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Development of a Second-Generation Vaccine against Mycoplasmosis: Preparation of a Fraction Candidate from *Mycoplasma bovis* and its Evaluation as a Vaccine

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Abstract: The present study aims, mainly, to fractionate a crude antigen preparation from *Mycoplasma bovis* and evaluate the abilities of the fraction candidate to stimulate B and/or T-cells mediated immunity for development of a more safe and effective vaccine against *M. bovis*. In this study, the fraction candidate was separated by DEAE-Sepharose and the protein content was estimated by SDS-PAGE. All Balb/c mice intramuscularly vaccinated with the obtained fraction either alone or combined with Freund's incomplete adjuvant (FIA). A significant level of specific anti-*M. bovis* IgG antibody titers was found in the sera of mice immunized with fraction candidate alone or combined with FIA, especially after the third immunization. Moreover, a significant reduction (50%) in macroscopic and microscopic lesions was obtained in the mice immunized with fraction candidate alone or combined with FIA. In conclusion, this study describes the design of a subunit vaccine that elicits a specific immune response against *M. bovis*.

Key words: *Mycoplasma bovis* · Subunit Vaccine · Immune Response

INTRODUCTION

Mycoplasma species cause highly contagious and serious diseases of livestock, such as contagious agalactia in small ruminants, contagious mastitis, contagious bovine and caprine pleuropneumonia, calf pneumonia, pleuritis in horses and enzootic pneumonia in pigs [1]. A number of mycoplasmas are suspected of causing disease in human, but only atypical pneumonia has been definitively related to a mycoplasma [2]. *Mycoplasma bovis* (*M. bovis*) is a significant world-wide pathogen of adult dairy cows as well as intensively reared dairy and beef calves. It is a main cause of calf pneumonia, mastitis and arthritis [3, 4]. In spite of the seriousness of these diseases there are few effective vaccines to combat them today. Indeed, those that are available are whole-cell vaccines, some of which are semi virulent, provide only partial or transient immune response and often induce unkind side effects. Furthermore and disturbingly, attempts at vaccine improvement have often led to exacerbation of diseases, due to their immune-pathological nature [5]. Moreover,

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the inability of chemotherapy to control *M. bovis* infections has focused attention on vaccine strategy [6]. The previous background invites, especially in view of the decreasing effectiveness of antibiotics in controlling mycoplasma infections, the need for reliable vaccines which has become even more urgent. Moreover, there is a critical need to develop improved preventative and treatment strategies for *M. bovis*-associated disease.

Currently, no commercial vaccines exist for *M. bovis* or are licensed for use in young dairy calves. There are several whole bacterin-based vaccines, including autogenous preparations that are available only in the USA [5]. A field trial was conducted to detect the efficiency of a common commercial *M. bovis* vaccine used in USA for the prevention of *M. bovis*-associated disease in calves [7]. This vaccine was not effective for the prevention of *M. bovis*-associated disease in young dairy calves. Vaccines against *M. bovis* have afforded partial protection from respiratory disease in European field trials [8]. A vaccine prepared with formalin-inactivated strains of *M. bovis* and *Mannheimiahaemolytica* reduced both losses from pneumonia and the cost of treatment in newly introduced feedlot calves [9]. More recently an inactivated vaccine containing saponin killed *M. bovis* was shown to be immunogenic, safe and protective against an experimental challenge with virulent *M. bovis*, but the duration of protection with this type of vaccine is very short [10].

Other recent research is focused on isolating immunogenic proteins that can be utilized in subunit or component vaccines. Additionally, recent advances in epitope mapping are making it much easier to recognize immunogenic proteins that may have uses in vaccine development [5]. Candidate vaccines would be required to induce mucosal immunity (to inhibit the growth of the pathogen or block its virulence factors) and to activate the specific T cells of the Th1 type. Ideally, these vaccines should be of the multicomponent subunit type, delivered directly to the mucosal respiratory surface where the mycoplasma recruits infection [11].

