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Review

Status of the development of a vaccine against *Mycoplasma bovis* ☆

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ABSTRACT

Mycoplasma bovis is an important pathogen of cattle and, despite numerous efforts an effective vaccine for control of the disease it causes remains elusive. Although we now know more about the biology of this pathogen, information is lacking about appropriate protective antigens, the type of immune response that confers protection and adjuvants selection. The use of conserved recombinant proteins, selected using in silico approaches, as components of a vaccine may be a better choice over bacterin-based vaccines due to the limited protection afforded by them and adverse reactions caused by them. More studies are needed on the characterization of host-pathogen interactions and to elucidate *M. bovis* products modulating these interactions. These products could be the basis for development of vaccines to control *M. bovis* infections in dairy farms and feedlots.

Contents

1. Introduction

Mycoplasma bovis is the causative agent of numerous diseases in cattle that have severe economic consequences for producers. The Chronic Pneumonia and Poly-arthritis Syndrome (CPPS) caused by *M. bovis* is associated with the Bovine Respiratory Disease (BRD) complex, an economically important disease in feedlot cattle [1]. In dairy cattle, *M. bovis* is probably the most common causative agent of mycoplasma mastitis although other mycoplasma species have been isolated from the milk of affected animals [2,3]. As a sequela of infection with *M. bovis*, arthritis and otitis media is sometimes observed in beef and dairy cattle. Affected animals present with clinical signs such as lameness, swelling of joints and ultimately weight loss as a consequence of impaired movement [1]. Keratoconjunctivitis, orchitis, infertility and decubital abscesses have been reported at lower frequency [1,4]. In a recent report, Gille et al. described post-surgical seromas as a new predilection site for *M. bovis* infections [5].

Due to their lack of a cell wall, the antibiotic arsenal available to treat *M. bovis* infections is limited, and numerous reports indicate that resistance to several antibiotics is on the rise (reviewed in [6]), compounding this problem, the cost of multiple antibiotic treatments adds considerable financial burden to the producer. This suggests that prevention and/or control of *M. bovis* infection by vaccination would be a valuable alternative. Research on *M. bovis* vaccines has been active for many years and this review is focused on the many vaccine candidate antigens identified so far; and the results of testing numerous experimental vaccines composed of bacterins, recombinant proteins, or live-attenuated strains.

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2. The challenges

There are numerous challenges that hinder the success of vaccines to prevent *M. bovis* infections. Among these challenges are colonization of young animals' upper respiratory tract, quality and modulation of the host immune response, the role of other respiratory pathogens, and the need for a challenge model that reproduces the disease seen in the field. The upper respiratory tract of young animals is colonized at a very early age by contact with shedding animals and also by ingestion of contaminated milk (reviewed in [4]). Adhesion to epithelial cells aids in the colonization but the adhesion capacity varies between isolates [7]. The variable-surface proteins (Vsps) and other surface proteins have been associated with attachment to host cells [7,8]. One complicating factor is that the capacity of Vsps for phase and antigenic variation [9]. The host-immune responses to *M. bovis* after natural or experimental infection provide important information that may help in designing a successful vaccine. In general, the immune response to *M. bovis* antigens is skewed to the Th-2 arm as they induce more IgG1 than IgG2 antibodies [10–12]. Activation of CD4+, CD8+ and γ/δ T-cells has been observed in response to heat-killed *M. bovis* [11], but not to live bacterial cells [13–17]. Although *M. bovis* is predominantly an extracellular pathogen, there is *in vivo* and *in vitro* evidence that suggests the potential for the bacterium to enter host cells [18–22]. Intracellular *M. bovis* can survive inside cells [13,20,21]; modulate cytokine expression [13,23–26] and apoptosis [13,20,27]; or directly play a role in pathogenesis [28]. Thus, because of the extra- and intracellular presence of *M. bovis* we believe that a vaccine that equally induces Th-1 and Th-2 responses would be more advantageous.

