Safety and Protective Efficacy of Intramuscular Vaccination with a Live aroA Derivative of Pasteurella multocida B:2 against Experimental Hemorrhagic Septicemia in Calves

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Three groups of five calves, namely, V1, V2, and V3, were immunized intramuscularly at 4 and 8 weeks of age with ca. 10⁸, 10⁹, and 10⁷ CFU, respectively, of a derivative of Pasteurella multocida B:2 wild-type strain 85020 containing a deletion in the aroA gene (strain JRMT12). The first and second vaccinations resulted in significantly (P < 0.01) higher rectal temperature responses in groups V1 and V2 than in group V3. Serum immunoglobulin M (IgM) and IgG titers did not increase in any group until after the second vaccination and were then significantly higher in groups V1 and V2 than in group V3 (P = 0.001 for both IgM and IgG). All vaccinated groups and three unvaccinated challenge control calves (group CC) were injected subcutaneously at 10 weeks of age with ca. 10⁷ CFU of strain 85020. Vaccinated calves survived the challenge, but two CC animals developed clinical disease and were killed for humane reasons. After challenge, mean serum amyloid A concentrations were significantly higher (P < 0.001) in the CC group than in the vaccinated groups. Postmortem examination revealed that calves in the CC group showed the most extensive range of bacteriologically positive tissues and gross and histopathological lesions. Overall, a clear dose-dependent response was present, with those receiving a higher vaccine dose being less affected clinically, bacteriologically, and pathologically by the wild-type challenge. The V2 treatment appeared to give the best combination of high immune response, protection, and safety.

Hemorrhagic septicemia (HS) affects cattle (family Bovidae) and buffaloes (Bubalus bubalis) and is caused by the gram-negative bacterium Pasteurella multocida. The disease has a major impact on the livestock industry in countries of South and Southeast Asia, where HS associated with serotype B:2 is distributed widely. It results in severe economic losses through morbidity and mortality and is ranked as the most important contagious disease of cattle and buffaloes by most countries in the region (1, 23), with young animals being most at risk of successive outbreaks (8).

Alum-precipitated or oil-adjuvant broth bacterins injected subcutaneously (s.c.) give some protection against HS, but these vaccines provide only short-term immunity and the high viscosity of oil-adjuvant vaccines makes them unpopular among field users (23). Multiple emulsion preparations have reduced the viscosity of these vaccines, making delivery easier, and immunity for up to 1 year has been reported (22). Attempts to elicit long-term immunity with live P. multocida vaccines have included the use of a streptomycin-dependent variant that gave variable protection for cattle and more reliable protection for buffaloes, although the long-term duration of immunity was not reported (7). A heterotypic live vaccine was developed from a serotype B:3,4 strain isolated from a fallow deer in the United Kingdom and showed promise, but the application of this vaccine resulted in mortality in a number of animals under 6 months of age and failed to induce immunity in a proportion of others (18). An intranasal version proved much safer, but some animals died of HS from a field-acquired infection within 11 months of vaccination (3).

Recently, we evaluated the efficacy of a live attenuated vaccine against experimental HS in calves, using a marker-free aroA deletion derivative of a virulent field isolate of P. multocida B:2 obtained from Sri Lanka (12). Calves that were immunized intramuscularly (i.m.) at 2 and 6 weeks of age with ca. 10⁶ CFU of the attenuated strain (JRMT12) were solidly immune to s.c. challenge at 8 weeks of age with ca. 10⁷ CFU of the wild-type parent strain (85020). The same dose of vaccine given intranasally failed to provide immunity to experimental challenge. A febrile response within 6 h of i.m. vaccination, probably caused by the endotoxin content of the vaccine preparation, prompted parenteral treatment of calves with a non-steroidal anti-inflammatory drug in case it signified the early stages of endotoxic shock. The objectives of the present work were to administer the same and lower doses of the attenuated vaccine strain via the i.m. route and to determine whether lower doses were nonreactogenic and protective against s.c. challenge with the virulent parent strain. The long-term aim of this work with a natural host species is to produce an effective,
easily administered vaccine for large-scale field use in all ages of cattle and buffaloes that will result in long-term immunity. This would enable control of HS and thereby improve the health and welfare of cattle and buffaloes throughout the affected regions and the communities dependent upon them.