Based on these findings, defining the mycoplasma antigens that are able to stimulate B and/or T-cell activation will be a first step towards the development of a more effective vaccine against mycoplasmas. The present study aims, mainly, to fractionate a crude antigen preparation from *M. bovis* and evaluate the abilities of fraction candidate to stimulate B and/or T-cells mediated immunity for development of a more safer and effective vaccine against *M. bovis*.

MATERIALS AND METHODS

All manipulations were in compliance with the guidelines for the welfare of animals and those of the concerned ethical authorities.

Animals: Specific pathogen-free (SPF) Balb/c mice aged 10–12 weeks, were obtained from Water life (Taif, KSA) and maintained under pathogen-free conditions in our animal house for use throughout these experiments. Experiments were performed in accordance to the rules of the Ethic Committee from Taif University.

Strain of *Mycoplasma bovis*: *Mycoplasma bovis* reference strain was obtained from Prof. Dr. Nicholas Robin, Veterinary Laboratory Agency (Weybridge), Addlestone, Surrey KT15 3NB, UK and used in this study.

Culture and identification of *M. bovis*: All mycoplasma cultures were carried out in bacto-brain heart infusion agar or broth supplemented with 20% horse serum, 10% (vol./vol.) fresh yeast extract, 0.02% (wt./vol.) DNA, 1000 IU/ml penicillin G sodium and 1% of 10% thallium acetate with the final pH adjusted to 7.6–7.8. Plates were incubated at 37 °C in 5% CO_2 and examined at 2, 4, 7 and 10 days for mycoplasmal growth.

Detection of Acholeplasma Contamination by Digitonin Disc Diffusion Assay: The digitonin disc diffusion assay was performed as previously describe [12]. Digitonin stock solution of 1.5% (w/v) in 95% ethanol was stored at 4°C. Digitonin disc was made by adding 25 µl of the stock solution to 6-mm blank paper disc. The digitonin discs were dried overnight at room temperature and stored at 4°C until use. Both reference and unknown mollicutes were cultured in PPLO broth and incubated in a 10% CO_2 environment for 4 days. Then, 200 µl of each culture was distributed on modified Hayflick agar plate. Digitonin discs were pressed gently on the surface of the agar after the inoculum had dried. Plates were incubated for 4–7 days at 37°C in a 10% CO_2 incubator. After incubation, all plates were examined under the stereomicroscope and the zones of inhibition were measured from the edge of the disc to the edge of clear zone of no growth (mm). Digitonin tests were considered positive when the clear zone was >5 mm, negative when the zone was <3 mm and

all else would normally be considered a vague result. In the analysis of the digitonin test, a threshold of >3 mm and >5 mm were both tested.

Preparation of Mycoplasma Antigen: One ml of an active growing *M. bovis,* approximately 10^7 CFU/ ml was estimated [13]. Culture of *M. bovis* was first made into 9 ml of broth (supplemented with horse serum, yeast extract DNA, penicillin G sodium and thallium acetate). After 72 hours of incubation, the contents were aseptically poured into 90 ml heart infusion broth. After incubation period of 72 hours, the contents of flasks (approx. 100ml) were transmitted to the final propagation flask containing 900 ml of broth. After another 72 hours of incubation, the Mycoplasma cells were harvested by centrifugation at 18000 rpm at 4°C for 1 hour. Sterility checks were routinely carried out with each passage of the growth to eliminate the bacterial contaminants. After two thorough washing in sterile PBS, cells were re-suspended in 15 ml of PBS/ 1000 ml of the original volume [14]. This stock suspension was sonicated (Bandelin electronic UW2070 Sonicator) at 20Kc/ min for 10 successive times on ice with one minute interval [15].

