Molecular characterization of bacterial microbiota associated withinfectious bovine keratoconjunctivitis in Michoacán, Mexico

Caracterización molecular de la microbiota bacteriana asociada con queratoconjuntivitis infecciosabovina en Michoacán, México

Caracterização molecular da microbiota bacteriana associada à ceratoconjuntivite bovina infecciosa emMichoacán, México

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Abstract

Background: The most common ocular disease affecting cattle worldwide is infectious bovine keratoconjunctivitis (IBK), which has been associated with *Moraxella bovis* bacterium.

Objective: To report the molecular characterization of the ocular bacterial microbiota and its relation to IBK in cattle in two dairy regions in Michoacán, Mexico.

Methods: A total population of 761 bovines were evaluated, of which 17 (2.23%) showed symptoms of IBK. Thirty-eight bacterial isolates from ocular samples of bovines with IBK were characterized by Gram-staining and antimicrobial sensitivity. In addition, isolates were identified by sequence comparisons of the 16S ribosomal gene.

Results: The genus *Moraxella* was one of the most abundant bacteria and *M. bovoculi* was the most predominant species. **Conclusion:** The bacterial isolates identified in eye lesions of cattle and associated to IBK are diverse. To the author´s knowledge, this is the first study on the subject in Mexico; therefore, more research is needed to estimate the incidence of IBK and determine its associated microbiota.

Keywords: *bacteria; bovine; corneal ulceration; dairy cattle; eye infection; IBK; keratoconjunctivitis; Moraxella bovis; Moraxella bovoculi; ocular bacteria; ocular disease; pink eye.*

Resumen

Antecedentes: la enfermedad ocular más común que afecta al ganado en todo el mundo es la queratoconjuntivitis infecciosa bovina (IBK), que se ha asociado con la bacteria *Moraxella bovis*.

Objetivo: reportar la caracterización molecular de la microbiota bacteriana ocular y su relación con IBK en ganado de dos regiones lecheras en Michoacán, México.

Métodos: se evaluó una población total de 761 bovinos de los cuales 17 (2,23%) mostraron síntomas de IBK. Se obtuvieron treinta y ocho aislamientos bacterianos de muestras oculares de bovinos con IBK, los cuales se caracterizaron por tinción de Gram y sensibilidad antimicrobiana. Además, los aislamientos se identificaron mediante comparaciones de secuencias del gen ribosomal 16S.

Resultados: el género *Moraxella* fue una de las bacterias más abundantes y *M. bovoculi* fue la especie más predominante.

Conclusión: los aislamientos bacterianos identificados en lesiones oculares de bovinos y asociados a IBK son diversos. Hasta donde sabemos, este es el primer estudio sobre el tema realizado en México; por lo tanto, es necesario ampliar esta investigación para estimar la incidencia de IBK y determinar la microbiota asociada con la misma.

Palabras clave: *bacterias; bacterias oculares; bovinos; conjuntivitis; enfermedad ocular; ganado lechero; infección ocular; Moraxella bovis; Moraxella bovoculi; queratoconjuntivitis; ulceración corneal.*

Resumo

Antecedentes: a doença ocular mais comum que afeta o gado no mundo é a ceratoconjuntivite bovina (IBK), que tem sido associada à bactéria *Moraxella bovis*.

Objetivo: relatar a caracterização molecular da microbiota bacteriana ocular e sua relação com a IBK em bovinos de duas regiões leiteiras de Michoacán, México.

Métodos: foi avaliada uma população total de 761 bovinos, más apenas 17 (2,23%) apresentaram sintomas de IBK. Trinta e oito isolados bacterianos de amostras de olho bovino com IBK foram caracterizados por coloração de Gram e sensibilidade antimicrobiana. Além disso, os isolados foram identificados por comparação de sequências do gene ribossômico 16S.

Resultados: a microbiota bacteriana associada à IBK foidiversa, sendo o gênero *Moraxella* uma das mais abundantes e *M. bovoculi* a espécie predominante.

Conclusão: de acordo como conhecimento dos autores, este é o primeiro estudo sobre o tema no México até o momento, portanto é necessário expandir essa pesquisa para estimar a incidência de IBK e determinar a microbiota associada à mesma.

