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Component Causes of Infectious Bovine Keratoconjunctivitis - The Role of Moraxella Species in the Epidemiology of Infectious Bovine Keratoconjunctivitis

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KEYWORDS

• Infectious bovine keratoconjunctivitis • *Moraxella bovis* • *Moraxella bovoculi* • *Moraxella ovis* • MALDI-TOF MS • Genomics • Pathogenesis

KEY POINTS

- *Moraxella bovis* can cause infectious bovine keratoconjunctivitis (IBK).
- The role of *M bovoculi* in IBK is not fully understood.
- *M bovis* and *M bovoculi* appear to undergo genetic recombination with each other or other members of the *Moraxellaceae.*
- Recombination complicates their classification and potential role(s) in IBK pathogenesis.
- MALDI-TOF MS is used to identify *M bovis*, 2 major strains or genotypes of *M bovoculi*, *M ovis,* and other members of the *Moraxellaceae.*
- Classification and determination of pathogenesis potential within *Moraxella* species may be better understood through whole genome sequencing.

INTRODUCTION

Bacterial pathogens have been associated with infectious bovine keratoconjunctivitis (IBK) or pinkeye, from some of the earliest descriptions of the disease. While investigating outbreaks of keratitis contagiosia in Nebraska cattle, one of the first

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documented reports of IBK, Billings described dense clusters of coccoid bacteria in corneal lesions. The organisms were subsequently isolated in pure culture; however, he was unsuccessful in reproducing the disease in healthy animals.[1] These early observations and experiments highlight some of the discoveries, knowledge gaps, and controversy that remain after 120 years of research on the causality of IBK. This article will focus on bacterial causes of IBK, specifically members of the genus *Moraxella*, likely the same organisms Billings observed in 1898.

Moraxella bovis (M bovis) is the most common bacterial species associated with the disease and appears well suited to cause IBK. *M bovis* possesses numerous adherence and colonization factors, secretes toxins and enzymes that damage bovine cells, and utilizes various mechanisms to avoid immune recognition and enable persistence in hosts. *M bovis* is the only pathogen to have reproduced IBK-like ocular lesions in various experimental models, including in gnotobiotic calves, indicating that it is capable of inducing disease in the absence of other potential pathogens and with cell-free supernatants, which is evidence for the role of secreted exotoxins in the pathogenicity of IBK.[2–4] For other members of the genus, primarily *Moraxella bovoculi (M bovoculi)*, direct causality appears less clear, as reproduction of the disease following experimental infection with pure culture has not yet been achieved.[5] However, the presence of virulence factors in *M bovoculi* similar in structure and function to *M bovis,* along with recent genomic data showing recombination between the 2 species, highlights the complexity of IBK pathogenesis and possible interactions. The role these pathogens play in causing and/or contributing to IBK is reviewed, including virulence factors, studies on causality, recent findings using genomics and mass spectrometry, and detection and differentiation of these organisms in clinical samples.

The Genus Moraxella

The genus *Moraxella* represents the type genus within the larger family *Moraxellaceae.*[6] *Moraxella* are gram-negative rods or cocci, which are oxidase and usually catalase positive.[7] There are 22 validly published species, most of which are parasites or commensals of mucous membranes of mammals.[7,8] *Moraxella* is among the most abundant organisms in the upper respiratory and ocular microbiome, and overall abundance of this genus was not found to be associated with risk of IBK in 1 study.[9,10] Three species within the genus have been studied in relation to IBK, and include *M bovis*, *M bovoculi*, and *Moraxella* (formerly *Branhamella* and *Neisseria*) *ovis (M ovis)*. There is likely taxonomic overlap between *M ovis* and *M bovoculi*. In the authors' view, *M ovis* is rare and mostly absent from ocular cultures of cattle, and reports or descriptions of *M ovis* from the eyes of cattle prior to the recognition of *M bovoculi* are likely *M bovoculi*. Both species can appear identical if utilizing only biochemical testing.[11] This view agrees with several large prevalence or descriptive epidemiology studies that found low numbers or no *M ovis* in cattle ocular cultures that have occurred since the description of *M bovoculi* as a separate species using polymerase chain reaction (PCR) or other nonbiochemical methods for identification.[12–15]

