Infectious bovine keratoconjunctivitis (IBK; ‘pinkeye’) is the most common ocular disease of cattle and manifests as corneal ulceration and oedema, ocular pain, photophobia and lacrimation. Animals that recover develop varying degrees of corneal scarring, while permanent blindness follows corneal rupture in severe cases. The only organism for which Koch’s postulates have been fulfilled with respect to IBK is Moraxella bovis (Henson & Grumbles, 1960). Nevertheless, other bacteria have been isolated in the absence of M. bovis from cattle with IBK. In 1966, Fairlie first reported the isolation of haemolytic Neisseria spp. from calves with severe keratitis and corneal ulceration (Fairlie, 1966). In Australia, Spradbrow isolated Neisseria spp. in 24 of 25 outbreaks of IBK; M. bovis was isolated in only two of those outbreaks (Spradbrow, 1967). In 1970, Wilcox reported Neisseria (Branhamella) catarrhalis from nearly 45% of IBK cases while M. bovis was isolated from only 28% of IBK cases (Wilcox, 1970). M. bovis and bacteria classified as species of the genus Neisseria have also been cultured from normal eyes of cattle (Barber, 1984; Barber et al., 1986). Moraxella ovis and Neisseria ovis were reported from IBK-affected cattle in Israel (Elad et al., 1988; Yeruham et al., 2001). Pederson investigated the pathogenicity of N. ovis inoculated into the eyes of experimental calves, however, lesions typical for IBK did not occur despite previous corneal irradiation (Pedersen, 1972). Haemolytic and cytolytic activity from culture filtrates of M. ovis isolated from cattle with IBK has been reported recently and this suggests a possible role for Gram-negative cocci in the pathogenesis of IBK (Cerny et al., 2006).

During an antibiotic efficacy trial in beef and dairy calves in northern California during summer 2002, swabs from 138 corneal ulcers were cultured from 3- to 9-month-old calves with clinical IBK. Haemolytic Gram-negative cocci were isolated from 68 ulcers; M. bovis was isolated from 29 ulcers. To characterize the haemolytic Gram-negative cocci and determine if they were related to known members of the genus Moraxella, biochemical characterizations and ribosomal/housekeeping gene sequence analyses were performed.

Ocular secretions from IBK-affected calves at two separate calf-rearing facilities (one beef cattle ranch and one dry-lot dairy) in northern California were collected from the inferior cul-de-sac onto sterile Dacron swabs. The swabs were used to inoculate 5% sheep blood agar plates which

**Moraxella bovoculi** sp. nov., isolated from calves with infectious bovine keratoconjunctivitis

John A. Angelos,1 Phillip Q. Spinks,2 Louise M. Ball1 and Lisle W. George1

1Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA
2Section of Evolution and Ecology, University of California, Davis, CA 95616, USA

Eighteen isolates of a Gram-negative coccus (strain 237T) were cultured from the eyes of dairy and beef calves affected with infectious bovine keratoconjunctivitis (IBK; ‘pinkeye’) in northern California, USA, during summer 2002. These isolates had near full-length (1397 bp) 16S rRNA gene sequences that clustered into three groups with 99.9% sequence similarity. On the basis of 16S rRNA gene sequence, the isolates were most closely associated with Moraxella bovis and Moraxella ovis in clade I of the classical moraxellae. Biochemically, the novel isolates could be distinguished from the other members of the genus Moraxella isolated from animals on the basis of phenylalanine deaminase activity. The results of partial sequence analysis of six housekeeping genes, the 16S–23S rRNA gene interspace region and partial 23S rRNA gene provide strong support for the inclusion of these isolates in a novel taxon, for which the name Moraxella bovoculi sp. nov. is proposed. The type strain is strain 237T (=ATCC BAA-1259T=CCUG 52049T).
were incubated at 35°C for 24 h. Colonies exhibiting β-haemolysis were subcultured for further study; one α-haemolytic isolate (2470-1) that exhibited colony morphology similar to the β-haemolytic isolates was also selected and subcultured. Isolates were stored frozen in 50% skim milk–glycerol media at −80°C for subsequent biochemical and molecular characterizations. The eighteen isolates were designated 212, 237T, 2467, 2470-1, 2471-2, 2473, 317, 371, 376, 380, 4624, 4773, 4785, 4786, 4787, 4794, 6170, 8342 and were from the ocular secretions of different calves. M. bovis was isolated concurrently with isolate 2471-2 from one calf. The isolates were propagated on 5% sheep blood agar plates and grown in either Luria–Bertani broth or brain heart infusion (BHI) broth supplemented with 1.5 mM calcium chloride.

