Cattle immunized against the pathogenic L-α-glycerol-3-phosphate oxidase of Mycoplasma mycoides subs. mycoides fail to generate neutralizing antibodies and succumb to disease on challenge

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1. Introduction

Mycoplasma mycoides subs. mycoides (Mmm) is the causal agent of contagious bovine pleuropneumonia (CBPP), a serious respiratory disease of cattle that causes major economic losses, especially in sub-Saharan Africa [1,2]. In addition to tissue damage caused by Mmm, pathogenesis of CBPP arises partially from uncontrolled inflammatory responses [3]. The most widely deployed vaccine against CBPP is based on the live attenuated strain T1/44, which induces short-lived immunity and causes severe post-vaccinal lesions at the site of inoculation [4]. Mmm belongs to the class Mollicutes, which comprises the smallest, and simplest of the free-living, self-replicating bacteria [5,6]. Because of their limited genetic resources, Mollicutes are almost entirely dependent on host biosynthetic activity and metabolism for survival and attach to host cells for optimal growth [7].

Several authors demonstrated that Mmm translocates glycerol from host interstitial fluid through membrane-associated ATP-binding cassette transporter proteins GtsA, GtsB and GtsC. Once taken up, glycerol is phosphorylated to glycerol-3-phosphate (G3P) and subsequently oxidized to dihydroxyacetone phosphate, with the simultaneous release of H₂O₂ [8–10]. Pilo et al. identified the trans-membrane L-α-glycerol-3-phosphate oxidase (GlpO) as the enzyme responsible for oxidation of glycerol-3-phosphate and generation of H₂O₂ and proposed that the metabolite and other reactive oxygen species are translocated into the cytoplasm of in-contact cells, resulting in cellular damage [9]. Importantly, European strains of Mmm of the 1992–2000 epidemic, which predominantly caused chronic CBPP with less severe clinical signs, do not possess the gtsB and gtsC genes and, consequently, release lower amounts of H₂O₂ in the presence of physiological concentrations of glycerol [10]. The pathogenicity of H₂O₂ arising from glycerol metabolism by Mmm has been confirmed in an in vitro model using embryonic calf nasal epithelial (ECaNEp) cells [11]. Pretreatment of Mmm with antibody binding fragments (Fab) derived from rabbit...
polyclonal serum raised against rGlpO neutralizes enzyme activity, as shown by inhibition of H$_2$O$_2$ release in the presence of glycerol and abrogation of the cytotoxic effect on ECAhEp cells [9].

The Mmm vaccine strain T1/44 has an intact glycerol uptake and metabolic system [11], and it is possible that H$_2$O$_2$ contributes to post-vaccinal reactions observed at the site of inoculation. In addition, H$_2$O$_2$ may trigger the marked inflammation observed in the lungs of Mmm infected–cattle [9]. Therefore, a vaccine that targets GlpO and inhibits production of H$_2$O$_2$ by Mmm would be desirable, since it would be unlikely to elicit site reactions and would protect immune cattle undergoing infection from H$_2$O$_2$-associated cytotoxicity. Such a vaccine could be produced in sub-unit form using rGlpO or the T1/44 strain could be genetically modified to produce a mutant devoid of the active enzyme, but retaining GlpO epitopes capable of inducing antibodies with inhibitory capacity. The latter approach was used to target the metabolic enzyme dihydropyridine dehydrogenase of Mycoplasma gallisepticum and yielded a vaccine with protection superior to that of three other commercial vaccines [12]. The potential of this approach in respect of CBPP will depend on whether antibodies can be induced in cattle that bind GlpO and neutralize its activity, and whether such antibodies are protective. We have therefore evaluated the capacity of bovine and mouse GlpO antibodies to inhibit H$_2$O$_2$ release by Mmm using an in vitro assay of enzyme function. We have also investigated whether immunization of cattle with recombinant GlpO confers protection against challenge with live Mmm.

