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Key Points

- Non-Moraxella organisms are associated with infectious bovine keratoconjunctivitis (IBK).
- *Mycoplasma bovoculi* can cause conjunctivitis and has a potential role in IBK pathogenesis.
- Other Non-Mycoplasma agents can cause disease that resembles IBK but is clinically different.
- Genomics and molecular technology are advancing research in this area.
- Classification and determination of pathogenesis potential of these organisms may be better understood through metagenomics and whole genome sequencing.

Keywords: IBK, Pink eye, Infectious bovine keratoconjunctivitis, *Mycoplasma bovoculi, Ureaplasma,* Bovine herpesvirus, Listeria, Chlamydia

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Introduction

Historically the role of bacterial organisms associated with infectious bovine keratoconjunctivitis (IBK) has fallen to members of the genus *Moraxella* (see John Dustin Loy and colleagues' article, "Component causes of IBK — The Role of *Moraxella* spp. in the epidemiology of Infectious Bovine Keratoconjunctivitis," *Veterinary Clinics of North America: Food Animal Practice,* 37:2, July 2021, pp 279-293; doi:10.1016/j.cvfa.2021.03.004). However, there are numerous other pathogens that have been described in association with either outbreaks or clinical cases of IBK with and without an association with *Moraxella*. The most significant of these include members of the genus *Mycoplasma*, specifically *Mycoplasma bovis* and *Mycoplasma bovoculi*, which have long been found associated with ocular infections in cattle. Other less understood potential pathogens include intracellular organisms such as *Chlamydia* spp. Viral causes such as bovine herpesvirus have also been associated with IBK. Other ocular diseases, including those caused by *Listeria monocytogenes,* may often have some clinical overlap with IBK-like diseases. Although none of these agents has strong support as a direct cause of IBK, there is evidence that infections with some may predispose animals to IBK. The role of these agents in association with IBK or IBK-like disease is reviewed later, including virulence factors, studies on causality, immune responses, and importantly detection and interpretation of diagnostic findings of these organisms in IBK cases.

Mycoplasma

Members of the genus *Mycoplasma* are small pleomorphic bacteria that lack a cell wall, are nutritionally fastidious, have a limited metabolism, and are composed of 124 species.[1] Most of the well-studied members of the genus are human or animal pathogens; however, about half of these exist as commensals or opportunistic pathogens that colonize fish, reptiles, and birds in addition to mammals.[1] There are at least 13 members of the genus *Mycoplasma* that infect cattle, and these include those that cause respiratory, reproductive,

mastitis, systemic diseases, and ocular infections.[2] *Mycoplasma* is one of the most abundant genera present in the bovine upper respiratory tract.[3–5] Evaluations of microbiota from calves with and without disease indicated that animals with otitis and pneumonia have a greater abundance of *Mycoplasma* sp than those without; and all conditions had a greater *Mycoplasma* sp abundance than healthy cohorts.[6] *Mycoplasma* has been studied by using 16S ribosomal sequencing to look at microbial communities within the bovine eye. Among, the top 10 genera identified, *Mycoplasma* showed a higher mean relative abundance in the non-IBK controls (26.86%) compared with the IBK cases (18.29%).[7] An in vitro co-culture study conducted with human sinus epithelial cells and donor respiratory microbiota determined that the inclusion of TH-1 macrophages shifted the microbial abundance from *Corynebacterium* spp, *Staphylococcus* spp, and *Dolosigranulum* spp to an abundance of *Moraxella* spp and *Mycoplasma* spp, providing some insight into how mammalian host immune status may alter microbial composition.[8] Another recently published study evaluated the ocular microbiome in calves over time and showed that *Mycoplasma* spp were detected at all time points with variation in abundance over the preweaning period. Significant differences were also observed in microbial communities before and after clinical IBK disease, with both *Mycoplasmataceae* and *Moraxellaceae* families increasing post-IBK infection[9]; this indicates that *Mycoplasmas* are likely part of the ocular flora and may change over time and in response to IBK. The nutritionally fastidious nature of *Mycoplasmas* makes isolation and study in the laboratory challenging. Recovered isolates require additional characterization to identify the species isolated, which often is only performed in reference laboratories. However, with the advent of molecular techniques such as polymerase chain reaction (PCR) and gene sequencing, more tools are now available to aid researchers and veterinarians in the study and diagnosis of these infections, and future research using these techniques may establish more definitive roles for these agents in association with IBK. The lack of available tools such as vaccines and effective treatments has also impeded successful mitigation of the disease-causing *Mycoplasma* spp in animals, including cattle, and these areas deserve additional research attention.[10]