*Moraxella caprae* (ATCC 700019T), *Moraxella boevrei* (ATCC 700022T) and *M. ovis* (ATCC 33078T) were obtained from the ATCC. *M. bovis* Tifton I is a strain of *M. bovis* that was originally isolated from a beef cow with IBK in Georgia, USA. Genomic DNA was isolated by the use of commercial DNA extraction kits (DNeasy kit; Qiagen). The 16S rRNA gene was amplified using primers NewEcoliDn1 (5′-GTATT-3′) and NewEcoliDn2 (5′-GCTGCATGGCTGTCGTCAG-3′). Ribosomal DNA from the end of the 16S rRNA gene, the 16S–23S interspacer region and a portion of the 23S rRNA genes was amplified with primers MorNeissDn5 (5′-GTTTGATCATGGCTCAGATTG-3′) and EcoliUp4 (5′-TTCC-CCTACGGTTACCTTGCTTT-3′). Oligonucleotide primers and PCR conditions that were used for the amplification of the six housekeeping genes are shown in Table 1. The housekeeping genes used were selected on the basis of previously described bacterial housekeeping genes (Christensen et al., 2004; Lim et al., 2003; Noller et al., 2003). In some instances, the deduced amino acid sequences of the amplicons generated using published housekeeping gene primers did not correspond to the expected protein sequences obtained from BLAST searches of GenBank (Altschul et al., 1997). In these cases, additional primers were designed from the aligned DNA sequence data and used for amplification and resequencing; subsequent BLAST searches of the resequenced amplicons were used for the amplification and partial sequencing of six housekeeping genes of *M. boevrei*. Reversed BLAST searches of the resequenced amplicons generated for the initial analysis, all were similar in length, which contained a 103 nt insert (with respect to all other sequences) that was excluded from the phylogenetic analysis.

The oligonucleotide primers and PCR conditions that were used for the amplification of the six housekeeping genes are shown in Table 1. The housekeeping genes used were selected on the basis of previously described bacterial housekeeping genes (Christensen et al., 2004; Lim et al., 2003; Noller et al., 2003). In some instances, the deduced amino acid sequences of the amplicons generated using published housekeeping gene primers did not correspond to the expected protein sequences obtained from BLAST searches of GenBank (Altschul et al., 1997). In these cases, additional primers were designed from the aligned DNA sequence data and used for amplification and resequencing; subsequent BLAST searches of the resequenced amplicons

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**Table 1. Primers (5′ to 3′) used for the amplification and partial sequencing of six housekeeping genes**

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RNA polymerase subunit B</strong></td>
<td>po82</td>
<td>GCACTGAAAGARTTCTTTGGTTC*</td>
</tr>
<tr>
<td><strong>3-Hydroxyacyl-CoA dehydrogenase</strong></td>
<td>FADB</td>
<td>GAYATTACCGAGTTTAYGGTTAT</td>
</tr>
<tr>
<td><strong>ATP synthase F1, epsilon subunit</strong></td>
<td>newATPD3</td>
<td>ACCTTAGGGTTTGGTTTGGTGAGC</td>
</tr>
<tr>
<td><strong>Histidine kinase</strong></td>
<td>hisA</td>
<td>GCAACGGGTCGGAGTATCAATGG</td>
</tr>
<tr>
<td><strong>Phospho-N-acetylmuramoyl-dipeptide transferase</strong></td>
<td>pentapeptide transferase</td>
<td>GCTTTATTGGTTATTTCAGCACAG</td>
</tr>
<tr>
<td><strong>Ferredoxin</strong></td>
<td>FesN</td>
<td>GTATTTACCGACGAGATGCGCTTT</td>
</tr>
</tbody>
</table>

*From Christensen et al., 2004. Indicate primer pairs that amplified only for *M. bovis* Tifton 1 DNA.*
matched the target proteins and were used to represent the housekeeping genes. The five-taxon housekeeping gene dataset was composed of up to 7121 nt. Despite extensive PCR optimizations, four housekeeping genes did not amplify from *M. bovis* Tifton I genomic DNA.