Fractionation of Sonicated Mycoplasma Suspension (SMS) with DEAE-Sepharose: Fractionation was performed at room temperature. SMS (3 ml of a solution of approximately 2 mg of protein per ml) was applied to a 20-ml column of DEAE-Sepharose (Pharmacia LKB, Uppsala, Sweden) then equilibrated with 0.1 M NaCl-0.01 M phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min. The column was then washed with 50 ml of the starting buffer (0.1 M NaCl, 0.01 M phosphate buffer, pH 7.2). Bound proteins were washed out in a different concentration of 0.3, 0.5 and 1 M NaCl in 0.01 M phosphate buffer (pH 7.2). Each elution contained 50 ml (2 ml per fraction). The protein concentrations were measured at A280. Peak fractions were pooled, concentrated and dialyzed.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE): The stacking and separating gels for SDS-PAGE consisted of 5 and 12% acrylamide, respectively. Samples were heated at 100°C for 3 min in 0.05 M Tris buffer (pH 6.5) containing 2% SDS, 10% 2-mercaptoethanol and 10% glycerolin water bath. Twenty microliters of each samples and marker was loaded then electrophoresis was carried out at 10 V/cm. The gels were stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, Calif.), dried and calibrated with broad molecular-weight standard mixtures (Bio-Rad).

Mice Immunization: The Mice (10 mice per group) were injected intramuscularly (i.m.), using syringes with $30_{1/2}$-gauge needles (Microlance; Becton Dickinson), both in the left and the right hind thigh muscle with 50 µg (100 µl in each muscle) of fraction candidate either alone or combined with 50 µg of Freund's Incomplete Adjuvant (FIA) (sc-3726; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), (vaccine groups), on days 0, 14 and 28. The control groups were composed of mice injected i.m. with 50 µg of FIA two times at intervals of 2 weeks and non-treated mice. Serum samples were collected from the peri-orbital venous sinus of the eye (Medial Canthus) by a fine-walled Pasteur pipette every 14 days for 8 weeks and stored at -20°C until analysis (each serum individually tested).

Measurement of Humoral Antibody Responses by Enzyme-linked Immunosorbent Assay (ELISA): Levels of antigen-specific IgG antibodies in serum samples were determined as previously described [16, 17]. The total antigen of *M. bovis* at 10µg/ml was used to coat microtiter plates. The antigen-specific antibody titer is given as the reciprocal of the highest dilution producing an absorbance (OD) that was 2.5-fold greater than that of the serum of control mice at the same dilution. Results are expressed as the means of $\log_2$titers±standard deviations (SD).

Challenge Infection: Stock cultures of *M. bovis* were grown, as previously mentioned in mycoplasma broth and frozen in 1-ml aliquots at -80°C. For inoculation, thawed aliquots containing 2 x 10^7 CFU of *M. bovis* per milliliter were diluted to 10^5 CFU/20 µl. Inoculations of 20 µl diluted mycoplasma were given orally for all mice [18]. One month after the challenge, mice were sacrificed for detection the macroscopic and microscopic lesions in different internal organs.

Histopathological Examination: The lungs of mice were collected from the different groups after one month of challenge. The samples were fixed in 10% buffered neutral formalin, then dehydrated in ascending grades of alcohols, cleared in xylene and embedded in paraffin. The samples were casted, then sliced into 5 µm in thickness

and placed onto glass slides. The slides were stained by hematoxyline and eosin (H&E) and examined microscopically [19].

Statistical Analysis: Levels of significance of the differences between groups of mice were determined by an analysis of variance test.

RESULTS

Identification of *M. bovis* and Detection of Acholeplasma Contaminationl: *Mycoplasma bovis* form colonies with a "fried-egg" appearance (Figure 1). All reference strains of Mycoplasma species showed zones of growth inhibition around the digitonin discs (Figure 2). Sizes of zones of growth inhibition ranged from 3 to 13 mm. No zones of growth inhibition were observed with Acholeplasma species (Figure 2).

SDS-PAGE Analysis of Eluted Proteins: The SMS was fractionated with DEAE-Sepharose. The fraction candidate was separated then concentrated. Different pattern of protein bands were seen in SDS-PAGE (Figure 3). The fraction candidate contained about 50% of the total protein. Sizes and numbers of proteins in this fraction was checked by SDS-PAGE separation (Figure 3). The fraction candidate had 2 dominant molecular mass bands (48 and 63 kDa).

