A haemolytic cell-free preparation of *Moraxella bovis* confers protection against Infectious Bovine Keratoconjunctivitis


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**Abstract:** Protection conferred by a cell-free preparation from a haemolytic *Moraxella bovis* isolate, UQV 148NF, was compared to an equivalent fraction from a non-haemolytic *M. bovis* isolate, Gordon 26L3, and to a recombinant DNA-derived pili vaccine. Three groups of ten calves were vaccinated twice with one of the three preparations and, together with ten non-vaccinated calves, challenged with virulent *M. bovis* isolate Dal 2d. Compared to the control group, significant protection was observed in the group receiving the pili vaccine and the group receiving the preparation from haemolytic isolate, UQV 148NF.

**Key words:** Moraxella bovis; Haemolysin; Vaccine; Infectious bovine keratoconjunctivitis

**Introduction**

*Moraxella bovis* is a Gram-negative bacterium responsible for the disease Infectious Bovine Keratoconjunctivitis (IBK). Following colonisation, the bacterium causes conjunctivitis and keratitis, with corneal ulceration a common sequel, leading to significant economic losses in cattle industries worldwide [1,2]. Only pilated and haemolytic isolates of *M. bovis* can cause disease [3], suggesting these features are important virulence traits. Pili mediate attachment [4], while the *M. bovis* haemolysin may contribute to the epithelial damage described in corneal lesions [5,6].

Strategies to prevent the disease could include vaccination with known virulence factors. In experimental challenges, immunisation with purified pili confers protection against colonisation and disease [7,8]; however, protection is serogroup-specific. Protection by a pili-based IBK vaccine therefore requires inclusion of pili from all seven serogroups of *M. bovis* [8,9] and may be limited by antigenic competition [10,11]. Although there are no reports of vaccination with...
purified *M. bovis* haemolysin, the presence of anti-haemolytic titres has been reported in naturally infected cattle, indicating that the haemolysin is antigenic [12]. Furthermore, sera from experimentally infected cattle possessed anti-haemolytic activity against all *M. bovis* isolates tested, suggesting conservation of epitopes on the haemolysin [12].

The haemolysin appears to be an ideal vaccine candidate; however, no data on protection have previously been collected. This study describes a challenge trial conducted to compare the antigenicity and immunogenicity of a partially purified cell-free preparation from a haemolytic isolate of *M. bovis* to: (i) an analogous preparation from a non-haemolytic isolate; and (ii) a recombinant DNA-derived pili vaccine.

**Materials and Methods**

*Preparation of recombinant DNA-derived pili vaccine antigen*

*P. aeruginosa* K/2Pfs containing plasmid pMD2d [13] was used to produce recombinant DNA-derived *M. bovis* Dal 2d pili as previously described [14]. Pili antigen was formulated in incomplete Freund’s adjuvant prior to vaccination.

*Preparation of vaccine antigens from haemolytic and non-haemolytic* *M. bovis* *strains*

The haemolytic *M. bovis* isolate UQV 148NF was used to prepare vaccine containing haemolysin antigen, while the non-haemolytic *M. bovis* isolate Gordon 26L3 was used to prepare vaccine lacking haemolysin antigen. Both isolates were non-piliated, eliminating the possibility of anti-pili antibody conferring protection, and no obvious differences were visible between the two isolates by SDS-PAGE. A partially purified and concentrated cell-free preparation from each isolate was obtained using a modification of the previously described technique [6]. Modifications included removal of whole cells by centrifugation at 15 000 × *g* for 30 min, and concentration and partial purification of supernatants by ultrafiltration (Minitan-S apparatus, Millipore) across a low-protein-binding cellulose membrane with a molecular mass cut-off value of 300 kDa. The ultrafiltration retentate was maintained at 4°C and examined for haemolytic activity by a haemolytic assay [6]. For vaccines, formaldehyde was added to concentrated cell-free preparations to a final concentration of 2% (which inactivated haemolytic capability) and each preparation formulated in incomplete Freund’s adjuvant.

**Vaccination schedules and challenge**

Forty Hereford calves between 4–6 months of age were randomly assigned to four groups. The animals were of similar size and weight and were reared at pasture until completion of vaccination when they were moved to a large, covered yard. Animals were fed hay and pellets with water ad libitum. Swabs were taken from the conjunctival sac 2 weeks before vaccination and cultured for *M. bovis*.