Palavras-chave: *bactérias; bactéria ocular; bovinos; ceratoconjuntivite, conjuntivite; doença ocular; gado leiteiro; infecção ocular; Moraxella bovis; Moraxella bovoculi; ulceração corneana.*

Introduction

Infectious bovine keratoconjunctivitis (IBK, also known as "pink eye") is a disease distributed worldwide and mainly associated to Gram-negative coccobacillus bacterium *Moraxella bovis*; but other agents such as *M. ovis*, *Mycoplasma bovoculi*, and *Clamydophila* spp., have also been implicated (Alexander, 2010). In addition, *Moraxella bovoculi* has been considered as a potential causal organism (Angelos *et al.*, 2007; Sosa and Zunino, 2013).

Corneal ulceration caused by IBK often heals without therapeutic intervention and cattle generally recover; however, corneal rupture canresult in complete and permanent loss of visionin severe cases, with marked ocular discomfort (Williams, 2010). Besides welfare implications, IBK also has a considerable economic impact, particularly due to reduced weight gain in calvessuffering from IBK at weaning, and the cost of antibiotics (Schnee *et al.*, 2015; Kowalski *et al.*,2017). Some studies have estimated losses of10 to 20 kg of BW per infected animal. In the United States, 10 million cattle have this disease(Hare *et al.*, 2008), causing losses of more than200 million dollars per year (Addison, 2011).

In México, IBK is considered an enzootic disease, and it is associated to environmental conditions and seasonal vectors in some geographical regions. In the North of Mexico, the intense solar radiation is thought to be the most important factor favoring the presence of IBK. In the Central region of the country, it is associated with the presence of flyes and dusty winds, whereas in the South it is attributed to vectors (Gasque, 2008). The impact of this pathology in animal production in Mexico is unknown (Infante *et al.*, 2000; Zamora *et al.*, 2010).

Studies about the eye bacterial microbiota of healthy or diseased bovines are scarce. In addition, identification of microorganisms associated with IBK is required to establish if the clinical presentation is caused by secondary colonization of the damaged eye or if such microorganisms participate in the pathogenesis (Spradbrow, 1967; Handool, 2013; Sosa andZunino, 2013). There is no information on the causal agent of IBK in Mexico; however, animals with eye lesions suggestive of IBK are frequently observed.

Therefore, the objective of this study was to report the molecular characterization of the ocular bacterial microbiota and their relationwith IBK in cattle from two dairy regions in Michoacán, Mexico.

Materials and Methods

The study was carried out from July to December of 2015 in the localities of Uruétaro (19° 48' North latitude and 101° 10' West longitude, 1,860 m.a.s.l.), and Villa Madero (19°59' North latitude and 103° 01' West longitude, 2,000 m.a.s.l.) in the Morelia-Queréndaro Valleydairy region, and Marcos Castellanos (19° 59' North latitude and 103° 01' West longitude, 2,000 m.a.s.l.), Sahuayo (20° 03' North latitude and 102° 44' West longitude, 1,530 m.a.s.l.), and Emiliano Zapata (20° 01' North latitude and 102° 36' West longitude, 1,540 m.a.s.l.) in the Ciénega de Chapala dairy region in Michoacán, under a hot subhumid climate.

Sampling

Samples were collected from 17 bovines (2.23%) showing ocular lesion, presumptive of IBK, from a population of 761 cattle. The animals were located in five localities and distributed in 11 herds. The number of affected animals and the number of herds sampled by locality were seven in Sahuayo (5 herds), four inMarcos Castellanos (3 herds), two in Uruétaro (1herd), two in Emiliano Zapata (1 herd), and twoin Villa Madero (1 herd). All were family herdsunder intensive (Uruétaro and Emiliano Zapata;18.18%), extensive (Villa Madero; 9.09%), andsemi-extensive systems (Sahuayo and Marcos Castellanos; 72.72%).

The animals were placed in a cattle chute designed to minimize stress during eye inspectionand sampling, and were observed for uni- or bilateral ocular symptoms suggestive of IBK. Before sampling, the periocular region of the eye was cleaned with a gauze soaked into a 10% benzalkonium chloride soap, and 0.9% sodium chloride solution. Samples were taken from theventral area of the eye, between the ocular globeand the conjunctival sac, using a sterile cotton swab, and then kept in a tube with Cary-Blair sterile medium (Copan Italia SpA, Brescia, Italy) until processing.