Near full-length (1397 bp) regions of the 16S rRNA gene of all 18 isolates were determined and were 99.9 % similar. At two locations, positions 443 and 472 (numbering based on the *Escherichia coli* 16S rRNA gene sequence, GenBank accession no. J01859), the 18 isolates differed, allowing them to be separated into three different groups. One isolate was selected to represent each group as follows (other isolates with identical 16S rRNA gene sequences are shown in parentheses): isolate 237T (212, 317, 376, 380, 4785, 4786, 6170 and 8342), isolate 2471-2 (2470-1) and isolate 371 (2467, 2473, 4624, 4773, 4787 and 4794).

To determine whether the representative isolates from each of the three groups were significantly different from other recognized species of the genus *Moraxella*, the following strategy was employed: (i) a BLAST search of GenBank (Altschul et al., 1997) using the 16S rRNA gene sequence from isolate 237T was performed to identify and select a set of 16S rRNA gene sequences that were most similar to the representative isolates; (ii) phylogenetic analysis was performed on this dataset and *M. bovis* and *M. ovis* were identified as the most closely related taxa to the representative isolates, and (iii) a five-taxon dataset composed of the representative isolates of each of the three groups (based on the near full-length 16S rRNA gene sequence), *M. bovis* and *M. ovis* was subjected to phylogenetic analysis using the 16S rRNA gene, the 16S–23S interspaced region and a portion of the 23S rRNA gene plus six housekeeping genes (Table 1). For both datasets, sequences were aligned in MacClade 4.06 (Maddison & Maddison, 2003). Missing data were coded as ‘?’ and gaps were coded as ‘−’. Phylogenetic trees were estimated with neighbour-joining (NJ) and maximum-likelihood (ML) using PAUP* 4.0b10 (Swofford, 2002). For the NJ distance metric, uncorrected *P* distances were used. Maximum-likelihood searches employed tree bisection reconnection (TBR) branch swapping with ten random-stepwise heuristic searches. Model parameter values were estimated using MODELTEST v3.06 PPC (Posada & Crandall, 1998) and selected under the Akaike information criteria. ML parameters conformed to the GTR + I + Γ model of sequence evolution. The statistical reliability of the NJ and ML trees was assessed using non-parametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates. The BLAST search identified 219 16S rRNA gene sequences, of which only the first 39 were downloaded. Redundant sequences were identified using MacClade 4.06 and these were excluded from the phylogenetic analysis. *Psychrobacter meningitidis* SGHS (GenBank accession no. AY057116) and *Psychrobacter faecalis* Iso-46^T^ (AJ421528) were selected in the BLAST search and included as outgroups. The 16S rRNA gene sequences for *Moraxella cuniculi* CCUG 2154^T^ (AF005188), *Moraxella osloensis* 5873 (AF005190), *Moraxella atlantae* 1922 (AF005191) and *Moraxella phenylpyruvica* 752/52 (AF005192) were not identified by the BLAST search and were downloaded from GenBank. The final 16S rRNA gene dataset was composed of 1453 nt from 27 taxa (TreeBASE accession no. S1634).

Based on the 16S rRNA gene sequence analysis, the three representatives of the groups of novel isolates were found to be most closely related to *M. ovis* and *M. bovis*; the gene sequence divergence between the novel strains and *M. ovis* was between 2.2 % and 2.4 %. The gene sequence divergence between the representative novel strains and *M. bovis* was between 2.1 % and 2.4 % (see Table 2). The ML searches recovered two equally likely trees, but the differences between the topologies were trivial. The NJ tree was not significantly different from the ML tree (Shimodaira-Hasegawa test *P* = 0.376), thus, Fig. 1(a) presents the ML tree with the NJ and ML bootstrap proportions as indicated. The results of phylogenetic analysis were congruent with previously defined phylogroups (Pettersson et al., 1998). The three groups of novel isolates formed a distinct, well-supported monophyletic unit that was most closely related to *M. ovis* and *M. bovis* and the novel isolates are therefore members of the *M. bovis* cluster. Uncorrected *P* sequence divergence between the novel strains and *M. ovis*/M. bovis was $\geq 1.75$ % (Table 3). A closest neighbour (based on near full-length 16S rRNA gene sequence) dataset was composed of up to 7121 nt for five taxa. ML parameters conformed to the TrN + I model of sequence evolution. The ML searches recovered a single tree in which the novel strains were

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**Table 2.** Percentage pairwise uncorrected *P* distances based on 16S rRNA gene sequence data

GenBank accession numbers are given in parentheses.

