (dendritic cells), which, after migrating to draining lymph nodes, will prime a MAP-specific T-cell response [32,33]. An *in vitro* study has shown that MAP infection can interfere with the ability of macrophages to produce reactive nitrogen and oxygen intermediates and with their capacity of phagosome–lysosome fusion [33]. Expression of IL-1α and the antiapoptotic molecule TNF receptor-associated factor (TRAF)1, involved in the signaling of TNF-α receptor superfamily members, is increased in MAP-infected bovine monocyte-derived macrophages, and this is another possible mechanism of MAP to subvert the bactericidal functions of infected macrophages and to survive [34].

Crosstalk between the host and MAP induces complex interactions between macrophages and lymphocytes, leading to an ensemble of cytokine secretion, recruitment of cells to the site of infection, activation of some cells and cell proliferation. Microscopic analysis of intestinal tissues from subclinically infected cows and goats has revealed the presence of diffuse granulomas containing acid-fast bacilli [35,36].

Role of B and T lymphocytes in the acquired immune response against MAP

The role of B cells and antibodies in immune protection against MAP infection is not fully understood. Indeed, upon disease progression, an early proinflammatory Th1-like immune response considered to be protective eventually gives way to a predominant antibody-based Th2-like immune response in diseased animals. However, there is some evidence for an antibody response against an unknown MAP protein in cattle as early as 3 weeks after infection [37]. Bannantine *et al.* have also demonstrated an early MAP-specific antibody response (70 days after infection) in experimentally infected calves using a recombinant protein array [38]. Along this line of thinking, Coussens proposed a model in which there was not an active switch from a proinflammatory response to a predominant IgG_1 response, but rather, a gradually increasing IgG_1 response dependent upon the dose of MAP and route of entry [39].

IFN-γ-producing $CD4^+$ T cells are key players in an effective immune response against intracellular mycobacteria such as MAP [37,40–42]. However, the protective mechanisms are complex and the fine-specificity of these IFN-γ-producing $CD4^+$ T cells is not fully understood. Thus, *in vitro* assays failed to demonstrate a direct correlation between IFN-γ and nitric oxide production by $CD4^+$ T cells and killing of intracellular MAP in peripheral blood-derived macrophages [43]. In addition, a commercial killed MAP vaccine (Mycopar®, strain 18) can induce a strong antigen-specific IFN-γ response by $CD4^+$ T cells from draining lymph nodes, but this vaccine was ineffective in decreasing MAP number in infected tissues [44,45]. On the other hand, live-attenuated vaccines provide a certain degree of protection against intestinal lesions in goats infected with MAP, and a flow-cytometric study has shown that close to all of the IFN-γ-producing cells in vaccinated goat kids are of the $CD4^+$ subset, while only a small number are $CD8^+$ T cells [46]. As for other mycobacterial infections, such as *Mycobacterium tuberculosis*, more work is needed to define the antigen specificity and the possible polyfunctionality of these IFN-γ-producing T cells needed for the protection against MAP [47].

Besides their cytolytic function, $CD8^+$ cytotoxic C lymphocytes may also exert an antimycobacterial effect through the secretion of cytokines, such as macrophage-activating IFN-γ and TNF-α [48]. Finally, numerous γδ T cells have been shown in granulomatous lesions after an experimental subcutaneous injection of MAP in calves [44] and in ileal and jejunal Peyer's patches and mesenteric lymph nodes from orally infected lambs [49]. *In vitro* assays showed that these sensitized γδ T cells do not have direct antimycobacterial activity [50], but rather, play a role in the formation and organization of granulomas [51].

Role of cytokines in immune defense against MAP infection

TNF-α is a key cytokine in granuloma formation through release of chemotactic factors, leading to attraction of activated immune cells [52,53]. A deficient TNF-α expression may lie at the basis of the diffuse granulomatous appearance of many MAP infection sites [34,39]. A comprehensive study of cytokine gene expression in peripheral blood mononuclear cells (PBMCs) and tissue of MAP-infected cattle confirmed this decreased TNF-α gene expression in PBMCs from infected cattle as compared with controls (uninfected cattle). In this study, expression of TGF-β, IL-4 and IL-12 was also

lower in PBMCs from infected cattle, whereas gene expression of IL-1α, IL-6, IL-8 (chemoattractant for neutrophils) and, particularly, IFN-γ was higher. Stimulation with MAP tended to reduce this differential expression in PBMC from infected and control animals, except for IL-10, which was consistently enhanced by MAP stimulation of PBMCs from subclinically infected cattle. In ileal tissues from MAP-infected cattle, expression of genes encoding IFN-γ, TGF-β, IL-5 and IL-8 was greater than the expression in comparable tissues from control, uninfected cattle, while expression of the gene encoding IL-16 (chemoattractant for $CD4^+$ T cells) was lower. In mesenteric lymph nodes cells, yet another expression pattern was observed with higher levels of IL-1α, -8, -2 and -10 and lower levels of TGF-β and IL-16 in infected animals [54]. Events leading to the gradual loss of mycobacteria-specific Th1-type CMI during progression to clinical disease are not fully understood, but increased levels of IL-10, rather than of the genuine Th2 cytokines IL-4 and IL-5, have been observed by many authors, suggesting that the appearance of regulatory T cells may be an important step in the progression to the symptomatic stage [55–60].

In conclusion, still more research is needed to define the triggers and mechanisms involved in the evolution from a controlled, presumably latent, mycobacterial infection toward an overt clinical disease. This is particularly important for the design of an efficacious vaccine.

Vaccination against Johne's disease

Vaccination against MAP was first reported in 1926 by Vallée and Rinjard [61]. Their vaccine consisted of a live nonvirulent strain of MAP adjuvanted in a mix of olive oil, liquid paraffin and pumice powder. During the 20th Century, a number of live-attenuated and killed whole-cell-based vaccines were developed both for bovine and ovine Johne's disease. Most efficacy trials of paratuberculosis vaccines have been field trials in regions with high incidence of Johne's disease, and design parameters and experimental conditions were not always fully controlled. Routinely, the vaccines, suspended in mineral oil, are inoculated subcutaneously in cattle within 30 days of birth in the brisket [62]. In goats, sheep and deer, vaccines are generally injected in the neck behind the ear (following manufacturer's instructions). Revaccination is not recommended [63].

Table 1 summarizes literature regarding these first-generation killed and live-attenuated vaccines against Johne's disease.

Killed whole-cell-based vaccines

Strain 18 used in a commercial vaccine (Mycopar) in the USA is actually composed of killed *M. avium* subsp. *avium* [64–66]. Strain ID-Lelystad vaccine, manufactured in The Netherlands, is composed of heat-killed MAP bacteria suspended in a water–oil emulsion [67,68]. Gudair™, strain 316F is a commercial vaccine developed in Spain by CZ Veterinaria for lambs and goat kids [69–71]. 5889 Bergey strain is an experimental vaccine developed in Hungary. This vaccine is composed of a heat-killed, oil-adjuvanted MAP 5889 Bergey strain (Phylaxia Veterinary Biologicals Company, Budapest, Hungary) [72].

Live-attenuated whole-cell-based vaccines

Neoparasec™, an oil adjuvanted, freeze-dried live modified 316 F strain of MAP (Rhone-Merieux, Lyon, France) was used until 2002 in New Zealand and in France for the vaccination of cattle, sheep and goats [73–77]. Neoparasec has also been used as a therapeutic, postexposure vaccine [78]. Paratuberkulose vaksine (Oslo, Norway), is based on two British reference strains of MAP (2E and 316F) adjuvanted in a mix of olive oil, liquid paraffin and pumice powder [46,79].

Improved whole-cell-based vaccines

Silirum® vaccine has recently been produced to vaccinate cattle against Johne's disease. It is produced by CZ Veterinaria of Spain, the same manufacturers of the Gudair vaccine. This vaccine is also a killed vaccine composed of MAP strain 316F combined with highly refined mineral oils to reduce the granuloma formation at the vaccination site. All vaccinated animals developed cellular and humoral immune responses to this vaccine and the percentage of animals with MAP lesions was significantly higher in the control group than in vaccinated animals. Reaction to bovine tuberculin was generally lower than to avian PPD, both for IFN-γ production and in skin testing, suggesting that this vaccine against MAP may not interfere with official diagnostic tests when comparative tests are used [80]. This vaccine is undergoing a follow-up field trial in Spain in cattle [81].