Humoral Immune Response Induced by Subunit Vaccination: All sera were tested by ELISA using total antigen of *M. bovis* (Figure 4). A significant level of specific anti-*M. bovis* IgG antibody titers was found in the sera of mice immunized with the fraction candidate either alone or combined with FIA, especially after the third immunization (the level of antibody titers increased with successive immunizations). The IgG antibody titer was not significantly greater in the sera of mice co-immunized with FIA than in the sera of mice immunized with the fraction candidate alone. In contrast, mice injected with control untreated mice or FIA did not generate antibodies against Fraction candidate (Figure 4).

Detection of the Microscopic Lesions of Balb/c Mice after One Month of the Challenge in Vaccinated and Non-vaccinated Groups: Pulmonary lesions were the most apparent lesions in infected Balb/c mice. Significant reduction (50%) in mice with microscopic lesions was

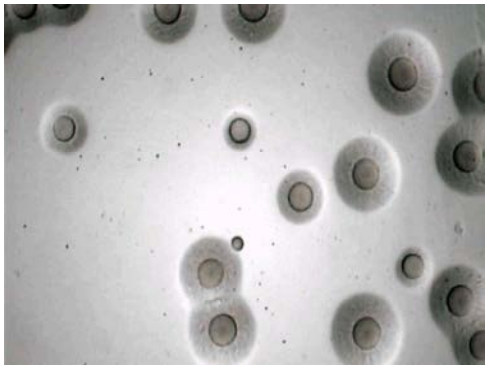

Fig. 1: Colonies with typical *Mycoplasma bovis* morphology.

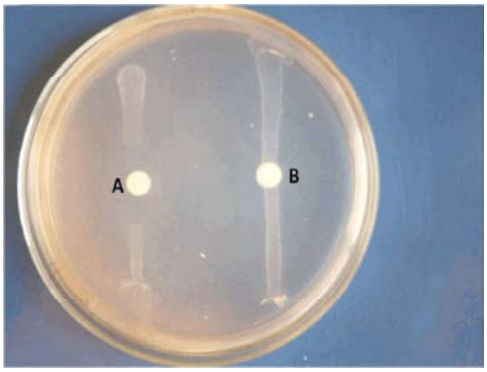

Fig. 2: Digitonin Senstivity Test, A: Mycoplasma, B: Acholeplasma.

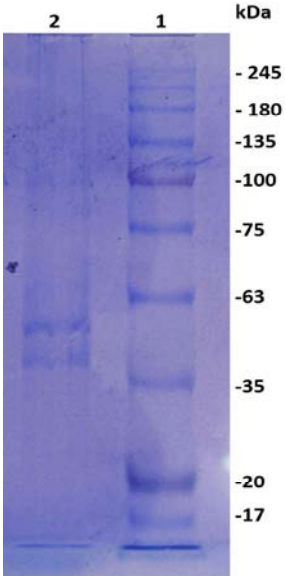

Fig. 3: SDS-PAGE (12% polyacrylamide) analysis of *M. bovis* fractions. Lane 1, protein molecular weight standards; Lane 2, fraction candidate (obtained by DEAE-Sepharose separation); Numbers indicate molecular weights in kDa.

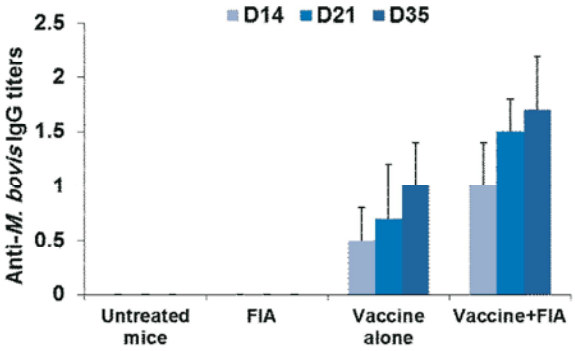

Fig. 4: Determination of specific anti-*M. bovis* antibody titers in the sera of Balb/c mice immunized with 50 µg of the fraction candidate either alone or combined with 50 µg of FIA (vaccine groups), on days 0, 14 and 28. Sera were collected on days 14, 21 and 35 and tested by ELISA using total antigen of *M. bovis*. The titer is given as the reciprocal of the highest dilution with an OD405 that was 2.5-fold greater than the OD of untreated mouse sera at the same dilution. Results are expressed as the mean $\log_2$ titers and SD and represent one of two similar experiments.