Culture of ocular samples

Culture was conducted within a laminar flowhood. The collected conjunctival swab samples were streaked on blood agar plates and grown 24 h at 37 °C. After incubation, colonies were observed under a microscope. The size, shape, edge, area, color, and presence of hemolysis in the colonies was evaluated. The gray-whitish, round, small convex colonies, with or without a hemolysis halo that could be associated with *Moraxella* were selected. The isolates were infused into a 15 ml Falcon tube containing 2.5 ml of Luria Bertani (LB) broth and incubated per 24 h at 37 °C under continuous stirring. One aliquot was mixed with 10% glycerol and stored at -80 oC. The remaining samplewas used to carry out the Gram-staining, antimicrobial testing, and DNA extraction.

DNA extraction

Samples of 1.5 ml culture from bacterial isolates were grown overnight in LB broth. The suspension was used for DNA extractionby CTAB (hexadecyltrimethylammonium bromide) protocol (Minas *et al.*, 2011). DNA was resuspended in deionized water and DNA integrity was verified by standard electrophoresis in 1% agarose gels.

Identification of bacterial isolates

In order to identify the bacterial isolates, a 1.5 kb fragment of the 16S ribosomal gene was amplified by PCR. Forward 5ʹ-AGAGTTTGATCCTGGCTGAG-3ʹ and reverse 5ʹ-GGTTCCTTGTTACGACTT-3ʹ oligonucleotides (Elim Biopharmaceuticals,Inc, Hayward, CA, USA) were used. PCR amplification was carried out using 50 ngof DNA and the Platinum PCR SuperMix High Fidelity (Invitrogen, California, USA) in a final volume of 20 µl. The same mix was used without DNA as a negative control. The amplification reaction was performed under thefollowing conditions: an initial step at 95 °C for 5 min, and then 30 cycles of the program, 30 s at 95 °C for DNA denaturalization, 30 sat 58 °C for oligonucleotides alignment, andan extension at 72 °C for 1.5 min. At the endof the final amplification, a one extension at 72 °C for 5 min was performed. The integrity of the PCR products was revised and analyzed by electrophoresis in 1% agarose gels.

The PCR products were sequenced by Sanger technique by Elim Biopharmaceuticals, Inc (Hayward, CA, USA). The electropherograms were analyzed using the Mega 7.0.7 (DNASTAR) program. The sequences obtained from the bacterial 16S gene were compared with those available in the NCBI data bank to identify the isolates using the BLAST option (https://www. ncbi.nlm.nih.gov/guide/sequence-analysis/).

Antimicrobials tests

All of the bacterial isolates were tested for antimicrobial susceptibility, which was determined using the disk diffusion method on Mueller-Hinton (MH) agar plates (Bioxon, Mexico). The following disks for Gram-negative bacteria (Gram Negatives II Bio-Rad) were used: amikacin, 30 µg; ampicillin, 10 µg; levofloxacin, 5 µg; cephalothin, 30 µg; cefotaxime, 30 µg; ceftriaxone, 30 µg; chloramphenicol 30 µg; gentamicin, 10 µg; netilmicin 30 µg; nitrofurantoin 300 µg; cefepime 30 µg; trimethoprim-sulfamethoxazole 25 µg. In addition, the following antimicrobials used against Gram-positive bacteria were evaluated (Gram-positive, Bio-Rad, México): ceftazidime, 30 µg; cefuroxime, 30 µg; dicloxacillin, 1 µg; erythromycin, 15 µg; pefloxacin, 5 µg; penicillin, 10 U; tetracycline, 30 µg. Isolates were classified as susceptible, intermediate and resistant according to the manufacturer's instructions.

An MH agar plate without antimicrobials was used as a control treatment. Plates were incubated at 37 °C for 24 h.

Statistical analysis

Data were analyzed using descriptive statitsitics based on frequencies.

Results

Seven hundred and sixty-one bovines from two dairy regions in Michoacán (México) were analyzed. According to symptoms, 17 animals (2.23%) showed IBK, mainly localized in one eye. Fifteen bovines showed unilateral lesions and only two showed lesions in both eyes. Based on the colony morphology, 38 colonies were isolated, of which 13 colonies were from samples of clinically healthy eyes, and 25 from cattle with morphological lesions (Table 1).