MATERIALS AND METHODS

Preparation of vaccination and challenge doses. To ensure uniformity of vaccination and challenge doses, all were prepared as described previously (12) and diluted in phosphate-buffered saline (PBS) as necessary to provide vaccination doses of ca. 10^6, 10^7, and 10^8 CFU and a challenge dose of ca. 10^10 CFU in 5 ml. Actual doses were determined retrospectively from viable counts after placing metered amounts onto sheep blood agar (blood agar base [Oxoid] containing 5% [vol/vol] sheep blood).

Animal procedures. P. multocida-free calves (n = 21) were selected and maintained in containment category 2 (UK Advisory Committee on Dangerous Pathogens), and waste was disposed of as described previously (12). All experimental protocols were approved by the Morison Research Institute Animal Experiments Committee and authorized under the UK Animals (Scientific Procedures) Act 1986. Calves to be vaccinated were allocated randomly to three different treatment groups (V1, V2, and V3), with each comprising five calves. Calves were immunized twice i.m. with the attenuated, aroA-deleted P. multocida strain JRMT12 in 5 ml of PBS injected into the semitendinosus muscle of each hind limb, first on the left side at 4 weeks of age (day 0), with a total of ca. 10^6, 10^7, or 10^8 CFU for group V1, V2, or V3, respectively, and then on the right side at 8 weeks of age (let 28), with the same dose. Groups of nonvaccinated calves (n = 3) were used as challenged (group CC) or unchallenged (group NC) controls. At 10 weeks of age (day 42), vaccinated and CC calves were injected s.c. with 1.35 × 10^7 CFU of P. multocida wild-type strain 85020 in 5 ml of PBS divided into two 2.5-ml doses, with one over each prescapular region. Experienced observers with access to veterinary advice and care at all times monitored rectal temperatures and the general demeanor (normal, dull, or depressed, as a score of 0, 1, or 2, respectively) of all calves at regular intervals in order to log changes and quickly identify any calf requiring veterinary treatment. Calves in group V1 were given intravenous fluorescein meglumine (Finazyme; Schering-Plough Animal Health, United Kingdom), a nonsteroidal anti-inflammatory drug, at 2.2 mg/kg body weight 4 to 7 h after each vaccination to control the pyrexia due to endotoxin at this high dose. Also, an option to give fluorescein meglumine to calves that became dull and/or showed an increase in rectal temperature above 1.5°C following challenge was available to observers. Challenged calves given but failing to respond to this treatment (continued high temperature and/or depressed demeanor) were killed for humane reasons within the containment facility by intravenous injection of 50 ml of sodium pentobarbital (approximately 200 mg/ml; Animal Care, United Kingdom).

Blood analysis. Blood samples (10 ml) were collected periodically from the jugular veins of all calves into both plain and heparinized Vacutainers (Becton Dickinson, Franklin Lakes, NJ) prior to vaccination and challenge and at 3- to 4-h intervals in between. Sera or plasma were separated and stored at −40°C until selected samples were analyzed for the acute-phase proteins serum amyloid A (SAA) and haptoglobin (Hp) by methods described previously (10, 17). Immunoglobulin M (IgM) and IgG antibody titers to P. multocida B2 envelope antigens were assessed by enzyme-linked immunosorbent assay (ELISA). ELISA for antibody to P. multocida B2 envelope antigens. The production of bacterial envelopes and ELISA methods were as described previously (12). Antibody titers were determined as ELISA U/ml of neat calf serum by comparison with values obtained for reference sera with intermediate IgG or high IgM titers, with each assigned arbitrarily the value of 1,000 ELISA U/ml. The antibody titers of all test samples, in ELISA U/ml, were calculated as the ratio of test serum optical density at 492 nm to reference serum optical density at 492 nm at a particular dilution × 1,000.