The current evidence strongly suggests that in cattle *M. bovis* is a secondary pathogen and that the contribution of other respiratory pathogens must be considered [29]. Immuno-suppressive viral pathogens such as bovine viral diarrhea virus (BVDV) and bovine herpes virus 1 (BHV-1) have long been associated with *M. bovis* respiratory disease in Canadian feedlots [30–34]. In 2014, Klima et al. reported the prevalence of BRD-associated pathogens in 68 cases in North American feedlots [35]. While *Mannheimia haemolytica* and BVDV-1 were the two pathogens most prevalent (91% and 69% respectively), the proportion of *M. bovis* detected by PCR was of 43%. The presence of BVDV-2 was only detected in Canadian animals however BHV-1 was not found. The authors suggest that this was due to limited sampling size and/or absence of viral DNA in the nasal mucus. The highest co-occurrence (16.2%) of pathogens was the combination of *Mannheimia* spp., BVDV-1 and *M. bovis*. In 11.2% of the cases, these three pathogens were also found with *Histophilus somni*. Finally, *Pasteurella multocida* was isolated from few samples. Thus, preventive measures against disease caused by *M. bovis* must take in consideration management of other respiratory pathogens by antimicrobial treatment and/or vaccination.

Testing of vaccine candidates greatly depends on the use of a challenge model that consistently reproduces the disease. Factors such as the age of the animals, the challenge dose, the challenge protocol and the role of other respiratory pathogens must be taken into account. A number of laboratories have reported success of their experimental vaccines after multiple challenges (up to three times) of young animals (ranging from 3 weeks to 5 months-old) with large doses of *M. bovis* (in the range of 10^9 to 10^{10} colony-forming units [cfu]) [36–38]. In these reports, the success of the challenges is associated with the onset of clinical signs such as dyspnea, nasal discharge, moderate fever, weight loss, the presence of characteristic macroscopic lesions, such as lung consolidation, adhesions, and caseonecrotic pneumonic lesions; microscopic lung lesions, such as suppurative bronchiolitis, lymphoid hyperplasia, intra-alveolar and intrabronchial exudates, and coagulative necrosis, and isolation of *M. bovis* from challenged animals. The clinical signs, gross and microscopic lung lesions, and isolation of *M. bovis* are consistent with the lesions seen in the feedlot animals. However the magnitude of these lesions, particularly the extent of lung involvement, and the number of caseonecrotic lesions and the degree of suppurative pneumonia [34,40,41] is less than seen in field cases. This could be due to the lesion age in feedlot animals or to the contribution of other respiratory pathogens.

In all these trials, the vaccines were solely tested against a *M. bovis* challenge but the role that other pathogens may have in the success or failure of the vaccines was not taken into account. Because of the association of *M. bovis* with other respiratory pathogens (see above), we wanted to establish a co-challenge model to test experimental vaccines. In 6 to 8 month-old Canadian feedlot cattle, a single intranasal challenge (5×10^8 cfu/ml) was sufficient to cause disease in animals previously exposed to BHV-1 [39]. We did not see disease in animals challenged with *M. bovis* only (intratracheal dose of 5×10^{10} cfu/ml) or in animals previously infected with BVDV-2 [39]. In this co-challenge model, the magnitude of the lesions more closely resembled the lesions seen in the feedlot animals [34,40,41].

