Effect of HEPES buffer systems upon the pH, growth and survival of 
*Mycoplasma mycoides* subsp. *mycoides* small colony (MmmSC) 
vaccine cultures

Emma R. Waite, John B. March *

Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, UK

Received 15 May 2001; accepted 7 June 2001

First published online 2 July 2001

**Abstract**

The use of a buffer system based on *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES), in conjunction with standard Gourlay’s culture medium was investigated for the growth and maintenance of *Mycoplasma mycoides* subsp. *mycoides* SC vaccine strain T144. When the initial pH of the culture medium was adjusted to 8.0, 0.075 M HEPES-NaOH was found to be sufficient to prevent the pH falling below 7.1 at any stage during the growth cycle, even in the presence of 0.5% glucose. Compared to growth in standard unbuffered Gourlay’s medium, the final culture titre was found to be one log10 higher, at 10^11 colour changing units (CCU) per ml, and considerably extended culture survival was observed at 37°C. The titre remained above 10^10 CCU ml^-1 for 4 days, and above 10^8 CCU ml^-1 in excess of 1 month. After 4 month’s storage at 37°C the titre had fallen to 5 x 10^4 CCU ml^-1. In contrast, no viable bacteria could be detected in standard unbuffered medium 3 days after the onset of stationary phase, at which point the pH had dropped to 5.4. No significant difference in growth rate between the two media was observed. Adoption of a HEPES-NaOH buffer system by African vaccine manufacturers should require minimal changes to current formulations and procedures, and should enhance both the final titre and thermostability of freeze-dried and liquid broth vaccines against contagious bovine pleuropneumonia (CBPP). © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Contagious bovine pleuropneumonia; Vaccine; *Mycoplasma mycoides* subsp. *mycoides* SC

1. **Introduction**

Contagious bovine pleuropneumonia (CBPP) is currently the most economically serious disease of cattle in Africa, with losses per annum estimated to be in the region of US$2 billion [1]. In the last decade there has been a substantial re-emergence of the disease, despite vaccination campaigns using freeze-dried broth cultures of live attenuated *Mycoplasma mycoides* subsp. *mycoides* small colony biotype (MmmSC) (strain T144 or T1SR). Observations from the field [2,3] and experimental studies [4,5] have indicated that the current vaccines do not effectively protect cattle from outbreaks of disease.

A major factor behind poor vaccine efficacy is likely to be suboptimum bacterial titres. The minimum OIE-recommended vaccinating dose for a live MmmSC vaccine (strain T1) is 10^7 colony forming units (CFU) per animal [6,7]. It has been reported that many vaccine production laboratories do not reach the OIE recommendation of delivering a vaccine at 10^8 viable mycoplasmas per animal dose (which allows for losses during lyophilisation, storage and transport [8,9]). Current vaccines are freeze dried to allow for longer term storage at −20°C and to reduce the requirement for cold chain transport in the field. However, following reconstitution they must be used within 2 h to avoid unacceptable titre loss in the high ambient temperatures found in African field conditions [10].

The pH of the growth medium is an important factor which affects bacterial viability [11]. MmmSC produces acidic metabolites during growth which lower the pH of
the medium. This eventually becomes inhibitory to growth and the titre falls. The best growth of MmmSC is achieved between pH 7 and 8, the optimum being pH 7.4; a decrease in pH to less than 6.5 causes cessation of growth and rapid death of cells [12]. Windsor [13] showed that it was the drop in pH and not a loss of nutrients during growth that was the cause of bacterial death following entry into stationary phase. Maintenance of a neutral pH by the intermittent addition of KOH during growth largely preserved the viability of the culture. Unfortunately, for reasons of sterility and practicality, continuous monitoring and correction of culture pH is not a realistic proposition, particularly following vaccine reconstitution in the field.