2. Materials and methods

2.1. Expression and purification of recombinant GlpO

The poly-histidine tagged full length 45–kDa recombinant GlpO protein was expressed and purified from transformed E. coli BL21 (DE3) harbouring gplO in the expression vector pETHIS-1 [13] and prepared as described by [9]. Briefly, transformed E. coli were grown and purified by Ni$^{2+}$ chelation chromatography as described for other recombinant proteins [14]. Fractions were analyzed by 12.5% SDS-PAGE using standard protocols and purified GlpO was dialysed (28,000 kDa cut-off) against PBS (pH 7.4) and quantified using the Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL, USA) as described by the manufacturer.

2.2. In vitro culture and quantification of Mmm

Lyophilized aliquots of the virulent Mmm isolate B237, which originated from an acute case of CBPP in central Kenya [15] were reconstituted in pre-warmed (37°C) Gourlay’s medium [13] and propagated as described [16]. Mmm for challenge infection was quantified on the basis of colour changing units/ml (CCU/ml) using the method of Spearman Karber [17] as described [16]. Mmm was considered suitable for inoculation on the third day of passage if the culture appeared filamentous and was at pH 6.5.

2.3. Cattle immunization and experimental infection

Cattle immunization was conducted following guidelines of the U.K. Animals (Scientific) Procedures Act, 1986 and was approved by the ethical review committees of the National Veterinary Research Centre, Muguga (Kenya) and the Moredun Research Institute (UK). Fourteen yearling Zebu steers from a CBPP-free zone of Kenya and sero-negative by the CBPP complement fixation test (CFT) were randomly assigned into 2 groups each comprising 7 experimental animals and an equal number of controls. The experimental group was inoculated sub-cutaneously with 200 µg of rGlpO emulsified in 1 ml Complete Freund’s adjuvant (CFA). Cattle were boosted after 4 weeks and again 6 weeks later with the same quantity of antigen emulsified in incomplete Freund’s adjuvant (IFA; Sigma). The control group was similarly inoculated with CFA and boosted with IFA emulsions in PBS respectively.

Challenge was performed by endobronchial inoculation with 60 ml of culture containing $\sim$10$^6$ CCU/ml of Mmm after sedation with 2 ml of 2% Xylazine (Bayer AG, Leverkusen, Germany). The inoculum was immediately followed by 30 ml of PBS and 15 ml of pre-warmed 10% low melting agar. Clinical signs were monitored daily and animals displaying continuous fever for ten days or recumbancy for 3 days were euthanized on ethical grounds. Surviving animals were euthanized at the end of the experiment on day 35. All animals were bled for preparation of sera by jugular venipuncture before challenge and prior to euthanasia.

2.4. Isolation of Mmm from experimental cattle

Lung tissue was immersed in 50 ml pre-warmed Gourlay’s medium containing phenol red indicator and incubated at 37°C in a humidified incubator with 5% CO$_2$ for 3 days. Upon change of colour to yellow, cultures were streaked on tryptose agar plates (Difco), and incubated at 37°C for 10 days. Four dilutions (1:10, 1:20, 1:30 and 1:40) of each broth culture were also prepared in Gourlay’s medium. Mmm isolation was considered positive if all dilutions of broth cultures changed from orange colour to yellow within 14 days and corresponding agar plates showed mycoplasmal morphological features.

2.5. Mouse polyclonal and monoclonal antibodies

Polyclonal mono-specific serum against recombinant GlpO was obtained by intra-peritoneal immunization of Balb/C mice with 50 µg of purified rGlpO in 0.25 ml of PBS, emulsified in an equal volume of CFA, followed by two booster immunizations with the same quantity of antigen emulsified in IFA 2 and 6 weeks after primary immunization. Three weeks after antigen was detectable in immunoblots with serum dilutions of 1:1000 or more, mice received a final intravenous boost with 50 µg of rGlpO in 250 µl PBS without adjuvant. Mice were euthanized three days later and the spleens were harvested for production of monoclonal antibodies, while blood was collected by cardiac puncture for preparation of polyclonal sera. Monoclonal antibodies were prepared by fusion of splenocytes with X63.Ag8.653 myeloma cells as previously described [14]. Supernatants from wells containing hybridoma colonies were screened for recognition of rGlpO by immunoblot. Hybridomas harvested from positive wells were cloned and the selection procedure repeated. A hybridoma lines, designated MUG 1, was obtained and further tested for specificity.