Two doses of a 2-ml vaccine were injected subcutaneously on opposite sides of the neck, behind the ear, at an interval of 28 days. Calves in group 1 received 0 haemolytic units of antigen prepared from non-haemolytic *M. bovis* isolate, Gordon 26L3 (800 μg total protein), group 2 received 1024 haemolytic units of antigen prepared from haemolytic *M. bovis* isolate, UQV 148NF (1 mg total protein), and group 3 received 15 μg of recombinant DNA-derived pili. Calves in group 4 were not vaccinated.

The challenge culture consisted of piliated *M. bovis* Dal 2d cells harvested from blood agar plates after overnight incubation and resuspended in chilled Trypticase soy broth to a concentration of not less than 10⁹ colony-forming units per ml as previously described [8]. Challenge culture was applied by cotton-tipped swab to the lower and upper palpebral surfaces of both eyes followed by instillation of 0.5 ml of the same culture into each conjunctival sac. The challenge procedure was performed as previously described [14].

**Assessment of infection and protection**

Clinical disease was assessed by examination of all eyes every second day for lachrymation, photophobia, iridospasm, blepharitis and kerati-
results, until day 10 after challenge. A definitive diagnosis of IBK was made only if a circumscribed corneal ulcer was present and the organism could be isolated from these eyes. Statistical analysis was performed using Fisher’s exact test.

Antibody response
Sera and tears were tested for antibody to the *M. bovis* haemolysin by a haemolysis inhibition assay [6]. Two-fold dilutions of serum were made and controls with no added serum were included. All titres were transformed to log₂ (antibody titre) to normalise the data. The geometric mean titre was calculated from the mean of the transformed titre data by back-transformation. Statistical analysis of post-vaccination titres was performed using analysis of co-variance, with pre-vaccination titres as the co-variate, to counter the presence of non-specific activity in normal serum.

**SDS-PAGE and Western blot analysis**
100 µg of haemolytic preparation from UQV 148NF was separated by SDS-PAGE with a 10% separating gel. Proteins were electro-transferred onto nitrocellulose filters and the blots probed with a 1:400 dilution of cattle sera or with purified IgG (dilutions specified in Fig. 1), obtained from group 1 and group 2 vaccinated animals. Total IgG was purified by affinity chromatography on Protein G Sepharose (Pharmacia LKB). Bound immunoglobulins were visualised by using peroxidase-labelled goat anti-cow immunoglobulins and ECL development reagent (Amersham).

**Results and Discussion**

**Evaluation of protection**
*M. bovis* Dal 2d was recovered from both eyes of 39 calves, and from one eye of one calf in group 2, 7 days following challenge. The number of animals that developed IBK lesions in each group by day 10 after challenge are reported in Table 1. In both the group that received antigen from haemolytic isolate, UQV 148NF (group 2) and the group that received recombinant DNA-derived pili antigen (group 3), the cumulative incidence of clinical disease in the period of observation was significantly lower (*P* = 0.0349 and 0.0027, respectively) than in the non-vaccinated controls (group 4). No significant difference (*P* = 0.2910) was found between the levels of protection observed in group 2 and group 3. Vaccination with the preparation from non-haemolytic isolate, Gordon 26L3 (group 1), showed no protection over non-vaccination (*P* = 0.3142).

A minor disparity in protein concentration was observed for the vaccine preparations used in groups 1 and 2, although both isolates were grown under the same conditions and concentrated to the same degree. However, this difference is unlikely to have been a contributing factor to observed protection as the preparations used were relatively crude and variation in individual protein concentration is likely to be insignificant. In addition, as haemolytic activity was deemed to be the important indicator for presence of

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Challenge strain</th>
<th>Animals challenged</th>
<th>Animals with IBK</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gordon 26L non-haemolytic</td>
<td>Dal 2d</td>
<td>10</td>
<td>6</td>
<td>20%</td>
</tr>
<tr>
<td>2. UQV 148NF haemolytic</td>
<td>Dal 2d</td>
<td>10</td>
<td>3</td>
<td>50%</td>
</tr>
<tr>
<td>3. Recombinant Dal 2d pili</td>
<td>Dal 2d</td>
<td>10</td>
<td>1</td>
<td>70%</td>
</tr>
<tr>
<td>4. Non-vaccinated control</td>
<td>Dal 2d</td>
<td>10</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

*a Percent protection is calculated by the formula [8]:

\[
P = \left( \frac{C_i - V_i}{C} \right) \times 100
\]

where *P*, percentage; *C*, number of non-vaccinated calves; *C_i*, number of non-vaccinated calves with IBK; *V*, number of vaccinated calves; *V_i*, number of vaccinated calves with IBK.
haemolysin, dilution of this activity appeared inappropriate.

