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RESEARCH

Genetic diversity, virulence genes, and antimicrobial resistance of *Moraxella bovoculi* and *Moraxella bovis* from infectious bovine keratoconjunctivitis: insights from MALDI-TOF-MS, 16S rRNA analyses, and other ocular bacteria

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Abstract

Background *Moraxella bovis* is the main etiological agent of infectious bovine keratoconjunctivitis (IBK). However, *Moraxella bovoculi* and other bacteria are also frequently isolated from IBK. The recurrent detection of *M. bovoculi* suggests a role in IBK, though its contribution remains unclear. This study aimed to characterize *M. bovoculi* and *M. bovis* isolates from IBK cases and evaluate the role of other ocular bacteria.

Methods Eye swabs from cattle with keratoconjunctivitis were cultured and identified using MALDI-TOF MS, with 16S rRNA sequencing for confirmation. Virulence gene analysis targeted six genes in *M. bovis* (*omp*79, *mbx*A, *fur*, *plb*, *pil*, *tol*C) and three in *M. bovoculi* (*pil*A, *mbv*A, *omp*79). Antimicrobial susceptibility was evaluated using the Kirby-Bauer disk diffusion method.

Results Forty-seven isolates were obtained, with *Moraxella* spp. predominant. Fifteen were identified as *M. bovoculi* and nine as *M. bovis*. Sequencing largely confirmed MALDI-TOF results with minor discrepancies. Virulence screening showed *M. bovoculi* consistently carried *pil*A and variably *mbv*A and *omp*79, whereas *M. bovis* harbored a broader set (*omp*79, *mbx*A, *fur*, *plb*, *pil*, *tol*C). Antimicrobial testing indicated a reduced susceptibility trend in *M. bovoculi*, particularly to fluoroquinolones, tetracyclines, and phenicols, whereas *M. bovis* exhibited a relatively higher susceptibility tendency. Furthermore, the isolation of non-Moraxella bacteria highlights their potential role as opportunistic pathogens in IBK.

Conclusion *Moraxella* isolates from IBK exhibit marked genetic diversity and distinct virulence repertoires, with *M. bovoculi* showing a trend toward decreased susceptibility. The detection of additional ocular bacteria highlights the multifactorial nature of IBK. These findings underscore the need for accurate diagnostics, antimicrobial stewardship, and exploration of alternative approaches such as vaccines for controlling IBK.

Keywords *Moraxella bovis* · *Moraxella bovoculi* · Infectious bovine keratoconjunctivitis · Virulence · Antimicrobial resistance · MALDI-TOF · Phylogenetic analysis

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Introduction

Infectious bovine keratoconjunctivitis (IBK), also known as pink eye, is a highly contagious ocular disease in cattle, characterized by ulcerative keratitis, corneal edema, and conjunctivitis (Kuibagarov et al. 2023). It can affect cattle of all ages, with herd prevalence reaching up to 80% within three weeks, causing major economic losses (Dima and Fikedu 2021). The global estimated prevalence is 2.78% (Dennis and Kneipp 2021). Multiple factors, including pathogens, environmental conditions, season, and host immunity, influence disease severity. Irritants such as wind, dust, sunlight, and flies increase susceptibility. Transmission occurs via ocular and nasal secretions, direct contact, or mechanically by flies. While asymptomatic carriage is common in older cattle, clinical disease is more frequent in young animals (Brown et al. 1998; Postma et al. 2008; Angelos 2015; Kneipp et al. 2021).

Although various bacteria can cause keratoconjunctivitis, *M. bovis* is recognized as the primary pathogen responsible for IBK in cattle (Kuibagarov et al. 2023; Postma et al. 2008). More recently, *M. bovoculi* has been identified as a novel species within the genus and is believed to contribute to IBK, as it has been isolated from clinical cases and carries a cytotoxin similar to that of *M. bovis* (Angelos et al. 2007a, b). The frequency of *M. bovoculi* isolation from IBK cases often exceeds that of *M. bovis*, whereas *M. ovis* is rarely detected (Galvão and Angelos 2010; Loy and Brodersen 2014; Ravikumaran et al. 2024). Before the description of *M. bovoculi*, many isolates previously classified as *M. ovis* were likely misidentified. Evidence also suggests horizontal gene transfer among *M. bovis*, *M. bovoculi*, and *M. ovis*, although the precise roles of *M. bovoculi* and *M. ovis* in cattle keratoconjunctivitis remain unclear (Ravikumaran et al. 2024; O'Connor et al. 2012). Historically, vaccines produced solely with *M. bovis* have been insufficiently protective in the field (Kneipp et al. 2023), likely due to the multifactorial etiology of IBK. In recent years, autogenous vaccines including *M. bovoculi* have been developed. However, a two-year controlled field trial of the *M. bovoculi* bacterin vaccine failed to demonstrate a protective effect against the incidence of IBK (O'Connor et al. 2012; O'Connor et al., 2019). Notably, *M. bovoculi* has also been isolated from equine keratoconjunctivitis, expanding its host range and suggesting a broader role in ocular pathology (Liu et al. 2014).

Virulence of *M. bovis* has been primarily associated with hemolytic and pilus-positive strains isolated from diseased cattle, while non-hemolytic and pilus-negative strains have been detected in the conjunctival flora of healthy animals (Billson et al. 2000; Angelos et al. 2007a; Angelos 2015). Pili mediate adherence to corneal epithelial cells, while hemolysin (cytotoxin) contributes to corneal ulceration. In strains with multiple virulence features, type IV fimbriae (Q and I pili) play a crucial role in adhesion and colonization, while hemolysis damages neutrophils and promotes corneal lesions. Additional factors such as phospholipases, outer membrane proteins, and hydrolytic enzymes also contribute to tissue damage (Sosa et al. 2015; Dickey et al. 2018; Kuibagarov et al. 2022, 2023). RTX toxins further cause corneal pore formation and neutrophil toxicity. Mutations in outer membrane proteins increase bacterial susceptibility to host defenses and antibiotics, highlighting their importance in virulence. In *M. bovis*, biofilm formation has also been linked to clinical manifestations (Prieto et al. 2013; Loy et al. 2021; Ravikumaran et al. 2024). Among other well-characterized virulence determinants in *M. bovis* are fimbriae, LPS, phospholipase B, TolC, and various outer membrane proteins (Angelos 2015; Sosa et al. 2015). While *M. bovoculi* harbors some of these factors, particularly fimbriae and outer membrane proteins, comparative genomics indicates its virulence gene repertoire is more limited and variable (Angelos et al. 2007a; Dickey et al. 2018; Wynn et al. 2022). Identifying these determinants remains essential for immunological stimulation and vaccine development (Bilbao et al. 2024).

The aim of this study was to characterize *M. bovoculi* and *M. bovis* isolated from cattle with IBK by molecular means and to evaluate the presence of various virulence factors. Oligonucleotide sequences belonging to the gene regions determined for all virulence traits were designed in this study. Simultaneously, the antibiotic susceptibilities of the bacteria identified as *Moraxella* spp. were also evaluated, and the current resistance status of these bacteria was aimed to be revealed. In addition, other pathogens that may be responsible for bovine keratoconjunctivitis cases were also determined.

Materials and methods

Sampling

Eye swab samples were collected from twelve distinct semi-open cattle farms in Muğla Province, western Türkiye, between July 2023 and July 2024. Forty-six swab samples were obtained from various cattle exhibiting signs (lacrimation, photophobia, eye closure, etc.) of keratoconjunctivitis at differing levels of severity by veterinarians working on the farms (Kasimanickam and Parish 2011), all of which had not yet undergone

antibiotic treatment. The samples were obtained from the inferior conjunctival fornix region of the affected eye using a sterile swab tool. The eye swabs, collected under aseptic conditions, were transported to the laboratory following a cold chain protocol and were subjected to analysis on the same day of collection (Kuibagarov et al. 2022).