Mycoplasma bovoculi

M bovoculi was first described following an outbreak of IBK that yielded an isolate that was biochemically different from previously described *Mycoplasmas*.[11,12] *M bovoculi* has a specific requirement for sterol in the culture media; use of standard *Mycoplasma* media may not support recovery of this agent and cause false-negative results.[11]

Subsequent studies on microbial flora of cattle eyes have shown *M bovoculi* to be highly prevalent in normal calves, and asymptomatic infections can occur at an early age, with spread to other animals occurring over time, including over the winter months when vectors are at lower levels.[13] The average prevalence of *M bovoculi* in repeated samplings in this study was more than 45%, which, given the challenges in isolating this organism, may indicate a much higher true prevalence. Given the apparent high prevalence in cattle eyes, it is difficult to evaluate *M bovoculi's* contribution as a risk factor to IBK. It seems likely that *M bovoculi*, if a pathogen, is an opportunistic one that may contribute to disease instead of directly causing IBK. Some studies summarized later show mild or absent disease when *M bovoculi* is found alone but describe potentiation of disease when administered or found with other pathogens, such as *Moraxella bovis*. There may be synergism between *Mycoplasma* and *Moraxella* species; however, the exact mechanisms of such a process have not yet been described.

Virulence Factors

Virulence factors and other characteristics of *M bovoculi* remain poorly understood. *M bovoculi* adheres to bovine epithelium in the absence of any specialized attachment structure and does not possess a capsule.[14] The adherence is tight and primarily to bovine conjunctival epithelial cells; organisms can be observed in infected animals when stained with specific florescent antibody.[15] Whole genome sequencing has been performed on the type strain of *M bovoculi*, which is the descendant of the original isolates used in the bacterial species description and which contains a 760,240 bp genome, 626 genes, and has 7 gene pairs potentially associated with adherence factors. [16] A novel contingency locus, that is, a region of hypermutable DNA, was

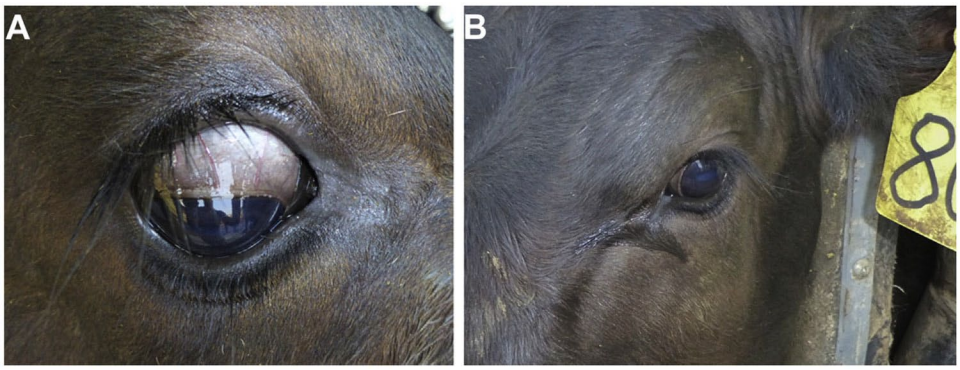

Fig. 1. (A and B) Calf with bilateral ocular discharge and mild conjunctivitis. PCR detected the presence of *Mycoplasma* spp in the absence of other agents. (Courtesy of Dr John Angelos, UC Davis.)

also identified, which has an array of 5 genes that indicate phase variation and combinatorial expression patterns, which may be involved in host immune evasion or other host adaptations of *M bovoculi* outer membrane proteins.[17]

Clinical Disease

Clinically, cattle with *M bovoculi*–associated signs do not appear ill, but 10% to 90% may have a unilateral or bilateral ocular discharge and conjunctival hyperemia (Fig. 1).[18] Field investigations of "epizootic conjunctivitis," a disease entity considered clinically different from classic IBK, isolated primarily *M bovoculi* in 11 out of 19 Iowa farms from affected animals.[15] In a different study ocular samples from 6 herds with cattle showing signs of conjunctivitis yielded recovery of *M bovoculi* from 50% to 100% of cattle but no recovery from two herds without signs of conjunctivitis.[18] Experimental infection studies with *M bovoculi* inoculation report mild conjunctivitis, and some have shown infections with *M bovoculi* may predispose animals to Moraxella-induced IBK. For example, under experimental inoculation, calves exposed to *M bovoculi* (n = 6) and *Ureaplasma* spp (n = 6) developed conjunctivitis and lacrimation 3 to 4 days following challenge and were recovered 1 to 2 months after infection.[18] *M bovoculi* was able to be re-isolated from the bovine eye following co-challenge with *M bovis*.[19] Another study describes a potential enhancement effect of *M bovoculi* on *M bovis* challenge in colostrum-deprived calves, where those that received *M*