AquaVax is composed of an aqueous suspension of live MAP strain 316F. In sheep, this vaccine induces low transient immune responses but confers very little protection after an experimental challenge (lesions present in 80% of vaccinated sheep) [42]. In farmed red deer, this vaccine induces a lower cell-mediated response and smaller nodules that regress more quickly than animals vaccinated with the oil-adjuvanted Gudair vaccine [77].

Killed commercial vaccine Strain 18/killed MAP field-isolate adjuvanted with human rIL-12

IL-12 is a key cytokine essential for the initiation and maintenance of Th1 immune responses [82]. Vaccination of cattle with these vaccines in phosphate-buffered saline alone or with rIL-12 was tested. Both strain 18 and the field-isolate based vaccines induced strong local, systemic and enteric IFN-γ responses. A significant reduction in mycobacterial colonization was observed when calves were vaccinated with field isolate prior to challenge (with the same field-isolate strain), but not following vaccination with *M. avium* strain 18 vaccine. Overall, the effect of rIL-12 on IFN-γ production and total mycobacterial load was not statistically significant [45].

Table 1. Summary of first-generation killed and live-attenuated vaccines against Johne's disease.

Vaccine type (vaccine strain)	Animal species	Infection type	Follow-up time	Age at vaccination	Effect of MAP vaccine on:		Interference with diagnostic tests	Comments	Ref.
					Clinical outcome	*Immune responses*			
Heat killed (Mycopar strain 18)	Cattle (15 vac., 5 cont.)	ND	15 months	5–35 days	ND	Antibody response (serum ELISA; 13 out of 15)	ELISA (based on strain 18 extract) due to seroconversion	Old study with small number of animals. Low herd incidence. Strain 18 actually *M. avium* subsp. *avium*	[65]
	Sheep (25 vac., 24 cont.)	Field follow-up	6 years	3 months	Histology negative control and vaccinated	Antibody response (serum ELISA and AGID) cellular immune response (skin test)	ELISA (Parachek). Positive skin test but comparative (bovine/avian) PPD permitted differentiation	Using *M. avium* subsp. *avium* strain. Low herd incidence. No analysis of fecal shedding. No culture on organs (viability)	[66]
Heat killed (ID-Lelystad)	Cattle (250)	Field follow-up	12–14 years	<1 month	No new cases of paratuberculosis detected	Persistent cellular immune response (IFN-γ) and antibody production (serum ELISA-CFT)	With serum ELISA (Idexx) up to 3 years; cellular IR (Bovigam®) against PPD-B	Herd with low incidence. Lack of histology. No control group	[68]
	Cattle (159 vac., 340 cont.)	Field follow-up	±6.5 years (cont.) 3.5 years (vac.)	Newborn	Reduced histopathology (5 vs 11.8%). Reduced number of clinical cases (1.8 vs 7.8%). Incidence of MAP-positive animals not affected (21.8 vs 25.9%)	ND	ND	Study performed in high incidence herd. Only newborn animals vaccinated. No info on immune response	[67]

AGID: Agar gel immunodiffusion; CFT: Complement fixation test; CIET: Counterimmunoelectrophoresis test; cont.: Control, nonvaccinated; FACS: Fluorescence-activated cell sorting; inf.: Infected; IR: Immune response; LTA: Lymphocyte transformation assay; MAP: *Mycobacterium avium* subsp. *paratuberculosis*; ND: Not described; OJD: Ovine Johne's disease; PPD: Purified protein derivative; Sc.: Subcutaneously; vac.: Vaccinated; ZN: Zielh Nielsen coloration.

Table 1. Summary of first-generation killed and live-attenuated vaccines against Johne's disease (cont.).

Vaccine type (vaccine strain)	Animal species	Infection type	Follow-up time	Age at vaccination	Effect of MAP vaccine on:		Interference with diagnostic tests	Comments	Ref.
					Clinical outcome	*Immune responses*			
Heat Killed (Gudair™)	Lambs (40 vac., 20 cont.). Goat kids (40 vac., 20 cont.)	Field follow-up	1.5 years	15 days (20/20) or 5 months (20/20)	No clinical cases in the study period in any of the groups	Higher immune response (IFN-γ Bovigam/PPD-A serum ELISA PPA-3) in 5-month-old goat kids than in 15-day-old animals. More rapid decrease of IR in goat kids than in lambs	With ovine and caprine IFN-γ diagnostic tests. With serum antibody detection	No established infection and hence no evaluation of protective effect possible. No histology studies. No fecal cultures	[69]
	Sheep (600 vac./600 cont. from three farms)	Field follow-up	4 years	1–3 months	Decreased mortality (90%). Decreased fecal shedding (90%). No decrease in proportion of subclinical cases. Some cases of multibacillary disease in vaccinates (7/600). High levels of shedding by vaccinates at some time points	Antibody production (ELISA/Parachek®). Cellular (IFN-γ/Bovigam) immune response against PPD-A and PPD-P	ND	Farms with high prevalence level. Lesions present at vaccine injection site (25% after 4 years). Controls vaccinated with saline. Data from this study enabled the registration of this vaccine in the national program for the control of OJD (Australia). Risk of self-inoculation very low (1 per 5,000,000 doses)	[70,71]
Heat killed (5889 Bergey)	Cattle (Herd 1: 866 vac. cows, 721 cont. bull; Herd 2: 800 cont. cows from a infected herd, 379 cont. newborn)	Field follow-up	5 years	1 month	Reduced fecal shedding determined by fecal microscopy (48 to 1.4%) but no elimination of the infection	Increased antibody levels (CFT and AGID) up to 3 years. then a decline. Increased number of animals positive in skin test	Interference with skin test to PPD-A and PPD-B	Very low number of culture-positive animals even in control group	[72]

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Table 1. Summary of first-generation killed and live-attenuated vaccines against Johne's disease (cont.).

Vaccine type (vaccine strain)	Animal species	Infection type	Follow-up time	Age at vaccination	Effect of MAP vaccine on:		Interference with diagnostic tests	Comments	Ref.
					Clinical outcome	*Immune responses*			
Live vaccine (Neoparasec-strain 316F)	Cattle (two single dosage, two double dosage, one cont)	Field follow-up	2 years	28 days	No shedding and no MAP colonization (PCR, culture and ZN) in organs in control or vaccinates	Early cellular (IFN-γ Idexx, proliferation and skin test) IR. Transient antibody (serum ELISA) IR	Positive or doubtful skin reactions against PPD-B	Only five animals. Large variations in antibody and proliferative responses. No quantification for IFN-γ production	[73]
	Goats (16 vac, four cont)	ND	1 years	1–2 months	ND	Specific cellular immune response (LTA) in 15 out of 16. No antibodies detected (CIET)	ND	Large fluctuations in cellular IR in time. No information regarding the status of animals. No information regarding histology or bacteriology	[74]
	Lambs (two cont., two vac. noninf., four nonvac. inf., eight vac. inf.)	Experimental	220 days	1 month	Partial protection (bacterial and histological). Isolation of bacteria only in infected groups in organs and tissues. No shedding found	Strong and immediate ELISA response (PPA-3) in vaccinates. In infected animals increased antibody response 6 weeks after infection	ND	No strain description used for challenge. No analysis of cellular immune response. No evaluation of damage at the site of injection	[76]
	Sheep (30 cont, 30 Neoparasec-inf, 30 Aquavax-inf, 30 inf)	Experimental	10–22 months	2.5–3 months	Partial protection (lesions). 28% in Neoparasec vaccinated vs 80% in unvaccinated or 75% Aquavax	Strong humoral (ELISA) and CMI response (LTA-ELISA IFN-γ Bovigam-FACS analysis)	Interference with PPD-B and PPD-A skin test	Good comparison of immune response induced by vaccination and infection. Comparison of two adjuvants (oil and water). Very complete study	[75]

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Table 1. Summary of first-generation killed and live-attenuated vaccines against Johne's disease (cont.).