2.6. Immunoblot

Immunoblotting was performed as described [18], with some modifications. Briefly, proteins were electro-blotted on nitrocellulose (NC) membrane with pore size 0.45 µm (Protran Whatman, Gmbh Germany) under constant voltage of 20V overnight at 4°C. Membranes were blocked in 5% BSA in PBS-T (PBS 0.05% Tween 20) for 1 h then washed in PBS-T before being incubated for 1 h with the relevant primary antibody diluted in PBS-T. After a further wash, membranes were incubated for 1 h in horseradish peroxidase-conjugated goat anti-bovine IgG or goat anti-mouse IgG (Sigma) as appropriate. Bands were visualized by incubation in 30% DAB and 1% H$_2$O$_2$ in PBS.

2.7. Neutralization of enzyme activity by antibodies

The method of [10] was used to measure the ability of anti-GlpO sera to inhibit production of H$_2$O$_2$ by Mmm strain B237
in vitro. Thirty ml of Mmm culture in exponential growth phase (5 x 10^6 cells/ml) was centrifuged at 8000 x g for 15 min. The supernatant was discarded and the pellet was washed by centrifugation using 10 ml sterile pre-warmed (37 °C) incubation buffer (0.1 M HEPES, 1.4 M NaCl, 0.1 M NaOH and 7 mM MgCl_2; pH 7.3). The culture was re-suspended in incubation buffer to a volume consistent with an OD_600 of 0.5–0.8 and 1 ml aliquots were dispensed in labelled 2 ml centrifuge tubes. After addition of 200 μl of diluted anti-GlpO serum, tubes were incubated for 1 h in a 37 °C water bath. Cells were then washed twice by centrifugation for 10 min at 8000 x g at 37 °C, resuspended in 2 ml incubation buffer containing glycerol at a final concentration of 100 μM and returned to the 37 °C water bath. Released H_2O_2 was quantified using Merckoquant Peroxidase test strips (Merck, Darmstadt, Germany). Starting 1 min after exposure to glycerol, a colorimetric strip was dipped into individual tubes for 1 s and the colour of the reaction zone was recorded. In this way, released H_2O_2 was measured at intervals of 5, 10, 15, 20 and 40 min. The experiment was repeated on three occasions and mean of each treatment was determined.

2.8. Bioinformatic analyses

Comparison of the GlpO protein sequence of Mmm (GenBank accession number CAE46341) with the EMBL/GenBank database was performed using the BLAST programme BLASTP [19]. Prediction of sub-cellular localizations was made with Psortb v3.0.2 (http://www.psort.org/psortb/; [20]). Repartition of detected proteins according to Cluster of Orthologous Group was performed with COGnitor (http://www.ncbi.nlm.nih.gov/COG/old/xognt.html).

3. Results

3.1. Immunoblot

SDS-PAGE analysis of eluates from the Ni-NTA matrix revealed the full-length 47 kDa rGlpO polypeptide along with additional minor bands of various sizes (Fig. 1a). A 100 ml-culture of E. coli yielded 2–4 mg of protein, of which rGlpO was the principal molecular component. Both mouse anti-GlpO serum (Fig. 1b) and serum prepared from GlpO-immunized cattle (Fig. 2b) reacted with native Mmm GlpO at a dilution of 1:100, although serum from animal #670 showed a comparatively weak signal. As expected, sera from cattle in the placebo group did not react with rGlpO (Fig. 2e). After challenge, in addition to antibody responses to other Mmm proteins, animals in both groups showed strong responses to native GlpO at a dilution of 1:100 (Fig 2c and d).

3.2. In vitro neutralization of Mmm H_2O_2 production by GlpO antisera

Release of H_2O_2 by untreated Mmm after addition of glycerol increased over 5–10 min to reach a plateau of 2–5 μg/ml, depending on the experiment (Fig. 3). Significant inhibition of peroxide release was observed in Mmm pretreated with varying dilutions of mouse anti-serum raised against full length rGlpO (Fig. 3a) before addition of glycerol, although inhibition was markedly reduced at a dilution of 1:5000. Release of H_2O_2 was not observed in control preparations consisting of Mmm and incubation buffer without glycerol.