3. *Mycoplasma bovis* vaccine candidates

3.1. Protein vaccine candidates

M. bovis cells display highly variable antigens on their surface. The most prominent of these are the variable surface proteins (Vsps). The Vsp family is composed of 13 lipoproteins that can generate a high degree of antigenic variation through genetic recombination [42,43]. Of the 13 Vsps identified, VspA, VspB and VspC are the most immunogenic [44] and thus they may be ideal targets for vaccines. However the high degree of antigenic variation in these lipoproteins may make the vaccines ineffective in the long run. Epitope mapping of the VspA, VspB, VspE and VspF proteins has identified several regions that are involved in adherence to embryonic bovine lung (EBL) cells [44]. The authors pointed out that because these epitopes were linear they may not be ideal targets for vaccines and as an alternative they suggested DNA vaccination with plasmids containing epitopes from variable and non-variable regions. To date, it is not clear whether such a DNA vaccination approach has been assessed. The surface expressed α-enolase protein of *M. bovis* has been characterized [45]. Its surface expression and binding to plasminogen combined with the fact that α-enolase of *Streptococcus iniae* has been shown to be protective in mice and zebra fish models [46,47], suggests that it has potential target for vaccine development in *M. bovis* but as yet, there have been no reports of the assessment of α -enolase in vaccine trials.

Numerous *M. bovis* proteins have been studied to evaluate their role in adherence. Sachse et al. described the capacity of a mAb against a 26 kDa *M. bovis* protein to inhibit adherence to EBL cells [48]. The mAb Mb4F6 was incubated with two strains of *M. bovis* that had differing adherence intensity. The mAb Mb4F6 more strongly inhibited adherence of the strain 454 than of the more adherent *M. bovis* strain 120. The strain 454 expressed less of the 26 kDa protein than the strain 120 suggesting that more mAb was able to bind to strain 454, resulting in more inhibition [48]. The identity of the 26 kDa protein remains unknown, but conceptually it could be used as a potential vaccine target.

Due to the high level of antigenic variation in *M. bovis*, the best vaccine targets are likely to be proteins that are conserved across strains. One example of such a protein is lipoprotein P48. Robino et al. reported that the P48 protein was detectable in all field isolates tested [49]. Compared to uninfected animals, antibody

responses to recombinant P48 were observed in greater magnitude in serum from animals that were either naturally exposed to or challenged with *M. bovis*. The authors proposed the use of P48 as a diagnostic marker, but not as a vaccine target. One drawback of this approach is that the P48 proteins of *M. agalactiae* and *M. bovis* are very similar so the diagnostic may not be specific where cattle and small ruminants comingle. Years later, Fu et al. developed a competitive ELISA test based on a mAb (10E) against the *M. bovis* P48 protein [50]. Based on the lack of recognition of the *M. agalactiae* P48 protein by the mAb 10E in Western blot assays and the failure to inhibit binding of the mAb 10E to *M. bovis* P48 by a rabbit polyclonal antibody against the *M. agalactiae* P48 protein, the authors concluded that this mAb 10E may be useful in a diagnostic test. Another protein proposed as diagnostic marker for *M. bovis* infection is the E1 β-subunit of the pyruvate dehydrogenase complex [51].

We employed the concept of using conserved proteins as vaccine targets using the *M. bovis* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein. Two different RFLP profiles of *gapC*, the *M. bovis* gene encoding GAPDH, were seen after digestion with *Hae*III or *Hinc*II when analyzing several clinical isolates [52]. Despite the genetic differences, the predicted GAPDH (GapC) protein sequences were the same in all isolates [52]. Beef cattle naturally exposed to *M. bovis* developed antibodies against recombinant GapC [52]. The recombinant *M. bovis* GapC protein fused to host-defence peptides (Gap-I) [17] was used in vaccination and challenge studies. Priming with DNA encoding Gap-I and boosting with recombinant Gap-I protein (rGap-I), and priming and boosting with the rGap-I alone, or in conjunction with *M. bovis* protein extracts, resulted in significant humoral immune responses against the antigens [14]. However vaccinated animals were not protected against challenge with three strains of *M. bovis*, and there was a suggestion of an adverse effect of vaccination as judged by the slightly higher, but not significant, proportion of the lungs with lesions in the groups inoculated with the Gap-I antigen [14]. As a continuation of this conserved protein approach, we assessed the immune responses to ten *M. bovis* proteins, PdhA, PepA, Tuf, P48, P81, OppA, LppA, PepQ, O256, and DeoB [53]. These proteins were all highly conserved, with identities ranging from 98% to 100% across the *M. bovis* strains PG45, HB0801, Hubei-1, and CQ-W70. This suggested that these proteins may be good targets for vaccines. We also formulated vaccines with *M. bovis* membrane fractions and cell extracts. Despite significant humoral immune responses to the antigens, the median proportion of the lung areas showing signs of *M. bovis* infection was 16.15% in the control group and 4.01% in the vaccinated group, a difference that was not significant [13]. Lack of protection using Triton X-114 membrane protein extracts and affinity-purified antigens was also reported elsewhere [54].