MORAXELLA BOVIS- VIRULENCE FACTORS

Repeats in Toxin

Exotoxins are a hallmark of many bacterial pathogens and repeats-in-toxin (RTX) type toxins found in numerous pathogenic bacteria. RTX toxins are thought to have originated in members of the *Pasteurellaceae*, and subsequently disseminated among other bacteria through horizontal gene transfer.[16] RTX toxins are large, pore-forming proteins with a common structure of glycine and aspartate-rich repeats, and are

secreted via a type I secretion system, a transporter necessary to enable secretion of toxin from the cytoplasm to the environment.[17] RTX toxins include *Escherichia coli* alpha hemolysin, *Mannheimia haemolytica* leukotoxin, and the Apx family of *Actinobacillus pleuropneumoniae* toxins.[18,19] *M bovis* RTX toxin, also called hemolysin, cytolysin, or cytotoxin, reacts with monoclonal antibodies to other RTX toxins and forms pores in bovine erythrocytes, demonstrating similarity in structure and function to other RTX toxins.[20,21] *M bovis* RTX toxin is toxic to bovine neutrophils and corneal epithelium, but not human neutrophils.[22,23] The host cell receptor for *M bovis* cytotoxin has not been definitively determined, but other closely related toxins bind to B_2 integrins on leukocytes, and some, like *M haemolytica* leukotoxin, bind to a host-specific CD18 signal peptide region.[18,24,25] This property makes the toxin highly specific to certain cell types, which likely leads to clinical signs observed in associated diseases. For example, *M bovis* RTX toxin is a necessary and sufficient virulence factor for IBK, where toxin-rich supernatants from hemolytic strains have been shown to reproduce IBK-like lesions in vivo, and nonhemolytic strains and supernatants are avirulent or nontoxic.[26,27] The RTX toxin is expressed in some *M bovis* strains in part by a gene (*mbx*A), which is absent in nonhemolytic strains and under the control of an operon, which forms a pathogenicity island.[28–30] Activity of the toxin is neutralized by rabbit antiserum raised against the carboxy terminus of *mbx*A.[28] In addition to *mbx*A, the RTX operon genes include *mbx*C (toxin activation protein), *mbx*B (transport protein), and *mbx*D (transport protein) and a flanking protein related to TolC, a secretion protein similar to other RTX toxin operons (type 1 secretion system).[17,29] A high degree of conservation in *mbx*A has been observed among isolates from diverse geographic origins, which makes this a potential vaccine antigen target.[31,32]

MORAXELLA BOVIS PILI

Type IV pili, also known as fimbriae or attachment pili, are small structural protein fibers made up of pilin proteins that serve numerous functions.[33] In *M bovis,* they facilitate attachment to corneal epithelium, a process that is inhibited by pili-specific antiserum. Pili are a necessary virulence factor, and strains lacking pili are unable to adhere and therefore cannot cause disease.[34–36] Inhibition of adherence by pili-specific antiserum is serogroup specific, with at least 7 different serogroups described.[37] Protection from IBK using pili-based vaccines was shown to be serotype specific in 1 study.[38] Selection and maintenance of pili in culture through selection and passage of agar-corroding colonies with pili enabled successful challenge and colonization models, as pili expression appears to decrease upon passage (**Fig. 1**).[39–41] The isolation frequency of piliated colony types from cattle changes based on seasonality and has been observed to positively correlate with levels of UV radiation.[42] *M bovis* also expresses 2 mutually exclusive forms of pilin, called Q (quick) and I (intermediate), in addition to serotype level variation conferred by pili.[43,44] The expression of pili is reversible and under the control of an inversion region under a single promoter.[44,45] The Q pili form has been shown to be more effective at binding to corneal epithelium than both I pili forms and non-piliated strains.[46] *M bovis* selectively attaches, presumably through pili, to dark cells in the cornea, those that are older and devoid of membrane ridges called microplicae, when viewed by scanning electron microscopy; the number of these cells is increased by UV light.[47] This observation has led to the hypothesis that other environmental factors or coinfections that increase the proportion of dark cells in the cornea may potentially enhance the ability of *M bovis* to colonize.[4,48,49] Pili are also involved in the formation of biofilms on both biotic and abiotic surfaces; this formation of biofilms seems to confer greater resistance to lysozyme.[50,51]