Necropsy. Postmortem examination was performed either 7 days after challenge or earlier, as dictated by the clinical status of the calves. Gross pathology was assessed, and selected lymph nodes (left and right retropharyngeal, submandibular, prescapular, axillary, subiliac, and prelomeral plus mesenteric and hepatic nodes) and the left and right tonsils were removed whole and dissected free from adjacent material prior to being weighed, fixed, and processed as described below. Representative samples from the lungs (left and right apical and caudal lobes), liver, kidney, spleen, brain, thymus, jejunum with Peyer’s patch, left and right vaccination sites (semitendinous muscle), and additional lymph nodes (mid- and caudal mediastinal and left and right bronchial nodes) were placed in 10% buffered formal-saline and prepared for histopathological examination by standard techniques (dehydrated through graded alcohols, embedded in paraffin wax, sectioned at 5 μm, mounted on glass microscope slides, and stained with hematoxylin and eosin).

Bacteriological examination. Small samples of tissue (~1-cm cubes; ~1 g) taken at postmortem examination from the lung, kidney, heart, spleen, liver, brain, left and right tonsils, and lymph nodes (left and right retropharyngeal and prescapular nodes) were homogenized in 9 ml of peptone water, and aliquots (10 μl) were spread on sheep blood agar containing vancomycin (1 mg/ml) to prevent the growth of gram-positive bacteria and incubated at 37°C for 16 to 20 h prior to phenotypic analysis. Peritoneal and tonsillar swabs and blood were also plated out, incubated, and analyzed as described above. Colonies were confirmed as P. multocida B2 by PCR, using genus-specific primers (21).

Statistical analyses. The rectal temperature and dullness score were measured at each intervention (vaccination or challenge), at four time points after each vaccination, and at five time points after challenge. For the temperature data, a separate repeated-measures model was fitted to each of these three periods, with the lack of independence between measurements made on the same animal modeled using a power model. For each period, the proportion of calves in each group that showed any sign of dullness (score greater than zero) was compared using Fisher’s exact test. IgM and IgG antibody titers were recorded on six occasions over the study period, and SAA and Hp levels were recorded at six time points after challenge for groups V1, V2, V3, and CC. Readings for IgM, IgG, and SAA showed increased variability with higher values, and thus a log transformation was used on the data before fitting them to the repeated-measures model. Serum Hp concentrations for all groups were often zero, and because of this, the small sample size, and a high level of correlation between successive time points, it was not sensible to fit a repeated-measures model either to the raw data or to the data treated as a binary variable. Instead, the effects of treatments on the Hp concentration were compared using a Kruskal-Wallis test based on the median of the maximum Hp values observed for the calves for each group.

Lymph node weights were corrected for body weight in order to identify animals that had heavier-than-expected lymph nodes. The relationship between a particular lymph node weight and body weight appeared to be consistent for groups NC, V1, and V2, and a simple linear regression model was fitted to the data for these three groups; this model should thus represent the underlying variability present in healthy calves. The fitted lines were used to estimate standardized residuals for the data from the V3 and CC groups, and r tests were used to determine whether any of the residuals were significantly higher than expected.

RESULTS

Clinical responses to vaccination with different doses of strain JRMT12. Following the first vaccination, two calves in the V1 group (at around 6 h after vaccination) and three calves in the V3 group (two at around 2 h and one from 4.5 until 18 h after vaccination) became dull. At 6 h, mean rectal temperatures (°C) ± standard errors of the means [SEM] had risen from time zero values of 38.8 ± 0.34, 38.7 ± 0.26, and 38.9 ± 0.31 to peak values of 40.3 ± 0.18, 40.2 ± 0.34, and 39.7 ± 0.18 for groups V1, V2, and V3, respectively (Fig. 1A). The patterns of change in mean rectal temperature over the monitoring period were similar for groups V1 and V2, with a peak at 6 h and a return to baseline by 18 h after vaccination. However, this may have been influenced by a single-treatment intervention with flunixin meglumine for each calf in the V1 group at between 4 and 6 h postvaccination to counteract the anticipated pyrogenic effect of high levels of endotoxin in the V1 dose. The mean temperature for the V3 group plateaued after 2 h and remained higher than the baseline for up to 24 h postvaccination, and this pattern of response was significantly different (P = 0.002) from that for groups V1 and V2. This was due to maintenance of a high temperature (about 40.5°C) in two of five V3 calves but was not seen after the second vaccination (Fig. 1B). After the second vaccination (at 7.5 h) (Fig. 1C),
1B), after which calves in group V1 were given flunixin meglumine for the same reasons as for the primary vaccination, maximum values for the mean rectal temperatures (°C) (± SEM) of the V1, V2, and V3 groups were 40.6 ± 0.13, 40.4 ± 0.19, and 39.6 ± 0.43, respectively, compared to their time zero values of 38.7 ± 0.09, 38.6 ± 0.13, and 39.1 ± 0.2, respectively. Mean rectal temperatures over time for calves in the V3 group were significantly lower ($P < 0.001$) than those for groups V1 and V2. The mean rectal temperatures of all calves in all groups had returned to baseline values by 21 h after the second vaccination. Following the second vaccination, only one animal, from group V3, was recorded as having a dull demeanor (at 2 h postvaccination).