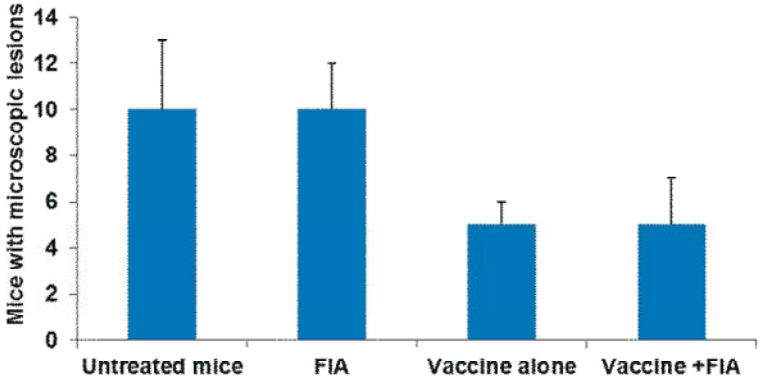

Fig. 5: Detection of the microscopic lesions of Balb/c mice after one month of challenge in vaccinated (fraction candidate either alone or combined with 50 µg of Freund's Incomplete Adjuvant (FIA)) and non-vaccinated (untreated mice and FIA) groups. Results are represent one of two similar experiments.

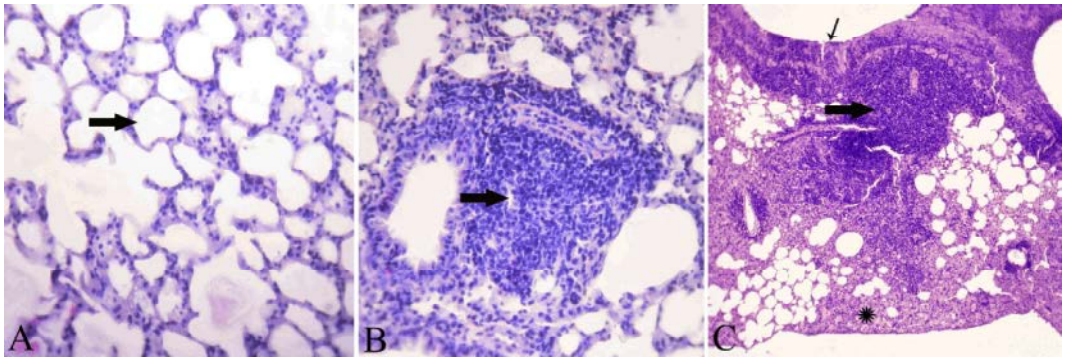

Fig. 6: Results of histopathologic examination. (A): Lung of vaccinated groups (fraction candidate either alone or combined with FIA) showed normal tissue architecture with normal alveolar wall in 50% of vaccinated mice (arrow) (HE X300). (B): Lung of vaccinated groups (the other 50% of mice) showed hyperplasia of peribronchial lymphoid aggregation (arrow) (HE X300). (C): Lung of non-vaccinated groups (untreated mice and FIA) showed intra-alveolar oedema with massive leukocytic infiltration in pulmonary tissue together with hyperplasia of bronchial epithelium (thin arrow) (HE X150).

obtained in the mice immunized with the fraction candidate alone (Figure 5). Co-administration of FIA did not potentiate the reduction of lesions (50%). In contrast, control untreated mice or FIA treated mice did not show any reduction of microscopic lesions (Figure 5). Lung of vaccinated groups (fraction candidate either alone or combined with FIA) showed normal tissue architecture with normal alveolar wall in 50% of vaccinated mice (Figure 6A). While the other 50% of vaccinated mice showed moderate lesions in the form of hyperplasia of peribronchial lymphoid aggregation (Figure 6B). Lung of non-vaccinated groups (untreated mice and FIA) showed intra-alveolar oedema with massive leukocytic infiltration in pulmonary tissue together with hyperplasia of bronchial epithelium (Figure 6C).