3.2. Bacterins

Early attempts at vaccination against *M. bovis* focused on the use of a vaccine composed of formalin-treated *M. bovis*, *M. dispar*, respiratory syncytial virus and parainfluenza type 3 (PI3) virus. This quadrivalent vaccine seemed to be effective against natural outbreaks of BRD, with a level of protection of 77% reported, based on a reduction from 38% to 25% of the proportion of animals that needed treatment for respiratory disease [55]. In another study with the same vaccine, there was a reduction of respiratory disease from 27% to 16.3% in the animals treated [56]. However, there was no direct evidence that this vaccine protected against *M. bovis* infection and it appears that this vaccine was never licensed for use. Later, a formalin-inactivated autogenous vaccine against *Mannheimia haemolytica* and *M. bovis* was capable of reducing the losses and costs associated with cattle pneumonia [57]. The first encouraging results on vaccination against *M. bovis* came from work with the *M. bovis* isolate 86B/96. This strain was treated with saponin and used as a single dose vaccine in 3–4 week-old calves [37]. The animals were challenged on two consecutive days with *M. bovis* strain 5063, a Hungarian isolate. The vaccine was deemed protective as judged by the decrease in lung necrosis, histopathological lesions signs and the number of animals containing *M. bovis*-infected tissues between the vaccinated and un-vaccinated groups [37]. This work showed that protection was possible in young animals using a heterologous challenge. Later, a saponin-inactivated vaccine was tested on one farm experiencing up to 25% mortality due to respiratory disease in three-month-old cattle [58]. A higher proportion of vaccinated animals had to be treated with antibiotics than in unvaccinated animals. The authors concluded that vaccination of cattle showing signs of *M. bovis* disease was not effective [58]. A saponin-inactivated *M. bovis* preparation was combined with a commercial adjuvant, Emulsigen® and tested for its capacity to induce protection. A Polish strain of *M. bovis* (KP795974) was used as a basis of the vaccine, which was administered to three to four-week-old cattle. *M. bovis*-specific antibodies were reported to increase up to 200% of a positive control and after vaccination, the animals were challenged with the same strain [36]. The vaccine was deemed to be protective after comparing clinical signs between the groups, but the pre-challenge clinical findings in the positive control group were not described. In addition, the authors do not explain the reason for increases in the respiratory rate and nasal discharge in the vaccinated group before challenge. Finally, differences in the lung lesions scores were reported for only two animals from each group [36].

Currently there are only two licensed vaccines for prevention of *M. bovis* infections in the USA namely, MpB Guard and Myco-Bac B. Two bacterin-based vaccines, Mycomune® R and Pulmo-Guard™ MpB were tested on veal calves in a controlled trial [59]. The authors inoculated four groups of 50 calves each with adjuvant A, saline solution, or one of the commercial products. The last round of inoculation was performed on the calves when they reached 56 days of age and the animals were followed for another 80 days. The IgM, IgG1, IgG2 and IgA titres were determined and the authors found that only the IgG1 titres continued increasing until the of the trial with the rest of the immunoglobulin isotype titres decreasing not long after the last inoculation [59]. There were no significant differences in the titres induced by the vaccines. At post-mortem, the number of animals lung lesions was 14 in the group inoculated with adjuvant A; 25 in the group inoculated with Mycomune® R, 24 in the group inoculated with saline solution, and 18 in the group inoculated with Pulmo-Guard™ MpB. While compared to adjuvant A, the number of lung lesions was significantly reduced in the animals receiving Mycomune® R, there were no significant differences between these two groups in the number of *M. bovis*-specific lung lesions. In addition, there were no significant differences on the number of total lesions and *M. bovis*-specific lesions between the animals that receive the saline solution and those that received Pulmo-Guard™ MpB [59]. The authors concluded neither of the vaccines was efficacious in reducing the number of *M. bovis* colonizing the upper respiratory track nor in reducing the number of *M. bovis*-specific lesions.