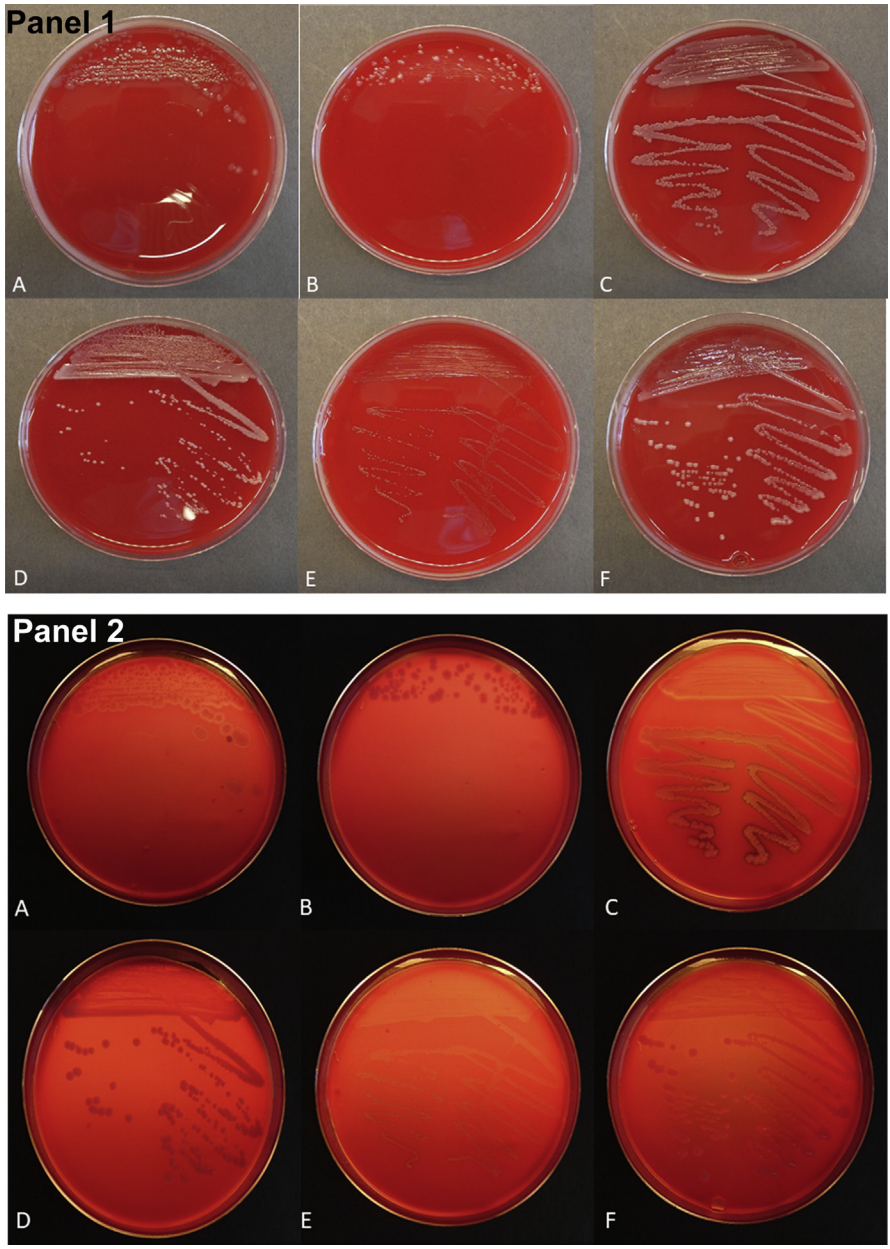

Fig. 1. Panel 1. Differences in *Moraxella* colony phenotypes. Images were captured after 48-hour growth in 5% CO_2 atmosphere on trypticase soy agar with 5% defibrinated bovine erythrocytes. (*A*) Strain Epp 63, *M bovis*, RTX +, beta hemolysis. (*B*) Isolate 0131, *M bovis* recombinant, RTX +, beta hemolysis. (*C*) Isolate Mb58081, *M bovoculi*, genotype 1, RTX +, beta hemolysis. (*D*) Isolate Mb58090, *M bovoculi*, genotype 1, RTX –, gamma hemolysis. (*E*) Mb58000, *M bovoculi,* genotype 1, RTX +, stop codon at codon 70 of PilA gene (no pili production), beta hemolysis. (*F*) Mb 68,535, *M bovoculi*, genotype 2, RTX -, gamma hemolysis. Panel 2. Same plates as panel 1 with backlighting to visualize hemolysis. (Images by Matthew Hille and Justin Lowery.)

MORAXELLA BOVIS OTHER POTENTIAL VIRULENCE FACTORS

M bovis possesses an outer membrane (OM) that consists of at least 3 distinct types of lipooligosaccharide (LOS), which are uniquely devoid of heptose.[52,53] The biologic role of LOS for *M bovis* was demonstrated, as mutants with truncated LOS (shortened and less functional) had increased susceptibility to the bactericidal activity of bovine serum, decreased adherence to mammalian cells, and increased sensitivity to detergents and some antibiotics.[54] Some *M bovis* strains also express a capsular polysaccharide.[54,55] OM proteins are the first line of defense against host immune defenses, and these studies provide supportive evidence that in *M bovis* they may serve as virulence factors.

M bovis strains produce an array of hydrolytic enzymes, which facilitate the breakdown of host protein, lipids, nucleic acids, carbohydrate and fat molecules into their simplest units, and therefore can cause damage to ocular tissues. These include esterases, lipases, phosphoamidase, phosphatase, and hyaluronidase. *M bovis* strains can also hydrolyze casein, produce fibrinolysin, and are agarolytic.