DISCUSSION

The inability of chemotherapy to control *M. bovis* infections has focused attention on vaccination. In the present study, we developed a subunit vaccine that elicits a specific immune response against *M. bovis* challenge. *M. bovis* formed colonies with a "fried-egg" appearance (Figure 1) because they grow into the agar surface at the center while spreading outward at the colony edges. Mycoplasma requires sterols in the growth media for incorporation into their cytoplasmic membranes. Cholesterol in particular, plays a key role in the maintenance and synthesis of the integrity of mycoplasma cell membranes. Cholesterol is supplied by animal serum that was added to mycoplasma growth media. Acholeplasma is able to synthesize cholesterol on its own and it can therefore grow in media without added cholesterol sources. Digitonin induces lysis of mycoplasma cells through the formation of digitonide-cholesterol precipitation complexes that lead to increased permeability of mycoplasma cell membranes. This increased leakiness of the membrane ultimately causes mycoplasma death. Therefore, growth of mycoplasma colonies on agar is inhibited by the digitonin while Acholeplasmas are unaffected [12].

The conventional approach for development of vaccine has been overtaken by two major successions of advances. The first was achieved through the identification of virulence factors, characterization of the immune response after infection and improved knowledge of the pathogenesis of microbial infections. Lessons from this led to a second generation of vaccines, which include the use of highly purified antigenic components, subunit vaccines produced with or without using recombinant DNA technology and rationally attenuated vaccine strains [20]. In this study, one immunogenic *M. bovis* fraction was evaluated as vaccine candidates. A similar method was reported for *M. hyopneumoniae* who proved that the protein cocktail vaccine of *M. hyopneumoniae* would be likely to induce a good immune response [21]. Analysis of serum antibody responses in mice vaccinated intramuscularly with the fraction candidate either alone or combined with FIA, elicit serum IgG responses in our study. Recent studies immunized the mice with protein cocktail vaccine include protein-C1 (P97/NrdF/P36/P46) and protein-C2 (P97R1/NrdF/P36/P46) and obtained strong serum IgG responses against each antigen [21, 22]. Moreover, mice immunized with a subunit vaccine containing P97R1 fused to the B subunit of the heat-labile enterotoxin of *E. coli* induced anti-P97R1 mucosal and Th1-biased immune responses when given intranasal and mucosal and Th2-biased immune responses when given intramuscularly.

Significant reduction (50%) in the microscopic lesions was obtained in the mice immunized with the fraction candidate alone (Figure 5). Co-administration of FIA did not potentiate the reduction of lesions (50%). Pulmonary lesions were the most apparent lesions in infected Balb/c mice. Lung of vaccinated groups showed normal tissue architecture with normal alveolar wall in 50% of vaccinated mice (Figure 6A). The other 50% of vaccinated mice showed moderate lesions in the form of hyperplasia of peribronchial lymphoid aggregation (Figure 6B). While the non-vaccinated groups showed severe lesions in the form of intra-alveolar oedema with massive leukocytic infiltration in pulmonary tissue together with hyperplasia of bronchial epithelium (Figure 6C). It is possible that the ongoing anti-*M. bovis* IgG responses success in reduction of the observed macroscopic and microscopic lesions in vaccinated groups [21]. Furthermore, hyper immune serum against a clonal variant of *M. bovis* PG45 was generated in Balb/c mice and measured for antibody activity [23]. An immunization study was carried out with the objective of comparing the immunological responses against the chimeric antigen to the individual subunits [24]. The study reported that the total IgG, IgG1 and IgG2a serum titres against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were significantly higher in the mice groups immunized with GAPDH and Vsp-GAPDH

compared to the titres in Placebo and vaccinated with Vsp conjugated to keyhole limplethemacyanin, respectively.