<table>
<thead>
<tr>
<th>isolate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Moraxella bovis</em> ATCC 10900^T^ (AF005182)</td>
<td>0.2774</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2. <em>Moraxella bovis</em> Tifton 1 (DQ647927)</td>
<td>0.2118</td>
<td>0.0707</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3. <em>Moraxella ovis</em> ATCC 33078^T^ (AF005186)</td>
<td>2.4965</td>
<td>2.7739</td>
<td>2.7587</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4. <em>Moraxella bovoculi</em> 237^T^ (DQ153089)</td>
<td>2.0824</td>
<td>2.3704</td>
<td>2.2978</td>
<td>2.2271</td>
<td>–</td>
</tr>
<tr>
<td>5. <em>Moraxella bovoculi</em> 2471-2 (DQ153093)</td>
<td>2.2265</td>
<td>2.3706</td>
<td>2.2975</td>
<td>2.3712</td>
<td>0.1437</td>
</tr>
<tr>
<td>6. <em>Moraxella bovoculi</em> 371 (DQ153085)</td>
<td>2.1549</td>
<td>2.2990</td>
<td>2.2263</td>
<td>2.2996</td>
<td>0.0718</td>
</tr>
</tbody>
</table>

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*

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monophyletic with respect to *M. bovis* and *M. ovis* with strong support (Fig. 1b) and had >4% gene sequence divergence from *M. bovis*/M. *ovis* (Table 3). In addition, NJ searches of the coding data converted to amino acids recovered the same topology, also with strong support. Based on these results, the 18 novel isolates are considered to

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**Fig. 1.** (a) ML reconstruction of 27 taxa and up to 1453 bp of the 16S rRNA gene. Estimated model parameter values conform to the GTR+I+Γ model of sequence evolution. −lnL=5513.85. Base frequencies were A=0.26, C=0.21, G=0.31 and T=0.22. Parameters for the substitution rate matrix were A–C=0.7086, A–G=2.7186, A–T=1.5815, C–G=0.6792, C–T=4.0333 and G–T=1. Proportion of invariable sites (I)=0.6757 and γ-shape parameter=0.5321. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per site. (b) Multi-gene analysis of the 16S rRNA gene, 16S–23S interspacer region, partial 23S rRNA gene and six housekeeping genes. ML reconstruction of the 5-taxon 7121 nt dataset. Estimated model parameter values conform to the TrN+I model of sequence evolution. −lnL=12202.0126. Base frequencies were A=0.28, C=0.21, G=0.26 and T=0.25. Parameters for the substitution rate matrix were A–C=1, A–G=3.2058, A–T=1, C–G=1, C–T=6.2479 and G–T=1. Proportion of invariable sites (I)=0.7197. Numbers above branches are ML bootstrap proportions while those below branches are NJ bootstrap proportions. Bar, 0.01 substitutions per site. The GenBank accession numbers for all the sequences generated for this analysis are DQ156147–DQ156148, DQ153081–DQ153098 and DQ647927–DQ647954 (see Supplementary Table S1 in IJSEM Online).
represent a novel taxon within the genus *Moraxella*, for which the name *Moraxella bovoculi* sp. nov. is proposed.

Biochemical testing of the 18 isolates was performed using microbiological testing media supplied by the University of California Biological Media Services, Davis, CA, USA, by Hardy Diagnostics and the by use of API 20NE, API NH and API ZYM test strips (bioMérieux) according to the manufacturer’s instructions. For individual biochemical test media, methods were performed according to standard testing methods as described online for the various test media (http://www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp). To assess the hydrolysis of Tween 80 (Sigma-Aldrich), Tween 80 opacity medium was prepared by dissolving 10 g Bacto peptone, 5 g NaCl, 0.1 g CaCl₂ and (Sigma-Aldrich), Tween 80 opacity medium was prepared by dissolving 10 g Bacto peptone, 5 g NaCl, 0.1 g CaCl₂ and 15 g agar in 1 l of ultrapure water. The medium was autoclaved, cooled to 50 °C and 5 ml of sterile Tween 80 was added. The medium was mixed and 25 ml was added to 100 × 15 mm sterile Petri plates. The plates were streaked with a heavy inoculum of bacteria, incubated at 35 °C and examined at 24 h intervals for the development of an opaque halo (positive test) adjacent to bacterial colonies. The results of the physiological characterization are provided in the species description. *Moraxella bovoculi* sp. nov. could be distinguished from other moraxellae isolated from animals on the basis of phenylalanine deaminase activity (see Table 4).