Vaccine type (vaccine strain)	Animal species	Infection type	Follow-up time	Age at vaccination	Effect of MAP vaccine on:		Interference with diagnostic tests	Comments	Ref.
					Clinical outcome	*Immune responses*			
Live vaccine (Neoparasec-strian 316F)	Red deer (15 nonvac, 15 twice vac, 15 aqueous formulation)	ND	42 weeks	5 months	ND	Cellular (LTA skin test) and humoral immune (ELISA) responses	Temporary interference with PPD-B and PPD-A skin test (negative at 36 weeks postvaccination). Interference with antibody ELISA	Aqueous MAP vaccine induced less crossreactivity. Presence of lesions more persistent in injection site using oil-adjuvanted vaccine than aqueous vaccine. No information on protection	[77]
	Lambs (14 inf., 14 inf. vac., 13 cont.)	Experimental	1 year	1–1.5 months postexposure	Decreased clinical signs (lesions) in vaccinated (14 vs 50% in control)	Early antibody (ELISA Paracheck) and IFN-γ response (Bovigam) more substantial in vaccinated animals	ND	Postexposure vaccination comparative histology between infected vaccinates and infected only	[78]
Live vaccine (OSLO-strain 316F/2E)	Goats (15,219)	Field follow-up	15 years	2–4 weeks	Decreased infection rate (53 vs 1%) during the study, based on postmortem examination	Antibody response (CFT)	ND	No information regarding cellular IR. Protective efficacy evaluated by posmortem examinations and culture. No control group (without vaccination) to validate the protective efficacy of the vaccine	[79]
	Goat kids (seven vac., three cont.)	ND	24 weeks	2–3 weeks	ND	Strong cellular immune response (IFN-γ ELISA [Bovigam]-FACS analysis)	ND	Three time points analyzed (0, 12 and 24 weeks). No information on protective efficacy	[46]

AGID: Agar gel immunodiffusion; CFT: Complement fixation test; CIET: Counterimmunoelectrophoresis test; cont.: Control, nonvaccinated; FACS: Fluorescence-activated cell sorting; inf.: Infected; IR: Immune response; LTA: Lymphocyte transformation assay; MAP: *Mycobacterium avium* subsp. *paratuberculosis*; ND: Not described; OJD: Ovine Johne's disease; PPD: Purified protein derivative; Sc.: Subcutaneously; vac.: Vaccinated; ZN: Zielh Nielsen coloration.

Killed vaccine based on spheroplast MAP (cell wall deficient) from a clinical goat isolate

The efficacy of cell wall competent (CWC) or spheroplast (cell wall deficient [CWD]) MAP vaccines adjuvanted in either alum or QS21 saponin was evaluated in goat kids. All four vaccines were associated with a persistent (up to 9 months) nonulcerative nodule at the injection site. Vaccines adjuvanted with QS21 had less systemic side effects than vaccines adjuvanted with alum. Interference with comparative skin tests and the IFN-γ (Bovigam®) test was also weakest with the CWC-QS21 vaccine. All kids presented some MAP lesions after experimental challenge with 6×10^9 organisms, indicating that these vaccines did not prevent infection. Best performances in protection and reduction of necropsy lesions at 9 months postchallenge were obtained with the CWC-QS21 vaccine. None of the vaccines had a significant effect on fecal shedding [83].

Killed vaccine based on a highly virulent MAP 'Bison-type' field strain

Recently, a highly virulent MAP strain isolated from a goat in India was compared, as a killed vaccine (adjuvanted in alum), with commercial Gudair (in Montanide adjuvant) in goat kids. After experimental challenge with 3×10^9 and 5×10^9 colony-forming units (CFUs) of 'Bison-type' MAP, fecal shedding was reduced in both vaccinated groups in comparison with the control group. Average body weight gains after challenge were highest in Bison-type-vaccinated kids. Histopathological evaluation of the lesions after infection was performed on a small number of animals (four from each group); only one out of the four of Bison strain-vaccinated animals presented with lesions in target tissue (mediastinal lymph nodes and intestine), two out of the four of Gudair-vaccinated animals and all four animals in the control group presented with lesions in the intestinal tissues [84].

All these whole-cell-based vaccines – particularly the ones adjuvanted in oil – have a number of major drawbacks: they interfere, to some extent, with the existing diagnostic tests for bovine tuberculosis as they induce a positive *in vitro* IFN-γ response and skin test to tuberculin/PPD, they interfere with the existing serodiagnostic tests for paratuberculosis, their administration is associated with a risk of accidental self-injection by the veterinarian and, finally, they induce granulomatous lesions at the vaccination site (this may be a problem at the abattoir). Moreover, the live vaccines are not really characterized with respect to their attenuation, making their use as marked vaccines impossible. It is, therefore, not surprising that animal health authorities and farmers are reluctant to use the existing vaccines [85]. It is clear that the development of marked vaccines, which are preferentially based on a few immunodominant, protective antigens that would not interfere with existing – or novel – diagnostic tests for MAP, is crucial to overcome these very important hurdles [86].

Subunit-based vaccines

The identification of immunodominant protein antigens inducing strong Th1-type immune responses during the first asymptomatic stage of the disease and the demonstration of their protective potential in experimental infection models (mouse and target species) will be crucial for the development of subunit-based vaccines. Immunization of animals with recombinant proteins in adjuvant or with DNA vaccines encoding immunogenic antigens would be an ideal procedure to overcome the interference issues linked to whole-cell-based vaccines. The entire genome sequence of the K-10 strain of MAP has recently become available and provides a precious tool for the study of MAP antigens to be used in more specific immunodiagnosis and more effective immunoprophylaxis. The genome of K-10 is a single circular chromosome composed of 4.83×10^6 bp coding for 4350 predicted open reading frames. *In silico* analysis has identified more than 3000 genes with homologs to *M. tuberculosis* and 161 unique regions coding for 39 previously unknown genes [87]. Characteristics of the MAP genome are its relative paucity in *PE*/*PPE* genes (implicated as virulence factors) and a deletion in the EntE domain of a salicyl-AMP ligase (the first gene in the mycobactin biosynthesis gene cluster) [87].

Immunodominant Th1 antigens identified so far

The three members of the Ag85 complex, Ag85A (MAP1609c), Ag85B (MAP0126) and Ag85C (MAP3531c), are highly conserved proteins with mycolyl-transferase activity present in all mycobacterial species and abundantly secreted in mycobacterial culture filtrate. The Ag85A and Ag85B components of *M. tuberculosis* are among the most promising vaccine candidates for human tuberculosis and are actually being tested in clinical trials either as a Hybrid1 fusion protein Ag85B–ESAT-6 [88] or as a recombinant modified vaccinia Ankara (MVA) virus encoding Ag85A in a Bacille Calmette–Guérin (BCG) prime–MVA-Ag85A boost protocol [89].

The immunodominant properties of the MAP antigens Ag85A, 85B and 85C have been reported in cattle and mice experimentally infected with MAP and also in mice vaccinated with recombinant protein, DNA or irradiated whole MAP bacilli [90–94]. Strong T-cell responses (proliferation and IL-2 and IFN-γ responses) can be detected against Ag85A and Ag85B and, to a lesser extent, to Ag85C in low and medium shedder animals, but not in culture-negative cows, whereas IL-4 levels are very low [91].

Heat-shock protein (Hsp)65 (GroEL) and Hsp70 (DnaK) can also induce specific immune responses in MAP-infected and MAP-vaccinated cattle. As for PPD responses, the mycobacterial Hsp70-specific CMI responses decrease upon progression to the clinical stage of the disease. Hsp65 induces less-prominent responses compared with Hsp70, but shows a similar pattern with regard to the stages of the disease [95].