bovoculi before *M bovis* challenge, 4/5 eyes developed conjunctivitis within 3 days versus only 1/3 that did not receive *M bovoculi* inoculation developing conjunctivitis.[20] However, the study lacked robust controls to evaluate the validity of this observed enhancement. Calves infected with *M bovoculi* in one study had longer colonization times and developed keratitis at higher rates when challenged with *M bovis* (n = 6/6 calves) when compared with those without mycoplasma exposure (0/4 calves).[21] *M bovoculi* also seemed to increase colonization levels of *Moraxella ovis* in calves. One study showed 0/3 calves had *M ovis* isolated from eyes 15 days after inoculation when compared with those that had been inoculated with *M bovoculi* before *M ovis* (3/3 calves). There were also significant differences in colony forming units/swab of *M ovis* isolated between these 2 treatment groups at earlier time points.[22] Even though several studies point toward a possible role of *Mycoplasma* in IBK, extrapolation of results from small experimental studies, which often lack blinding and randomization of study animals, requires confirmation of findings in field trials. Difficulty arises from the fact that prevalence of *M bovoculi* infection in eyes of cattle populations seems to be very high, which may actually obscure its potential role as a risk factor.

Immunity

Immune responses to *M bovoculi* seem robust in recovered animals and include immunoglobulin A (IgA) in lacrimal and nasal secretions in addition to serum IgG and IgM and cellular immune responses.[23,24] Previous natural exposure to these organisms seems to provide protection against colonization. One study showed that in calves with evidence of prior *M bovoculi* colonization that were vaccinated and subsequently challenged, most cleared the organism by day 3 and all were negative by day 10 postchallenge. None of these animals showed evidence of conjunctivitis. In contrast, protection from *M bovoculi* challenge in gnotobiotic calves was not induced by administration of killed *M bovoculi* antigens (either from membrane extracts or killed whole organism); all animals developed conjunctivitis and had *M bovoculi* recovered throughout the study period (15 days).[24] *M bovoculi* strains have similar, but not identical, protein profiles, with antigenic differences apparent when evaluated by immunoblotting

with serum from recovered calves. However, a 94 kDa outer membrane protein that seems antigenically identical across multiple strains and monoclonal antibodies raised against this protein interact with other *Mycoplasma* species.[25,26] Ocular challenge with *M bovoculi* seems to result in enhanced systemic natural killer (NK) cell activity as well as to induce NK migration into bovine eyes following acute infection.[27] A small study with 65 cattle from India found serum IgG antibodies that reacted to sonicated *M bovoculi* antigens in 44% of cattle with IBK and 15% of nonclinical cattle, indicating an association between immune response and clinical disease.[28]

Epidemiologic Studies

Some associations between the presence of *M bovoculi* and IBK have been found, but the evidence has to be interpreted with caution. Schnee and colleagues (2014) examined the point prevalence of IBK-associated pathogens in cattle from 4 different herds in Europe representing 4 different clinical stages of IBK. The investigators found that herds early in the course of IBK had a higher prevalence of *M bovoculi* detected by PCR than those recovering from or without clinical IBK and hypothesized that herds with higher *M bovoculi* prevalence are predisposed to acute outbreaks of IBK, possibly due to synergism with *Moraxella* spp. However, further studies are needed to confirm the findings of the cross-sectional study that may have been confounded by different breeds, housing types, or other management factors.[29] Other surveys indicate that the prevalence of *M bovoculi* in cattle eyes seems quite high in both clinical and nonclinical animals. One study mentioned earlier demonstrated that in a group of calves followed from 1 week to 15 months of age without clinical ocular disease, *M bovoculi* recovery rate by culture was initially low but increased over time with an overall prevalence of 45% in normal eyes.[13] Using a recently developed real-time PCR approach to detect IBK-associated pathogens, Zheng and colleagues found very high prevalence of *M bovoculi* in case submissions to a diagnostic laboratory (159/179; 88%). Although diagnostic submissions represent a biased sample, these results indicate that *M bovoculi* may be an underdetected component of IBK, and PCR testing may reveal it is present at high levels in diagnostic submissions and during outbreaks.[30]