In a first approach, the bacterial isolates were identified using the Gram-staining. The results showed that 68.98% of the bacterial isolates were Gram-positive and 31.56% were Gram-negative. Furtherly, bacterial isolates were identified using the sequences of the 16S ribosomal RNA. In the Gram-positive samples, the most abundant bacterial microbiota corresponded to *Staphylococcus saprophyticus* (15.78%), *Staphylococcus agnetis* (10.25%), *Streptococcus uberis* (7.89%), *Staphylococcus chromogenes*, and *Arthrobacter luteolus* (5.26%). *Staphylococcus haemolyticus*, *Streptococcus dysgalactiae*, *Streptococcus suis*, *Enterococcus mundtii*, *Bacillus aerius*, *Bacillus toyonensis*, *Bacillus pumilus*, *Rothia nasimurium*, *Arthrobacter gandavensis*, *Peptoniphilus indolicus*, and *Corynebacterium aquilae* were present in 2.63%, each species. In relation to the Gram-negative isolates, the most abundant species was *Moraxella bovoculi* (10.52%), whereas the remaining identified microorganisms (*Pseudomonas aeruginosa*, *Pseudomonas zhaodongensis*, *Mannheimia granulomatis*, *Acinetobacter schindler*, *Enterobacter mori*, and *Moraxella equi*) showed frequencies of 2.63% (Table 2).

Antimicrobial sensitivity of bacteria isolates and their resistance patterns are shown in Table 3. Multi-resistance was observed for different groups of antibiotics. The 88.8% of isolates was resistant to dicloxacillin, 77.7% to ceftazidime, 55% to penicillin, 22.2% to tetracycline and ampicillin, and only one isolate was resistant to erythromycin.

The highest resistance rate of Gram-positive isolates was toward doxycycline (75%; 18/24), ceftazidime and penicillin (54.1%; 13/24). Interestingly, *Staphylococcus* isolates showed resistance mainly to

ceftazidime, dicloxacillin, and penicillin. In the same way, isolates of *Streptococcus uberis* showed 100% resistance to dicloxacillin. Regarding to the genus *Arthrobacter*, isolates showed 100% resistance to penicillin, 66.6% to pefloxacin, and 33.3% to doxycycline. In addition, *A. gandavensis* showed resistance to cephalothin, ceftazidime, erythromycin, ampicillin, and doxycycline.

Seven Gram-negative isolates were observed, of which only *M. granulomatis* showed sensitivity to all antimicrobials. The remaining isolates showed resistance to antimicrobials with different patterns (Table 3). Noteworthy, *P. aeruginosa* (case 2) showed resistance to nitrofurantoin, chloramphenicol, ceftriaxone, ampicillin, trimiteprim sulfamethoxazole, cefotaxime, cephalothin and cefepime. Also, *P. zhaodongenisis* (case 6) only showed resistance to ampicillin and levofloxacin. Finally, *E. mori* (case 14), *A. schindler* (case 17), *M. equi* (case 8) and *M. bovoculi* (cases 5 and 11) showed resistance to ampicillin and cephalothin.

Discussion

Bacterial isolates from injured eyes were diverse and mainly Gram-positive (68.98%), similar to other reports in bovines (Sosa and Zunino, 2013), and humans with conjunctivitis (Hernández and Quintero, 2003). Presence of Gram-positive bacteria could be attributed to its resistance to adverse and dry conditions because they contain a thick cell-wall, rich in peptidoglycan (Russell, 2003). For the Gram-negative bacteria, the predominant genus was *Moraxella*.

Table 1. Microorganisms obtained from ocular samples of bovines with presumptive infectious keratoconjunctivitis (IBK) in Michoacán, Mexico.