P22 (22 kDa) is an exported MAP protein belonging to the LppX/LprAFG family of putative mycobacterial lipoproteins. The IFN-γ response against this protein could be detected in sheep vaccinated with the live-attenuated Neoparasec vaccine. Also, antibodies against this protein were detected by western blot analysis in ten out of 11 vaccinated sheep, in two out of two clinically affected cows and in 11 out of 13 subclinically infected cows [96]. The P22 protein was also shown to induce good IFN-γ and antibody responses when administered as a recombinant protein in a water-in-oil emulsion [97]. Another lipoprotein, the 19-kDa (MAP0261c) protein, was also reported to stimulate strong humoral but weak IFN-γ production in infected cattle [98].

Two MAP proteins belonging to the PPE family, that is, MAP1518 and MAP3184, were described to elicit significant IFN-γ levels in PBMC cultures of experimentally infected Holstein calves [99]. Some of these PPE protein family members of *M. tuberculosis* are promising vaccine candidates against human tuberculosis [100].

Superoxide dismutase (SOD) is a 23-kDa intracellular protein of virulent mycobacteria that is exported by a bacterial protein secretion system. SOD is considered to be a virulence factor, interferes with macrophage bactericidal properties and has anti-apoptotic properties [101]. Vaccination of mice with recombinant MAP SOD was reported to induce a mixed Th1/Th2 response (IFN-γ, IL-6 and TNF-α), significant antibody production and a DTH reaction [102]. In cattle, SOD strongly induces $\gamma\delta^+$ T cells, thought to be important in the early stages of infection and in granuloma formation [91].

Another antigen that could be involved in the innate immune response to MAP is MPP14, a 14-kDa secreted MAP protein specific of the group *M. avium–intracellulare–scrofulaceum* (MAIS) complex. This protein can induce strong IFN-γ response, both in experimentally infected and uninfected calves, responses that may interfere with diagnostic testing using the IFN-γ test [103].

Alkyl hydroperoxide reductases C (AhpC) and D (AhpD) are constitutively expressed by MAP *in vitro* and homologous antigens can be detected in *M. tuberculosis* during exposure to oxidative stress but not in *M. avium* subsp. *avium*. In experimentally infected goats, antibodies against AhpC but not against AhpD could be detected and both these antigens elicited a strong IFN-γ response [104].

The definition of virulence factors can also provide targets for vaccine development. For this purpose, Shin *et al.* screened a library of insertional mutants of MAP with deficient *in vivo* growth characteristics, which enabled the identification of eleven virulence factors involved in iron, tryptophan and mycolic acid metabolic pathways [105]. Allelic exchange in MAP has been very difficult until now because of the slow multiplication rate of the bacteria (2 months before colonies can be counted visually). Recently, techniques for allelic exchange in MAP have been improved, and three genes encoding virulence factors, that is *pknG, relA* and *lsr2*, have been rationally deleted using this approach [106]. The use of these rationally attenuated MAP mutants may be an alternative approach to subunit-based vaccination, although it is not clear for the moment to what extent this type of vaccines would interfere with existing diagnostic tests as they are composed of whole bacteria.

Another important avenue that needs further exploration in the context of vaccine development is the issue of latent paratuberculosis. Recently, Wu *et al.* have reported on the so-called stressome of MAP, characterized by gene-expression profiling of MAP exposed to different stress conditions or shed in cow feces [107]. This study identified a novel set of putative virulence genes, which is very important for a better understanding of the pathology of MAP. The extent that some of these stress-induced proteins are also involved in the cellular immune control during the first asymptomatic stage of the MAP infection still remains to be determined. In this context, it is important to note that similar stress-induced proteins of *M. tuberculosis* are strong T-cell antigens in healthy PPD-positive donors [108] , as well as in DNA-vaccinated and tuberculosis-infected mice [109].

Testing vaccine candidates in experimental mouse models

Although the mouse is not a target species for Johne's disease, it is a valuable tool for the preclinical testing of vaccine candidates due to the wide range of immunologic tools, the various genetic backgrounds of inbred strains and the low costs needed for purchase and maintenance [18]. Protective efficacies are generally demonstrated by comparing bacterial replication in control versus vaccinated animals. DNA vaccines are very effective (particularly in small rodents) in inducing humoral and cellular immune responses needed for the protection against intracellular mycobacterial pathogens [110,111]. Furthermore, DNA vaccines do not require a cold chain and are very stable. This technique is very effective for screening large numbers of vaccine candidates since it does not require the purification of the protein antigens. Promising results have been obtained with veterinary DNA vaccines against several infectious diseases and, so far, three DNA vaccines for veterinary use have been licensed (against West Nile encephalitis in horses, against melanoma in dogs and against infectious hematopoietic necrosis virus in salmon) [86].

Using expression library immunization, Huntley *et al.* reported in 2005 on the protective potential of a plasmid mix (encoding 26 MAP antigens) that conferred significant protection of BALB/c mice against intraperitoneal challenge with 10^8 CFUs of MAP. Genes in the protective clone were identified as coding for transport/binding, membrane and virulence proteins and mycobactin/polyketide synthases, but further analysis of the respective antigens has not been performed to our knowledge [112].

Using DNA vaccination, we have recently evaluated the vaccine potential of two MAP proteins, MAP0586c and MAP4308c, previously identified by postgenomic and immunoproteomic analysis of the MAP secretome, as novel serodiagnostic antigens [113]. Immunizations of mice with plasmid DNA encoding MAP0586c and MAP4308c induced strong Th1-type immune responses, whereas antibody responses were only induced upon immunization with DNA encoding MAP4308c. MAP-infected BALB/c mice also generated strong MAP0586c-specific T-cell responses and could be partially protected against infection following DNA vaccination, indicating that this putative transglycosylase is an interesting vaccine candidate that warrants further investigation [114].

Recombinant viral vectors have also been used to study the vaccine potential of MAP antigens. Bull *et al.* reported on recombinant adenovirus 5 and MVA virus, expressing a 95-kDa fusion protein, consisting of fragments of two secreted (MAP 1589c/AphC and MAP 1234/Gsd) and two cell surface (MAP2444c and MAP 1235/Mpa) proteins. Significant IFN-γ ELISPOT responses were observed in vaccinated C57BL/6 mice and an Ad5 prime–MVA boost protocol conferred some protection against subsequent challenge, as measured by quantitative PCR in spleen and liver [115].

As mentioned previously, vaccine potential is often measured by monitoring bacterial replication in spleen and liver using fastidious and expensive CFU plating on Middlebrook 7H11 agar. We have described that this method of vaccine testing can be replaced by quick and cheap luminometry, using a luminescent MAP isolate [94]. Luminometry has also allowed us to formally demonstrate the role of the *Slc11a1* gene in innate resistance and susceptibility to MAP of different mouse strains. In BALB/c, congenic BALB.B10-H2<b> (BALB/c background, H-2^b), C57BL/6 and beige C57BL/$6^{bg/bg}$ mice (all *Slc11a1*^{s}) bacterial numbers in spleen and liver remained constant during the first 4 weeks of infection, whereas in DBA/2, congenic C.D2 (both *Slc11a1*^{r}) and (C57BL/6 × DBA/2) F_1 (*Slc11a1*$^{s/r}$) mice, the bacterial number had decreased more than tenfold during the same period in both male and female mice [27]. At later time points, additional differences in bacterial replication were observed between the susceptible mouse strains, particularly in the liver. Whereas bacterial numbers in the liver gradually decreased more than 100-fold in C57BL/6 mice between weeks 4 and 12, bacterial numbers were stable in liver from BALB/c, BALB.B10 and beige C57BL/$6^{bg/bg}$ mice during this period. Vaccination of BALB.B10 mice with BCG or γ-irradiated MAP ATCC19698 resulted in significant reductions in relative light unit and CFU counts in spleen following challenge with luminescent MAP, showing that some susceptible mouse strains are valuable models for vaccine testing.