[56] Some strains express and secrete phospholipase, which is conserved and has been considered by some as a potential vaccine antigen.[57] Other strains carry a plasmid encoding filamentous haemagglutinin-like proteins (*flp*A and *flp*B), which may facilitate host colonization, similar to virulence factors found in *Bordetella pertussis.*[58] Iron acquisition is a critical function for pathogens to replicate and maintain infections, as iron is tightly regulated in the host environment. Some *M bovis* strains also possess iron-repressible outer membrane proteins similar to the human pathogen *N meningitidis* (*lrp*A)[59] and have robust iron acquisition machinery, including fur homologues, and are capable of acquiring iron from bovine lactoferrin (lbp) and transferrin (tbp).[60,61]

STUDIES ON *MORAXELLA BOVIS* AND CAUSALITY

Experimental studies have definitively demonstrated that *M bovis* can cause IBK-like lesions compared with uninfected controls.[5,62–64] However, insults to the cornea such as ultraviolet (UV) radiation or corneal scarification may be necessary in experimental infection studies to induce IBK.[5,65] Lesions have also been produced in the absence of corneal insults, and whether they are required may depend on the infectious dose or the strain virulence and immune status of the host.[63] Experimental infections with some strains appear to be enhanced by coinoculation with *M bovoculi*.[66] Evidence for the causal role of *M bovis* in IBK came through experimental infections with strains of known virulence in gnotobiotic calf models, which have reproduced clinical and lesions consistent with IBK, including histopathological changes in the cornea and conjunctiva in the absence of other factors such as trauma or coinfections with other bacterial species.[3,4,67] Purified fractions of *M bovis* cell-free supernatants were shown to reproduce IBK lesions in calves, implying a role for a specific excreted *M bovis* virulence factor to cause IBK, which we now understand to be the RTX toxin encoded by *mbx*A.[2]