Effect of challenge with *P. multocida* wild-type strain 85020 on clinical responses of calves vaccinated with different doses of strain JRMT12. Mean rectal temperatures rose in groups V1, V2, V3, and CC after challenge (Fig. 2). The patterns of response between the three vaccinated groups differed significantly ($P < 0.001$), with the V1 group showing the lowest increase in mean rectal temperature postchallenge and the V2 and V3 groups showing progressively greater responses. Means of the maximum observed increases in rectal temperature dur-
ing the period of 0 to 12 h postchallenge were 0.8, 1.0, 1.3, and 1.6°C for groups V1, V2, V3, and CC, respectively.

There was a significant difference (P = 0.027) in general demeanor after challenge between groups V1, V2, and V3 compared to that for the CC group. All CC animals became dull and pyrexic by 11.5 h postchallenge. One calf in the V1 group became dull briefly at 11.5 h postchallenge, and four calves in the V3 group became dull (one briefly at 15 h, one between 4.5 and 11.5 h, one between 7.5 and 28 h, and one between 7.5 and 11.5 h postchallenge).

Two calves from group V3 and two from group CC were treated with flunixin meglumine (at 11.5 h; one CC calf was treated at 23 h postchallenge) to relieve pyrexia and dullness. Both CC calves were unresponsive to this treatment, necessitating euthanasia, at 48 and 55 h postchallenge. This resulted in highly variable results for rectal temperatures for the CC group, and the data were excluded from the statistical analysis.

**Acute-phase proteins.** SAA and Hp concentrations were measured after the first vaccination in calves receiving 10⁸ CFU (group V2). Concentrations of both proteins in the sera of individual calves varied widely. The median (range) SAA concentrations increased from 16.2 (11.8 to 85.5) µg/ml pre-vaccination to 89.5 (61.5 to 122.8) µg/ml at 24 h postvaccination. Two calves showed a further increase by 36 h postvaccination, and three showed a decrease; the median (range) at 36 h was 81.0 (50.8 to 262.5) µg/ml. The mean Hp concentrations increased from an undetectable level prevaccination to medians (range) of 0.01 (0.01 to 0.03) mg/ml at 24 h and 0.03 (0.02 to 0.11) mg/ml at 36 h postvaccination (first vaccination). Values were not available after the second vaccination.

SAA and serum Hp concentrations were measured in groups V1, V2, V3, and CC at intervals after challenge. The distribution of SAA concentrations (Fig. 3) was skewed within groups at each time point, and there was increasing variability with increasing values; statistical analyses were therefore performed after transformation to log₁₀ values. After challenge on day 42, all vaccinated groups showed increasing mean concentrations of SAA until 23 h postchallenge. The mean SAA levels appeared to be returning to normal in all of these groups by 72 h postchallenge. For the nonvaccinated CC group, the mean SAA concentration increased markedly over the whole time. There is strong evidence that over the period until 72 h postchallenge, mean concentrations of SAA were significantly (P < 0.001) higher in the CC group than in the vaccinated groups. However, by 72 h postchallenge, two of the calves in the CC group had been euthanized, and the value at this time point is based on the one remaining animal only.

Serum Hp concentrations were undetectable in all groups until 7.5 h postchallenge, after which the Hp concentrations increased in all but one calf (V3 group), reaching a maximum value at around 48 h postchallenge. Once an animal showed a positive reading, it generally had positive readings for the rest of the study period. Groups V1, V2, and V3 had low median maximum Hp concentrations over the whole period of 0.01, 0.01, and 0.22 mg/ml, respectively, whereas the CC group had a median maximum of 0.86 mg/ml. These observed differences were not statistically significant using a Kruskal-Wallis test; larger samples would be needed to determine if the observed low levels in groups V1, V2, and V3 were a genuine effect of vaccination.

