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Vaccination with recombinant *Mycoplasma bovis* GAPDH results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with *M. bovis*☆

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ABSTRACT

Mycoplasma bovis continues to cause significant disease in feedlots and dairy farms. The ability of the micro-organism to evade the immune system of the host combined with the lack of effective vaccines makes this disease difficult to control. Bacterin-based vaccines have not been successful in field trials and in some cases enhance the disease. In an attempt to develop a sub-unit vaccine, we used the conserved *M. bovis* glyceraldehyde-3-phosphate (GAPDH) protein in combination with a protein extract prepared from three *M. bovis* isolates to immunize feedlot animals. After challenge with a combination of three *M. bovis* isolates, there were differences in the proportion of weight loss between the control and vaccinated groups but no differences in rectal temperature and survival rate in all the groups. In addition, there were no significant differences between the proportions of lungs lesions in all the groups despite the percentages of lesions being higher in the vaccinated groups. These findings indicate that the *M. bovis* GAPDH protein is not a suitable antigen for a vaccine against this pathogen.

1. Introduction

Mycoplasma bovis is an important pathogen of beef and dairy cattle. Amongst the most economically important diseases caused by this micro-organism are chronic bronchopneumonia and polyarthritis syndrome (CPPS) and mastitis. Because of the importance of these diseases, numerous studies have focused on developing protective vaccines with no success to date. Potential factors for vaccine failure may include the ability of *M. bovis* to vary its surface antigen makeup, resistance to antibiotics [1] and also the capacity to invade peripheral-blood mononuclear cells and erythrocytes [2]. While some experimental vaccines showed promising results [3–6], field testing of vaccines did not provide protection and in some cases exacerbated the disease (Reviewed in Ref. [7]). This suggests that bacterins may not be ideal antigens thus a different approach in the form of recombinant antigens may be needed. Some *M. bovis* proteins have the potential of being targets for vaccine development. Amongst these are the variable surface proteins [8], the conserved P26 [9] and P48 [10] lipoproteins, and heat-shock proteins [11] but to date, no experimental vaccines containing these antigens have been tested.

One protein that has received attention as a potential vaccine target in many species is the conserved glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein. In addition to its role in glucose metabolism, GAPDH had been shown to bind cellular matrixes [12–16] and it has been postulated to be a virulence factor [17–20]. These properties of GAPDH suggest that this protein could be used as a protective antigen. In several trials however, vaccination with this protein has resulted in mixed outcomes. Immunization with the *Streptococcus suis* GAPDH does not protect pigs from an experimental challenge with *S. suis* [21]. On the other hand, vaccination of dairy cattle with the GAPDH protein from environmental streptococci protects animals from a challenge with either *Streptococcus uberis* [22] or *Streptococcus dysgalactiae* [23].

Our laboratory is working on a protective vaccine against *M. bovis*. Because of our experience with the environmental streptococci GAPDH protein, we focused our attention on the *M. bovis* GAPDH product. Animals naturally infected with *M. bovis* developed an immune response to GAPDH [24]. As part of our studies we also investigated the use of novel adjuvants for vaccine formulation. Amongst the adjuvants we chose were the host-defence peptide indolicidin [25] and synthetic oligonucleotides

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known as CpG [26]. These adjuvants were chosen because they promote a balanced Th1/Th2 immune response (reviewed in Ref. [27])

In this study we chose to use Gap-I a protein chimera composed of the *M. bovis GAPDH* and the host-defence peptide indolicidin that retained the properties of the individual components [28]. We used vaccines composed of a plasmid encoding the Gap-I protein formulated in PBS, the Gap-I protein and cell extracts of *M. bovis* formulated with CpG2007 [29] to investigate whether these vaccines could protect feedlot cattle from an experimental challenge. The results show that vaccination with Gap-I in the form of protein or DNA or mixed with a *M. bovis* extract failed to protect feedlot cattle. The results also suggest that vaccination with Gap-I may enhance lung pathology.

2. Materials and methods

2.1. Bacterial and virus strains

The *M. bovis* isolates used in this study were Mb1, obtained from a case of poly-arthritis [24], and two field isolates, Mb160 and Mb163 recovered from clinical cases in the Provinces of Alberta and Saskatchewan respectively [30]. The *M. bovis* isolates were grown in modified Hayflick's medium containing 20% inactivated horse serum at 37 °C in 5% CO_2. Bacteria were collected by centrifugation at 10,000 × g and suspended in the same medium at the highest concentration as determined by counting the bacteria in a Petroff-Housser counter after staining the bacteria with a 0.015% solution of crystal violet for 1 min. The bacterial counts were confirmed by plating on modified Hayflick's agar. Challenge doses were prepared by serial dilutions of the original suspension in the same medium. The BHV-1 strain 108 was grown in MDBK cells in MEM supplemented with 5% fetal bovine serum (FBS Gibco). Cells were lysed as before [30] and aliquots were diluted in 4 ml of tissue-culture medium to $1 \times 10^{5.5}$ tissue-culture cells infective dose 50 (TCID50)/ml.