In field studies, *M bovis* is often isolated from IBK-affected cattle, but it is also found in normal healthy eyes, with variations in isolation rates observed depending on seasonality and animal age.[42,68,69] One study showed high prevalence of isolation of *M bovis* in the absence of other agents in outbreaks that were diverse in space and time, but did not include cultures from nondiseased eyes for comparison.[70] A study of an outbreak during winter, winter pinkeye, had a high prevalence of *M bovis* isolated in the absence of other agents, and the isolates were able to reproduce IBK in calves, but this study also did not culture healthy eyes for comparison.[71] More recent studies using PCR methods showed only a weak temporal association between *M bovis*

detection and IBK in naturally occurring outbreaks of IBK.[14] The observation that *M bovis* is isolated from nonclinical animals at similar rates as those from IBK cases has been documented numerous times in epidemiologic studies, thus complicating the picture of a single infectious agent that is alone necessary and sufficient for causality.[72,73]

The mechanism for why discrepancies between the presence of *M bovis* and clinical cases of IBK are observed could be comparable to what is understood about bovine respiratory disease (BRD). Many of the pathogens associated with BRD can be isolated from the respiratory tract of healthy animals. Immunomodulatory effects of common bovine viral infections or stress allow for these opportunistic pathogens to replicate, invade deeper into the respiratory tract, secrete toxins, and cause disease.[74] Likewise, *M bovis* may only be able to cause IBK if other conditions are met, such as stress, physical damage, UV radiation, or face fly irritation. Also, with BRD there are well-defined strains, or genotypes, of *M haemolytica* that differ by their armament of virulence factors and outer membrane proteins, and by their association with BRD.[75,76] The same could be true for *M bovis* and IBK, because of changes in alleles throughout the genome that alter or enhance virulence. More detailed discussion about causal models for IBK is available elsewhere in this issue.

MORAXELLA BOVIS GENOMICS

Genetic diversity among *M bovis* has been examined through DNA fingerprinting with enterobacterial repetitive intergenic consensus (ERIC), and random amplified polymorphic DNA (RAPD).[53,77] Recently, the first complete whole-genome sequence of the Epp63 strain, used in many studies, was published.[78] Aside from having known virulence factors including an RTX operon, multiple pilin and prepilin genes, and other genes involved with adhesion, the Epp63 genome has numerous repetitive regions. These include 2 repeat regions associated with CRISPR and 5800 repeat regions consisting of dinucleotide to decanucleotide units, ranging in size from 8 to 422 bases. The repeats are located throughout the genome, either in noncoding regions, coding regions, or both, which suggests that *M bovis* may employ slipped strand mispairing coupled to phase variation as a way to adapt to particular niche environments including changing host immune responses directed at select *M bovis* antigens. Multiple pathogens employ this technique, which may enable *M bovis* strains to vary their pathogenicity by the phase state of their genomes.[79] On a practical level, multiple repeat regions probably explain why there is a paucity of fully assembled *M bovis* genomes in public databases at present, as short reads of a sequence commonly produced by most sequencing technologies would not span these large repeat regions, which would interfere with the assembly of a complete *M bovis* genome. Additional complete genomes will be required to understand *M bovis* genomic diversity better, as well as strain pathogenicity or lack thereof, and the extent of recombination between *M bovis* and other members of the *Moraxellaceae.*