**IgM and IgG antibody titers to* P. multocida* B:2 envelopes.** Mean IgM titers decreased in groups V1, V2, V3, and CC after the first vaccination and continued at a low level until after the second vaccination (day 28) (Fig. 4). The mean IgM titers then increased for all three vaccinated groups, but the patterns of response were significantly different (P < 0.001) between the three vaccine groups, with the mean level in the V3 group rising at a somewhat lower rate up to day 42 (Fig. 4). The mean

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**FIG. 2.** Rectal temperatures (means ± SEM) following challenge of vaccinated and CC calves 2 weeks after the second vaccination by s.c. inoculation of 10⁷ CFU of the wild-type parent* P. multocida* 85020 strain.
IgM titer for the CC group remained fairly constant over the course of the experiment.

There was a significant difference in the patterns of change in mean IgG titers between groups V1, V2, V3, and CC (P < 0.001) (Fig. 5). The mean IgG titers for the vaccinated groups increased steadily until after the second vaccination (day 28), when they increased rapidly for the V1 and V2 groups before leveling out after challenge. Mean IgG titers continued to increase steadily in the V3 group from the second vaccination to 3 days postchallenge. The mean IgG titer of the CC group remained fairly static over the time course of the experiment and was at a lower level than those of the vaccinated groups.

After challenge, the titer increased slightly over the next 2 days but remained well below those of groups V1, V2, and V3.

**Bacteriology.** There was no evidence of bacteremia in samples taken at various time points postchallenge, including immediately prior to necessary or scheduled euthanasia, except in one CC animal that was euthanized for humane reasons at 48 h postchallenge. Bacteriological examination postmortem found *P. multocida* B2 in various tissues from all animals except the three NC calves (Table 1). The calves in the CC group that developed overt disease and were euthanized showed the most extensive range of bacteriologically positive tissues. However, infected tissues were also found in group V1, V2, and V3.
calves, but with a much more restricted and variable distribution (Table 1).

Necropsy. Postmortem examination revealed a range of gross and histological lesions in the calves from each of the groups, except for the NC calves, in which no gross or histological lesions were present (Table 2). Some calves in the CC group and one animal in the V3 group had significantly heavier (enlarged) prescapular, hepatic, and axillary lymph nodes than those of animals in the other groups with comparable body weights ($P < 0.01$ for each lymph node). There was no obvious difference between groups with respect to the weights of the submandibular, mesenteric, subiliac, or retropharyngeal lymph nodes or tonsils.

Severe suppurative lymphadenitis, as denoted by infiltration of polymorphonuclear neutrophils (PMN), was present in the prescapular lymph nodes of the CC group. The vaccinated groups appeared to show a graded response in that there was an absence of PMN in the prescapular lymph nodes of the V1 group compared to mild and moderate suppurative lymphadenitis in the V2 and V3 groups, respectively. Unvaccinated, unchallenged negative control animals (NC group) all had quiescent, nonhemorrhagic, nonsuppurative prescapular lymph nodes.

The effects of challenge on axillary lymph nodes were similar to those for the prescapular lymph nodes, but with a somewhat less convincing pattern of histopathology due to the presence of hemorrhage.

![Figure 5](http://iai.asm.org/)

**FIG. 5.** Total IgG titers (means ± SEM) in sera from vaccinated and CC calves. Titers were assessed by ELISA with sera collected at the times described in the legend to Fig. 4.

<table>
<thead>
<tr>
<th>Tissuea</th>
<th>V1 (n = 5)</th>
<th>V2 (n = 5)</th>
<th>V3 (n = 5)</th>
<th>CC (n = 5)</th>
<th>NC (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonsil swab</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>0</td>
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<tr>
<td>Right tonsil</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Left tonsil</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Right retropharyngeal LN</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Left retropharyngeal LN</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Right forelimb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Left forelimb</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Peritoneal swab</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Right prescapular LN</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Left prescapular LN</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Lung (left caudal lobe)</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Blood</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

a LN, lymph node.

b Note that positive tissues were not necessarily present in the same animal within a group.