Supernatant obtained from the MUG1 hybridoma (IgG2b) raised against rGlpO was also tested for its ability to inhibit H_2O_2 release by Mmm (Fig. 3b). This supernatant significantly blocked H_2O_2 release at a dilution of 1:10, while partial blocking was apparent at 1:100. However, the blocking capacity of this supernatant was minimal at 1:1000, with released H_2O_2 reaching near baseline levels after 15 min incubation. As expected, supernatants from mAb PK2, which binds Mmm membrane polysaccharide [21] did not inhibit H_2O_2 release.

Although seroconversion of cattle immunized with rGlpO was confirmed by immunoblot analysis, none of these sera inhibited H_2O_2 production by Mmm, before or after challenge infection (Fig. 3c). Similarly, sera derived from non-immunized cattle after challenge failed to inhibit H_2O_2 production by Mmm (data not shown).

3.3. Impact of GlpO immunization on the outcome of challenge

Clinical signs, antibody titres before euthanasia, survival, size of lung lesions and mycoplasma isolation are shown in Table 1. In both experimental and placebo groups, post-challenge clinical signs were consistent with acute CBPP (3 vaccinees and 1 placebo) and chronic CBPP (all remaining animals). Cattle displaying acute CBPP and were euthanized before the end point. While 6 vaccinates and 4 placebo animals developed fever (≥39.5 °C) within 2 weeks after challenge, no significant association was evident between immunization status and the likelihood of developing fever (Fisher’s exact test, p = 0.55). Four vaccinees displayed CBPP lesions while only 1 placebo animal had a regressing lesion. Three immunized and five placebo animals had single or multiple sequestra (Table 1).

3.4. Bioinformatic analysis

Bioinformatic analysis revealed no GlpO homologue in cattle. The closest structurally related orthologue of GlpO of Mmm in cattle and other mammals is 1,2-hydroxy glutarate dehydrogenase (L2HDH; Acc. no. BC151577), an enzyme belonging to the FAD-dependent superfamily of malate: quinone oxidoreductases of Bos
taurus and Bos grumiensis, showing a maximum amino-acid (a.a.) identity of 26% over a coverage of 87% of the GlpO a.a. sequence, with an E-value of $2 \times 10^{-24}$. The only similarity between these two orthologues was found within the FAD binding site. Comparing the a.a. sequence of the FAD binding site of Mmm GlpO with the corresponding sequences of L2HDH of cattle (Bos taurus L2HDH GenBank accession no. BC151577), mouse (Mus musculus L2HDH GenBank accession no. AK152450) and rabbit (Oryctolagus cuniculus L2HDH GenBank accession no. XM_002718232) reveals that the bovine L2HDH FAD binding site is most closely related to FAD of GlpO and to a lesser extent that of mouse, followed by that of rabbit (Fig. 4).

Table 1
Summary of clinical, serological, pathological and bacteriological outcome of experimental challenge in GlpO vaccinated and placebo groups.

| Animal ID | Respiratory distress | Fever | Survival (days) | Lung lesion (cm) | Mmm isolation |
|-----------|----------------------|-------|-----------------|------------------|---------------|
| (a) GlpO vaccinated group | | | | | |
| 652       | Yes                  | Yes   | 35              | $13 \times 116.5 \times 3$ (s) | +ve |
| 656       | No                   | Yes   | 35              | $9.3 \times 8.5$ (s) | +ve |
| 658       | Yes                  | No    | 35              | No               | −ve |
| 662       | No                   | Yes   | 15              | $10 \times 19 \times 12$ | +ve |
| 664       | No                   | Yes   | 35              | $10 \times 17 \times 2$ (s) | +ve |
| 668       | No                   | Yes   | 20              | $15 \times 22$ | +ve |
| 670       | No                   | Yes   | 15              | $26 \times 15$ | +ve |
| (b) Placebo group | | | | | |
| 651       | Yes                  | No    | 35              | $12 \times 11$ (s) | +ve |
| 654       | No                   | No    | 35              | $1.8 \times 0.8$ (ms) | +ve |
| 657       | No                   | Yes   | 35              | $7.1 \times 2$ (s) | +ve |
| 659       | No                   | No    | 35              | No               | −ve |
| 663       | Yes                  | Yes   | 20              | $7 \times 9$ (rl) | +ve |
| 667       | Yes                  | Yes   | 35              | $10 \times 9$ (s) | +ve |
| 669       | No                   | Yes   | 35              | $4.8 \times 4.3$ (s) | +ve |