It must of course be admitted that the experimental MAP infection model in mice is not fully satisfactory, as it does not present with the classical intestinal pathology observed in ruminants. On the other hand, experimental infections of target species are very expensive and, moreover, they require specific containment measures, as MAP can survive for a very long time on pastures in the environment. An alternative to the mouse model may be the rabbit. Indeed, paratuberculosis has been described in wild rabbits in Scotland, and it has been hypothesized that lagomorphs could play a role as a wildlife reservoir for MAP [116]. Oral infection of newborn New Zealand white rabbits was extensively documented by Mokresh *et al.* in 1989, describing a 60% take of the infection, as indicated by histopathology and culture [117]. This work was recently revisited and confirmed to some extent by Vaughan *et al.* in adult and juvenile animals [118]. Therefore, rabbits may become a new reasonably priced disease model for the study of paratuberculosis in ruminants.

Subunit vaccine trials in target species

To our knowledge, only two trials have reported on the use of subunit vaccines in target species of Johne's disease. DNA vaccines encoding Ag85A from *M. bovis* BCG and *M. avium* subsp. *avium,* and Hsp65 from MAP have been evaluated for protection against a MAP infection in lambs of Sarda breed. In total, 25 lambs, divided into five groups of five animals each, were vaccinated intramuscularly three times (0, 20 and 40 days) at 5 months of age. At 90 days after vaccination, DNA encoding Ag85A-*avium* and Hsp65-MAP induced higher IFN-γ levels than the two other vaccines, as measured by real-time PCR. A total of 90 days after oral challenge with 20×10^8 MAP linda (a strain originally derived from a patient with Crohn's disease), highest IFN-γ levels were found in animals vaccinated with Ag85A-BCG and Hsp65-MAP. Histopathology of postmortem tissue sections after 1 year revealed the absence of lesions in all three DNA-vaccinated groups, whereas, in the control group, lesions were readily observed [119].

Koets *et al.* reported on vaccination experiments with recombinant MAP Hsp70 protein mixed with dimethyl dioctadecyl ammonium bromide (DDA) adjuvant in 40 female calves [120]. Results demonstrated that rHsp70 can be used successfully as a subunit vaccine against bovine paratuberculosis, significantly reducing shedding of bacteria in feces during the first 2 years following experimental infection with an admittedly low dose (~2×10^4 CFU). This vaccine has little direct and long-term side effects and enables the serological differentiation between vaccinated and infected animals, as infection is reported to induce only weak Hsp70-specific antibodies. The interference of this Hsp70-based vaccine with tuberculin skin testing and its vaccine potential against natural infection still needs to be evaluated in long-term protection experiments [120].

Conclusion & expert commentary

An 'ideal' vaccine against paratuberculosis or Johne's disease should generate sterilizing immunity, or at least abolish fecal shedding. The vaccines currently available reduce clinical symptoms but cannot avoid the contamination due to fecal shedding. A better understanding of the molecular and immunological processes involved in the progression to clinical paratuberculosis may help to develop more efficient vaccines. Finally, the development of

'marker' vaccines will require the further identification and discrimination of MAP antigens with either a strong immunodiagnostic potential (humoral or T-cell based) or, on the other hand, a strong protective potential. Begg *et al.* have related that ovine or bovine MAP strains produce different immunological profiles in experimentally infected animals and, therefore, target species (and geographic region) also need to be taken into account in the development of new vaccines [121].

New experimental infection models to test vaccine efficacy are needed. If possible, evaluation of the new vaccines should use experimental challenge conditions with dose and inoculation route similar to natural infection. Due to the slow progression of the disease, a compromise must be found between the length of the follow-up to validate a potential protective efficacy and the cost-management involved in this study. As stated during the 9th International Congress on Paratuberculosis, held in Tsukuba Japan, in November 2007, diagnostic tests (and tests used to evaluate vaccines) need to be more standardized and optimized for their sensitivity and specificity. With respect to this, Hines *et al.* have reported in detail on experimental challenge models for Johne's disease and proposed international guidelines [18].

Five-year view

Molecular and immunological tools will give us more insight into the immune processes involved in the control of MAP infection and the eventual progression to clinical Johne's disease. The characterization of immunodominant MAP-specific antigens will also be expanded and lead to the development of specific and early diagnostic tests and, possibly, of noninterfering Th1-inducing (protein or plasmid-based) subunit vaccines. Characterization of novel, latency-associated antigens from the MAP stressome may prove to be a useful strategy.

In view of the high prevalence of MAP infection, the 'tuberculin test and cull' strategy applied for the control of bovine tuberculosis is currently not a realistic option for paratuberculosis, particularly in light of the existence of possible wildlife reservoirs. Finally, more insight will be generated with respect to the possible involvement of MAP in Crohn's disease, and this may give a further impetus to the funding of research into better MAP vaccines, for domestic and nondomestic ruminants.

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Key issues

- Paratuberculosis, or Johne's disease, is a chronic granulomatous enteritis of the small intestine, affecting cattle, sheep and goats and also nondomestic ruminants, such as deer and bison.
- *Mycobacterium avium* subsp. *paratuberculosis* (MAP) may be one of the bacterial triggers involved in the development of Crohn's disease.
- The disease is transmitted by the fecal–oral route when young animals ingest feces – or milk contaminated with feces – from MAP-shedding adult animals.
- Initially, the infection is controlled by an effective Th1-type immune response, which decreases upon progression to the clinical stage of the disease. MAP-specific antibodies appear during the clinical stage.
- Existing vaccines composed of killed or live-attenuated whole bacteria delay the fecal shedding and progression to clinical disease, but do not protect against the infection.
- These whole-cell-based vaccines interfere with existing diagnostic tests of bovine tuberculosis (tuberculin skin testing) and with the serodiagnosis of paratuberculosis.
- Recently, a number of immunodominant B- and T-cell antigens specific for MAP have been identified.
- The combination of a sensitive species-specific immune diagnosis (serology or T-cell based) with a marked rationally attenuated or subunit vaccine may, ultimately, lead to an efficient immunoprophylaxis of Johne's disease.

References

Papers of special note have been highlighted as:

- • of interest
- •• of considerable interest

1 Harris NB, Barletta RG. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clin. Microbiol. Rev.* 14(3), 489–512 (2001).

•• **Good starter course.**

2 Hasanova L, Pavlik I. Economic impact of paratuberculosis in dairy cattle herds: a review. *Vet. Med. Czech.* 51, 193–211 (2006).

3 Sweeney RW. Transmission of paratuberculosis. *Vet. Clin. North Am. Food Anim. Pract.* 12(2), 305–512 (1996).

4 Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW. Johne's disease in Canada. Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Can. Vet. J.* 47(9), 874–882 (2006).

5 Buergelt C, Williams E. *In utero* infection of pregnant cattle by *Mycobacterium avium* subspecies *paratuberculosis* detected by nested polymerase chain reaction. *Intern. J. Appl. Res. Vet. Med.* 1, 4 (2003).

6 Buergelt CD, Donovan GA, Williams JE. Identification of *Mycobacterium avium* subsp. *paratuberculosis* by polymerase chain reaction in blood and semen of a bull with clinical paratuberculosis. *Intern. J. Appl. Res. Vet. Med.* 2(2), 130–134 (2004).

7 Whittington R, Marshall D, Nicholls P, Marsh I, Reddacliff L. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Appl. Environ. Microbiol.* 70(5), 2989–3004 (2004).

8 Chacon O, Bermudez LE, Barletta RG. Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. *Annu. Rev. Microbiol.* 58, 329–363 (2004).

•• **Provides a clear picture of a controversial issue.**

9 Bull TJ, McMinn EJ, Sidi-Boumedine K *et al*. Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J. Clin. Microbiol.* 41(7), 2915–2923 (2003).

10 Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet* 364(9439), 1039–1044 (2004).

11 Economou M, Pappas G. New global map of Crohn's disease: genetic, environmental, and socioeconomic correlations. *Inflamm. Bowel Dis.* 14(5), 709–720 (2008).

12 Whitlock RH, Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet. Clin. North Am. Food Anim. Pract.* 12(2), 345–356 (1996).

13 Rideout B, Brown S, Davis WC *et al*. *Diagnosis and Control of Johne's Disease*. The National Academies Press, Washington, DC, USA (2003).

14 Chiodini RJ, Van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet.* 74(3), 218–262 (1984).

15 Feola RP, Collins MT, Czuprynski CJ. Hormonal modulation of phagocytosis and intracellular growth of *Mycobacterium avium* ss. *paratuberculosis* in bovine peripheral blood monocytes. *Microb. Pathog.* 26(1), 1–11 (1999).