2.2. Construction of synthetic genes encoding GAPDH and derivatives

To allow for more efficient expression of recombinant proteins, the gene encoding a chimeric protein composed of GAPDH coupled to the host-defence peptide indolicidin were synthesized by GeneArt AG (Regensburg, Germany). The codon usage was optimized for efficient expression in *E. coli* and the synthetic gene cloned into the histidine (his)-tag expression vector pQE-30 (Qiagen, Mississauga ON, Canada). The resultant plasmid pQE-sGap-I (s stands for synthetic gene) was used for protein purification after transformation into *E. coli* JM109. For DNA immunizations, the gene encoding Gap-I was codon-optimized for expression into bovine species (GeneArt AG, Regensburg, Germany) and cloned into pMASIA [31] to generate pMASIA-bGap-I (b stands for bovine-optimized codons).

2.3. Expression and purification of recombinant proteins

The *E. coli* strain JM109 carrying the plasmid pQE-sGap-I was grown in 1 l of Luria-Bertrani (LB) media at 37 °C with shaking. The A_{600} was measured over time on aliquots of the cultures. The Gap-I protein was purified by Ni-NTA affinity chromatography as described before [24]. The Gap-I protein was solubilized in sterile PBSA (137 mM NaCl, 2.7 mM KCl, 7 mM Na_3PO_4 and 1.5 mM KH_2PO_4) and stored at −20 °C until use.

2.4. Preparation of M. bovis cell extracts

To prepare the extracts, three flasks each containing100 ml of Hayflick's medium were inoculated with the three *M. bovis* strains (one strain per flask) and incubated at 37 °C in 5% CO_2 for 48 h. The bacterial cells were collected by centrifugation at 6000 × g for 15 min at 4 °C. The cells were washed twice in 100 ml PBS and collected again by centrifugation. The cell pellet was suspended in 500 μl of lysis buffer (9 M urea, 2% CHAPS, 1% DTT, 2% pharmalyte 3-10, and protease inhibitors). The cells were disrupted by sonication and the preparation was centrifuged at 30,000 × g for 1 h at 4 °C. The cleared extracts were stored at −80 °C until use.

2.5. Humoral and cell-mediated immunity assays

Determination of serum and nasal secretion titres was carried out as described before [24]. Briefly, 96-well plates were coated with the antigens used in the immunizations and incubated with serial dilutions of sera or nasal secretions. For *M. bovis* whole-cell ELISA assays, 0.1 ml/well of a 5×10^8 cfu/ml suspension in 0.1 M carbonate buffer pH 9.6 was used to coat the plates, After overnight incubation at 4 °C, the plates were washed and serial dilutions of serum or nasal secretions were added. After incubation with alkaline phosphatase (AP)-coupled secondary antibodies, AP substrate PNPP was added and the plates read at 405 nm with a referenced filter set at 490 nm. Titres were calculated by the intersection of least-square regression of A_{405} versus logarithm of dilution. Purification of bovine peripheral-blood mononuclear cells (PBMC) was performed as described before [2].

The proliferation of PBMCs after stimulation with Concanavalin A (ConA) (Sigma–Aldrich, Oakville, ON) or with the equal proportions of the recall antigens (*M. bovis* GAPDH or *M. bovis* extracts) at a concentration of 2 μg/ml was determined by seeding 96-well tissue-culture plates at a concentration of 3×10^5 PBMCs/well. Cells were incubated at 37 °C in 5% CO_2 in the presence of the recall antigens for 72 h in triplicate. A solution containing 0.4 μCi/well of ^{3}H-thymidine (GE Healthcare, Mississauga, ON) was added and the cells incubated for 18 h. Cells were harvested (Packard, Filtermate Harvester) and the amount of incorporated ^{3}H-thymidine was determined in a scintillation counter (Packard, Top Count NXT™). The stimulation index was determined by dividing the treated cell counts by medium counts.

To determine the effect of the recall antigens on the ability of PBMCs to produce IFN-γ, ELISPOTs assays were conducted in triplicate. ELISPOT plates (Millipore HA plate) were coated overnight (24 h) at 4 °C with an in-house produced anti-bovine IFN-γ mouse monoclonal antibody (1:3000 in sterile coating buffer). The plates were washed 4 times with sterile PBSA and blocked at 37 °C for 2 h using 1% bovine albumin (Cal Biochem) in PBSA. A suspension of purified PBMCs (0.1 ml of 1×10^7 PBMC/ml) was incubated with the recall antigens. Control wells included medium or ConA at 1 μg/ml. The PBMC/antigen mixes were incubated overnight at 37 °C and the plates washed with ddH_20 to lyse the cells. Following these washes, the plates were incubated at room temperature for 2 h with a rabbit anti-bovine IFN-γ antibody (Cedarlane, ON) (1:3000 in 1% albumin/PBSA). After washes with PBST (0.1 M PBS + 0.1% Tween 20); an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (KPL, ON) (1:1500 in 1% albumin/PBSA) was added and incubated for 2 h at room temperature. Following this incubation, the plates were washed with PBST, the BCIP/NBT substrate (Sigma–Aldrich, Oakville, ON) added and the cells secreting IFN-γ (spots) developed for 30 min. The spots were then counted under a light microscope.