The size shown is the longest dimensions (L × W) of the area covered by the lesion s, sequestrum; ms, multiple sequestra; rl, lesion in recession.
Fig. 3. The capacity of anti-GlpO mouse polyonal sera, MUG1 monoclonal antibodies and bovine sera to inhibit production of $\text{H}_2\text{O}_2$ by $\text{Mmm}$. $\text{Mmm}$ were starved in PBS at 37°C and simultaneously pre-incubated with serum or hybridoma supernatants before addition of 100 $\mu$M of glycerol and measurement of the quantity of $\text{H}_2\text{O}_2$ (y-axis) by $\text{Mmm}$ at the intervals shown (a) Mouse polyclonal sera against GlpO: +glyc: +glyc control: $\text{Mmm}$ that did not receive glycerol after starvation; +glyc; $\text{Mmm}$ that was not incubated with serum but received glycerol after starvation; +glyc+serum: $\text{Mmm}$ incubated with serum at the dilution shown and also received glycerol after starvation. (b) Hybridoma supernatant containing anti-GlpO Mab: +glyc neg control: $\text{Mmm}$ that did not receive glycerol after starvation; +glyc: $\text{Mmm}$ that was not incubated with serum but received glycerol after starvation; +glyc+PK2: $\text{Mmm}$ that was incubated with hybridoma supernatant containing antibodies against a the $\text{Mmm}$ capsular polysaccharide (PK2) before addition of glycerol; +glyc+Mab: $\text{Mmm}$ that was incubated with hybridoma supernatant containing Mabs against GlpO at the dilutions shown before addition of glycerol. (c) Bovine sera against GlpO (dilution 1:1): +glyc neg control: $\text{Mmm}$ that did not receive glycerol after starvation; +glyc: $\text{Mmm}$ that was not incubated with serum but received glycerol after starvation; +glyc+pre-challenge sera: $\text{Mmm}$ that was incubated with sera from vaccinated but uninfected cattle before addition of glycerol; +glyc+post-challenge sera: $\text{Mmm}$ that was incubated with vaccinated and infected cattle sera before addition of glycerol. For each data point, $n = 7$, and the error bars represent the means of standard deviation. (x-axis).

4. Discussion

We have evaluated the capacity of a recombinant form of $\text{Mmm}$ GlpO to protect cattle against challenge with the organism. Using an immunization regimen based on Freund’s adjuvant formulations, we found no evidence of protection in immunized animals, despite evident sero-conversion. Indeed, vaccines seem to show increased pathological damage in comparison to challenge control cattle. This suggests either that GlpO is not a protective antigen, or that inappropriate immune responses were generated using Freund’s adjuvant. No information is currently available on the nature of protective immunity in CBPP, although more favourable outcomes have been associated with $\text{Mmm}$-specific IFN-γ secreting CD4+ T cells [22,23] and, independently, with specific serum IgA responses [24]. We did not collect data on these parameters in our study.

With the exception of lung lesions in the immunized group and sequestration in the placebo group, no clear differences were apparent between the experimental and control groups with regard to post-challenge clinical and pathological parameters. However, immunized animals seemed to exhibit a more adverse outcome. Three of these animals were euthanized on humane grounds before the end of the experiment. Nonetheless, given the relatively low number of animals involved, no definitive conclusion is possible on whether immunization with recombinant GlpO results in a more severe outcome of disease following experimental infection. Enhanced pathology has been reported previously in cattle challenged with $\text{Mmm}$ following immunization with a recombinant form of the LppQ protein of the organism formulated in ISCOMs [25]. The authors did not determine the underlying causes of the enhanced pathology and, given that a different adjuvant system was used, it is difficult to make direct comparisons between that study and our observations.

