Original Article

Diversity of *Moraxella* spp. strains recovered from infectious bovine keratoconjunctivitis cases in Uruguay

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Abstract

Background: Infectious bovine keratoconjunctivitis (IBK) is the most common ocular disease that affects cattle throughout the world and it has a very significant economic impact. IBK is caused by members of the genus *Moraxella* and therapeutic and preventive measures have shown limited success. Vaccines, most of them chemically inactivated bacterins, generally induce a limited protection.

Methodology: In this study, the genetic diversity of Uruguayan clinical *Moraxella bovis* and *Moraxella bovoculi* isolates was assessed by RAPD-PCR, ERIC-PCR and BOX-PCR fingerprinting. Also, antibiotic resistance of the *Moraxella* spp. isolates was assessed utilizing the disk diffusion method.

Results: When interspecific molecular diversity was assessed, different bands patterns were observed even within a single outbreak of IBK, showing the coexistence of different genotypes of *Moraxella* spp. The high genetic diversity within *M. bovis* and *M. bovoculi* isolates did not permit to correlate isolates DNA fingerprints with geographical origins, dates or even with both different *Moraxella* species. Antibiotics resistance patterns showed significant differences between *M. bovis* and *M. bovoculi*.

Conclusions: This is the first study of diversity that includes *M. bovis* and *M. bovoculi* associated to IBK cases. Genetic diversity did not allow to correlate DNA fingerprints of the isolates with geographical origins, isolation dates or even both different *Moraxella* species. Antibiotics resistance patterns showed differences between *M. bovis* and *M. bovoculi*. This remarkable variation within isolates could explain the partial protection induced by commercial vaccines. All these findings could be important for the design of prevention or treatment strategies against IBK.

Key words: Infectious bovine keratoconjunctivitis, *Moraxella bovis, Moraxella bovoculi,* diversity, DNA fingerprinting

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Introduction

Infectious bovine keratoconjunctivitis (IBK), commonly known as pink eye, is the most common ocular disease of cattle including symptoms like corneal ulceration and edema, blepharospasm, photophobia and lacrimation. Its economic impact is significant since prevention and treatment strategies are often ineffective [1].

Moraxella bovis has been traditionally recognized as the etiologic agent of IBK. However, the recently characterized species *Moraxella bovoculi,* has been recovered from corneal ulcers of beef and dairy calves in USA [2] and in Uruguay [3].

Many vaccines available nowadays contain mainly different strains of *M. bovis*. Although these vaccines have shown partial protection, they are not completely effective. This fact may be due to intraespecific diversity of *M. bovis* [4]. This irregular protection conferred by vaccines against IBK has led several authors to investigate the biology of the etiologic agents associated with the disease. Several features related to the biology of *M. bovis* have been studied, including analyses of outer membrane proteins (OMP), lipopolysaccharides (LPS), pili and cytotoxin or antibiotic susceptibility [4-7].

Phenotypic typing based on expression of cellular characteristics may vary according to culture or experimental conditions; moreover, these methods involve time-consuming processes [8-10]. The relatively recent development of molecular techniques to assess bacterial genetic diversity has introduced the possibility to characterize genetic differences among isolates. However, reports regarding genetic typing of *M. bovis* and *M. bovoculi* collections are scarce.

Rep-PCR is a molecular biology based method suitable for the grouping of microorganisms. This technique is based on DNA amplification and has been usually considered as an extremely reliable, reproducible, rapid and highly discriminatory method

[11]. Rep-PCR genomic fingerprinting is based on the use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most microorganisms [12]. Three families of repetitive sequences have been identified, including the enterobacterial repetitive intergenic consensus (ERIC) sequence, and the BOX element [11]. The repetitive elements may be present in both orientations, and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in ERIC, and from the boxA subunit of BOX, in the PCR [11]. The use of these primers and PCR lead to the selective amplification of distinct genomic regions located between ERIC or BOX elements.

Randomly Amplified Polymorphic DNA (RAPD) has been also frequently used to assess genetic diversity among isolates. Only one primer is used in RAPD analysis, its sequence is arbitrarily chosen and sequence knowledge of the genomic DNA is not required. This results in a number of anonymous, not previously determined, but reproducibly amplified fragments [13].

So far, RAPD and ERIC fingerprint have been used to study differences between small numbers of only *M. bovis* strains in studies focused on IBK epidemiology [4,14].

The main objective of the present study was to evaluate the genetic diversity of a wide collection of *M. bovis* and *M. bovoculi* isolates recovered from outbreaks of IBK between 1983 and 2009 in Uruguay using RAPD-PCR, ERIC-PCR and BOX-PCR. Also, antibiotic susceptibility of the strains was assessed in order to add information about diversity of *Moraxella* spp.