Nevertheless, other attempts to vaccinate against *M. bovis* have been less successful in relevant species. Thus, the severity of pneumonia in calves was enhanced by mixtures of affinity purified antigens and by Triton X-114 membrane protein extracts [25]. In a calf arthritis model, vaccination was shown not to be protective, although high titres of antibody were detected before challenge [26]. Another former vaccine has been efficacious against arthritisin experimental challenge studies [27]. Experimental vaccines against mycoplasma mastitis have been shown to exacerbate the disease [28]. We hope that our vaccine candidate would succeed in relevant species and overcome these problems.

CONCLUSION

In conclusion, this study describes the design of a subunit vaccine that elicits specific immune response against *M. bovis* challenge. This study recommended the used of the designed subunit vaccine as a model for other type of mycoplasmosis in animals and humans. In addition, a lot of work is required to obtain a better understanding of its activity and mechanism of protection with trail for using in relevant animals as calves to avoid the disadvantages of other trailed vaccines.

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REFERENCES

1. Radostits, O.M., C.C. Gay, K.W. Hinchcliff and P.D. Constable, 2007. Diseases associated with Mycoplasma spp. In: Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats. 10thed. Saunders/Elsevier, pp: 1123-1156.
2. Waites, K.B. and D.F. Talkington, 2004. Mycoplasma pneumoniae and its role as a human pathogen. C. M. R., 17: 697-728.
3. Nicholas, R.A. and R.D. Ayling, 2003. *Mycoplasma bovis*: disease, diagnosis and control. Research in Veterinary Science, 74: 105-112.
4. Gagea M.I., K.G. Bateman, R.A. Shanahan, T. van Dreumel, B.J. McEwen, S. Carman M. Archambault and J.L. Caswell, 2006. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. Journal of Veterinary Diagnostic Investigation, 18: 29-40.
5. Nicholas, R.A.J., R.D. Ayling and L. McAuliffe, 2009. Vaccines for Mycoplasma Diseases in Animals and Man. Journal of Comparative Pathology, 140: 85-96.
6. Ayling, R.D., S.E. Baker, M.L. Peek, A.J. Simon and R.A. Nicholas, 2000. Comparison of in vitro activity of danofloxacin, florfenicol, oxytetracycline, spectinomycin and tilmicosin against *Mycoplasma bovis*. Veterinary Record, 146: 745-747.
7. Maunsell F.P., G.A. Donovanb, C. Risco and M.B. Brown, 2009. Field evaluation of a *Mycoplasma bovis* bacterin in young dairy calves. Vaccine, 27: 2781-2788.
8. Stott E.J., L.H. Thomas, C.J. Howard and R.N. Gourlay, 1987. Field trial of a quadrivalent vaccine against calf respiratory disease. Veterinary Record, 121: 342-7.
9. Urbaneck, D., F. Liebig, T. Forbrig and B. Stache, 2000. Experiences with herd-specific vaccines against respiratory infections with *M. bovis* in a large feedlot. Praktische Tierarzt, 81: 756-63.
10. Nicholas R.A.J., R.D. Ayling and L. Stipkovits, 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. Vaccine, 20: 3569-3575.
11. Dedieu-Engelman, L., 2007. Contagious bovine pleuropneumonia: a rationale for the development of a mucosal sub-unit vaccine. Comparative Immunology, Microbiology and Infectious Diseases, 31: 227-238.
12. Tully, J.G., S. Razin and J.G. Tully, 1983. Section E5. Tests for digitonin sensitivity and sterol requirement. In: Methods in mycoplasmology I, ed. J.G. Tully, S. Razin, pp: 355-362. Academic Press, New York, NY.
13. Rodwell, A.W. and R.F. Whitcomb, 1983. Methods for direct and indirect measurement of mycoplasma growth. In Methods in Mycoplasmology Vol. I Mycoplasma characterization edited by Razin, S. and Tully, J.G. Academic Press.
14. Ruhnke H.L. and S. Rosendal, 1989. Useful protocols for diagnosis of animal mycoplasmas. WAVLD Mycoplasma Workshop Vet. Microbiol. andImmunol. Dept. Ontario Vet. Collage, Guelph, Ontario, Canada NIC2W1.