**Description of *Moraxella bovoculi* sp. nov.**

*Moraxella bovoculi* (bov.occuli i. L. n. *bovoculi* - an ox, a bull, a cow; L. n. *culus* - an eye; N.L. gen. n. *bovoculi* of the eye of a cow, from where the type strain was isolated).

All 18 isolates grow on 5 % sheep blood agar at 25 and 35 °C under aerobic conditions; 13 of 18 isolates grow on 5 % sheep blood agar incubated at 42 °C (type strain 237T is negative). Seven of 18 isolates grow on nutrient agar with 0 % NaCl (strain 237T is positive). Bacteria grown on 5 % sheep blood agar form ≤1 mm diameter colonies that are white to off-white, circular and convex with an entire margin and a moist-looking surface. Seventeen of 18 isolates have a zone of β-haemolysis around or under (when picked) each colony (strain 237T is β-haemolytic); isolate 2470-1 exhibits α-haemolysis. Gram-negative diplococci with occasional cocci; the cell diameters are 0.7–1.3 μm (Fig. 2) and adjacent sides appear flattened. Growth does not occur on MacConkey agar, Salmonella–Shigella agar, Simmons’ citrate agar, sodium acetate agar or nutrient agar with 6 % NaCl. Sixteen of 18 isolates are nitrate-reduction positive (strain 237T positive) and 9 of 18 isolates show proteolysis of Löffler medium (strain 237T negative). Fourteen of 18 isolates are positive for leucine arylamidase activity (strain 237T positive); 9 of 18 isolates are positive for DNase activity (methyl green agar; strain 237T negative). Seventeen of 18 isolates are positive for hydrolysis of Tween 80 (96 h result; strain 237T positive). Seventeen of 18 isolates were negative for alkaline phosphatase (strain 237T negative). All 18 isolates have positive test reactions for the following: penicillin sensitivity, oxidase, lipase (hydrolysis of 5-bromo-3-indoxyl-caprate), catalase, phenylalanine deaminase, C4 esterase (hydrolysis of 2-naphthyl butyrate) and C8 esterase lipase (hydrolysis of 2-naphthyl caprylate). All 18 isolates have negative reactions for motility, Litmus milk reaction, hydrogen sulfide production, indole production from tryptophan, sugar fermentation (glucose, xylose, mannitol, lactose, fructose, maltose and sucrose), aesculin hydrolysis, gelatin hydrolysis, β-galactosidase, tests for carbon assimilation (glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, caproic acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid), arginine dihydrolase, urease, ornithine decarboxylase, proline arylamidase, γ-glutamyl transferase, C-14 lipase (hydrolysis of 2-naphthyl myristate), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid

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**Table 3.** Percentage pairwise uncorrected *P* distances based on the combined 16S rRNA gene plus the 16S–23S interspacer region and a portion of the 23S rRNA gene (values below diagonal)

The values above the diagonal are distances based on the six housekeeping genes.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Moraxella bovis</em> Tifton I*</td>
<td>9.62</td>
<td>15.69</td>
<td>15.08</td>
<td>15.59</td>
<td></td>
</tr>
<tr>
<td>2. <em>Moraxella ovis</em> ATCC 33078T</td>
<td>3.00</td>
<td>4.02</td>
<td>4.02</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>3. <em>Moraxella bovoculi</em> 237T</td>
<td>3.20</td>
<td>1.75</td>
<td>1.02</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>4. <em>Moraxella bovoculi</em> 371</td>
<td>3.20</td>
<td>1.78</td>
<td>0.06</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>5. <em>Moraxella bovoculi</em> 2471-2</td>
<td>3.34</td>
<td>2.16</td>
<td>0.55</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

*For *bovis* Tifton 1, the distances were based on two housekeeping genes only: RNA polymerase subunit B and 3-hydroxyacyl-CoA dehydrogenase (see text).*
phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, x-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The type strain, strain 237T (= ATCC BAA-1259T = CCUG 52049T), was isolated from ocular secretions of a calf with infectious bovine keratoconjunctivitis in Brown’s Valley, California, USA.

### Acknowledgements

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### References