<table>
<thead>
<tr>
<th>Lesiona</th>
<th>No. of positive animals in groupa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhagic prescapular LN</td>
<td>0</td>
</tr>
<tr>
<td>Significantly enlarged left prescapular LN*</td>
<td>0</td>
</tr>
<tr>
<td>Significantly enlarged right prescapular LN*</td>
<td>0</td>
</tr>
<tr>
<td>Significantly enlarged hepatic LN*</td>
<td>0</td>
</tr>
<tr>
<td>Significantly enlarged axillary LN*</td>
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</tr>
<tr>
<td>Hemorrhagic mesentery</td>
<td>1</td>
</tr>
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<td>Serosanguinous peritoneal fluid</td>
<td>1</td>
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<td>Fibrous lung adhesions</td>
<td>1</td>
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<tr>
<td>Multiple foci of consolidation in lung</td>
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<tr>
<td>Nonsuppurative nephritis</td>
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<tr>
<td>Focal suppurative hepatitis</td>
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<tr>
<td>Increased hepatocellular apoptosis</td>
<td>0</td>
</tr>
<tr>
<td>Edema of vaccination site</td>
<td>0</td>
</tr>
</tbody>
</table>

a LN, lymph node; * $P < 0.01$.

b Note that tissue lesions were not necessarily present in the same animal within a group.
of PMN in some samples from the NC animals. There were no other observable differences between the five groups in terms of the presence or absence of histopathological lesions in any of the other tissues examined.

**DISCUSSION**

This study showed that primary and booster vaccinations with 10⁸ or 10⁷ CFU of JRMT12 could be administered safely i.m. to calves and that all calves given two i.m. vaccinations 4 weeks apart, starting early at 4 weeks of age, with any of the three chosen doses of JRMT12 (ca. 10⁹, 10⁸, and 10⁷ CFU) survived s.c. challenge with ca. 10⁷ CFU of wild-type *P. multocida* B2 (strain 85020). However, vaccine dose-dependent trends were apparent for most of the parameters measured in response to vaccination and challenge. Significant dose-dependent differences were found in the patterns of mean rectal temperature responses to the primary and secondary vaccinations, whereby the greater the number of bacteria in the dose, the greater was the rise in mean rectal temperature. Conversely, the greater the number of bacteria in the vaccine dose, the smaller was the temperature rise in response to challenge. The lower mean maximum temperature of the V3 group following immunization than those of the V1 and V2 groups suggested that the pyrexic effect was related directly to the antigenic load. The lack of significant difference between the V1 and V2 groups was probably due to the administration of flunixin meglumine to group V1 calves. This was used previously (12) to reduce an anticipated risk of endotoxin shock at the high vaccination dose. A preparation of live gram-negative bacteria in the log phase of growth, such as the JRMT12 strain used in this study, will contain free endotoxin (19) in amounts proportional to the number of bacteria present, but attempts to remove the endotoxin by washing the bacteria in PBS followed by centrifugation were of little success and significantly reduced the viability of the preparation (J. C. Hodgson, unpublished observation).

In previous work, it was shown that mean SAA concentrations in calves vaccinated i.m. with 10⁸ CFU of the *P. multocida* JRMT12 strain peaked at 24 h postvaccination and then declined (12), and a similar trend was also apparent in the present experiment after vaccination with 10⁶ CFU. The low mean SAA concentrations and the lack of any significant differences in concentrations among the vaccinated groups after challenge suggest that vaccination with any of the three vaccine preparations was of similar efficacy in reducing the acute-phase protein response. Serum concentrations of Hp were initially very low after challenge and continued to be low at many subsequent time points in all animals, including the challenge control group. Consequently, comparisons of Hp concentrations were of little use in differentiating calf responses.