CBPP was eradicated in Australia in the late 1960s following many decades of vaccination [14] using liquid broth cultures in which bacteria remained viable for long periods under adverse conditions (up to 3 months at 37°C, e.g. [15,16]). The broth used to grow the vaccine was made in-house from a meat liver digest with added ox serum and a buffer system made up of dibasic (Na₂HPO₄) and monobasic (KH₂PO₄) phosphate salts (BVF-OS [15]). Current vaccine media (e.g. Gourlay's [17] and F₆₆ [18]) are made predominantly of desiccated manufacturer-supplied components (e.g. bacto-tryptose and yeast extract), a dibasic (Na₂HPO₄) phosphate salt only, together with the addition of a dedicated carbon source (glucose). Glucose is a reducing sugar, and its metabolism directly leads to the large drop in pH observed during MmmSC growth. Glucose can be omitted from the culture medium, thereby reducing the drop in pH (and leading to an increased longevity of the culture), but its absence also leads to a reduction in the final yield of MmmSC [11,19]. Addition of phosphate buffer to the growth medium at concentrations that can be tolerated by osmotically sensitive mycoplasma was found to be unable to maintain a neutral pH when standard glucose concentrations were also present in the growth medium [11,19]. Thus a trade-off is apparent between the need to maintain a neutral pH for bacterial longevity (i.e. glucose absent) and the conflicting requirement for a dedicated carbon source to achieve a high final titre (i.e. glucose present), whilst minimising any osmotic damage due to the presence of an excessive buffer salt concentration.

This study was designed to investigate an osmotically benign buffer system that would maintain the pH above 7.0 when standard glucose concentrations were present in the growth medium, thus prolonging bacterial viability and at the same time allowing a high final titre. To increase the likelihood of uptake by vaccine producers, changes to current media formulations and growth protocols were minimised. A buffer system based on N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), in conjunction with a starting pH of 8.0 rather than 7.6 was assessed in detail for a mycoplasmal culture over a 4-month period at 37°C.

2. Materials and methods

2.1. Mycoplasma strains and growth conditions

T₄4 [20] vaccine strain of MmmSC (batch number 6PRV316, obtained from the Botswana Vaccine Institute, Gaborone, Botswana) was grown in the following media: (a) Standard Gourlay’s medium pH 7.4 (modified Newing’s tryptose broth [17]). Autoclaved (121°C, 15 min) portion (weights/volumes are per litre): bacto-tryptose (Difco) 20 g, glucose 5 g, NaCl 5 g, Na₂HPO₄ 2.5 g, glycerol 5 ml, yeast extract (Difco) 1 g, distilled water 750 ml. When cooled to 47°C, the following were added: heat-inactivated horse serum 200 ml, thallous acetate (10% w/v) 2.5 ml, ampicillin (100 mg ml⁻¹) 2.5 ml, phenol red (0.4% w/v) 15 ml. (b) HEPES-buffered Gourlay’s medium (buffered Gourlay’s) was produced by adding HEPES to the above medium at a concentration of 0.075 M (17.9 g l⁻¹), and adjusting to pH 8.0 using concentrated NaOH.

Growth of culture: 1 ml of stock T₄4 (from −70°C storage) was inoculated into 100 ml of standard Gourlay’s medium and incubated at 37°C overnight. When the culture was at the late logarithmic phase of growth (indicated by an orange colour or drop of pH to ca. pH 6.8 [21]) an aliquot was diluted 1:50 into 100 ml of fresh medium (either standard or buffered Gourlay’s) and grown and maintained at 37°C in a stopped 250-ml glass bottle over a 4-month period.

2.2. Determination of the pH and titre

Samples were taken every 4 h for the first day and once daily thereafter for the first week, then approximately weekly until the end of the experiment (4 months). The pH of 1-ml samples was measured using a pH meter (Mettler, Delta 345). The titre (colour changing units (CCU) per ml) was determined using a microtitration method adapted from [22]. Briefly, 50 µl of the growing culture was added to the first column of a sterile 96-well microtitre plate. Each well of the plate contained 200 µl of fresh, unbuffered Gourlay’s medium. Serial 5-fold dilutions were made across the plate using an eight-channel pipette (eight replicates). The plate was incubated at 37°C for 1 week and the estimated titre was calculated by counting the number of wells positive for growth, as indicated by a colour change from red to yellow.