Animal	Type of lesion	Injured eye	Sampled eye	Sample ID	Identified microorganism
1	Bilateral	Right, left	Left	M1	Staphylococcus saprophyticus
		Right, left	Left	M2	Rothia nasimurium
		Right, left	Right	M3	Staphylococcus saprophyticus
		Right, left	Right	M4	Staphylococcus saprophyticus
2	Unilateral	Right	Left	M5	Bacillus toyonensis
		Right	Left	M6	Pseudomonas aeruginosa
3	Unilateral	Left	Right	M7	Staphylococcus saprophyticus
		Left	Left	M8	Staphylococcus saprophyticus
4	Unilateral	Left	Right	M9	Staphylococcus saprophyticus
		Left	Right	M10	Enterococcus mundtii
5	Bilateral	Right, left	Right	M11	Staphylococcus agnetis
		Right, left	Right	M12	Staphylococcus agnetis
		Right, left	Left	M13	Moraxella bovoculi
		Right, left	Left	M14	Moraxella bovoculi
		Right, left	Left	M15	Streptococcus uberis
6	Unilateral	Right	Right	M16	Mannheimia granulomatis
		Right	Right	M17	Streptococcus uberis
		Right	Right	M18	Streptococcus uberis
7	Unilateral	Right	Right	M19	Arthrobacter luteolus
		Right	Left	M20	Bacillus aerius
8	Unilateral	Right	Left	M21	Moraxella equi
		Right	Right	M22	Arthrobacter gandavensis
9	Unilateral	Right	Left	M23	Arthrobacter luteolus
10	Unilateral	Right	Left	M24	Streptococcus dysgalactiae
		Right	Right	M25	Peptoniphilus indolicus
11	Unilateral	Left	Left	M26	Bacillus pumilus
		Left	Right	M27	Moraxella bovoculi
12	Unilateral	Right	Left	M28	Pseudomonas zhaodongensis
		Right	Right	M29	Moraxella bovoculi
13	Unilateral	Left	Right	M30	Staphylococcus agnetis
		Left	Left	M31	Corynebacterium aquilae
14	Unilateral	Right	Right	M32	Enterobacter mori
		Right	Right	M33	Staphylococcus chromogenes
		Right	Left	M34	Streptococcus suis
15	Unilateral	Right	Right	M35	Staphylococcus chromogenes
16	Unilateral	Right	Right	M36	Staphylococcus haemolyticus
		Right	Right	M37	Staphylococcus agnetis
17	Unilateral	Right	Left	M38	Acinetobacter schindler

In addition, more bacterial isolates were obtained from injured eyes in comparison with healthy eyes. This could be explained by the fact that defense mechanisms are affected in the injured cornea favoring the invasion of opportunist infectious agents.

One of the main predisposing factors for the presentation of IBK is the environment. A previous study by Takele and Zerihun (2000) in South-east Ethiopia showed an incidence of 2.10% IBK in local zebu and crossbreed dairy animals, which is similar to what was observed here. In that study, the researchers reported unilateral presentation in 85.5% of the cases, whereas bilateral infection was 14.5%. In our study 88.2 and 11.76% of unilateral and bilateral afections were observed, respectively. Aditonally, in 80% of reported IBK cases M. bovis has been isolated together with other bacteria such as *Actinomyces piogenes*, *Staphylococcus aureus*, *Pasteurella haemolytica*, *Escherichia coli*, and *Proteus* spp (Takele and Zerihun, 2000). In our study, the ocular bacterial microbiota was diverse, probably related with the environment and production system of each farm (intensive or semi-intensive), which may favor dissemination or growth of different bacteria populations. It is important to highlight that in this study, although we found presumptive symptomatology to IBK, this was associated with the presence of *M. bovoculi* and not to M. bovis as reported by Takele and Zerihun (2000). According to the above comments, it is necessary to conduct studies in Mexico's tropical areas to determine if M. bovis is the causal agent of IBK.

Studies in cattle where bacterial microbiota was identified show some of the species of bacteria reported here;

i.e., *Acinetobacter* spp. (Wilcox, 1970; Hare *et al.*, 2008; Sosa and Zunino, 2013), *Bacillus* spp., *Corynebacterium* (Spradbrow, 1967; Wilcox, 1970), *Streptococcus* ssp. (Sosa and Zunino, 2013), *A. gandavensis*, *A. luteolus*, *Pseudomonas* spp. (Hare *et al.*, 2008, Sosa and Zunino, 2013), *Arthrobacter* (Sosa and Zunino, 2013), and *M. bovoculi* (Blood and Radostits, 1992; Angelos *et al.*, 2007; Libardoni et al., 2007). Differences between studies could be attributed to geographical locations, which are expected to have different environmental conditions. In the same way, some of the bacteria isolated in this study have been associated with etiological agents of bovine conjunctivitis and bovine keratosis. However, other opportunistic bacteria living in the skin and nasal cavities are commonly found in the conjunctivae of the eyes of healthy animals (Handool, 2013), favored by farm environmental and management conditions.

Table 2. Frequency of bacterial isolates associated to presumptive infectious bovine keratoconjunctivitis (IBK) in catle in Michoacán, México.