16 Kennedy DJ, Benedictus G. Control of *Mycobacterium avium* subsp. *paratuberculosis* infection in agricultural species. *Rev. Sci. Tech.* 20(1), 151–179 (2001).

17 Larsen AB, Merkal RS, Cutlip RC. Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *Am. J. Vet. Res.* 36(3), 255–257 (1975).

18 Hines II ME, Stabel JR, Sweeney RW *et al*. Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Vet. Microbiol.* 122(3–4), 197–222 (2007).

•• **Reviews experimental infection models for Johne's disease, important for harmonization of future vaccine studies.**

19 Morein B, Blomqvist G, Hu K. Immune responsiveness in the neonatal period. *J. Comp. Pathol.* 137(Suppl. 1), S27–S31 (2007).

20 Morein B, Abusugra I, Blomqvist G. Immunity in neonates. *Vet. Immunol Immunopathol.* 87(3–4), 207–213 (2002).

21 Pravieux JJ, Poulet H, Charreyre C, Juillard V. Protection of newborn animals through maternal immunization. *J. Comp. Pathol.* 137(Suppl. 1), S32–S34 (2007).

22 Barrington GM, Parish SM. Bovine neonatal immunology. *Vet. Clin. North Am. Food Anim. Pract.* 17(3), 463–476 (2001).

23 Moore M, Tyler JW, Chigerwe M, Dawes ME, Middleton JR. Effect of delayed colostrum collection on colostral IgG concentration in dairy cows. *J. Am. Vet. Med. Assoc.* 226(8), 1375–1377 (2005).

24 Kelleher SL, Lonnerdal B. Immunological activities associated with milk. *Adv. Nutr. Res.* 10, 39–65 (2001).

25 Tuboly S, Bernath S. Intestinal absorption of colostral lymphoid cells in newborn animals. *Adv. Exp. Med. Biol.* 503, 107–114 (2002).

26 Reddacliff LA, Beh K, McGregor H, Whittington RJ. A preliminary study of possible genetic influences on the susceptibility of sheep to Johne's disease. *Aust. Vet. J.* 83(7), 435–441 (2005).

27 Roupie V, Rosseels V, Piersoel V, Zinniel DK, Barletta RG, Huygen K. Genetic resistance of mice to *Mycobacterium paratuberculosis* is influenced by *Slc11a1* at the early but not at the late stage of infection. *Infect. Immun.* 76(5), 2099–2105 (2008).

28 Mortensen H, Nielsen SS, Berg P. Genetic variation and heritability of the antibody response to *Mycobacterium avium* subspecies *paratuberculosis* in Danish Holstein cows. *J. Dairy Sci.* 87(7), 2108–2113 (2004).

29 Gonda MG, Kirkpatrick BW, Shook GE, Collins MT. Identification of a QTL on BTA20 affecting susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* infection in US Holsteins. *Anim. Genet.* 38(4), 389–396 (2007).

30 Sigurethardottir OG, Valheim M, Press CM. Establishment of *Mycobacterium avium* subsp. *paratuberculosis* infection in the intestine of ruminants. *Adv. Drug Deliv. Rev.* 56(6), 819–834 (2004).

31 Sigurdardottir OG, Bakke-McKellep AM, Djonne B, Evensen O. *Mycobacterium avium* subsp. *paratuberculosis* enters the small intestinal mucosa of goat kids in areas with and without Peyer's patches as demonstrated with the everted sleeve method. *Comp. Immunol. Microbiol. Infect. Dis.* 28(3), 223–230 (2005).

32 Kuehnel MP, Goethe R, Habermann A *et al*. Characterization of the intracellular survival of *Mycobacterium avium* ssp. *paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cell Microbiol.* 3(8), 551–566 (2001).

33 Woo SR, Heintz JA, Albrecht R, Barletta RG, Czuprynski CJ. Life and death in bovine monocytes: the fate of *Mycobacterium avium* subsp. *paratuberculosis*. *Microb. Pathog.* 43(2–3), 106–113 (2007).

34 Chiang SK, Sommer S, Aho AD *et al*. Relationship between *Mycobacterium avium* subspecies *paratuberculosis*, IL-1α, and TRAF1 in primary bovine monocyte-derived macrophages. *Vet. Immunol. Immunopathol.* 116(3–4), 131–144 (2007).

35 Corpa JM, Garrido J, Garcia Marin JF, Perez V. Classification of lesions observed in natural cases of paratuberculosis in goats. *J. Comp. Pathol.* 122(4), 255–265 (2000).

36 Buergelt CD, Hall C, McEntee K, Duncan JR. Pathological evaluation of paratuberculosis in naturally infected cattle. *Vet. Pathol.* 15(2), 196–207 (1978).

37 Waters WR, Miller JM, Palmer MV *et al*. Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infect. Immun.* 71(9), 5130–5138 (2003).

38 Bannantine JP, Bayles DO, Waters WR, Palmer MV, Stabel JR, Paustian ML. Early antibody response against *Mycobacterium avium* subspecies paratuberculosis antigens in subclinical cattle. *Proteome Sci.* 6, 5 (2008).

39 Coussens PM. Model for immune responses to *Mycobacterium avium* subspecies *paratuberculosis* in cattle. *Infect. Immun.* 72(6), 3089–3096 (2004).

40 Koo HC, Park YH, Hamilton MJ *et al*. Analysis of the immune response to *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected calves. *Infect. Immun.* 72(12), 6870–6883 (2004).

41 Stabel JR. Production of γ-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. *J. Vet. Diagn. Invest.* 8(3), 345–350 (1996).

42 Begg DJ, Griffin JF. Vaccination of sheep against *M. paratuberculosis*: immune parameters and protective efficacy. *Vaccine* 23(42), 4999–5008 (2005).

• **Comprehensive study of immune responses developed in sheep during vaccination and infection.**

43 Hostetter J, Zhang W, Simutis F. *Mycobacterium avium* subspecies *paratuberculosis* infection of cattle does not diminish peripheral blood-derived macrophage mycobactericidal activity. *Immunol. Lett.* 107(1), 76–79 (2006).

44 Simutis FJ, Cheville NF, Jones DE. Investigation of antigen-specific T-cell responses and subcutaneous granuloma development during experimental sensitization of calves with *Mycobacterium avium* subsp *paratuberculosis*. *Am. J. Vet. Res.* 66(3), 474–482 (2005).

45 Uzonna JE, Chilton P, Whitlock RH, Habecker PL, Scott P, Sweeney RW. Efficacy of commercial and field-strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* 21(23), 3101–3109 (2003).

46 Hasvold HJ, Valheim M, Berntsen G, Storset AK. *In vitro* responses to purified protein derivate of caprine T lymphocytes following vaccination with live strains of *Mycobacterium avium* subsp *paratuberculosis*. *Vet. Immunol. Immunopathol.* 90(1–2), 79–89 (2002).

47 Beveridge NE, Price DA, Casazza J *et al*. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific $CD4^+$ memory T lymphocyte populations. *Eur. J. Immunol.* 37, 3089–3100 (2007).

48 Coussens PM. *Mycobacterium paratuberculosis* and the bovine immune system. *Anim. Health Res. Rev.* 2(2), 141–161 (2001).

49 Beard PM, Rhind SM, Sinclair MC *et al*. Modulation of γδ T cells and CD1 in *Mycobacterium avium* subsp. *paratuberculosis* infection. *Vet. Immunol. Immunopathol.* 77(3–4), 311–319 (2000).

50 Simutis FJ, Jones DE, Hostetter JM. Failure of antigen-stimulated γδ T cells and $CD4^+$ T cells from sensitized cattle to upregulate nitric oxide and mycobactericidal activity of autologous *Mycobacterium avium* subsp. *paratuberculosis*-infected macrophages. *Vet. Immunol. Immunopathol.* 116(1–2), 1–12 (2007).