Mycoplasma bovis

M bovis causes a wide array of significant bovine diseases that include pneumonia, mastitis, arthritis, otitis, and keratoconjunctivitis.[31] It possesses a variety of virulence factors involved in adherence, antigenic variation, host cell invasion, immune modulation, and biofilm formation.[31] *M bovis* has been shown to suppress host immunity via altered cytokine (interferon gamma and tumor necrosis factor alpha) expression, depressed oxidative burst from neutrophils, and suppressed lymphocyte proliferation. [31] In a retrospective study from a diagnostic reference laboratory in the United Kingdom, *M bovis* was the most frequently isolated *Mycoplasma* sp from bovine cases annually, representing 52% of all *Mycoplasmas* isolated from cattle over a 10-year period, which were primarily cultured from the lung or upper respiratory tract but also from eyes with IBK lesions.[32] *M bovis* was investigated as a cause in an outbreak of IBK in a beef herd that subsequently developed pneumonia and arthritis.[33] *M bovis* isolates were recovered during the outbreak investigation. All were identified as a single strain that had similarity to other European *M bovis* strains and possessed variable membrane surface lipoprotein (*vsp*) genes found in *M bovis* but not in other *Mycoplasma* spp.[34] *M bovis* has been reported in outbreaks of IBK in 1-year-old beef calves and a group of Holstein cattle in the absence of other pathogens.[35,36] *M bovis* has also been found in mixed *Mycoplasma* outbreaks of IBK (along with *M bovoculi*) in Holstein calves following morbidity with respiratory disease that had 30/40 affected.[37]

Other Mycoplasmas

Other *Mycoplasmas*, including *Mycoplasma bovirhinis* and *Mycoplasma bovigenitalium* in a mixed infection with bovine herpesvirus-1 (BoHV-1), have been reported associated with IBK. The significance of these findings is unknown because the role of the individual agents was not evaluated and the findings stem from a series of case reports.[38] Experimental inoculation of calves with *Mycoplasma conjunctivae* or *Acholeplasma laidlawii* did not result in IBK.[39]

Ureaplasma

Ureaplasma, originally classified as T-strain *Mycoplasmas*, are small pleomorphic bacteria that lack a cell wall and are very similar to *Mycoplasma*, with the exception of a requirement for urea for growth.[33] Similar to *Mycoplasma,* all of the members of the genus are obligate commensals or opportunistic pathogens of vertebrate hosts, primarily of mucosal surfaces. There are 7 species of *Ureaplasma*, and the species associated with bovine hosts is *U diversum*, of which there are 3 distinct serologic clusters.[40] Infections with *Ureaplasma* spp in cattle are typically associated with reproductive infections or fetal/neonatal pneumonia.[41] However, there have been some reports of *Ureaplasma*-associated ocular infections. Large colony and T-strain mycoplasmas (Ureaplasmas) have been isolated from ocular secretions, along with *M bovis* in cases of calves with IBK, but not in healthy calves; however, these results are from a single publication 50 years ago and have not been reproduced.[42] Case reports suggest fetal infections with *Ureaplasma spp* can induce extensive nonsuppurative conjunctivitis and goblet cell metaplasia throughout the eyelid epithelium.[43] *M bovoculi* and *Ureaplasma* sp have been recovered from outbreaks of epizootic conjunctivitis.[44] Inoculation of calves with *Ureaplasma* spp caused diffuse conjunctivitis and lacrimation, and organisms were able to be recovered from inoculated eyes up to 2 months postinfection.[18] Overall, the evidence for the role of *Ureaplasma* spp in IBK is limited at present to a few older studies, and its contribution to the disease complex is likely minor.