3. Results and discussion

The titre, pH and survival of cultures of MmmSC grown and maintained in standard (unbuffered) Gourlay’s medium, and Gourlay’s medium buffered by the addition of 0.075 M HEPES pH 8.0 at 37°C are shown in Fig. 1. The pH of standard Gourlay’s medium dropped from 7.4 to 5.5 during growth, and this decline was mirrored by a
rapid drop in the titre of MmmSC. The maximum titre of $10^{10}$ CCU ml$^{-1}$ was observed between 23-42 h post-inoculation. No viable bacteria could be detected 48 h later (90 h after the initial inoculation of the medium, when the pH had dropped to 5.4). In contrast, the pH of HEPES-buffered Gourlay’s medium dropped from 8.0 to 7.1 during growth, and both the final bacterial titre and culture survival were increased compared to unbuffered medium. Maximum titre was at least one log$_{10}$ higher at $10^{11}$ CCU ml$^{-1}$ at 24-48 h post-inoculation, while the titre was maintained above $10^8$ CCU ml$^{-1}$ for up to 1 month at 37°C. Even after storage for 4 months at 37°C the residual titre remained at $5 \times 10^4$ CCU ml$^{-1}$ in a stoppered glass bottle.

In this study, several buffer systems were tested, including phosphate (di-sodium hydrogen phosphate (0.056 M) and potassium di-hydrogen phosphate (0.015 M) [15]), Tris-HCl (0.05 M) and HEPES-NaOH. Both Tris-HCl and phosphate buffer systems were found to be unable to maintain medium pH above 7.0 following mycoplasmal growth (final pH of 6.7 and 6.6 respectively from a starting pH of 8.0; data not shown). HEPES was chosen because previous work had shown it to be an effective buffer for growth of a related organism, Acholeplasma laidlawii [12], although previous studies had not yielded encouraging final pH values following growth of MmmSC in HEPES-buffered media containing glucose. Rice et al. [23] observed final pH values of 6.1, 6.3 and 6.7 with 30, 90 and 120 mM of HEPES added to PRM media, respectively (starting pH of 7.6), suggesting that osmotically damaging levels of HEPES might be needed to prevent the pH from dropping below neutrality. The alternative approach adopted here was to alter the starting pH to 8.0 rather than 7.6, and to use a relatively low level of HEPES (75 mM). No significant reduction in growth rate compared to standard Gourlay’s medium was observed, either from the data shown in Fig. 1, or from a variety of replicate growth curves comparing the two media (data not shown).

Previous studies [11,13,19,24] examining the effect of final medium pH on MmmSC survival have almost exclusively concentrated upon the storage of liquid cultures at 4°C, rather than at 37°C as reported here. The use of HEPES-buffered medium with a starting pH of 8.0 offers several advantages compared to current vaccine formulations. Using HEPES-buffered medium, the time of freeze drying is not as vital since considerable latitude is apparent before the culture titre starts to decay (no significant drop was observed over a period of several days; Fig. 1). The considerably enhanced survival of the culture, particularly at higher temperatures, suggests that a return to liquid broth cultures may be feasible (thought by some to exhibit greater protective efficacy [25], although currently exhibiting greater cold chain dependence than freeze-dried vaccines). The higher titre observed using HEPES-buffered medium should reduce both production costs and the incidence of vaccine failure. In addition, the immediate use of reconstituted freeze-dried vaccine should not be necessary in the field, since HEPES-buffered cultures exhibit considerable thermostability at temperatures up to 37°C. This should increase both the ease of use in the field, and lead to a reduction in wastage.

Minimal changes are required compared to current protocols, with only the addition of a small amount of relatively inexpensive HEPES to current media formulations, with no other alterations to current procedures and protocols. In a continent where control and eradication of CBPP depends largely on successful vaccination programmes, the production of a consistently potent and stable vaccine is essential in the fight against the disease.
Acknowledgements

This research was supported by a grant from the Animal Health Program of the UK Department for International Development. For their help, advice, support and useful discussions thanks are extended to Roger Windsor, Duncan Brown, Ian Maudlin, Jason Clark, Rachel Loiselet, Willie Donachie, Martyn Jeggo and Joseph Litamoi.

References