15. Krogsgaard, A.J., 1971. Indirect hemagglutination with mycoplasma antigens: Effect of pH on antigen sensitization of tanned fresh and formalinized sheep erthrocytes. Journal of Applied Microbiology, 22: 756-759.
16. Ismael, A.B., D. Sekkai, C. Collin, D. Bout and M.N. Mévélec, 2003. The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. Infection and Immunity, 71: 6222-6228.
17. Chessa, B., M. Pittau, M. Puricelli, R. Zobba, E. Coradduzza, P. Dall'ara S. Rosati, G. Poli and A. Alberti, 2009. Genetic immunization with the immunodominant antigen P48 of *Mycoplasma agalactiae* stimulates a mixed adaptive immune response in BALB/c mice. Research in Veterinary Science, 86: 414-420.
18. Jones, H.P., L. Tabor, X. Sun, M.D. Woolard and J.W. Simecka, 2002. Depletion of CD8+ T Cells Exacerbates CD4+ Th Cell-Associated Inflammatory Lesions During Murine Mycoplasma Respiratory Disease. Journal of Immunology, 168: 3493-3501.
19. Suvarna, S.K., C. Layton and J. Bancroft, 2013. Bancroft's Theory and Practice of Histological Techniques, 7thEdition, London: Churchill Livingstone.
20. Movahedi, A.R. and D.J. Hampson, 2008. New ways to identify novel bacterial antigens for vaccine development. Veterinary Microbiology, 131: 1-13.
21. Chen, A.Y., S.R. Fry, G.E. Daggard and T.K. Mukkur, 2008. Evaluation of immune response to recombinant potential protective antigens of *Mycoplasma hyopneumoniae* delivered as cocktail DNA and/or recombinant protein vaccines in mice. Vaccine, 26: 4372-4378.
22. Conceicao, F.R., A.N. Moreira and O.A. Dellagostin, 2006. A recombinant chimera composed of R1 repeat region of *Mycoplasma hyopneumoniae* P97 adhesin with Escherichia coli heat-labile enterotoxin B subunit elicits immune response in mice. Vaccine, 24: 5734-5743.
23. Rosengarten, R., A. Behrens,A.Stetefeld, M. Heller, M. Ahrens, K. Sachse, D. Yogev and H. Kirchhoff, 1994. Antigen heterogeneity among isolates of *Mycoplasma bovis* is generated by high-frequency variation of diverse membrane surface proteins. Infection and Immunity, 62: 5066-5074.
24. Perez-Casal, J. and T. Prysliak, 2007. Detection of antibodies against the *Mycoplasma bovis* glyceraldehyde-3-phosphate dehydrogenase protein in beef cattle. Microbial Pathogenesis, 43: 189-197.
25. Bryson, T.D.G., H.J. Ball, F. Foster and N. Brice, 2002. Enhanced severity of induced *Mycoplasma bovis* pneumonia in calves following immunization with different antigenic extracts. Research in Veterinary Science, 72(Suppl. A), 19.
26. Poumarat, F., P. Belli and D. Calavalas, 2000. Immunoprophylaxis of experimental *Mycoplasma bovis* arthritis in calves. In: Mycoplasmas of Ruminants: Pathogenicity, Diagnostics, Epidemiology and Molecular Genetics, Vol. 4, D. Bergonier, X. Berthelot and J. Frey, Eds, European. Commission, Brussels, Belgium, pp: 75-78.
27. Chima, J.C., B.N. Wilkie H.L. Ruhnke R.B. Truscott and R.A. Curtis, 1980. Immunoprophylaxis of experimental *Mycoplasma bovis* arthritis in calves-protective efficacy of live organisms and formalinized vaccines. Veterinary Microbiology, 5: 113-122.
28. Ross, R.F., 1993. Mycoplasma-animal pathogens. In: Rapid Diagnosis of Mycoplasmas, I. Kahane and J. R. A. Adoni, Eds, Plenum Press, New York, pp: 69-109.