Methodology

Bacterial strains, media and growth conditions

Fifty four *Moraxella* spp. strains comprising forty five Uruguayan field isolates (21 *M. bovis* strains and 24 *M. bovoculi* strains), 3 reference strains (ATCC Epp63 *M. bovis*, ATCC BAA1259 *M. bovoculi* and ATCC 33078 *M. ovis*) and six *Moraxella* spp. strains obtained from IBK cases in Kansas, USA and kindly provided by Dr. Brad Fenwick (Kansas State University) were evaluated. The field isolates were obtained from several Uruguayan provinces between 1983 and 2009 (Table 1).

All isolates had been identified and characterized in a previous study [3].

Table 1. *Moraxella* spp. strains used in the present study.

Isolates	Origin	Homology
Epp63	ATCC	*M. bovis*
BAA1259	ATCC	*M. bovoculi*
33078	ATCC	*M. ovis*
5120	Uy, Paysandú (2009)	*M. bovis*
5148	Uy, Paysandú (2009)	*M. bovis*
64	Uy (2009)	*M. bovis*
771A	Uy, Florida (2009)	*M. bovis*
BM3A	Uy, Cerro Largo (2005)	*M. bovis*
CANII	Uy, Canelones (2009)	*M. bovis*
CANIIIA	Uy, Canelones (2009)	*M. bovis*
CANIIIB	Uy, Canelones (2009)	*M. bovis*
EV121	Uy, Maldonado (2006)	*M. bovis*
EV250	Uy, Maldonado (2006)	*M. bovis*
EV345	Uy, Maldonado (2006)	*M. bovis*
EV366	Uy, Maldonado (2006)	*M. bovis*
EV378	Uy, Maldonado (2006)	*M. bovis*
EV450A	Uy, Maldonado (2006)	*M. bovis*
EV450B	Uy, Maldonado (2006)	*M. bovis*
Fs327	USA, Kansas	*M. bovis*
Fs328	USA, Kansas	*M. bovis*
Fs330	USA, Kansas	*M. bovis*
LC190	Uy, Flores (2006)	*M. bovis*
SJ01	Uy, San José (2007)	*M. bovis*
SJ02	Uy, San José (2007)	*M. bovis*
SJ03B	Uy, San José (2007)	*M. bovis*
SJ07	Uy, San José (2007)	*M. bovis*
SJ08	Uy, San José (2007)	*M. bovis*
Sp346	USA, Kansas	*M. bovis*
1965	Uy, San José (2008)	*M. bovoculi*
1A1	Uy, Treinta y Tres (2008)	*M. bovoculi*
2358	Uy, Salto (2004)	*M. bovoculi*
2419	Uy, Florida (1983)	*M. bovoculi*
2439-2	Uy, San José (1983)	*M. bovoculi*
2693	Uy (2008)	*M. bovoculi*
3346-II	Uy, Mercedes (1983)	*M. bovoculi*
3A8	Uy, Treinta y Tres (2008)	*M. bovoculi*
771B	Uy, Paysandú (2009)	*M. bovoculi*
Art	Uy, Florida (1983)	*M. bovoculi*
BM3B	Uy, Cerro Largo (2005)	*M. bovoculi*
CANIA	Uy, Canelones (2009)	*M. bovoculi*
EV458	Uy, Maldonado (2006)	*M. bovoculi*
Fs343	USA, Kansas	*M. bovoculi*
Fs347	USA, Kansas	*M. bovoculi*
Fs467	USA, Kansas	*M. bovoculi*
LC010	Uy, Flores (2006)	*M. bovoculi*
LC2747	Uy, Flores (2006)	*M. bovoculi*
M1	Uy, Rocha (2009)	*M. bovoculi*
M3	Uy, Rocha (2009)	*M. bovoculi*
NIN1	Uy, Tacuarembó (2007)	*M. bovoculi*
NIN9	Uy, Tacuarembó (2007)	*M. bovoculi*
PRO	Uy, Canelones (2007)	*M. bovoculi*
SES	Uy, Salto (2004)	*M. bovoculi*
SJ03A	Uy, San José (2007)	*M. bovoculi*
TT3	Uy, Treinta y Tres (2007)	*M. bovoculi*

DNA amplification by PCR

Genomic DNA of the different isolates was used as a template for RAPD, ERIC and BOX-PCR analysis. For DNA extraction, bacterial isolates were grown in Brain Heart Infusion (BHI) incubated at 37 ºC for 24 h. Genomic DNA from each strain was obtained using a genomic DNA extraction kit (Sigma

Table 2. In vitro resistance of *M. bovis* and *M. bovoculi* isolates determined by agar disk diffusion.