Isolation and bacterial identification with MALDI-TOF MS

The swab samples (n:46) were cultured on blood agar medium enriched with 5–7% sheep blood. The culture medium underwent incubation at 37 °C for 24–48 h under aerobic conditions. Following the incubation period, Gram staining and catalase and oxidase tests were conducted on all colonies that exhibited growth. Each distinct colony type was then analyzed by MALDI-TOF MS for identification, allowing both *Moraxella* and non-*Moraxella* bacterial species to be detected and recorded. Additional biochemical tests (gelatinase, oxidation–fermentation (OF) reactions, and growth on MacConkey agar, etc.) were applied to colonies characterized by a gray coloration, smooth edges, a diameter of 1–2 mm, beta-hemolytic properties, and positive results for the oxidase and catalase tests, which microscopically exhibited diplococcus or coccobacillus morphology. These phenotypic features are consistent with previous descriptions of *Moraxella* species (Garrity et al. 2004; Sosa and Zunino 2012; Sotnikova et al. 2021; Angelos et al. 2021). Pure cultures were obtained from each bacterial isolate for identification via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The MALDI-TOF MS analyses were conducted at the Mustafa Kemal University Plant Health Clinic Application and Research Center (Türkiye). The analysis was performed according to the ethanol-formic acid protocol previously reported by Uysal et al. (2019). Following the analysis of these proteins, spectra obtained with the instrument's FlexControl software program (Biotyper 3.0; Microflex LT; Bruker Daltonics GmbH, Bremen, Germany) were compared with the MALDI Biotyper Real-Time Classification (RTC) software (version 12) to identify bacteria at the genus and species levels. Data determined as yellow/green between 1,700 and 3,000 were considered reliable scores (Uysal et al. 2018; Uysal, 2019). Following the analysis, high-probability species identification was assessed using scale values between 2,000 and 3,000 (green), while genus-level and probable species-level identifications were assessed using scale values between 1,700 and 1,999 (yellow) (Uysal et al. 2018, 2019; Ponderand et al. 2020; Park et al. 2021).

DNA isolation and molecular identification of *Moraxella* spp. Isolates

Genomic DNAs (gDNA) from bacteria belonging to the *Moraxella* genus were isolated utilizing a commercially available DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Scientific, Waltham, MA, USA), following the manufacturer's guidelines. The isolated gDNAs were subsequently stored at −20 °C until their application in PCR analyses.

For molecular identification, the 16S rRNA gene regions of bacteria were amplified by PCR using universal primers 27 F and 1492R. The 16S rRNA PCR was performed using the same protocol as described in our previous study (Yalcin et al. 2024). Services were acquired for the purification and sequencing analysis of the amplicons (BM Labosis, Türkiye). The results of the sequence analysis were compared to the GenBank database at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic Local Alignment Search Tool (BLAST). Accession numbers were assigned to the base sequences of the 16S rRNA gene region obtained through sequencing (Sosa and Zunino 2012; Hadi et al. 2021).

The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (−4294.72) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 37 nucleotide sequences. There were a total of 1,566 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021). Furthermore, the phylogenetic tree incorporates 16S rRNA sequences from *Moraxella bovoculi* 237 (NR_043583.2) and *Moraxella bovis* strain L-3 (NR_028668.1) as reference sequences, while the 16S rRNA sequence from *Gordonia polyisoprenivorans* was utilized as an outgroup.

Determination of some virulence factors of *Moraxella* isolates

Eight primers were designed in this study to detect virulence genes by PCR. The NCBI gene bank obtained the sequences of *mbx*A, *omp*79, *tol*C, *fur*, *plb*, *pil*, *mbv*A, and *pil*A virulence genes (Table 2). The amino acid sequences of the proteins they encode were obtained from The Gene Database of NCBI (https://www.ncbi.nlm.nih.gov/gene) (Accessed November 27, 2024). Information on the conserved

domains of virulence proteins was obtained from Conserved Domains NCBI (Wang et al. 2023) (Accessed March 20, 2025). The predicted subcellular locations of the virulence genes were determined using the online server DeepLoc-1.0 (https://services.healthtech.dtu.dk/services/DeepLocPro-1.0/) (Accessed March 20, 2025). Additionally, the characteristics of virulence proteins are presented in Table 1.

Three different multiplex–PCR reactions (Rxn) were performed for bacteria *M. bovis* (Rxn1, Rxn2, Rxn3). Two different PCR reactions (Rxn4, Rxn5) were performed for bacteria *M. bovoculi* (Table 2). The PCR mix was used for multiplex PCR processes (DreamTaq Hot Start Green PCR Master Mix, Thermo Scientific, Waltham, MA, USA). All PCR reactions were programmed as follows: initial denaturation min at 95 °C; 35 cycles, denaturation: 30 s at 95 °C, binding: 30 s at 54 °C, extension: 1 min at 72 °C, and final extension: 10 min at 72 °C. The PCR products were used for electrophoresis in a 1% agarose gel and visualised.

Antimicrobial susceptibility analyses of *Moraxella* spp. Isolates

Antibiotic susceptibility testing was conducted on bacteria identified as *Moraxella* spp. utilizing the Kirby-Bauer disk diffusion method. The tests employed Müller Hinton Agar (MHA) as the growth medium. Bacterial inocula were prepared to achieve a turbidity of 0.5 according to the MacFarland standard and then spread onto the surface of the agar in 100 µl volumes. The medium was subsequently incubated at 37 °C for a duration of 24 h. Following the incubation period, the diameters of the inhibition zones formed around the antibiotic discs were measured. In the assessment of antibiotic susceptibilities, antibiotic discs used included ofloxacin 5 µg (OFX), ciprofloxacin 5 µg (CIP), oxytetracycline 30 µg (TE), amoxicillin/clavulanic acid 30 µg (AMC), sulfamethoxazole/trimethoprim 25 µg (SXT), and chloramphenicol 30 µg (C) (Bioanalyse, Türkiye).

Antimicrobial susceptibility testing was performed using the disk diffusion method. Since no specific clinical breakpoints are available for *M. bovis* or *M. bovoculi* in either EUCAST or CLSI guidelines, the interpretation of inhibition zone diameters in this study was based on the clinical breakpoints defined for *Moraxella catarrhalis* by EUCAST (v.15.0, 2025). Similarly, previous studies assessing the antimicrobial susceptibility of *M. bovis* and *M. bovoculi* have also used *M. catarrhalis* breakpoints as a reference (Ozavci and Seferoglu 2023; de Carvalho et al. 2025).

Results

MALDI-TOF MS isolation results

A total of 47 bacterial isolates were identified using the MALDI-TOF MS method from swab samples collected from 46 eyes affected by keratoconjunctivitis, each belonging to distinct cattle. The predominant genus among the identified strains was *Moraxella* ($n=21$). *Corynebacterium xerosis* was identified with the highest incidence ($n=7$), while another 7 strains represented various species within the *Staphylococcus* genus. *Acinetobacter* species were recovered from three separate samples. Additionally, one *Rothia nasimuri*um, *Aerococcus viridans*, *Pseudomonas koreensis*, and *Escherichia coli* were identified in the eye swab samples, alongside three isolates of *Micrococcus terreu*s and *Bacillus* spp. Furthermore, three strains

Table 1 Examined virulence features and associated regions

Bacteria	Gene	Protein name	Number of amino acids	Conserved domain (amino acid)	Subcellular location
M. bovis	*mbx*A	MbxA	927	RTX family hemolysin (25–901)	Extracellular
	*omp*79	79 kDa outer membrane protein	748	OM_channels super family (55–746)	Outer membrane
	*tol*C	TolC	434	tolC super family (24–427)	Outer membrane
	fur	ferric uptake regulator	150	HTH_CRP super family (3–140)	Cytoplasmic
	plb	PLB	616	Triacylglycerol_lipase_like (28–322)	Outer membrane
	pil	pilin	156	Pilin (35–154)	Extracellular
M. bovoculi	*omp*79 *	hypothetical protein *	744	OM_channels super family (51–742)	Outer membrane
	*mbv*A	MbvA	927	RTX family hemolysin (25–901)	Extracellular
	*pil*A	PilA	152	Pilin (37–149)	Extracellular