MORAXELLA BOVOCULI

Virulence Factors

Several virulence factors homologous to those found in *M bovis* have been found within *M bovoculi,* which provides partial support for the hypothesis that it may play a role in IBK pathogenesis, despite not having been able to replicate IBK-like lesions in experimental infections. The 2 most significant homologous virulence factors are a complete RTX operon and type IV pilin proteins.[80,81] The *mbvA* gene, responsible for the RTX toxin (also called cytotoxin A) within *M bovoculi* is highly conserved with

99.3% nucleotide and 98.8% corresponding amino acid homology within the species.[32] Similar to *M bovis*, the RTX toxin of *M bovoculi* is necessary for hemolytic activity (**Fig. 2**).[80,82] Although the RTX toxins produced by *M bovis* and *M bovoculi* are similar in structure and function, within an *in vitro* model using bovine erythrocytes, polyclonal serum raised against *M bovoculi* RTX toxin did not neutralize corresponding *M bovis* toxin activity encoded by *mbxA,* but did neutralize *M ovis* RTX toxin, thus indicating sufficient differences between *M bovis* and *M bovoculi* toxins in structure to require additional immune responses.[80] Some of these RTX-related operon sequences found in *M bovoculi* demonstrate what appears to be interspecies mosaicism, where recombination with *M bovis* RTX-related sequences (*mbvB* and *mbvD*) is apparent in some sequences.[83]

Sequence analysis of *M bovoculi* pilin (pilA) appears to be substantially different than that of *M bovis,* with as little as 38% sequence homology between the 2 species.[81] However, *M bovoculi* pilin sequences show limited intraspecies diversity in contrast to *M bovis*.[83,84] The large diversity between species suggests that any cross-protection to pilin epitopes would be minimal. The potential importance of the few variable regions within *M bovoculi* pilin sequences is unknown. If these variable regions prove to be important clinically, it could explain the failures thus far to produce disease experimentally and/or provide an effective immune response using a limited number of isolates, as the variable regions may be important determinants of initial ocular colonization and disease initiation, as observed with *M bovis.*

Like *M bovis, M bovoculi* has also been shown to form biofilms on abiotic surfaces such as polystyrene microplates.[51] Biofilms have a well-established association with disease severity in numerous microbial diseases of people and animals.[85,86] Biofilm formation is variable within *M bovoculi,* and also could potentially contribute to variation in virulence. For example, the type strain of *M bovoculi* (BAA1259) has moderate biofilm-forming capacity, where more recently isolated field strains of *M bovoculi* used in the study had stronger biofilm-forming capability.[51] Given the relatedness of the species, the reliance on type IV pilus for biofilm formation would seem likely, but this has not been examined within *M bovoculi*.

CHALLENGE MODELS

In 1 study, *M bovoculi* did not cause IBK in conventionally reared calves, even though a hemolytic isolate representing the type strain was used for the challenge. In this same study, controls using *M bovis* strain Epp63 did cause disease consistent with

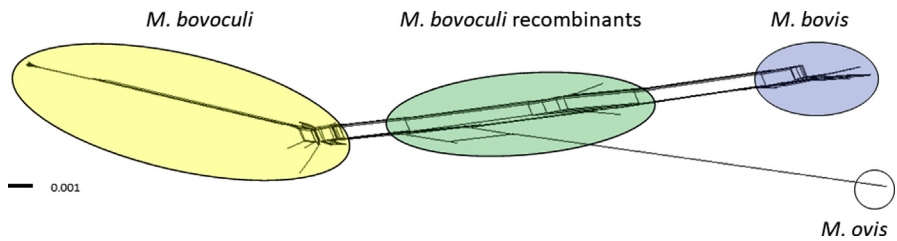

Fig. 2. Neighbor-joining network made in SplitsTree of full length 16S sequences. The network contains 18 *M bovis* sequences (contained in the blue ellipse and described by Robbins and colleagues*),* 253 *M bovoculi* non-recombinant and recombinant sequences (contained in the yellow and green ellipses, respectively, and described by Dickey and colleagues, GenBank# SRP070887) and 2 *M ovis* sequences (contained in the white circle, GenBank # AF005186 and NR_028670). The scale bar equals substitutions per site.