Herpesviruses

Infections with BoHV-1, the causative agent of infectious bovine rhinotracheitis (IBR) can cause ocular disease that resembles IBK. Outbreaks of IBK-like diseases, some with an absence of corneal ulcers, had virus isolated from nasal and ocular secretions.[45–50] The virus isolated from early outbreaks was determined to be a herpesvirus indistinguishable from that causing IBR. In experimental infections, BoHV-1 produced conjunctivitis but not keratitis in challenged calves, thus indicating

infection with this herpesvirus caused a disease distinct from IBK.[51,52] However, BoHV-1 has shown some association with IBK. Challenge with BoHV-1 alone in more recent studies caused conjunctivitis and blepharitis but not keratitis.[53] Pugh described higher prevalence of IBK in animals that were exposed to BoHV-1 before challenge with *M bovis* versus those that were exposed to BoHV-1 after *M bovis* challenge.[53] George and colleagues demonstrated that vaccination with a modified live IBR vaccine either intranasally or intraocularly increased lesion scores and isolation rates of *M bovis* in calves challenged with *M bovis* when compared with nonvaccinated, but challenged, controls.[54] A high seroprevalence (60.1%) to BoHV-1 was detected in a yak (*Poephagus grunniens*) farm experiencing an outbreak of abortion and keratoconjunctivitis; no animals had previously been vaccinated against BoHV-1.[55] However, more recent work looking at an outbreak of IBK in a beef herd did not show an association between BoHV-1 status and IBK.[56] A potential mechanism to explain the association of IBR with IBK is that BoHV-1 causes immune depression characterized by inhibition of polymorphonuclear cell migration and cell-mediated cytotoxicity, which could predispose the host to superinfection with bacterial pathogens.[57] BoHV-1 also produces host immunosuppression by inhibiting the production of interferon beta, which may also result in secondary infections in the host.[58]

Other alphaherpesviruses have been associated or experimentally shown to cause infectious keratoconjunctivitis-like lesions in a variety of other ruminant species, including mule deer and semidomesticated reindeer and seem to be the primary cause of these lesions in some of these cases.[59–63]

Malignant Catarrhal Fever

Malignant catarrhal fever (MCF) is caused by 1 of 2 γ-herpesviruses: alcelaphine herpesvirus 1 found in wildebeest and ovine herpesvirus 2 found in sheep, both of which can cause MCF in cattle, bison, deer, pigs, and other ungulates.[64–66] The reservoir hosts are inapparent carriers, but susceptible species show fever, depression, ocular and nasal discharge, diarrhea, and frequently do not survive. Cattle become infected through direct contact with a host or through aerosol exposure.[67] Cattle are considered dead-end hosts that do not transmit the

virus to herdmates.[68] Although variable in clinical presentation, a common ocular lesion in MCF cases is corneal edema, which may mimic IBK, although cattle with MCF are systemically sick, which is typically not observed in IBK. Other hallmarks of the disease that preclude IBK include the pattern of ocular opacity, with a fine line that spreads centripetally from the limbus, and the presence of concurrent signs such as persistent high fever, salivation, purulent nasal discharge, and generalized lymph node enlargement.[69] One study of chronic and recovered cattle infected with sheep-associated MCF observed the most obvious clinical sign was bilateral ocular lesions.[70] Severity of corneal edema at the time of diagnosis is not correlated with clinical outcome in cases of MCF; however, cases of MCF that did not survive had no improvement of corneal edema during hospitalization and treatment in a prospective study.[71] Other common clinical signs shared with IBK are blepharospasm, ocular discharge, corneal vascularization, conjunctival hyperemia, and miosis. Corneal ulceration, although uncommon in cases of MCF, does occur.[71,72] Anterior uveitis is a common ocular clinical sign in cases of MCF that is usually absent in cases of IBK.[71] Histologically, MCF ocular lesions are characterized by mitotic figures in lymphoblasts, which is not a finding in IBK lesions.[72]

Listeria monocytogenes

L monocytogenes can cause ocular infections that may resemble IBK, which is characterized by keratoconjunctivitis and uveitis, frequently called "silage eye."[73,74] An excellent review with more detail can be found on this condition.[74] Briefly, Listeria has been shown experimentally to directly penetrate corneal epithelial cells and cause ocular infections, demonstrating that contact of ocular epithelium with concentrated bacteria, potentially through feed, may be a route of entry, thus the common name of silage eye.[75,76] The ability to infect bovine conjunctiva seems in some strains to be associated with resistance to lysozyme.[77] Exposure keratitis secondary to facial nerve palsy caused by infection also seems to be involved.[78] Clinically, ocular infections with *L monocytogenes* differ from IBK, whereas in the former, the conjunctivitis is nonpurulent, the cornea has minimal changes, and lesions are usually unilateral.[79] Outbreak descriptions involve slow spread over several weeks with an intraherd prevalence that ranged between 7%

and 29%.[79] Three out of 170 cases submitted to a diagnostic laboratory were bovine ocular infections with 4 different subtypes isolated.[80,81] Outbreaks have also been associated with baleage-fed animals.[82] *L monocytogenes* infections in cattle associated with silage may be more complex than initially thought. One study looking at an outbreak of *L monocytogenes* (which primarily caused reproductive infections) in a large system using genomic analysis demonstrated that 3 distinct strains were isolated from animals and only one strain matched those found in silage sources.[83]