* The DNA sequence of this protein was amplified with the *omp*79 primer

Table 2 The virulence gene primers designed and investigated in this study

Bacteria	Gene	Primer sequence (5'−3')	Amplicon size (bp)	Gen Bank ID
M. bovis	*mbx*A	F- GCAAAAGCTG GCAATGACGA R- GTGCCATTGAC CCAACTAGC	943	EF436243.1
	*omp*79	F- AGGTAAGCCA AAGGCTGGTGCC R- TGTACCATCAG CAATATCAGCA	607	AB106521.1
	*tol*C	F- GGTTTGCTAGT AGCTCTACTGC R- TTACAGATTGC CTTCCAATCCC	1259	AF205359.3
	fur	F- ATGGCTTTTAC CAACAAAGATT R- TTAGCTTTTGT TAGCAGGGTCA	447	AB079375.1
	plb	F- CGCAAATCACC GACCATTTG R- CATCAAGCGA ACCCCAAGCG	1487	AY032849.1
	pil	F- ATGAACGCTCA AAAAGGTTTC R- CTAAGCTTTTG TGCAACCAG	471	M92155.1
M. bovoculi	*omp*79	F- AGGTAAGCCA AAGGCTGGTGCC R- TGTACCATCAG CAATATCAGCA	607	CP011374.1
	*mbv*A	F- AATGCTGGTGC TGGTAACGA R- TGGTTGCAGG GTATTGGAGC	990	DQ155440.1
	*pil*A	F- ATGATTACTC AGAAAGGTTTT ACC R- CTACGCTGCAC CACATCCTTG	459	MT333689.1

bp: base pair; F: forward; R: reverse. PCR reactions (Rxn) for *M. bovis*: Rxn1:.*mbx*A, *fur*; Rxn2: *omp*79, *tol*C; Rxn3: *plb*, *pil*; PCR reactions (Rxn) for *M. bovoculi*: Rxn4: *mvb*A, *pil*A; Rxn5: *omp*79

identified as *Moraxella* during preliminary biochemical analyses could not be conclusively identified as a low score was obtained in the MALDI-TOF identification process. While 13 of the *Moraxella* isolates obtained from twenty swab samples were identified as *M. bovoculi*, 7 strains were identified as *M. bovis*. One strain was identified as *M. ovis*. According to MALDI TOF results, the second closest identification in the strain identified as *M. ovis* (H1-2) showed a high confidence level match with *M. bovoculi*. However, in the result showed a very small difference between the *M. ovis* and *M. bovoculi* scores (0.050). According to MALDI-TOF MS results, the strains identified as *M. bovoculi* with a high score matched the species *M. ovis* with the second highest score. All *Moraxella* isolates were identified from keratoconjunctivitis cases in different cattle, and only one sample contained both *M. bovis* and *M. bovoculi*. Information on the samples and the identified microorganisms is given in Table 3.

16S rRNA gene region sequence analysis, identification, and phylogenetic analysis of *Moraxella* isolates

16S rRNA sequence analysis was conducted on 21 bacterial strains identified as *Moraxella* through MALDI-TOF MS, alongside three strains (H34-2, H37-2, and H42-2) that were unable to be scored by this technique. Following the comprehensive sequence analysis of a total of 24 *Moraxella* strains, results were obtained for 22 strains. Based on the findings, three strains that were not identifiable by MALDI-TOF MS analysis were classified as *M. bovis*, whereas two strains (H25-3 and H41-1), although initially identified through MALDI-TOF MS as *M. bovoculi*, could not be conclusively identified via 16S rRNA sequence analysis due to insufficient sequence data. A strain (H1-2) identified as *M. ovis* by MALDI-TOF MS was identified as *M. bovoculi* by 16S rRNA sequence analysis. Detailed information and similarity rates pertaining to the strains identified through sequence analysis are presented in Table 4.

Accession numbers were assigned to the sequences obtained from DNA sequence analysis of the 16S rRNA gene region of 22 *Moraxella* spp. bacteria. The accession numbers were provided (NCBI, GenBank) as *M. bovoculi* strain H1-2 (PV088719.1), *M. bovoculi* strain H3-2 (PV088722.1), *M. bovoculi* strain H10-2 (PV088725.1), *M. bovoculi* strain H13-1 (PV088740.1), *M. bovoculi* strain H18-2 (PV088743.1), *M. bovoculi* strain H23-2 (PV088747.1), *M. bovoculi* strain H28-1 (PV088905.1), *M. bovoculi* strain H29-2 (PV088908.1), *M. bovoculi* strain H30-1 (PV088909.1), *M. bovoculi* strain H33-1 (PV088915.1), *M. bovoculi* strain H32-1 (PV088916.1), *M. bovoculi* strain H35-1 (PV088918.1), *M. bovoculi* strain H43-1 (PV089009.1), *M. bovis* strain H21-1 (PV088744.1), *M. bovis* strain H22-2 (PV088745.1), *M. bovis* strain H24-3 (PV088901.1), *M. bovis* strain H25-2 (PV088902.1), *M. bovis* strain H27-1 (PV088904.1), *M. bovis* strain H34-2 (PV088917.1), *M. bovis* strain H37-2 (PV088919.1), *M. bovis* strain H42-2 (PV088946.1), *M. bovis* strain H44-1 (PV089051.1). Based on the obtained sequences, the strains were compared using a similarity analysis method that produced phylogenetic trees. The phylogenetic tree was drawn from the 16S rRNA sequences of 37 bacterial species using the MEGA11 program. As illustrated in Fig. 1, the *M. bovoculi* species cluster in the same phylogenetic branch

Table 3 Identification results of bacteria isolated from eyes with keratoconjunctivitis using MALDI-TOF-MS

Farm Cod	Sample No	Bacteria	MALDI-TOF MS best skor value (second skor value)
F1	H1	*Bacillus altitudinis*	*1.805*
		**Moraxella ovis (M. bovoculi)*	*2.055 (2.005)*
	H2	*Acinetobacter indicus*	*1.856*
		Bacillus sp. (Bacillus pumilus)	*1.982 (1.719)*
	H3	*Moraxella bovoculi (M. ovis)*	*2.119 (1.798)*
	H5	*Acinetobacter indicus*	*1.828*
		Micrococcus luteus	*2.215*
F2	H6	*Escherichia coli*	*2.31*
		Bacillus sp.	*1.879*
		Corynebacterium xerosis	*2.393*
F3	H7	*Staphylococcus saprophyticus*	*1.877*
		Micrococcus terreus	*2.018*
		Corynebacterium xerosis	*1.86*
	H8	*Staphylococcus haemolyticus*	*2.066*
		Corynebacterium xerosis	*1.767*
	H10	*Staphylococcus hominis*	*2.007*
		Moraxella bovoculi (M. ovis)	*2.074 (1.728)*
	H11	*Acinetobacter towneri*	*1.993*
		Staphylococcus epidermidis	*2.214*
F4	H12	*Pseudomonas koreensis*	*2.182*
	H13	*Moraxella bovoculi (M. ovis)*	*2.154 (1.821)*
F5	H16	*Corynebacterium xerosis*	*1.929*
	H17	*Corynebacterium xerosis*	*2.305*
	H20	*Corynebacterium xerosis*	*1.923*
F6	H18	*Moraxella bovoculi (M. ovis)*	*2.115 (1.936)*
F7	H21	*Moraxella bovis*	*1.993*
F8	H22	*Moraxella bovis*	*2.16*
		Rothia nasimurium	*2.096*
	H23	*Moraxella bovoculi (M. ovis)*	*2.036 (1.865)*
	H24	*Moraxella bovis*	*2.136*
	H25	*Moraxella bovis*	*2.232*
		Moraxella bovoculi (M. ovis)	*1.832 (1.733)*
	H26	*Staphylococcus simulans*	*1.757*
		Aerococcus viridans	*2.021*
	H27	*Moraxella bovis*	*1.972*
	H28	*Moraxella bovoculi (M. ovis)*	*2.065 (1.735)*
	H44	*Moraxella bovis*	*2.201*
F9	H29	*Moraxella bovoculi (M. ovis)*	*2.23 (1.723)*
	H31	*Staphylococcus haemolyticus*	*1.736*
	H32	*Moraxella bovoculi*	*1.94*
	H33	*Moraxella bovoculi*	*2.125*
	H41	*Moraxella bovoculi*	*2.233*
	H43	*Moraxella bovoculi (M. ovis)*	*2.229 (1.794)*
F10	H30	*Moraxella bovoculi*	*1.951*
	H35	*Moraxella bovoculi (M. ovis)*	*1.87 (1.712)*
	H37	*Staphylococcus xylosus*	*1.943*
F12	H45	*Corynebacterium xerosis*	*1.773*

(): The values shown in parentheses represent the second-best match scores obtained from the MALDI-TOF MS device, along with the corresponding bacterial species indicated in parentheses. Second scores are not provided for strains where the second-best result corresponds to the same bacterial species

*This strain was identified as *M. ovis* by MALDI-TOF MS

as the reference strain *M. bovoculi* 237. Similarly, *M. bovis* strains were found in the same branch as the reference strain *M. bovis* L-3.