Bovine mastitis caused by *M. bovis* causes results in considerable losses in the dairy industry. The bacterium is transmitted from infected cows and control measures include antimicrobial treatment, segregation and/or culling of infected animals (reviewed in [2]). *M. bovis* can persist in the mammary gland for extended periods possibly, even for more than one milking cycle [60]. After an experimental challenge, milk production per quarter can be as low as 15.4% of the normal level of production in un-vaccinated cows [61]. In animals vaccinated with a *M. bovis* bacterin, the lowest level of production was 15% of normal and remained much

lower than the un-vaccinated group for a few weeks after the challenge [61]. Three rounds of weekly subcutaneous inoculations followed by two weekly rounds of intramammary inoculations resulted in high IgG1 and IgG2 titres in serum. The same IgG1 and IgG2 increases were detected in the milk with IgA titres increasing only after challenge [62]. Despite these increases in the antibody levels, there was no protection against challenge [62]. Cell-mediated immune responses against *M. bovis* were measured and no increase of *M. bovis*-specific lymphocyte proliferation was detectable although circulating lymphocytes from vaccinated animals responded better to other mitogens such as phytohaemagglutinin and concanavalin A. Similar findings were obtained with milk lymphocytes [63]. A trial using Pulmo-Guard™ MpB was carried out to determine the IgG1 levels in milk and colostrum after vaccination [64]. The authors concluded that serum IgG1 titres against *M. bovis* decreased before parturition and higher IgG1 levels were present in milk than in colostrum. However, the study did not determine whether the colostral or milk antibodies protected the animals against mastitis, as the animals were not challenged after vaccination [64]. Thus, in spite of all the considerable amount of work on bacterin-based *M. bovis* vaccines, there is no direct proof that these vaccines are effective under field conditions.

3.3. Live-attenuated vaccines

Live attenuated vaccines are used to control disease caused by a range of mycoplasmas. A list of these vaccines is provided in Table 1. A live-attenuated vaccine against *M. hyopneumoniae* is only available in China [65]. While these vaccines have been licensed and accepted by swine, poultry, and cattle producers in many countries, it remains to be seen whether North American or European producers, especially dairy farmers, would accept this kind of vaccine against *M. bovis*. The Chinese *M. bovis* strain HB0101 (CCTCC #M2010040) has been passaged *in vitro* at 41 °C and two variant strains, P150 and P180 (passages 150 and 180, respectively), were selected [38]. A trial was conducted on 5–6 month-old female cattle to assess protection after immunization with the P150 or P180 strains. The animals were divided into four groups of five - positive control group, a group receiving P150, a group receiving P180, and a negative control group. The animals in the P150 and P180 groups were immunized intranasally with 10 ml cultures of the strains at a dose of 10^8 cfu/ml. The animals were challenged 46 days later on three consecutive days by intra-tracheal inoculation of an exponential-phase culture of HB0101 at a dose of 10^{10} CFU each day. The serum IgG responses to the attenuated strains increased after vaccination but after a brief increase, the IgA responses had reduced by the end of the trial. Gross lung lesions and lung lesion scores were significantly reduced in the groups vaccinated with P150 or P180, with a slightly greatly reduction in the group vaccinated with P150 [38].