Chlamydia spp

Chlamydia spp are nonmotile obligate intracellular bacteria with small genomes that can replicate in a wide variety of host cells.[84] Although the nomenclature of members of this genus has been in flux, there are 10 valid species described, 2 of which infect cattle: *Chlamydia pecorum* and *Chlamydia abortus,* which were previously classified as *Chlamydia psittaci*.[85–88] Infections with *Chlamydia* spp in cattle typically involve mucosal cells or penetration of mucosal surfaces to establish systemic infection, and infectious elementary bodies are shed in feces, nasal, ocular, and reproductive exudates.[89] Diseases in cattle include enteric disease, respiratory disease, polyarthritis-serositis, and sporadic bovine encephalomyelitis.[89] However, asymptomatic infections seem common with one study finding 61% prevalence in normal calves.[90] The age of the calf when exposed in addition to the virulence of the strain seem to have impact on the type and severity of disease observed.[91]

Given the infection of mucosal surfaces, *Chlamydia* spp have long been associated with conjunctivitis in lambs and reindeer among other species, although the relationship with conjunctivitis in cattle is less clear.[92,93] However, it is thought that infections in cattle are likely to resemble other species. Calves experimentally inoculated with *Chlamydia* that resulted in systemic infection and polyarthritis also developed conjunctivitis and subsequent blindness that involved the retina and optic nerve.[94] One report describes 3 outbreaks of IBK-like disease in cattle, one of which lasted 5 months and had morbidity of 100% where the only agent detected was *Chlamydi*a sp using PCR.[95] Seven out of 47 conjunctival biopsy samples collected from Kansas cattle

with signs consistent with IBK, representing 35 herds, had detectable Chlamydial proteins by enzyme-linked immunosorbent assay.[96] In a study of ruminants in India, 2 out of 8 cattle with clinical keratoconjunctivitis were positive by PCR for *Chlamydia* spp (*C abortus* and *C psittaci*).[97] In another survey, high percentages of both clinical (88%) and nonclinical (68%) cattle were found to be positive for *C psittaci* by PCR in a study conducted in Egypt.[98] A study conducted in India revealed that 3% of ocular infections in cattle with conjunctivitis showed evidence of Chlamydia inclusions in conjunctival smears.[99] Other outbreaks have indicated *C psittaci* detection in association with *M bovis*.[100]

Much remains unclear about the role of *Chlamydia* spp in bovine disease in general, including ocular disease. Diagnosis of chlamydial infections is challenging, and there may be underdiagnosis and underreporting of this pathogen in cattle.[101] However, as methods and technologies advance to detect and sequence these organisms, more is being understood. For example, a specific multilocus sequence type of *C pecorum* (ST23) is associated with ocular infections in sheep and seems to be widespread in livestock including cattle.[102,103] Cattle infected with *Chlamydia* have been reported to have growth reduction; however, they seem to be largely subclinical or nonclincal.[88,90,104,105]

Summary

Although IBK has classically been associated with *Moraxella* organisms, there has been work exploring associations with other pathogenic and opportunistic bacteria and ocular lesions in cattle. Many of these pathogens are extremely challenging to study due to their minimal ability to survive outside of hosts and study in vitro. However, as technological advances progress, tools are becoming available to study them using molecular and next-generation sequencing and other approaches. Additional research needs to be conducted into the association of these challenging microbes, in particular *Myco bovoculi,* and their association and prevalence in both animals with IBK and normal animals.

Clinics Care Points

- Determination of the cause of outbreaks is challenging, especially when they may involve poorly understood or uncommon pathogens or opportunistic infections with commensals.
- Organisms other than *Moraxella* spp have been implicated as causes of IBK-like clinical disease.
- *Mycoplasma* spp, *Ureaplasma* spp, *Chlamydia* spp, *L monocytogenes,* BoHV-1, and *γ*-herpesviruses can produce ocular lesions in cattle.
- Syndromic PCR panels that include non-*Moraxella* agents may help rule out some of the less common etiologic agents.[30]
- Advanced technologies including molecular characterization of potential pathogens and metagenomics tools will help elucidate the roles other agents play in IBK.

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