Furthermore, sequences from *M. bovoculi* and *M. bovis* strains, as reported by Wynn et al. in 2022, were also included in the tree drawing. Comparisons were made with genotype 1 and genotype 2 reference sequences from both bacterial species. Some of our *M. bovis* isolates (H34-2, H37-2, and H42-2) were positioned in a branch phylogenetically close to the genotype 1 references, while the remaining *M. bovis* isolates were associated with the genotype 2 group. *M. bovoculi* isolates were also located near the genotype 1 references. Since 16S rRNA analysis alone does not allow for definitive genotyping, these results were interpreted as reflecting phylogenetic relatedness rather than precise genotype assignment. The phylogenetic tree indicates that the molecularly identified isolates have close relationships with other *Moraxella* species in the GenBank database.

The virulence factors of *Moraxella* spp. Strains

The virulence factors of 24 identified strains, classified as *M. bovis* or *M. bovoculi* through MALDI-TOF MS and/or 16S rRNA PCR analyses, were evaluated using PCR analyses. *M. bovoculi* strains were assessed for the genes *mbv*A, *pil*A, and *omp*79, whereas *M. bovis* strains were analyzed for the presence of the *omp*79, *mbx*A, *fur*, *tol*C, *plb*, and *pil* gene regions. The analysis confirmed the presence of the *pil*A gene region across all 15 strains of *M. bovoculi*. Additionally, the *omp*79 gene region was identified in two strains, while the *mbv*A gene region was found in nine strains of *M. bovoculi*. All examined virulence genes were detected in 6 of 9 *M. bovis* strains; however, strains H34-2, H37-2, and H42-2, which were identified exclusively through sequence analysis of the 16S rRNA gene region, lacked most or all of the tested virulence genes (Table 5; Figs. 2 and 3).

The amino acid sequence of the 79 kDa outer membrane protein of *M. bovis* and the amino acid sequence of the hypothetical protein of *M. bovoculi* were aligned using the Multiple Sequence Alignment (MSA) by the CLUSTALW tool (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) (Accessed March 21, 2025) in Fig. 4. In addition, as a result of BLAST analysis, it was determined that the similarity between these two proteins was 95.12% (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Accessed March 21, 2025).

Antimicrobial susceptibility test results of *Moraxella* spp. Strains

The antibiotic susceptibilities of all strains identified as *M. bovis* or *M. bovoculi* through MALDI-TOF-MS and/or 16S rRNA PCR analysis were examined (n:24 strains).

Table 4 Similarity rates of *Moraxella* spp. Strains identified based on 16S rRNA gene sequence analysis

Isolate no	Identified strain	Our strain's length (bp)	Similarity rate	Alignment identities ***(Query/Subject)	*Accession numbers
H1-2	**Moraxella bovoculi*	1430	99.86%	1417/1419(99%)	CP011374.1
H3-2	*Moraxella bovoculi*	1432	99.57%	1391/1397(99%)	DQ153083.1
H10-2	*Moraxella bovoculi*	1428	99.36%	1388/1397(99%)	DQ153083.1
H13-1	*Moraxella bovoculi*	1419	99.79%	1416/1419(99%)	GU181221.1
H18-2	*Moraxella bovoculi*	1417	99.86%	1408/1410(99%)	CP011374.1
H21-1	*Moraxella bovis*	1423	99.93%	1417/1418(99%)	CP087771.1
H22-2	*Moraxella bovis*	1439	99.86%	1429/1431(99%)	CP087771.1
H23-2	*Moraxella bovoculi*	1432	99.86%	1421/1423(99%)	CP011374.1
H24-3	*Moraxella bovis*	1434	99.72%	1404/1408(99%)	JN001942.1
H25-2	*Moraxella bovis*	1430	99.37%	1414/1423(99%)	CP087771.1
H27-1	*Moraxella bovis*	1431	99.44%	1420/1428(99%)	CP087771.1
H28-1	*Moraxella bovoculi*	1428	99.72%	1416/1420(99%)	GU181221.1
H29-2	*Moraxella bovoculi*	1386	99.78%	1378/1381(99%)	GU181221.1
H30-1	*Moraxella bovoculi*	1425	99.79%	1421/1424(99%)	CP011380.2
H32-1	*Moraxella bovoculi*	1441	99.93%	1425/1426(99%)	CP011381.2
H33-1	*Moraxella bovoculi*	1425	99.51%	1415/1422(99%)	CP011381.2
H34-2	*Moraxella bovis*	1418	99.72%	1414/1418(99%)	CP087863.1
H35-1	*Moraxella bovoculi*	1420	99.65%	1417/1422(99%)	CP011380.2
H37-2	*Moraxella bovis*	1430	99.65%	1419/1424(99%)	CP087863.1
H42-2	*Moraxella bovis*	1434	99.65%	1419/1424(99%)	CP087863.1
H43-1	*Moraxella bovoculi*	1424	99.58%	1413/1419(99%)	CP195440.1
H44-1	*Moraxella bovis*	1434	99.65%	1414/1419(99%	CP087771.1

* Accession numbers of strains with similarities identified in the NCBI GenBank database

** This strain was identified as *M. ovis* by MALDI-TOF MS and as *M. bovoculi* by 16S rRNA gene sequence analysis

*** Query: This study/Subject: NCBI GenBank database

When all *Moraxella* strains were evaluated together according to EUCAST criteria, the highest resistance trend was observed for the fluoroquinolones ofloxacin and ciprofloxacin (87.5%). This was followed by tetracycline and chloramphenicol, with a similar resistance trend (83.3%) for both antibiotics. For trimethoprim-sulfamethoxazole, the resistance trend was 75%, and the highest susceptibility trend was determined for amoxicillin-clavulanic acid (91.7%). Twenty-one of the 24 *Moraxella* strains (87.5%) showed resistance tendency to three or more antibiotic groups. This was considered as decreased susceptibility to multiple antibiotics (MDR trend). Table 6 presents the distributions of inhibition zone diameters and comparative resistance-susceptibility trends for all *Moraxella* strains tested against the antibiotics.