IBK using the same randomized and blinded challenge model.[5] These findings are in agreement with a smaller study from 25 years prior in which *M ovis* (likely *M bovoculi*) did not cause corneal lesions in a small group of calves.[87] Gould and colleagues[60] recognized this older study was inadequate because of a lack of a positive control, meaning it was not possible to determine if the organism did not cause disease or the model was incapable of causing disease. Regardless, *M bovoculi* has not demonstrated a causal relationship to IBK under experimental conditions. However, genomic diversity within *M bovoculi* further complicates interpreting this challenge work, as only limited strains have been used for experimental inoculations.[83,88]

MORAXELLA BOVOCULI AND CAUSALITY

Despite not having shown to reproduce the disease experimentally, a hallmark of infectious disease causation in veterinary science, there are some findings that support a potential contributive or associative role for *M bovoculi* in IBK pathogenesis. Prior to the characterization of *M bovoculi*, isolates classified as *M ovis* (likely *M bovoculi)* were isolated three times more often than *M bovis* from clinical samples of IBK cases submitted to a diagnostic laboratory.[82] More recently, *M bovoculi* was the only bacteria isolated from most (64%) of individual animal cases of IBK over a 4-year timeframe with only 22% of cases isolating only *M bovis*.[13] In this same study, the authors found when examining cases where more than 1 bacterial species was isolated, *M bovoculi* and *M bovis* were isolated from 74% and 32% of cases, respectively.[13] Another study utilizing real-time PCR detection on diagnostic laboratory submissions found similar results, where 75.9% of samples (136/179) had *M bovoculi* detected.[89] In studies looking at prevalence of *Moraxella,* one study showed that only the detection of *M bovoculi* by PCR methods was associated with clinical signs of IBK. This same study also showed that prevalence of *M bovoculi* may be associated with likelihood of undergoing an acute IBK outbreak.[68] Another study showed the recovery of *M bovoculi* and *M bovis* was more frequent from eyes with IBK lesions than unaffected eyes.[14] It also showed that calves infected with *M bovis* had a higher subsequent risk of developing IBK than calves infected with *M bovoculi*.[14] Caution should be taken to avoid overinterpretation of postlesion microbiological data, which does not establish temporality between presence of pathogens and occurrence of lesions.[5] Documenting that exposure occurs before disease is essential for establishing causation in future studies.

MORAXELLA BOVOCULI GENOMICS

M bovoculi has recently been classified into 2 major strain types, or genotypes (1 and 2), based on whole-genome sequencing of over 200 isolates.[83,88] The genotypes differ in several ways. Both genotype 1 and 2 *M bovoculi* have been isolated from the eyes of cattle without IBK, while only genotype 1 *M bovoculi*, which includes the type strain, has been isolated and identified from the eyes of cattle with IBK. However, the frequency and potential role, or lack thereof, of genotype 2 *M bovoculi* in IBK remain poorly understood. Genotype 2 *M bovoculi* has only recently been described, and isolates may have been inadvertently misclassified as non-*bovoculi Moraxella* in the past based on a PCR typing method instead of biochemical for *M bovoculi* that was developed prior to their discovery.[88,90] The PCR-based typing method only identifies genotype 1 strains, as genotype 2 strains do not contain the restriction enzyme binding site targeted in the method to distinguish *M bovoculi* from *M ovis* and other *Moraxella* species.[83,90,91] Thus, the difference between the genotypes in IBK-positive or -negative eyes needs to be tested in more studies that do not have biases in isolate

identification. Recently, a MALDI-TOF biomarker method, which uses mass spectrometry peaks that are genotype specific to classify *M bovoculi* isolates, was developed that would make these data easier to generate, as these instruments are now available in many diagnostic laboratories.[92]

Genotype 1 *M bovoculi* appears to be more diverse than genotype 2. Over 127,000 SNPs have been identified within and across the 2 *M bovoculi* genotypes, with over 80% characterizing diversity exclusively in genotype 1 strains.[83] The 2 genotypes also appear to differ in their RTX profiles. To date, no genotype 2 strains have been found to have an RTX operon versus 85% of genotype 1 strains isolated from IBK eyes that do, including the type strain.[83] Thus, current studies suggest that *M bovocul*i genotypes may differ in their propensity to cause or contribute to the development of IBK, with genotype 1 isolates more likely to have RTX, and extensive genetic diversity, and genotype 2 isolates having genomic profiles more consistent with commensals.