4. Conclusions

Research on the development of protective vaccines against *M. bovis* has been active for many years and although there has been some, we still lack information about many areas including the key protective antigens, the type of immune response needed (Th-1, Th-2, Th-17 or a combination of all three), and optimal adjuvant formulations. Some of the bacterin-based vaccines have shown some efficacy, which may be related to the strain for vaccine production. Using an autogenous vaccine may result in better protection in a closed herd, as the antigenic variation shown by *M. bovis* may suggest that a vaccine produced from one isolate may not confer full protection to cattle exposed to other isolates. This is particularly important in feedlot operations, where cattle are sourced from multiple farms with differing biosecurity measures. The use of recombinant proteins that are conserved in all the *M. bovis* isolates is a better option. Traditional approaches to recombinant protein vaccine development based on their reaction to antibodies from exposed cattle may not yield the optimal protective antigens. More advanced approaches, such as reverse vaccinology [66], may be needed as this approach assesses all possible antigens for their potential. Using this approach, we were able to identify recombinant proteins from *Mycoplasma mycoides* subsp. *mycoides* that conferred protection against an experimental challenge [67,68]. The host immune responses to *M. bovis* seem to be skewed towards a humoral (Th-2) response [10,11]. In a recent publication, Th-17 (IL-17) responses against *M. pulmonis* were reported and these responses increased the clearance of *Listeria monocytogenes* [69]. The role of Th-17 responses in protection against *M. bovis* infections remains to be determined.

Adjuvants enhance the immune responses to antigens. Saponin was used as an adjuvant in the first experimental *M. bovis* vaccine that showed promising results [37] and a combination of saponin and Emulsigen® was used in later trials [36], also with encouraging results. The ability of *M. bovis* to invade and persist intracellularly, suggests that balanced Th-1/Th-2 response may be a better choice [70,71]. Numerous research groups have focused recently on studying the interaction of *M. bovis* and its bovine host [13,18–21,23–27]. A strong push from the industry is needed to encourage funding organizations to invest more in such studies, as they are important for the understanding of how *M. bovis* modulates the host immune system. Elucidation of these mechanisms may pave

Table 1
Current *Mycoplasma* sp. attenuated vaccines.[a]

Name	Vaccine strain	Manufacturer	Countries of distribution
Contavax	*M. mycoides* subsp. *mycoides* T1/44	KEVEVAPI	Kenya, Rwanda, Sudan, Uganda
PERIBOV	*M. mycoides* subsp. *mycoides* T1/44-2	Botswana Vaccine Institute	Botswana, Namibia, Zimbabwe
Cevac MG-F	*M. gallisepticum* F	Cevac Sante Animale	Brazil, Malaysia, Philippines, Vietnam
Nobilis MG 6/85	*M. gallisepticum* 6/85	MSD Animal Health	Egypt; Arab Rep., France, Germany, Russian Federation, South Africa, Thailand, Ukraine, United Kingdom, Venezuela
Vaxsafe MG	*M. gallisepticum* ts-11	Bioproperties Ptl Ltd.	Australia, Brazil, Bulgaria, China, Egypt; Arab Rep., Hungary, India, Indonesia, Iran; Islamic Rep. of, Italy, Japan, Korea; Rep. of, Malaysia, Pakistan, Philippines, Poland, Romania, South Africa, Thailand, Turkey
M. synoviae MH-S vaccine	*M. synoviae*	Pharmsure Ltd	European Union
Mycovax MS-H	*M. synoviae*	Merial	Brazil
Vaxsafe MS	*M. synoviae*	Bioproperties Ptl Ltd.	Argentina, Australia, Brazil, Indonesia, Iran; Islamic Rep. of, Japan, Mexico, Philippines, South Africa, Thailand, Turkey
M. hyopneumoniae 168	*M. hyopneumoniae* 168	China	China

[a] *Source:* Vetvac.org.

the way for the development of control measures in the form of new treatments or more effective vaccines.

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