Discussion

This study utilized both MALDI-TOF MS and 16S rRNA gene sequencing to identify 47 bacterial isolates obtained from cattle afflicted with keratoconjunctivitis. According to MALDI-TOF MS results, the genus *Moraxella* was the most frequently isolated group ($n=21$), including 13 *M. bovoculi*, 7 *M. bovis*, and one *M. ovis*. In a single case, both *M. bovis* and *M. bovoculi* were simultaneously isolated. For some isolates, the second-best match in MALDI-TOF analysis corresponded to a different *Moraxella* species with high similarity scores; for example, isolate H1-2 was identified as *M. ovis*, although the second-highest score was for *M. bovoculi*, with only a 0.050 difference. This observation aligns with prior reports indicating that interspecies spectral overlap can complicate species-level discrimination (Strejček et al. 2018). Among the 24 *Moraxella* isolates subjected to 16S rRNA sequencing, 22 strains were identified at the species level. Although isolates H25-3 and H41-1 were identified as *M. bovoculi* by MALDI-TOF, the 16S sequencing data did not offer sufficient resolution to confirm species-level identification. Conversely, three isolates (H34-2, H37-2, H42-2), which could not be reliably identified by MALDI-TOF due to low scores, were classified as *M. bovis* via 16S sequencing. In cases of bovine keratoconjunctivitis, the prevalence of *M. bovoculi* often exceeds that of *M. bovis*; however, reports of *M. ovis* in IBK are rare, and most earlier positive findings are believed to have resulted from misclassification prior to the recognition of *M. bovoculi*. The close genetic relationship among these three species has been associated with the potential for horizontal gene transfer. Furthermore, both *M. ovis* and *M. bovoculi* have been reported to produce highly similar lipooligosaccharide

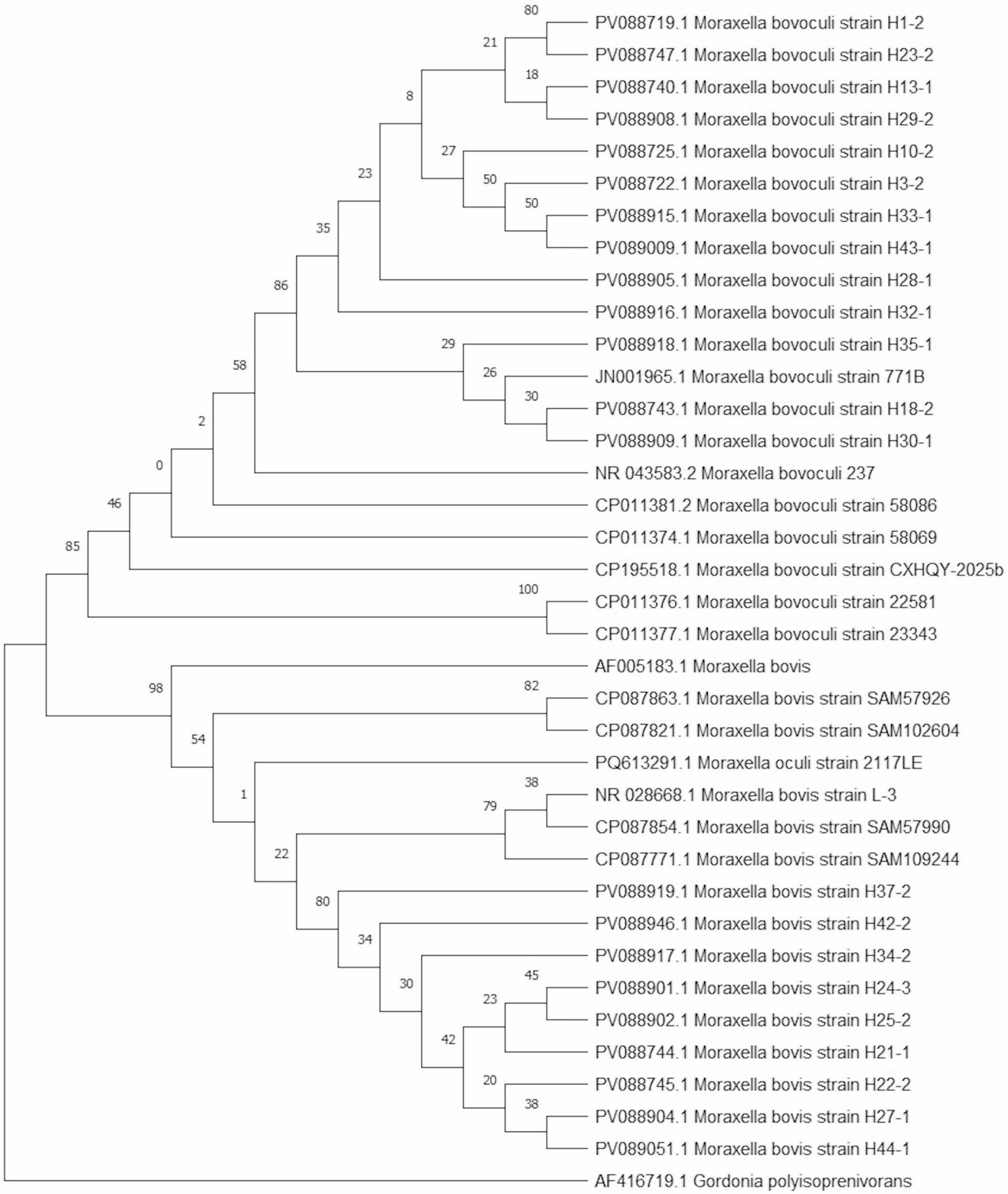

Fig. 1 The Phylogenetic tree shows the taxonomic position of identified bacterial species according to the 16S rRNA sequence. The 16S rRNA sequences of *M. bovoculi* 237 (NR_043583.2) and *M. bovis* strain L-3 (NR_028668.1) strains were used as reference sequences, and the 16S rRNA sequence of *G. polyisoprenivorans* was used as an outgroup. The evolutionary history was inferred using the Neighbor-Joining method. The phylogenetic tree was visualized using iTOL

Table 5 Virulence factors of *Moraxella* spp. Strains

No	Strain no	Bacteria	*mbv*A	*pil*A	*omp*79	*mbx*A	*fur*	*tol*C	*plb*	*pil*
1	H1-2	**M. bovoculi*	-	+	+	NA	NA	NA	NA	NA
2	H3-2	*M. bovoculi*	-	+	+	NA	NA	NA	NA	NA
3	H10-2	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
4	H13-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
5	H18-2	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
6	H23-2	*M. bovoculi*	-	+	-	NA	NA	NA	NA	NA
7	H25-3	***M. bovoculi*	-	+	-	NA	NA	NA	NA	NA
8	H28-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
9	H29-2	*M. bovoculi*	-	+	-	NA	NA	NA	NA	NA
10	H30-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
11	H32-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
12	H33-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
13	H35-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
14	H41-1	***M. bovoculi*	-	+	-	NA	NA	NA	NA	NA
15	H43-1	*M. bovoculi*	+	+	-	NA	NA	NA	NA	NA
16	H21-1	*M. bovis*	NA	NA	+	+	+	+	+	+
17	H22-2	*M. bovis*	NA	NA	+	+	+	+	+	+
18	H24-3	*M. bovis*	NA	NA	+	+	+	+	+	+
19	H25-2	*M. bovis*	NA	NA	+	+	+	+	+	+
20	H27-1	*M. bovis*	NA	NA	+	+	+	+	+	+
21	H34-2	****M. bovis*	NA	NA	-	-	-	-	-	-
22	H37-2	****M. bovis*	NA	NA	+	-	-	-	-	-
23	H42-2	****M. bovis*	NA	NA	-	-	-	-	-	-
24	H44-1	*M. bovis*	NA	NA	+	+	+	+	+	+

*This strain was identified as *M. ovis* by MALDI-TOF MS and as *M. bovoculi* by 16S rRNA gene sequence analysis