51 Tanaka S, Itohara S, Sato M, Taniguchi T, Yokomizo Y. Reduced formation of granulomata in γδ T cell knockout BALB/c mice inoculated with *Mycobacterium avium* subsp. *paratuberculosis*. *Vet. Pathol.* 37(5), 415–421 (2000).

52 Adams JL, Collins MT, Czuprynski CJ. Polymerase chain reaction analysis of TNF-α and IL-6 mRNA levels in whole blood from cattle naturally or experimentally infected with *Mycobacterium paratuberculosis*. *Can. J. Vet. Res.* 60(4), 257–262 (1996).

53 Alzuherri HM, Woodall CJ, Clarke CJ. Increased intestinal TNF-α, IL-1 β and IL-6 expression in ovine paratuberculosis. *Vet. Immunol. Immunopathol.* 49(4), 331–345 (1996).

54 Coussens PM, Verman N, Coussens A, McNulty AM. Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with *Mycobacterium avium* subsp *paratuberculosis* evidence for inherent proinflammatory gene expression pattern. *Infect. Immun.* 72, 1409–1422 (2004).

55 Khalifeh MS, Stabel JR. Effects of γ interferon, interleukin-10, and transforming growth factor β on the survival of *Mycobacterium avium* subsp. *paratuberculosis* in monocyte-derived macrophages from naturally infected cattle. *Infect. Immun.* 72, 1974–1982 (2004).

56 Weiss DJ, Evanson OA, Souza CD. Expression of interleukin-10 and suppressor of cytokine signaling-3 associated with susceptibility of cattle to infection with *Mycobacterium avium* subsp *paratuberculosis*. *Am. J. Vet. Res*. 66, 1114–1120 (2005).

57 Buza JJ, Hikono H, Mori Y *et al*. Neutralization of interleukin-10 significantly enhances γ interferon expression in peripheral blood by stimulation with Johnin purified protein derivative and by infection with *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected cattle with paratuberculosis. *Infect. Immun.* 72(4), 2425–2428 (2004).

58 Weiss DJ, Evanson OA, Souza CD. Mucosal immune response in cattle with subclinical Johne's disease. *Vet. Pathol.* 43(2), 127–135 (2006).

59 Koets A, Rutten V, Hoek A *et al*. Progressive bovine paratuberculosis is associated with local loss of $CD4^+$ T cells, increased frequency of γδ T cells, and related changes in T-cell function. *Infect. Immun.* 70(7), 3856–3864 (2002).

60 Stabel JR. Cytokine secretion by peripheral blood mononuclear cells from cows infected with *Mycobacterium paratuberculosis*. *Am. J. Vet. Res.* 61(7), 754–760 (2000).

61 Vallée H, Rinjard P. Etudes sur l'entérite paratuberculeuse des bovidés. *Res. Gen. Med. Vet.* 35(1) (1926).

62 Bakker D, Willemsen PT, van Zijderveld FG. Paratuberculosis recognized as a problem at last: a review. *Vet. Q.* 22(4), 200–204 (2000).

63 Gilmour NJ. The pathogenesis, diagnosis and control of Johne's disease. *Vet. Rec.* 99(22), 433–434 (1976).

64 Chiodini RJ. Abolish *Mycobacterium paratuberculosis* strain 18. *J. Clin. Microbiol.* 31(7), 1956–8 (1993).

65 Spangler E, Heider LE, Bech-Nielsen S, Dorn CR. Serologic enzyme-linked immunosorbent assay responses of calves vaccinated with a killed *Mycobacterium paratuberculosis* vaccine. *Am. J. Vet. Res.* 52(8), 1197–1200 (1991).

66 Nedrow AJ, Gavalchin J, Smith MC *et al*. Antibody and skin-test responses of sheep vaccinated against Johne's Disease. *Vet. Immunol. Immunopathol.* 116(1–2), 109–112 (2007).

67 Wentink GH, Bongers JH, Zeeuwen AA, Jaartsveld FH. Incidence of paratuberculosis after vaccination against *M. paratuberculosis* in two infected dairy herds. *J. Vet. Med. B*. 41(7–8), 517–522 (1994).

68 Muskens J, van Zijderveld F, Eger A, Bakker D. Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. *Vet. Microbiol.* 86(3), 269–278 (2002).

69 Corpa JM, Perez V, Garcia Marin JF. Differences in the immune responses in lambs and kids vaccinated against paratuberculosis, according to the age of vaccination. *Vet. Microbiol.* 77(3–4), 475–485 (2000).

70 Reddacliff L, Eppleston J, Windsor P, Whittington R, Jones S. Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. *Vet. Microbiol.* 115(1–3), 77–90 (2006).

71 Windsor P. Research into vaccination against ovine Johne's disease in Australia. *Small Rum. Res.* 62, 139–142 (2006).

72 Kormendy B. The effect of vaccination on the prevalence of paratuberculosis in large dairy herds. *Vet. Microbiol.* 41(1–2), 117–125 (1994).

73 Kohler H, Gyra H, Zimmer K *et al.* Immune reactions in cattle after immunization with a *Mycobacterium paratuberculosis* vaccine and implications for the diagnosis of *M. paratuberculosis* and *M. bovis* infections. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* 48(3), 185–195 (2001).

74 Molina JM, Anguiano A, Ferrer O. Study on immune response of goats vaccinated with a live strain of *Mycobacterium paratuberculosis*. *Comp. Immunol. Microbiol. Infect. Dis.* 19(1), 9–15 (1996).

75 Begg DJ, O'Brien R, Mackintosh CG, Griffin JF. Experimental infection model for Johne's disease in sheep. *Infect. Immun.* 73(9), 5603–5611 (2005).

76 Juste RA, Garcia Marin JF, Peris B, Saez de Ocariz CS, Badiola JJ. Experimental infection of vaccinated and non-vaccinated lambs with *Mycobacterium paratuberculosis*. *J. Comp. Pathol.* 110(2), 185–194 (1994).

77 Mackintosh CG, Labes RE, Griffin JF. The effect of Johne's vaccination on tuberculin testing in farmed red deer (*Cervus elaphus*). *NZ Vet. J.* 53(4), 216–222 (2005).

78 Gwozdz JM, Thompson KG, Manktelow BW, Murray A, West DM. Vaccination against paratuberculosis of lambs already infected experimentally with *Mycobacterium avium* subspecies *paratuberculosis*. *Aust. Vet. J.* 78(8), 560–566 (2000).

79 Saxegaard F, Fodstad FH. Control of paratuberculosis (Johne's disease) in goats by vaccination. *Vet. Rec.* 116(16), 439–441 (1985).

80 Munoz M, Garcia Marin JF, Garcia-Pariente C *et al.* Efficacy of a killed vaccine (Silirum) in calves challenged with MAP. In: *Eighth International Colloquium on Paratuberculosis*. Manning EJ, Nielsen SS (Eds). International Association for Paratuberculsois, Inc., Copenhagen, Denmark 208–217 (2005).

81 Garrido JM, Geijo MV, Molina E *et al.* Preliminary evaluation of a field trial on the use of vaccination in dairy cattle farms with paratuberculosis. In: *Nine International Colloquium on Paratuberculosis*. Nielsen SS (Ed.). International Association for Paratuberculsois Inc., Tsukuba, Japan 204–207 (2007).

82 Park AY, Scott P. Il-12: keeping cell-mediated immunity alive. *Scand. J. Immunol.* 53(6), 529–532 (2001).

83 Hines ME 2nd, Stiver S, Giri D *et al.* Efficacy of spheroplastic and cell-wall competent vaccines for *Mycobacterium avium* subsp. *paratuberculosis* in experimentally-challenged baby goats. *Vet. Microbiol.* 120(3–4), 261–283 (2007).

84 Singh SV, Singh PK, Singh AV, Sohal JS, Gupta VK, Vihan VS. Comparative efficacy of an indigenous 'inactivated vaccine' using highly pathogenic field strain of *Mycobacterium avium* subspecies *paratuberculosis* 'bison type' with a commercial vaccine for the control of Capri-paratuberculosis in India. *Vaccine* 25(41), 7102–7110 (2007).

