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

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

Caracterização molecular da microbiota bacteriana associada à ceratoconjuntivite bovina infecciosa em Michoacán, 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.*

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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, mas 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 foi diversa, sendo o gênero *Moraxella* uma das mais abundantes e *M. bovoculi* a espécie predominante. **Conclusão:** de acordo com o 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 can result in complete and permanent loss of vision in 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 calves suffering from IBK at weaning, and the cost of antibiotics (Schnee *et al.*, 2015; Kowalski *et al.*, 2017). Some studies have estimated losses of 10 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 than 200 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 and Zunino, 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 relation with 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 (19º 48' North latitude and 101º 10' West longitude, 1,860 m.a.s.l.), and Villa Madero (19º 59' North latitude and 103º 01' West longitude, 2,000 m.a.s.l.) in the Morelia-Queréndaro Valley dairy region, and Marcos Castellanos (19º 59' North latitude and 103º 01' West longitude, 2,000 m.a.s.l.), Sahuayo (20º 03' North latitude and 102º 44' West longitude, 1,530 m.a.s.l.), and Emiliano Zapata (20º 01' North latitude and 102º 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 in Marcos Castellanos (3 herds), two in Uruétaro (1 herd), two in Emiliano Zapata (1 herd), and two in Villa Madero (1 herd). All were family herds under intensive (Uruétaro and Emiliano Zapata; 18.18%), extensive (Villa Madero; 9.09%), and semi-extensive systems (Sahuayo and Marcos Castellanos; 72.72%).

The animals were placed in a cattle chute designed to minimize stress during eye inspection and 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 the ventral area of the eye, between the ocular globe and 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 flow hood. 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 sample was 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 extraction by 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 5'-AGAGTTTGATCCTGGCTGAG-3' and reverse 5'-GGTTCCTTGTTACGACTT-3' oligonucleotides (Elim Biopharmaceuticals, Inc, Hayward, CA, USA) were used. PCR amplification was carried out using 50 ng of 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 the following 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 s at 58 °C for oligonucleotides alignment, and an extension at 72 °C for 1.5 min. At the end of 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 ºC 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*
15	Unilateral	Right	Left	M34	*Streptococcus suis*
		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 catle 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
Streptococcus uberis	5	Villa Madero	CAZ, AM, DC, PE
	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
Moraxella equi	5	Villa Madero	AM, CF
	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 confers resistance 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), such as Gram-negative bacilli, mainly enterobacteria, are generally multi-resistant, especially beta-lactams. Bacterial resistance is also attributed to the common use of these drugs for the treatment of several infectious diseases in cattle (Ochoa *et al.*, 2008). Presumably, the selective pressure derived from the use and abuse of new antibiotics has 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

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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.

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