Gram-classification	Microorganism	Frequency (%)
Negative	*Acinetobacter schindler, Pseudomonas aeruginosa, Mannheimia granulomatis, Bacillus aerius, Pseudomonas zhaodongenisis, Corynebacterium aquilae, Enterobacter mori.*	2.63
	Moraxella equi	2.63
	Moraxella bovoculi	10.52
Positive	*Rothia nasimurium, Bacillus toyonensis, Enterococcus mundtii, Arthrobacter gandavensis, Streptococcus dysgalactiae, Peptoniphilus indolicus, Bacillus pumilus, Streptococcus suis, Staphylococcus haemolyticus.*	2.63
	Arthrobacter luteolus, Staphylococcus chromogenes	5.26
	Streptococcus uberis	7.89
	Staphylococcus agnetis	10.52
	Staphylococcus saprophyticus	15.78

Table 3. Antimicrobial sensitivity of isolates associated with infectious bovine keroconjuntivitis (IBK) in cattle in Michoacán, México.

Isolates	Clinical case	Location	Antimicrobial resistance pattern
Staphylococcus saprophyticus	1	Uruétaro	CAZ, DC, PE
	2	Uruétaro	CAZ, E, DC, PE
	2	Uruétaro	CAZ, E, DC, PE
	3	Emiliano Zapata	CAZ, AM, DC, PE
	3	Emiliano Zapata	CAZ, TE, DC, PE
	4	Emiliano Zapata	CAZ, TE, DC, PE
	5	Villa Madero	CAZ, AM, DC, PE
Staphylococcus agnetis	5	Villa Madero	CAZ
	13	Sahuayo	CAZ, DC
	16	Marcos Castellanos	DC
Staphylococcus chromogenes	15	Marcos Castellanos	CAZ, DC
	5	Villa Madero	CAZ, AM, DC, PE
Streptococcus uberis	6	Villa Madero	DC
	6	Villa Madero	DC
Arthrobacter luteolus	7	Sahuayo	PEF, PE
	9	Sahuayo	PEF, DC, PE
Arthrobacter gandavensis	8	Sahuayo	CF, CAZ, E, AM, DC, PE
Rothia nasimurium	1	Uruétaro	CAZ, DC, PE
Bacillus toyonensis	2	Uruétaro	CAZ, DC, PE
Bacillus aerius	7	Sahuayo	CAZ, CTX, DC
Bacillus pumilus	11	Sahuayo	CMX, DC
Peptoniphilus indolicus	10	Sahuayo	DC, PE
Corynebacterium aquilae	13	Sahuayo	CF, CAZ, E, AM, PEF, DC, PE
Pseudomonas	2	Uruétaro	NF, CL, CRO, AM, STX, CTX, CF, FEP
aeruginosa	12	Sahuayo	AM, LEV
Pseudomonas zhaodongenisis	14	Marcos Castellanos	AM, CF
Enterobacter mori	17	Marcos Castellanos	AM, CF
Acinetobacter schindler	8	Sahuayo	AM, CF
	5	Villa Madero	AM, CF
Moraxella equi	5	Villa Madero	AM, CF
	11	Sahuayo	AM, CF

CF: Cephalothin 30 µg, CAZ: Ceftazidime 30 µg, E: Erythromycin 15 µg, AM: Ampicillin 10 µg, TE: Tetracycline 30 µg, STX: Trimethoprim sulfamethoxazole 25 µg, CTX: Cefotaxime 30 µg, GE: Gentamicin 10 µg, CMX: Cefuroxime 30 µg, PEF: Pefloxacin 30 µg, DC: Dicloxacillin 1 µg, PE: Penicillin 10U. NF: Nitrofurantoin 300 µg, CL: Chloramphenicol 30 µg, CRO: Ceftriaxone 30 µg, FEP: Cefepime 30 µg, LEV: Levofloxacin 5 µg.

Antimicrobial sensitivity tests showed that IBK-associated isolates possess extensive resistance to β-lactams, mainly penicillin, ampicillin, and doxycycline. Many Gram- negative bacteria have a naturally occurring chromosome-mediated β-lactamase that confersresistance to this group of antibiotics and the use of new β-lactams resistant to the hydrolytic action of β-lactamases has caused the emergence of new β-lactamases that favors resistance selection to those drugs (Bradford, 2001). Strains producing extended-spectrum beta-lactamases (ESBL), suchas Gram-negative bacilli, mainly enterobacteria,are generally multi-resistant, especially beta- lactams. Bacterial resistance is also attributed tothe common use of these drugs for the treatment of several infectious diseases in cattle (Ochoa *et al.*, 2008). Presumably, the selective pressurederived from the use and abuse of new antibioticshas selected for new variants of β-lactamase.In this regard, multi-resistant isolates were observed. For example, isolates of the species *A. gandavensis*, *C. aquilae* and *P. aeruginosa* showed resistance to more than 50% of the tested antimicrobials. These resistance patterns are most often associated with the integration of new enzymes obtained by conjugation, transformation, or transduction (Navarro *et al.*, 2010). Although this could explain the frequency of