CLASSIFICATION OF *MORAXELLA BOVIS* AND *MORAXELLA BOVOCULI*

MALDI-TOF assays that enable identification of *M bovis* and the 2 genotypes of *M bovoculi* may be improved or enhanced as more is learned about the 2 species and the extent of recombination that may have taken place between them. There is compelling evidence that *M bovoculi* has recombinant 16S rDNA alleles (see **Fig. 2**) that were possibly acquired from *M bovis*, and *M bovis* has recombinant alleles within the internal transcribed spacer region of the ribosomal locus.[91] Given the high conservation of the ribosomal locus throughout the evolution of bacteria, it is not unexpected that these species have extensive recombination elsewhere throughout their genomes.[83] This observation challenges assignment of IBK causality, as these mixed chimeric genomes may not be easily assigned to 1 species. However, it is possible to distinguish *M bovis*, *M bovoculi*, and *M bovoculi* recombinants from each other with full-length 16S rDNA sequences (see **Fig. 2**). Full-genome sequences will ultimately be much more powerful in classifying these bacteria as an identity -by -state combination of alleles acquired through vertical descent and horizontal lateral gene transfer events. The same may be true for other members of the *Moraxellaceae*, and methods that classify members of this family using full, or particularly partial 16S sequences, may be shown to conflate members of the family. Examples include *M ovis* and members of the closely related genus *Psychrobacter,* which are also found in cattle and have been isolated from bovine eye swabs by the authors.[93,94]

SUMMARY AND FUTURE WORK

Whole-genome sequencing of *M bovis* and *M bovoculi* strains isolated from the eyes of cattle with or without IBK, followed by genome comparisons, may reveal core genetic determinants that are necessary for virulence and further understanding of the diversity and contributions of these organisms to IBK.

CLINICS CARE POINTS- DIAGNOSIS AND ESTABLISHMENT OF *MORAXELLA* ETIOLOGY IN INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

- Determining the etiology in individual outbreaks is challenging, because *Moraxella* species have been isolated from cases of IBK and normal eyes, and the isolation of an agent after the appearance of a lesion does not establish causation.
- For microbiological sample collection, the ocular conjunctival sac is aggressively swabbed to culture for pathogens while avoiding contamination. Use of flocked

swabs in liquid transport media offers enhanced collection and release of bacteria into the media.[95]
- False-negative culture results can occur if there is contamination or multiple pathogens.
- PCR as a culture independent modality can be useful, although caveats and challenges related to classification and misclassification of *Moraxella*, as discussed, also apply to PCR diagnostic methods.
- A multiplexed real-time PCR is available that targets the toxin genes for *M bovis* (*mbx*A) and *M bovoculi (mbv*A).[89] Similar approaches for bovine respiratory disease pathogens have been shown to increase co-detections over culture alone.[96]
- The authors routinely see case submissions from field outbreaks of IBK where only *M bovoculi* is isolated by culture, but similar levels (Ct values) of *M bovis* toxin genes are detected by real-time PCR, which indicates the potential for false-negative *M bovis* cultures.
- Newly developed MALDI-TOF assays provide quick, accurate, and economic methods to identify *Moraxella* species from culture. These methods also allow differentiation of *M bovoculi* isolates traditionally associated with disease versus those that likely represent normal flora.[91,92]

DISCLOSURE

The authors have nothing to disclose.

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