**These strains were identified only by MALDI-TOF MS

*** These strains were identified solely through analysis of the 16S rRNA gene sequence

NA: Not analyzed

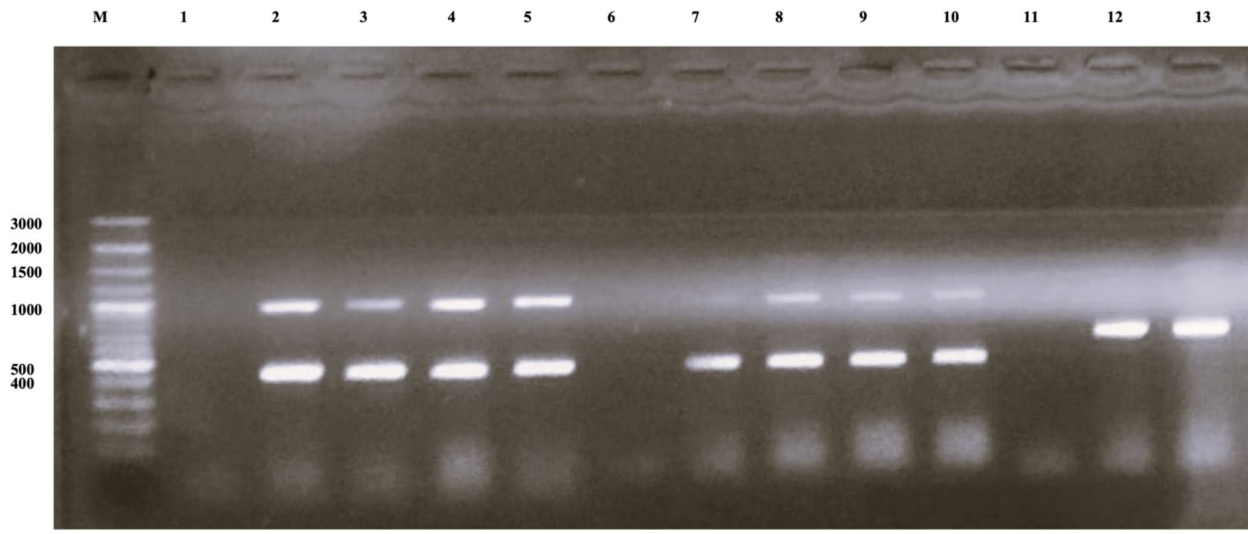

Fig. 2 M: Marker; 1: R4 negative control; 2: H13-1 (R4: *pil*A and *mbv*A positive); 3: H18-(R4: *pil*A and *mbv*A positive); 4: H28-1 (R4: *pil*A and *mbv*A positive); 5: H32-1 (R4: *pil*A and *mbv*A positive); 6: R1 negative control; 7: H21-1 (R1: *mbx*A and *fur* positive); 8: H22-2 (R1: *mbx*A and *fur* positive); 9: H25-2 (R1: *mbx*A and *fur* positive); 10: H44-1 (R1: *mbx*A and *fur* positive); 11: R5 negative control; 12: H1-2 (R5: *omp*79 positive); 13: H3-2 (R5: *omp*79 positive)

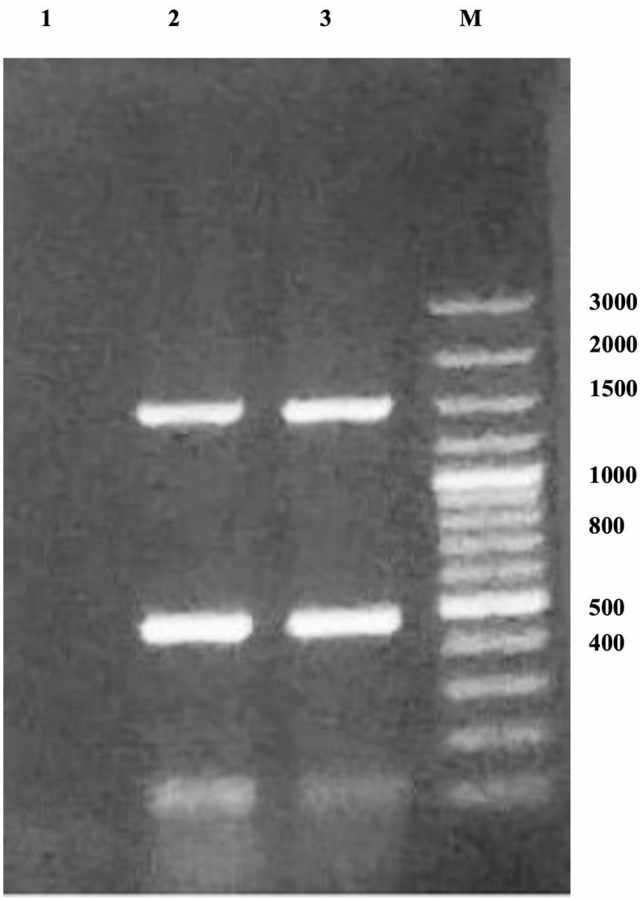

Fig. 3 1: R3 negative control; 2: H24-3 (R3: *plb* and *pil* positive); 3: H27-1 (R3: *plb* and *pil* positive); M: Marker

(LOS) structures, and known *M. ovis* strains have been shown to cluster genetically with *M. bovoculi* genotype 2 (Ravikumaran et al. 2024).

These findings emphasize the limitations inherent in biochemical methods and underscore the critical importance of integrating molecular sequencing with advanced spectral analyses to achieve accurate species differentiation. The 16S rRNA gene, due to its highly conserved nature, is a reliable molecular tool for both accurate bacterial identification and phylogenetic analysis (Makharita et al. 2020; Girlich et al. 2012). Some hemolytic Gram-negative cocci isolated from calves with keratoconjunctivitis could not be biochemically distinguished from *M. ovis*, but their 16–23 S ISR DNA sequences were identical to those of *M. bovoculi* sp. nov., supporting the existence of intraspecies genetic variability (Angelos and Ball 2007). Consistently, *M. bovoculi* has been shown to comprise two genetically distinct groups: genotype 1, predominantly isolated from IBK-affected eyes and harboring the RTX toxin gene (*mbvA*), and genotype 2, mainly recovered from the nasopharynx of asymptomatic cattle and lacking this operon (Dickey et al. 2018).

Although *M. bovis* has traditionally been regarded as the primary etiological agent of IBK, recent studies indicate that *M. bovoculi* is more frequently isolated from affected eyes, although its direct causal role remains unconfirmed (Kuibagarov et al. 2023). In addition to *Moraxella* species, other bacterial agents such as *Mycoplasma bovis* and *Mycoplasma bovoculi* have been associated with ocular infections. Intracellular organisms such as *Chlamydia* species, as well as bovine herpesvirus, are also regarded as potential pathogens. Certain ocular diseases caused by agents such as *Listeria monocytogenes* can present with clinical signs resembling IBK. Furthermore, opportunistic microorganisms are frequently isolated from these infections. Although these agents have not been conclusively proven to be direct causes of IBK, there is evidence suggesting that some infections may increase susceptibility to the disease (Laishevtcev et al. 2016; Loy et al. 2021).

A recent study characterizing the colonization patterns of the bovine ocular microbiota demonstrated that marked changes occur in the microbiome before and after the onset of IBK. Using both culture-based methods and 16S rRNA amplicon sequencing, the study showed that dysbiosis in one eye could also affect the contralateral eye. Moreover, the slow recovery of microbial balance was noted to facilitate the establishment of opportunistic pathogens, thereby supporting the progression of IBK (Bartenslager et al. 2021). *M. bovis* and *M. bovoculi* can also be isolated from the eyes of clinically healthy cattle, indicating that IBK is not solely the result of bacterial pathogens but is influenced by a combination of environmental and host-related factors. External risk factors such as ultraviolet (UV) radiation, face flies, and foreign bodies are known to play a role in the development of the disease, whereas host-associated internal factors remain insufficiently understood (Anis et al. 2023).