85 Emery DL, Whittington RJ. An evaluation of mycophage therapy, chemotherapy and vaccination for control of *Mycobacterium avium* subsp. *paratuberculosis* infection. *Vet. Microbiol.* 104(3–4), 143–155 (2004).

86 Meeusen EN, Walker J, Peters A, Pastoret PP, Jungersen G. Current status of veterinary vaccines. *Clin. Microbiol. Rev.* 20(3), 489–510 (2007).

87 Li L, Bannantine JP, Zhang Q *et al.* The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc. Natl Acad. Sci. USA* 102(35), 12344–12349 (2005).

•• **Complete sequence of the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) K-10 genome.**

88 Andersen P, Gicquel B, Huygen K. Tuberculosis vaccine science. In: *Tuberculosis Science (2nd Edition).* Rom WN, Garay SM (Eds). Lippincott Williams & Wilkins, PA, USA 885–898 (2004).

89 McShane H, Pathan AA, Sander C *et al.* Recombinant modified Vaccinia Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat. Med.* 10, 1240–1244 (2004).

90 Rosseels V, Marche S, Roupie V *et al.* Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infect. Immun.* 74(1), 202–212 (2006).

91 Shin SJ, Chang CF, Chang CD *et al.* *In vitro* cellular immune responses to recombinant antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infect. Immun.* 73(8), 5074–5085 (2005).

92 Mullerad J, Michal I, Fishman Y, Hovav AH, Barletta RG, Bercovier H. The immunogenicity of *Mycobacterium paratuberculosis* 85B antigen. *Med. Microbiol. Immunol.* 190(4), 179–187 (2002).

93 Rosseels V, Scanlan V, Vanonckelen A *et al.* Development of plasmid DNA based *Mycobacterium avium* subsp. *paratuberculosis* vaccine encoding immunodominant T cell antigens identified in mycobacterial culture filtrate. In: *Seventh International Colloquium on Paratuberculosis*. Juste RA, Geijo MV, Garrido JBM (Eds). International Association for Paratuberculosis Inc., Bilbao, Spain 108–113 (2002).

94 Rosseels V, Roupie V, Zinniel DK, Barletta RG, Huygen K. Development of luminescent *Mycobacterium avium* subsp. *paratuberculosis* for rapid screening of vaccine candidates in mice. *Infect. Immun.* 74(6), 3684–3686 (2006).

95 Koets AP, Rutten VP, Hoek A *et al.* Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis. *Vet. Immunol. Immunopathol.* 70(1–2), 105–115 (1999).

96 Dupont C, Thompson K, Heuer C, Gicquel B, Murray A. Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subsp *paratuberculosis*. *J. Med. Microbiol.* 54(Pt 11), 1083–1092 (2005).

97 Rigden RC, Jandhyala DM, Dupont C *et al.* Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *J. Med. Microbiol.* 55(Pt 12), 1735–1740 (2006).

98 Huntley JF, Stabel JR, Bannantine JP. Immunoreactivity of the *Mycobacterium avium* subsp. *paratuberculosis* 19-kDa lipoprotein. *BMC Microbiol.* 5(1), 3 (2005).

99 Nagata R, Muneta Y, Yoshihara K, Yokomizo Y, Mori Y. Expression cloning of γ interferon-inducing antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infect. Immun.* 73(6), 3778–3782 (2005).

100 Vipond J, Vipond R, Allen-Vercoe E *et al.* Selection of novel TB vaccine candidates and their evaluation as DNA vaccines against aerosol challenge. *Vaccine* 24, 37–39 (2006).

101 Hinchey J, Lee S, Jeon BY *et al.* Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. *J. Clin. Invest.* 117(8), 2279–2288 (2007).

102 Mullerad J, Hovav AH, Fishman Y, Barletta RG, Bercovier H. Antigenicity of *Mycobacterium paratuberculosis* superoxide

dismutase in mice. *FEMS Immunol. Med. Microbiol.* 34(1), 81–88 (2002).

103 Olsen I, Storset AK. Innate IFN-γ production in cattle in response to MPP14, a secreted protein from *Mycobacterium avium* subsp. *paratuberculosis*. *Scand. J. Immunol.* 54(3), 306–313 (2001).

104 Olsen I, Reitan LJ, Holstad G, Wiker HG. Alkyl hydroperoxide reductases C and D are major antigens constitutively expressed by *Mycobacterium avium* subsp. *paratuberculosis*. *Infect. Immun.* 68(2), 801–808 (2000).

105 Shin SJ, Wu CW, Steinberg H, Talaat AM. Identification of novel virulence determinants in *Mycobacterium paratuberculosis* by screening a library of insertional mutants. *Infect. Immun.* 74, 3825–3833 (2006).

106 Park KT, Dahl JL, Bannantine JP *et al.* Demonstration of allelic exchange in the slow growing *Mycobacterium avium* subsp. *paratuberculosis* and the generation of deletion mutants at the *pknG, relA* and *lsr2* loci. *Appl. Environ. Microbiol.* 74(6), 1687–1695 (2008).

•• **First report on targeted generation of deletion mutants of MAP by allelic exchange.**

107 Wu C-W, Schmoller SK, Shin SJ, Talaat AM. Defining the stressome of *Mycobacterium avum* subsp. *paratuberculosis in vitro* and in naturally infected cows. *J. Bact.* 189(21), 7877–7886 (2007).

•• **Transcriptional profiling of MAP exposed to different stress conditions.**

108 Leyten EM, Lin MY, Franken KL *et al.* Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect.* 8(8), 2052–2060 (2006).

109 Roupie V, Romano M, Zhang L *et al.* Immunogenicity of eight dormancy (DosR) regulon encoded proteins of *Mycobacterium tuberculosis* in DNA vaccinated and tuberculosis-infected mice. *Infect. Immun.* 75, 941–949 (2007).

110 Huygen K. Plasmid DNA vaccination. *Microbes Infect.* 7(5–6), 932–938 (2005).

111 Huygen K. DNA vaccines against mycobacterial diseases. *Future Microbiol.* 1, 63–73 (2006).

112 Huntley JF, Stabel JR, Paustian ML, Reinhardt TA, Bannantine JP. Expression library immunization confers protection against *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infect. Immun.* 73(10), 6877–6884 (2005).

113 Leroy B, Roupie V, Noel-Georis I *et al.* Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. *Proteomics* 7(7), 1164–1176 (2007).

114 Roupie V, Leroy B, Rosseels V *et al.* Immunogenicity and protective efficacy of DNA vaccines encoding MAP specific antigens MAP0586c and MAP4308c of *Mycobacterium avium* subsp. *paratuberculosis* in mice. *Vaccine* (2008) (In Press).

115 Bull TJ, Gilbert SC, Sridhar S *et al.* A novel multi-antigen virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis*. *PLoS ONE* 2(11), e1229 (2007).

116 Grieg A, Stevenson K, Hendserson D *et al.* Epidemiological study of paratuberculosis in wild rabbits in Scotland. *J. Clin. Microbiol.* 37, 1746–1751 (1999).

117 Mokresh AH, Czuprynski CJ, Butler DG. A rabbit model for study of *Mycobacterium paratuberculosis* infection. *Infect. Immun.* 57(12), 3798–3807 (1989).

118 Vaughan JA, Lenghaus C, Stewart DJ, Tizard ML, Michalski WP. Development of a Johne's disease infection model in laboratory rabbits following oral adminsitration of *Mycobacetrium avium* subsp *paratuberculosis*. *Vet. Microbiol.* 105, 207–213 (2005).

119 Sechi LA, Mara L, Cappai P *et al.* Immunization with DNA vaccines encoding different mycobacterial antigens elicits a Th1 type immune response in lambs and protects against *Mycobacterium avium* subspecies *paratuberculosis* infection. *Vaccine* 24(3), 229–235 (2006).

•• **First report on DNA vaccination against paratuberculosis in sheep.**

120 Koets A, Hoek A, Langelaar M *et al.* Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. *Vaccine* 24(14), 2550–2559 (2006).

•• **First report on subunit protein vaccination against paratuberculosis in cattle.**

121 Begg DJ, Whittington RJ. Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. *Vet. J.* 176(2), 129–145 (2008).

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