Immunoblot analysis confirmed that immunization with rGlpO induced detectable antibody responses against the native GlpO molecule, although these antibodies did not confer protection in vivo. Anti-GlpO polyclonal mouse serum and a mAb against rGlpO inhibited $\text{H}_2\text{O}_2$ $\text{Mmm}$ production. This is consistent with previous reports, which demonstrated that sera from GlpO-immunized rabbits or mice could neutralize enzymatic activity of GlpO [9,26]. However, in our hands, sera from cattle immunized with rGlpO, or from cattle infected with CBPP, failed to inhibit $\text{H}_2\text{O}_2$ production by $\text{Mmm}$. In addressing this dichotomy, we have identified a
single amino-acid substitution in the flavin-adenine-dinucleotide (FAD)-binding site (one of the main active sites of GlpO) of MmM
GlpO, as compared with the closest orthologue in cattle, mice and rabbits, L2DH.D. It is tempting to speculate that adaptation of MmM to mimic neutralizing epitopes of bovine orthologues is responsible for absence of neutralizing antibodies to GlpO of MmM in cattle immunized with rGlpO. Such an eventuality might arise from a requirement to avoid immune recognition of neutralizing epitopes in cattle in order to retain enzyme function, in line with the importance of GlpO in its metabolism of glycerol [9].

In conclusion, our results indicate that induction of a humoral response to rGlpO of MmM fails to protect cattle against challenge with the organism, with immunized animals showing apparently enhanced pathology. The evident lack of GlpO neutralizing activity in sera from immunized cattle when compared with that obtained from similarly immunized mice is intriguing, particularly in the light of a closer homology of GlpO with the bovine orthologue. Further work will be required to determine whether this truly reflects an adaptation of the organism to avoid the generation of a neutralizing response against the enzyme in cattle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.08.100. These data include Google maps of the most important areas described in this article.