In the present study, MALDI-TOF MS identification of bacterial isolates obtained from conjunctival swabs of cattle with keratoconjunctivitis revealed that, in addition to *Moraxella* species, various other bacterial agents were detected. Although MALDI-TOF MS scores in the range of 1.7–1.99 have certain limitations for species-level identification, in this study, in order to provide a more comprehensive evaluation of the bacterial diversity associated with IBK cases, isolates within this range were also evaluated at the species level, as supported by the literature (Uysal et al. 2018, 2019; Ponderand et al., 2020; Park et al. 2021). Among these, *Moraxella* represented the most frequently isolated group, followed by *Staphylococcus* species and *Corynebacterium xerosis*. Less frequently identified bacteria included *Rothia nasimurium*, *Aerococcus viridans*, *Pseudomonas koreensis*, *Escherichia coli*, *Micrococcus terreus*, and *Bacillus* spp. *C. xerosis* is normally regarded as a commensal organism found on the skin and mucous membranes of humans and

Fig. 4 CLUSTALW analysis between the amino acid sequences of the 79 kDa outer membrane protein of *M. bovis* and the amino acid sequences of a hypothetical protein of *M. bovoculi*. Asterisks denote fully conserved residues. BAC92729.1: 79 kDa outer membrane protein of *M. bovis*; AKG19301.1: hypothetical protein of *M. bovoculi*

animals; however, it has been isolated from a variety of clinical cases in animals (Hernández-León et al. 2016; Palacios et al. 2010). In humans, *Corynebacterium* species are generally present on the ocular surface as commensals, usually in association with other bacteria. Nevertheless, in immunocompromised individuals, *Corynebacterium* species on the ocular surface have been reported as potential pathogens (Aoki et al. 2021). A study investigating the ocular microbiota of cats infected with herpesvirus and calicivirus reported predominant colonization by *Staphylococcus intermedius*, *Streptococcus agalactiae*, and *Staphylococcus epidermidis*, among which *C. xerosis* was also detected (Mohammadzadeh Vazifeh et al. 2024). Similarly, a study on conjunctival samples from cattle with keratoconjunctivitis found that the most frequently isolated organisms were *Staphylococcus* and *Streptococcus* species, as well as members of the *Enterobacteriaceae* family, including *E. coli*, *Proteus*, and *Enterobacter* species, alongside *Bacillus* spp. Importantly, although *M. bovis* and *M. bovoculi* were also detected at lower frequencies, a notable proportion of isolates belonged to *C. xerosis* within the *Corynebacterium* genus, as well as *Acinetobacter*, *Pseudomonas*, and *Klebsiella* species. Due to the lack of sufficient data in the literature, these bacteria were regarded as opportunistic pathogens (Laishevtcev et al. 2016). A recent metagenomic study reported that *R. nasimurium* was detected at significantly higher levels in ocular samples from IBK-affected cattle compared with healthy eyes, suggesting a possible association with the

Table 6 Inhibition zone diameters and susceptibility tendencies of *Moraxella* spp. Strains

No	Strain no	Bacteria	OFX	CIP	TE	AMC	SXT	C	MDR
1	H1-2	**M. bovoculi*	R(23)	R(28)	R(21)	S(27)	R(3)	R(10)	4
2	H3-2	*M. bovoculi*	R(25)	R(24)	R(10)	S(31)	R(3)	R(12)	4
3	H10-2	*M. bovoculi*	R(13)	R(17)	R(12)	S(28)	R(2)	R(13)	4
4	H13-1	*M. bovoculi*	R(18)	R(19)	R(17)	S(31)	R(10)	R(13)	4
5	H18-2	*M. bovoculi*	R(16)	R(19)	R(18)	S(28)	R(6)	R(22)	4
6	H23-2	*M. bovoculi*	R(14)	R(20)	R(7)	S(28)	R(9)	S(32)	3
7	H25-3	***M. bovoculi*	R(22)	R(26)	S(24)	S(28)	R(6)	R(23)	3
8	H28-1	*M. bovoculi*	R(16)	R(16)	R(17)	S(26)	R(2)	R(19)	4
9	H29-2	*M. bovoculi*	R(27)	R(18)	R(18)	S(30)	R(7)	R(24)	4
10	H30-1	*M. bovoculi*	R(14)	R(20)	R(18)	S(26)	R(4)	R(28)	4
11	H32-1	*M. bovoculi*	R(14)	R(15)	R(10)	S(28)	R(4)	R(26)	4
12	H33-1	*M. bovoculi*	R(20)	R(19)	R(21)	S(28)	R(2)	R(22)	4
13	H35-1	*M. bovoculi*	R(21)	R(18)	R(17)	R(10)	R(6)	R(26)	5
14	H41-1	***M. bovoculi*	R(27)	S(36)	R(22)	S(30)	S(20)	R(30)	3
15	H43-1	*M. bovoculi*	R(10)	R(14)	R(10)	S(24)	R(2)	R(28)	4
16	H21-1	*M. bovis*	R(26)	R(22)	R(18)	S(34)	R(9)	R(16)	4
17	H22-2	*M. bovis*	S(40)	R(26)	S(26)	S(38)	S(22)	S(34)	1
18	H24-3	*M. bovis*	R(20)	R(24)	R(15)	S(28)	S(18)	R(26)	3
19	H25-2	*M. bovis*	R(21)	R(25)	R(20)	S(36)	R(10)	R(28)	4
20	H27-1	*M. bovis*	R(20)	R(26)	R(20)	S(28)	R(8)	R(24)	4
21	H34-2	****M. bovis*	S(36)	S(38)	S(28)	S(32)	S(20)	S(38)	0
22	H37-2	****M. bovis*	R(27)	R(26)	R(21)	R(6)	S(30)	R(28)	4
23	H42-2	****M. bovis*	S(38)	S(38)	S(28)	S(32)	S(32)	S(39)	0
24	H44-1	*M. bovis*	R(19)	R(22)	R(18)	S(26)	R(8)	R(26)	4
Susceptiblity *(%)*			3 *(12.5)*	3 *(12.5)*	4 *(16.7)*	22 *(91.7)*	6 *(25.0)*	4 *(16.7)*	MDR = 21 *(87.5%)*
Resistance *(%)*			21 *(87.5)*	21 *(87.5)*	20 *(83.3)*	2 *(8.3)*	18 *(75.0)*	20 *(83.3)*	

(): Measured zone diameters of the strains (milimeter); MDR: Multi-drug resistance; OFX: Ofloxacin; CN: Gentamicin; CIP: Ciprofloxacin; TE: Tetracycline; AMC: Amoxicillin/Clavulonic acid; SXT: Sulfamethoxazole/Trimethoprim; C: Chloramphenicol

Antibiotic susceptibility status was evaluated according to the *M. catharalis* criteria in the EUCAST Clinical Breakpoint Tables v. 15.0, valid from 2025-01-01 document

*This strain was identified as *M. ovis* by MALDI-TOF MS and as *M. bovoculi* by 16S rRNA gene sequence analysis

**These strains were identified only by MALDI-TOF MS

*** These strains were identified solely through analysis of the 16S rRNA gene sequence

Note The abbreviations MDR (Multidrug Resistance), S (susceptible), and R (resistant) were used, based on inhibition zone diameter trends, to facilitate ease of interpretation for the reader. Since EUCAST defines clinical breakpoints only for *M. catarrhalis*, the approach described by de Carvalho et al. (2025) was followed, and the results were interpreted as resistance–susceptibility tendencies rather than confirmed clinical resistance

disease state (Gafen et al. 2023). In our study, the detection of diverse bacterial agents beyond *Moraxella* species from bovine ocular samples highlights the microbial diversity of the ocular surface and suggests that different species may contribute to the infection process. Particularly noteworthy was the culture-based identification of *R. nasimurium* and *C. xerosis*.

In the current study, PCR assays were conducted using primers specifically designed in our laboratory to target virulence-associated genes in *M. bovis* and *M. bovoculi*. For *M. bovis*, six genes (*mbx*A, *omp*79, *pil*, *plb*, *tol*C, and *fur*) were analyzed, while in *M. bovoculi*, three genes (*mbv*A, *omp*79, and *pil*A) were investigated. *M. bovis* produces the RTX cytotoxin *mbxA*, which is responsible for host cell lysis, while *M. bovoculi* expresses the related variant *mbv*A; both belong to the RTX family of extracellular toxins (Angelos and Ball 2007; Bilbao et al. 2024; Wang et al. 2023). PCR amplification also confirmed a 607 bp *omp*79 fragment in both species, corresponding to an outer membrane protein containing OM_channels superfamily domains, consistent with porin family proteins that facilitate small molecule transport (Nikaido 2003). *tol*C, another detected factor, is a type I secretion outer membrane protein involved in efflux and toxin export (Sosa et al. 2015). The Fur protein, encoded by the *fur* gene, regulates iron uptake and coordinates expression of toxins, enzymes, and adhesins during

infection (Kakuda et al. 2003). The *plb* gene encodes a phospholipase B enzyme of the GDSL family, potentially contributing to the pathogenesis of IBK (McConnel and House 2005). Finally, pilin proteins facilitate adhesion to the ocular epithelium and represent a key virulence factor in *M. bovis* (Kuibagarov et al. 2022).