Specie of *Moraxella*	Number of strains tested	% of isolates resistant to*												
		CX1	C30	N30	T30	PB300	SXT25	E15	TE30	NOR10	FD300	CR30	RA5	G300
M. bovis	22	14	0	2	2	2	6	0	6	2	3	0	0	7
M. bovoculi	25	3£	2	0	0	2	0£	2	0£	0	3	0	0	4
P-value		2.4x10^{-4}£	0.18	0.12	0.12	0.90	0.005 £	0.18	0.005 £	0.12	0.87			0.20

T: oxytetracycline (30 µg), G: sulfisoxazole (300 µg), FD: nitrofurantoin (300 µg), C30: chloramphenicol (30 µg), CX1: cloxacillin (1 µg), N30: neomycin, PB300: polymyxin B (300 µg), TE30: tetracycline (30 µg), SXT25: cotrimoxazole (25 µg), E15: erythromycin (15 µg), NOR10: norfoxacin (10 µg), CR30: cephalotin (30 µg), RA5: rifampicin (5 µg).

* Isolates were considered resistant when growth was not inhibited at all with the disks containing the drug.

£ Significant differences were observed between *M. bovis* and *M. bovoculi* ($P < 0.05$).

Aldrich, St. Louis, USA) according to the manufacturer's instructions.

The primers used in this study to amplify RAPD sequences were JWP1 (5'-GCACTGAAGTGACCAAGCGG-3') and JWOPA7 (5'-GAAACGGGTG-3') [15]. The amplification program included an initial step at 94 ºC for 3 min, followed by 43 cycles of 1 min at 94 ºC, 1 min at 36 ºC and 2 min at 72 ºC, with a final extension cycle at 72 ºC for 7 min. Primers used to amplify ERIC sequences were ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAGTGACTGGGGTGAGCG-3') while the primer sequences used for BOX-PCR was BOXA1R (5'-CTACCGCAAGGCGACGCTGAC-3') [16, 17]. The program used in both cases included an initial denaturation step at 94 ºC for 5 min, followed by 40 cycles at 94 ºC for 1 min, 40 ºC for 2 min and 65 ºC for 8 min, with a final extension cycle at 65 ºC for 16 min.

Reactions were performed using a DNA thermal cycler T1 (Biometra, Gottingen, Germany).

Eight µl of each PCR reaction mixture were analyzed by gel electrophoresis in a 0.8 % agarose gel and stained with ethidium bromide. A 1 kb DNA ladder (Fermentas Thermo Scientific, Waltham, USA) was used to determine molecular size. The gels were photographed under UV light to record the results.

Analysis of genetic diversity

The DNA fingerprints obtained by RAPD, ERIC and BOX-PCR reactions were firstly visually analyzed. A positive response (a score '1') was defined as the presence of a visible band of a given size, while a negative response (a score '0') was defined as the absence of any band of the same size. These scores were then merged in a Microsoft Excel spreadsheet and then inserted in the PAST Version 2.1 computer software for the construction of a dendogram using Paired Group to determine the relatedness of the *Moraxella* spp. isolates. Fingerprinting pattern types were considered to be distinct if they differed by more than one band.

Disk diffusion susceptibility

Antimicrobial resistance was assessed by the classic disk diffusion method following a slightly modified protocol [18]. Briefly, colonies of 24 h-old cultures on BHI agar containing 5 % sheep blood, were used to generate bacterial suspensions containing approximately 1.5 x 10^8 colony-forming units (cfu) per ml, which served as the inoculum for the agar disk diffusion. Mueller-Hinton agar supplemented with 5 % sheep blood was used for the agar disk diffusion procedure. Disks containing oxytetracycline (T, 30 µg), sulfisoxazole (G, 300 µg), nitrofurantoin (FD, 300 µg), chloramphenicol (C30, 30 µg), cloxacillin (CX1, 1 µg), neomycin (N30), polymyxin B (PB300, 300 µg), tetracycline (TE30, 30 µg), cotrimoxazole (SXT25, 25 µg), erythromycin (E15, 15 µg), norfoxacin (NOR10, 10 µg), cephalotin (CR30, 30 µg) and rifampicin (RA5, 5 µg), were used [19]. This assay was done in triplicate. Since there are not standardized criteria available to correlate inhibition zones around disks with susceptibility or

Figure 1. ERIC-PCR fingerprints of *Moraxella* spp. isolates used in the present study.