resistance observed to β-lactams in our study, molecular studies are needed to identify if they have this type of enzymes. Different resistance patterns may indicate the preferred use of antimicrobials to treat IBK in each region (Loy and Brodersen, 2014), and the bacterial microbiota associated with this pathology can be related to the frequency and pattern of use of antibiotics in dairy systems.

In conclusion, normal bacterial microbiota of the conjunctivae has been poorly studied, lacking phenotypic and genotypic indicators to compare the bacterial microbiota of the clinically healthy eye and animals with IBK. In this study, the bacterial isolates identified in eye lesions of cattle and associated to IBK was diverse. This is the first study on the subject conducted in Mexico. More studies on IBK are required under the conditions of Michoacán and other Mexican regions.

Declarations

Conflicts of interest

The authors declare that they have no conflicts of interest with regard to the work presented.

Author contributions

Ana M Rios-Alanis, conducted most of the experimental work to obtain her Master´s degree in agricultural sciences. Joel E. López-Meza, adviced on the development of the experimental work and interpretation of molecular tests to identify bacterial isolates. Alejandra Ochoa- Zarzosa, contributed with development of primers and determination of DNA sequences. Jose C Segura-Correa, contributed to the statistical analysis and revision of the manuscript. José Herrera-Camacho, thesis director of Rios-Alanis, wrote the manuscript.

References

Addison B. Research shows new emerging strain of pinkeye. Progressive Cattleman 2011; 5:22–23. https://addisonlabs.com/wp-content/uploads/2016/02/CattlemanPinkeye.pdf

Alexander D. Infectious bovine kerato- conjunctivitis: A review of cases in clinical practice. Vet Clin Food Anim 2010; 26:487–503. DOI: https://doi:10.1016/j.cvfa.2010.09.006

Angelos JA, Spinks PQ, Ball LM, George LW. *Moraxella bovoculi* sp. nov., isolated from calves with infectious bovine keratoconjunctivitis. Int J Syst Evol Microbiol 2007; 57:789–795. DOI: https://doi.org/10.1099/ijs.0.64333-0

Blood DC, Radostits OM. Enfermedades causadas por bacterias. En: Medicina Veterinaria. Séptima edición. Vol I. Madrid Interamericana. McGraw-Hill, 1992; 750–753.

Bradford P. A. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbol Rev 2001; 14:933–951. DOI: https://doi.org/10.1128/CMR.14.4.933-951.2001

Gasque R. Queratoconjuntivitis infecciosa bovina. Enciclopedia Bovina. Enfermedades de los bovinos. Facultad de Medicina Veterinaria y Zootecnia-UNAM. www.fmvz.unam.mx/fmvz/e_bovina/ 2008. Fecha de consulta: 12 de enero 2015.

Handool H. Isolation and identification of some genera and species of bacteria and fungi from conjunctiva in cattle in Al-Diwaniya city. AL-Qadisiya J Vet Med Sci 2013; 12:55–62. DOI: https://doi.org/10.29079/vol12iss2art258

Hare WR, Hoyt PG, Hohn C, Higgins JA. Ribosomal RNA-based analysis of the bacterial flora from the conjunctivae of cattle with bovine keratoconjunctivitis (BKC). Vet Microbiol 2008; 131:358–368. DOI: https://doi.org/10.1016/j.vetmic.2008.04.018

Hernández P, Quintero G. Etiología bacteriana de infecciones oculares externas. Nova Publicación Científica 2003; 1:57–64. DOI: https://doi.org/10.22490/24629448.1056

Infante FM, Flores G, Falcón NA, Infante AF. Control de un brote de queratoconjuntivitis infecciosa bovina mediante el control de las moscas en estadio larvario. Med Vet 2000; 17:273–276. DOI: https://pdfs.semanticscholar.org/6810/9cc3670cfcbf69cf0df258e2c46ebf9ae6bd.pdf?_ga=2.212 58299.1286023369.1582899306-648624553.1559616002