In this study, clear differences were observed between *M. bovoculi* and *M. bovis*. All *M. bovoculi* isolates carried *pil*A, nine were positive for *mbv*A, and only two for *omp*79. In contrast, six *M. bovis* isolates harbored the full virulence repertoire, while three isolates (H34-2, H37-2, H42-2) showed limited or completely negative profiles. In *M. bovoculi*, the analysis was limited to *pil*A, *mbv*A, and *omp*79. Other loci were excluded because conserved regions suitable for primer design could not be identified from available GenBank sequences. This limitation is consistent with the high genetic diversity and genotype-specific differences reported in the literature (Bilbao et al. 2024; Dickey et al. 2018; Wynn et al. 2022), suggesting that certain virulence genes may indeed be absent in *M. bovoculi* or that sequence variability hindered primer design. In the current study, two independent data lines were evaluated to reveal phylogenetic and gene-level similarities: target gene PCR results (specifically, *mbv*A/*mbx*A, *pil*A, and *omp*79) and phylogenetic analysis using reference sequences reported by Wynn et al. (2022). The detection of the *pil*A gene in all *M. bovoculi* isolates indicates the adhesion and colonization capacity of this species, while a heterogeneous distribution of the *mbv*A and *omp*79 genes was observed. The phylogenetic analysis based on 16S rRNA sequences revealed that some *M. bovis* isolates (H34-2, H37-2, and H42-2) were located in a branch phylogenetically close to the genotype 1 references, while the remaining *M. bovis* isolates were associated with the genotype 2 group. *M. bovoculi* isolates also occupied positions adjacent to the genotype 1 references. Notably, although strains H34-2, H37-2, and H42-2 showed phylogenetic proximity to genotype 1, almost all of the virulence genes investigated were undetected in these isolates. This suggests the presence of intra-genotypic heterogeneity within *M. bovis* genotype 1 or the loss of some virulence genes. Some isolates may lose virulence genes during adaptation to the host environment (Sokurenko et al. 1999). These findings suggest that phylogenetic relatedness in *Moraxella* species represents genealogical relationship but does not directly reflect the level of virulence. In conclusion, the presence of the RTX gene region, although frequently associated with genotype 1, is not a universal marker; Furthermore, the absence of the gene does not reliably indicate genotype 2 (Dickey et al. 2018; Hille et al. 2021). Therefore, in the current study, the positivity or negativity of these genes and their placement within the 16S clade were considered indicators of phylogenetic relatedness; however, definitive genotype classification was not established. Future research employing higher-resolution methods, such as whole-genome sequencing, are anticipated to be valuable in corroborating these genetic relationships.

In this study, the antimicrobial susceptibility trends of *M. bovis* and *M. bovoculi* strains isolated from cattle with keratoconjunctivitis were also examined. A previous study based on United States data reported that all *M. bovis* isolates were resistant to cloxacillin and that 68% of the hemolytic strains were resistant to streptomycin (Webber et al. 1982). Subsequent studies conducted in the USA and Australia showed that isolates in some regions remained susceptible to many antibiotics (for example, all isolates in Australia were susceptible to tetracycline, florfenicol, and cloxacillin). However, more new research has reported an increasing trend of reduced susceptibility, particularly to sulfonamides (McConnel et al. 2007; Angelos et al. 2011). Pimenov et al. (2024) retrospectively evaluated data obtained from the USA, Australia, Brazil, and Russia, and reported that antibiotic susceptibility in *Moraxella* species has diversified over time at the species level. In their study, *M. bovoculi* isolates were found to be non-susceptible to oxacillin, methicillin, carbenicillin, and lincomycin, whereas *M. bovis* isolates were non-susceptible only to oxacillin and methicillin. These findings suggest that *M. bovoculi* may generally exhibit a higher tendency toward decreased susceptibility. For both species, high susceptibility to fluoroquinolones and cephalosporins was reported, and ampicillin and benzylpenicillin were among the most effective agents. However, a geographical "drift" was observed for some antibiotics; notably, doxycycline was reported to have lost efficacy in many regions and become widely ineffective. Maboni et al. (2015) evaluated the antimicrobial susceptibility profiles of *M. bovis*, *M. bovoculi*, and *M. ovis* strains using the microdilution method and reported significant differences among species. According to interpretive criteria, 9% of *M. bovoculi* isolates showed reduced susceptibility to penicillin, while 20% of *M. bovis* strains showed reduced susceptibility to oxytetracycline. The highest MIC values were observed in *M. bovis* isolates, whereas the lowest modal MIC values were detected in *M. bovoculi* in the study. For both species, the most effective antibiotics were reported to be ampicillin, ceftiofur, enrofloxacin, florfenicol, and gentamicin. The researchers emphasized that the decreased susceptibility to oxytetracycline in *M. bovis* may have increased over time under selective pressure from irregular antibiotic use.

In the present study, since only *M. catarrhalis* has defined breakpoints in the EUCAST documents, an approach similar to that reported by de Carvalho et al. (2025) was adopted to interpret inhibition zone diameters. Accordingly, zone diameters were evaluated as comparative indicators of susceptibility trends based on the reference values defined for

M. catarrhalis; the results were interpreted not as clinical evidence of resistance but as population-level indicators of susceptibility trends. In the current study, among *M. bovoculi* isolates, narrowing of inhibition zones was particularly observed for fluoroquinolones (ofloxacin 100%, ciprofloxacin 93.3%), tetracycline (93.3%), trimethoprim-sulfamethoxazole (93.3%), and chloramphenicol (93.3%). In contrast, zone diameters against amoxicillin-clavulanate were largely preserved (93.3%). In *M. bovis* isolates, zone diameters were generally larger, indicating a greater tendency toward susceptibility. Moderate-to-high degrees of zone narrowing were recorded for fluoroquinolones (ofloxacin 66.7%; ciprofloxacin 77.8%), tetracycline (66.7%), and chloramphenicol (66.7%), while for trimethoprim-sulfamethoxazole, this rate was 44.4%. Wide inhibition zones for amoxicillin-clavulanate were preserved in 88.9% of isolates. The findings indicate a tendency toward reduced susceptibility (MDR tendency) in both species, though the trend was more pronounced in *M. bovoculi* isolates.

In conclusion, this study found that multidrug resistance was prevalent among both *M. bovis* and *M. bovoculi* isolates, but *M. bovoculi* exhibited a higher propensity. The findings in this study, when considered alongside previous studies, emphasize that antibiotic selection should not be based solely on the literature but should be supported by regular regional resistance surveillance. However, further molecular and phenotypic studies are needed to better understand the dynamics of antimicrobial susceptibility in IBK pathogens.

Conclusion

This study demonstrates that *Moraxella* species isolated from cattle with ocular infections exhibit marked differences in virulence and antibiotic resistance propensities. Notably, all *M. bovoculi* strains possessed the *pil*A gene, which promotes their ability to adhere to ocular tissue. Propensity for resistance to fluoroquinolones and tetracyclines was particularly prevalent in *M. bovoculi*, which exhibited a more frequent propensity for multidrug resistance, while *M. bovis* maintained a relatively higher propensity for susceptibility. These findings highlight the importance of accurate species identification, as resistance propensity patterns vary among species. The increasing trend in antibiotic resistance underscores the need to develop new control measures alternative to antibiotics, to effectively manage IBK infections.

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Author contributions **SY: ** Conceptualization, Study design, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Data curation, Supervision, Funding acquisition, Project administration. **ISC: ** Investigation, Sample collection, Laboratory work (isolation), Data curation, Project administration, Funding. **AO: ** Primer design, Phylogenetic analysis, Figure preparation, Writing – review & editing, Writing – original draft, Supervision. **SC: ** Laboratory assistance, Investigation, Writing – review & editing.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval This study was approved by the Local Ethics Committee of Animal Experiments, Muğla Sıtkı Koçman University, Directorate of the Experimental Animals Application and Research Center (Approval no: 2023/01).

Competing interests The authors declare no competing interests.

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