The lack of significant difference in protection conferred on calves in group 2 and group 3 is important. The pili of the challenge isolate (Dal 2d) were homologous to the vaccine used in group 3 calves; however, the challenge isolate was heterologous to the isolate used for vaccine preparation in group 2 calves. Therefore, cross-protective immunity may be induced by antigen(s) prepared from a haemolytic isolate of \textit{M. bovis}, possibly the haemolysin. In contrast, cross-protective immunity between different serogroups has not been demonstrated for \textit{M. bovis} pili vaccines.

\textbf{Antibody response}

Collected sera were examined for anti-haemolytic activity by the haemolytic inhibition assay and the geometric mean anti-haemolytic titres for each group post-vaccination are shown in Table 2. Anti-haemolytic titres of animals in group 2 (receiving vaccine prepared from the haemolytic \textit{M. bovis} isolate) were significantly higher than all other groups ($P<0.05$). Although anti-haemolytic activity of tears was tested, no significant difference of post-vaccination titres was observed (data not shown) when compared to pre-vaccination levels ($P>0.05$). Demonstration of a systemic anti-haemolytic antibody response and the lack of detectable anti-haemolytic antibody in tears may be due to a natural dilution effect resulting in relatively low antibody titres in tears compared with those in serum [8]. The haemolytic inhibition assay may not be sensitive enough to detect the lower level of anti-haemolytic antibody occurring in tears. Alternatively, the haemolysin may not adequately stimulate a local immune response, with systemic IgG in the cornea being more important in providing protection.

The absence of significant protection with a lack of anti-haemolytic antibodies (group 1) is suggestive of a role for those antibodies in preventing disease. However, the possible role of other antibodies in protection cannot be excluded, as several proteins were identified by Western blot analysis.

\begin{table}[h]
\centering
\begin{tabular}{lllc}
\hline
\textbf{Group} & \textbf{Vaccine antigen} & \textbf{Number of cattle} & \textbf{Geometric mean titres} \\
\hline
1 & Gordon 26L non-haemolytic preparation & 10 & 62.4 \\
2 & UQV 148NF haemolytic preparation & 10 & 818.7 \\
3 & Recombinant Dal 2d pili preparation & 10 & 58.8 \\
4 & Non-vaccinated control & 10 & 63.2 \\
\hline
\end{tabular}
\caption{Anti-haemolytic titre, determined by haemolytic inhibition assay, of each group of cattle following vaccination}
\end{table}

\textbf{Western blot analysis}

Western blot analysis using specific polyclonal anti-Dal 2d antibodies confirmed the absence of Dal 2d pili in the other vaccine antigens (data not shown). Analysis of haemolytic and non-haemolytic \textit{M. bovis} preparations by Western blot was performed using different dilutions of IgG purified from sera of all group 2 calves after vaccination. An example of the resulting blotting pattern is provided (Fig. 1). Although a common 45-kDa Fig. 1. Western blot analysis of antigens of non-haemolytic (tracks 1 and 2) and haemolytic (tracks 3 and 4) vaccine preparations (30 \textmu g protein each). Antigen preparations were separated by SDS-PAGE and analysed by Western blotting as described in Materials and Methods. Total IgG purified from haemolytic vaccinate sera by affinity chromatography was used in dilutions 1:1000 (tracks 1 and 3) and 1:500 (tracks 2 and 4). Bound immunoglobulins were visualised with an enhanced chemiluminescence system (Amersham).
protein was recognised in both preparations, a
group of 4–5 proteins with molecular masses
ranging from 65–97 kDa was also observed in the
antigen from the haemolytic isolate, UQV 148NF
(Fig. 1, lanes 3 and 4). These bands were not
apparent in the equivalent fraction from the
non-haemolytic strain, Gordon 26L3 (Fig. 1, lanes
1 and 2).

The presence of immunoreactive protein with
molecular masses of 65–97 kDa in the haemolytic
preparation suggests that protection could be in-
duced by one of these proteins, one of which may
be the haemolysin. Although several proteins were
recognised, it is possible the different bands are
degradation products of the one protein, as pro-
tease inhibitors were not used in the preparation
of the vaccine.

Further studies are required to determine the
specific role of haemolysin protein in the ob-
served protection. Although antibodies in im-
mune serum actively inhibit haemolytic activity, it
cannot be ruled out that these antibodies are not
responsible for protection. Experiments are cur-
rently being conducted to purify the haemolysin
and to further define the role of this protein in
the pathogenesis of IBK and in protection against
disease.

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