Kowalski AP, Maboni G, Gressler LT, Espíndola JP, Balzan C, Tasca C, Guizzo JA, Conceição F R, Frandoloso R, de Vargas AC. Antigenic characterization of *Moraxella bovis*, *Moraxella bovoculi* and *Moraxella ovis* strains with potential use in vaccines. Vet Microbiol 2017; 210:56–63. DOI: http://dx.doi.org/10.1016/j.vetmic.2017.08.016

Libardoni F, Scherer Ch, Farias L, Vielmo A, Balzan C, Vargas A. *Moraxella bovoculi* in cases of infectious bovine keratoconjunctivitis in Rio Grande do Sul. Brazil. Pesq Vet Bras 2007; 32:743–746. DOI: https://doi.org/10.1590/S0100-736X2012000800011

Loy JD, Brodersen BW. *Moraxella* spp. isolated from field outbreaks of infectious bovine keratoconjunctivitis: a retrospective study of case submissions from 2010 to 2013. J Vet Diag Invest 2014; 26:761–768. DOI: https://doi.org/10.1177/1040638714551403

NCBI (National Center for Biotechnology Information). https://www.ncbi.nlm.nih.gov/ guide/sequence-

analysis/. Fecha de consulta: diciembre 2016.

Minas K, McEwan N, Newbold CJ, Scott KP. Optimization of a high-throughput CTAB-based protocol for the extraction of qPCR- grade DNA from rumen fluid, plant and bacterial pure cultures, FEMS Microbiology Letters 2011; 325 (2)2:162–169. DOI: https://doi.org/10.1111/j.1574-6968.2011.02424.x

Navarro JM, Tormo A, Martínez-García E. Stationary phase in gram-negative bacteria, FEMS Microbiol Rev 2010; 34:476–495. DOI: https://doi.org/10.1111/j.1574-6976.2010.00213.x

Ochoa A, Loeza P, Torres F, Loeza H, Mascot N, Sánchez S, López JE. Antimicrobial susceptibility and invasive ability of *Staphylococcus aureus* isolatesfrommastitisfromdairybackyardsystem. Antonie Leeuwenhoek 2008; 94:199–206. DOI: https://doi.org/10.1007/s10482-008-9230-6

Russell A. Bacterial outer membrane and cell wall penetration and cell destruction by polluting chemical agents and physical conditions. Sci Prog 2003; 86(4):283–311. DOI: https://doi.org/10.3184/003685003783238608

Schnee C, Heller M, Schubert E, Sachse K. Point prevalence of infection with *Mycoplasma bovoculi* and *Moraxella* spp. in cattle at different stages of infectious bovine keratoconjunctivitis.Vet J 2015;203:92–96. DOI:https://doi.org/10.1016/j.tvjl.2014.11.009

Sosa V, Zunino P. Diversity of *Moraxella* spp. Strains recovered from infectious bovine Keratoconjunctivitis cases in Uruguay. J Infect Dev Ctries 2013; 7:819–824. DOI: https://doi.org/10.3855/jidc.3458

Spradbrow PB. A microbiological study of bovine conjunctivitis and Keratoconjunctivitis. Aus Vet J 1967; 43:55–58. DOI: https://doi.org/10.1111/j.1751-0813.1967.tb15063.x

Takele G, Zerihun A. Epidemiology of infectiouskeratoconjunctivitis in cattle in South-east Ethiopia. J. Vet. Med. A 2000; 47:169–173. DOI: https://doi.org/10.1046/j.1439-0442.2000.00274.x

Wilcox GE. Bacterial flora of the bovine eye with special reference to the *Moraxella* and *Neisseria*. Aust Vet J 1970; 46:253–256. DOI: https://doi.org/10.1111/j.1751-0813.1970.tb15771.x

Williams DL. Welfare issues in farm animal ophthalmology. Veterinary Clinics of North America: Food Anim Prac 2010; 26:427–435. DOI: https://doi.org/10.1016/j.cvfa.2010.08.005

Zamora QMA, Aguilar AJ, Sumano L. Eficaciaclínica del florfenicol oftalmico *vs* florfenicol parenteral en el tratamiento de queratoconjuntivitis infecciosa bovina. Vet Méx 2010; 41:219–25. DOI: http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0301-50922010000300006