References

[1] Epegwu GO, Nicholas RAJ, Ameh JA, Bashidurin JB. Contagious bovine pleuro-
pneumonia: an update. Vet Bull 1996;66(9):877–88.
[2] Manso-Silvan L, Vilei EM, Sachse K, Djordjevic SP, Thiaucourt F, Frey J. Mycoplasma leachi sp. nov. as a new species designation for Mycoplasma sp. bovine group 7 of leach, and reclassification of Mycoplasma mycoplas. mycoplas LA as a serovar of Mycoplasma mycoplas subsp. capri. Int J Syst Evol Microbiol 2009;59(Pt 6):1353–8.
[3] Provost A, Perreau P, Breaud A, le Geoff C, Martel JL, Cottew GS. Contagious bovine pleuropneumonia. Revue scientifique et technique. Office Int des Epizootes 1987;6:625–79.
[4] Thiaucourt F, Yaya A, Wesonga H, Huebschle OJ, Tulasne J, Provost A. Contagious bovine pleuropneumonia. A reassessment of the efficacy of vaccines used in Africa. Ann NY Acad Sci 2000;916:71–80.
[5] Abu-Groun EA, Taylor RR, Varsani H, Wadher BJ, Leach RH, Miles RJ. Bio-
chemical diversity within the Mycoplasma mycoplas cluster. Microbiology 1994;140(8):2033–42.
[6] Pollack JD, Williams MV, McElhaney RN. The comparative metabolism of the mollicutes (Mycoplasmas): the utility for taxonomic classification and the relationship to itsputative gene annotations and putative biological function in the smallest free-living cells. Crit Rev Microbiol 1997;23(4):269–354.
[7] Rotter S. Interaction of mycoplasmas with host cells. Physiol Rev 2003;83(April 2):417–32.
[8] Polo P, Frey J, Vilei EM. Molecular mechanisms of pathogenicity of Mycoplasma mycoplas subsp. mycoplas SC. Vet J 2007;174(November (3)):513–21.
[9] Polo P, Vilei EM, Peterhans E, Bonvijn-Kloetz L, Stoffel MH, Doblarlebera D, et al. A metabolomic view as a primarily one factor of Mycoplasma mycoplas subsp. mycoplas small colony. J Bacteriol 2005;187(October (19)):6824–31.
[10] Vilei EM, Frey J. Genetic and biochemical characterization of glycerol uptake in Mycoplasma mycoplas subsp. mycoplas: its impact on H2O2 production and virulence. Clin Diagn Lab Immunol 2001;8(January (1)):85–92.
[11] Bischof DF, Janis C, Vilei EM, Berton G, Frey J. Cytotoxicity of Mycoplasma mycoplas subsp. mycoplas small colony type to bovine epithelial cells. Infect Immun 2008;76(January (1)):263.
[12] Gates AE, Frasca S, Nyaoko A, Corton TS, Silburt DK, Geary SJ. Comparative assessment of a metabolically attenuated Mycoplasma gallisepticum mutant as a live vaccine for the prevention of avian respiratory mycoplasmosis. Vaccine 2008;26(April (16)):2010–9.
[13] Brown RD, Gourlay RN, Macleod AK. The production of T1 broth cul-
ture contagious bovine pleuropneumonia. Bull Epizoot Dis Afr 1965;13(June):149–55.
[14] Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, Maclunes JI, et al. Characterization of apxIVA, a new RTX determinant of Actinobacillus pleu-
ropneumoniae. Microbiology 1999;145(August Pt (8)):2105–16.
[15] Jones J, Nikand I, Sterner-Kock A, Haider W, Poole J, Unger H, et al. Assessment of in vitro interferon-gamma responses from peripheral blood mononuclear cells of cattle infected with Mycoplasma mycoplas subsp. mycoplas small colony type. Vet Immunol Immunopathol 2008;124(July (1–2)):192–7.
[16] Litamo JK, Palya VI, Sylla D, Rweyemamu MM, editors. Quality control test-
ing of contagious bovine pleuropneumonia live attenuated vaccine; standard operating procedures, Rome, Italy; FAO: 1996.
[17] Villegas P, editor. Titration of biological suspension. 4th ed. Krennet Square, American Association of Avian Pathologists Inc.: 1998.
[18] Goncalves R, Regalla J, Nicolet J, Frey J, Nicholas R, Bashiruddin J, et al. Antigen heterogeneity among Mycoplasma mycoplas subsp. mycoplas SC iso-
lates: discrimination of major surface proteins. Vet Microbiol 1998;63(August (1)):13–28.
[19] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215(3 Oct 5):403–10.
[20] Yang YW, Wagner JR, Laird MR, Melli G, Rey S, Lo R, et al. PSORTb 3.0: improved protein subcellular localization prediction with refined localized subcategories and predictive capabilities for all prokaryotes. Bioinformatics (Oxford, England) 2010;26(13):1608–15.
[21] Kiarse MN, Rurangirwa FR, Perryman LE, Jasmer DP, McGuire TC. Mono-
clonal antibodies to surface-exposes proteins of Mycoplasma mycoplas subsp. mycoplas (small-colony strain), which causes contagious bovine pleuropne-
pneumonia. Clin Diagn Lab Immunol 1995;3(6 November 1):746–52.
[22] Dedieu L, Balcer-Rodrigues V, Yaya A, Hamadou B, Cisse O, Dlalo M, et al. Gamma interferon-producing CD4 T-cells correlate with resistance to Mycoplasma mycoplas subsp. mycoplas SC infection in cattle. Vet Immunol Immunopathol 2005;107(3–4):217–33.
[23] Totte P, Rodrigues V, Yaya A, Hamadou B, Cisse O, Dlalo M, et al. Analysis of cellular responses to Mycoplasma mycoplas subsp. mycoplas small colony biotype associated with control of contagious bovine pleuropneumonia. Vet Res 2008;39(1):8.
[24] Niang M, Dlalo M, Cisse O, Kone M, Doucoure M, Roth JA, et al. Pulmonary and serum antibody responses elicited in zebu cattle experimentally infected with Mycoplasma mycoplas subsp. mycoplas SC by contact exposure. Vet Res 2006;37(5 Sep–October(733–44.
[25] Nicholas R, Tijpuara-Zaire G, Mbulo R, Scacchia M, Mettler F, Frey J, et al. An inactivated whole cell vaccine and LppQ subunit vaccine appear to exacerbate the effects of CBFP in adult cattle. In: Proceedings of the 3rd meeting of the FAO-
OECD-IAUR-IAEA consultative group on CBFP 2003 Rome, 12–14 November 2003, p. 91–7.
[26] Bischof DF, Vilei EM, Frey J. Functional and antigenic properties of GlpO from Mycoplasma mycoplas subsp. mycoplas SC: characterization of a flavin adenine dinucleotide-binding site deletion mutant. Vet Res 2009;40(4):35.