The acute-phase protein response and the increase in rectal temperature after the primary vaccination with the JRMT12 strain indicated that the attenuated bacteria persisted long enough to promote an inflammatory response. The high and variable IgM titers present on the day of primary vaccination presumably represented variations in the content and intake of maternally derived colostral IgM. Infection of the upper respiratory tract of cattle with *P. multocida* serotype A is common in the United Kingdom (10), such that antibodies to this would be present in colostrum, and previous work has shown cross-reaction of a *P. multocida* B:2 envelope antigen preparation with antisera raised to a *P. multocida* A:3 strain (12). The apparent association of different mean IgM titers with groups preceded any differences in treatment and was coincidental. There was no significant increase in either serum IgG or IgM concentrations during the days following primary vaccination with any of the vaccine doses, in agreement with our previous work (12) and with the observations of others (4, 13, 20). If anti-*P. multocida* serogroup A:3 IgM antibody was present, it may have reacted with the vaccine dose, a phenomenon reported previously (2), thereby reducing the titer, as seen at day 14, and thus minimizing the humoral response to vaccination. However, no such effect was observed after the second vaccination. In this case, concentrations of serum IgM and IgG rose markedly within 6 days and, in the case of IgG, had risen further by the day of challenge. There was, however, a significant difference in the patterns of increase of the titers between the three groups of vaccinated calves, with the highest dose of the JRMT12 strain administered, ca. 10⁹ CFU, promoting higher IgM and IgG responses than the ca. 10⁶ dose, which in turn created better responses than those in calves receiving the ca. 10⁷ dose. These dose effects were particularly apparent with the IgG response, where the mean titer in sera from calves given the ca. 10⁷ dose was 4-fold higher on day 42 than on day 0, whereas those of the ca. 10⁸ and ca. 10⁹ doses were 7-fold and 15-fold higher, respectively. If the primary vaccination was affected by maternally derived cross-reacting antibodies, these results raise the possibility that a single vaccination with JRMT12 at around 8 weeks of age or older might confer adequate protection. A single-vaccination regimen would be worthy of investigation, as it would be highly advantageous for use in the field.

The relative contributions of cellular and humoral immunity to protection have not been investigated in this study, but a strong correlation between the induction of humoral immunity and active protection in buffaloes vaccinated with killed whole-cell vaccines has been reported (4), which prompted that study’s authors to suggest that there may be a minimum threshold of antibody necessary for protection. Our data showed that all vaccinated calves were protected against challenge, including those calves receiving ca. 10⁷ CFU of the JRMT12 strain, where the IgM and IgG responses were significantly lower than those in calves given ca. 10⁸ or ca. 10⁹ CFU. This may indicate that cell-mediated immunity can contribute to protection.

Bacteriological examination postmortem found the *P. multocida* B:2 challenge strain in the blood of only the most severely affected control animal, which developed early signs of disease. This is consistent with the natural disease, where bacteremia is seen only as a terminal phenomenon (6). The presence of the organism in the nasopharynges of healthy carrier animals is well established (6, 24), and virulent HS-causing bacteria can be recovered from the tonsils and lymph nodes of the head and neck of natural (25) and experimentally induced (14) carrier animals, despite these animals exhibiting a strong and protective immune response to the organism (9). In the current work, bacteria were also recovered from tonsillar tissue and the retropharyngeal lymph nodes, including those from some animals in all three vaccinated groups (V1, V2, and V3), showing that vaccination, although protective, did not induce...
sterile immunity. This supports the relevance of our bovine model of experimentally induced HS with respect to the manifestations of HS observed in the field and highlights the tonsil as a possible immunologically privileged site enabling the organisms to survive.

This study showed that the most suitable vaccination dose of JRMT12 for use in calves against experimental HS was ca. 10^6 CFU, which conferred protection and a temporary pyrexia that resolved without treatment. However, our challenge regimen, although useful for experimental purposes, may be extreme, as it bypasses all mucosal defenses and delivers a large number of bacteria as a single dose, a situation unlikely to occur under natural conditions. Vaccination with ca. 10^5 bacteria, which results in significantly less pyrexia, may be sufficient for total protection in the field, particularly for buffaloes, one of the main target species for vaccination against HS, which are more sensitive than cattle to the effects of parenterally administered bacteria as a single dose, a situation unlikely to occur under natural conditions. Vaccination with ca. 10^7 bacteria, which bypasses all mucosal defenses and delivers a large number of organisms to survive.

It is important to note that the duration of immunity and single-dose vaccination, which are critical with respect to the usefulness of any new vaccine to protect against HS.

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