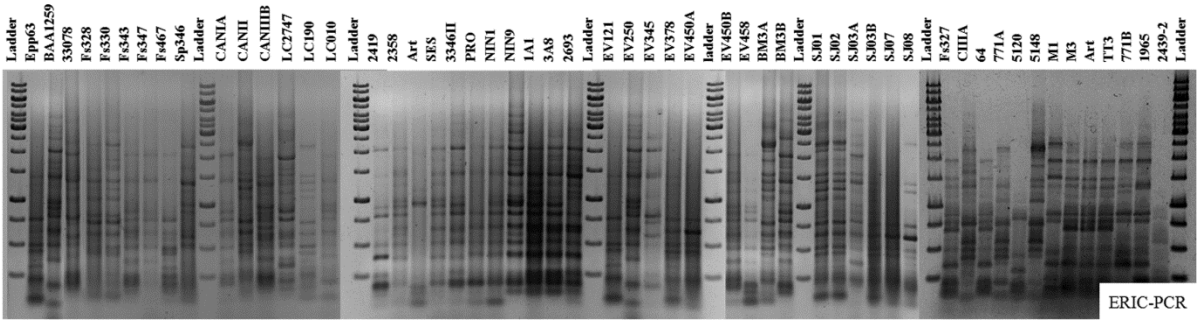
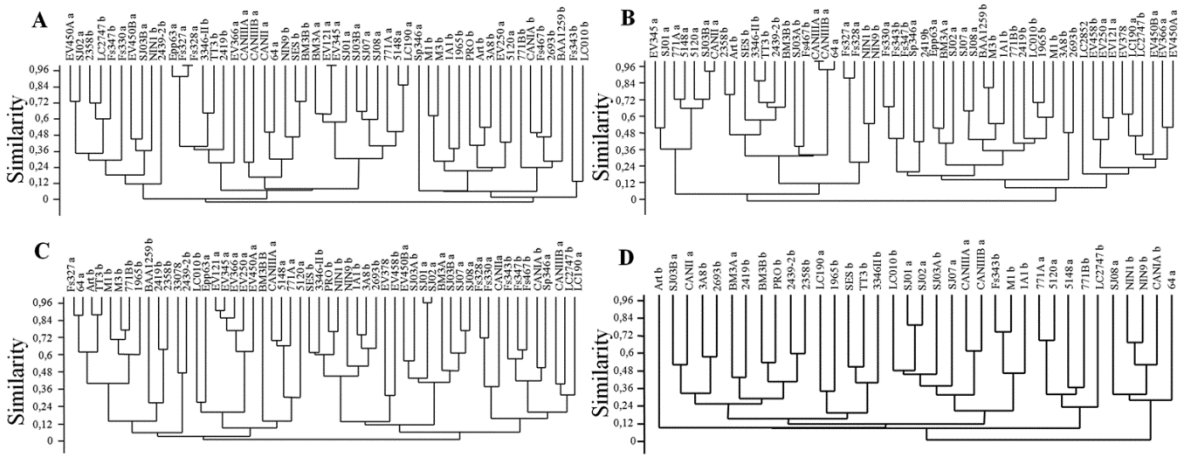


Figure 2. Dendrogram for the *M. bovis* and *M. bovoculi* isolates collection based on RAPD-PCR using primer JWOPA7 (A), RAPD-PCR using primer JWP1 (B), ERIC-PCR (C) and BOX-PCR (D) fingerprints.



resistance against antimicrobial drugs, *Moraxella* spp. strains were just considered resistant when growth was not at all inhibited.

To assess differences in antibiotic resistance associated with *M. bovis* and *M. bovoculi* the chi-square test was used.

Results

DNA-fingerprints of Moraxella spp. strains

The DNA fingerprints of *Moraxella* spp. isolates generated by RAPD, ERIC and BOX-PCR were complex. In general, fingerprint patterns obtained with RAPD, ERIC and BOX-PCR were very diverse among the collection of isolates. As a representative example, Figure 1 shows the ERIC-PCR fingerprinting patterns of the strains used in this study.

The number of polymorphic bands went from 1 to 12 bands in fingerprints obtained by RAPD, ranging between 5000 and 250 bp for primer JWP1 and 6000 to 250 pb for primer JWOPA7.

In the case of ERIC-PCR, the numbers of generated bands were among 3 and 20 ranging between about 250 bp and up to 5000 bp. Patterns of bands obtained by BOX-PCR 5 and 21 bands ranging from approximately 250 bp up to 6000 bp.

DNA fingerprint for *M. bovis* and *M. bovoculi* isolates generated with two RAPD primers (JWP1 and JWOPA7) showed different patterns between isolates except a few that had the same profile. In the case of primer JWOPA7 the isolates that showed the same profile were Fs327/Fs328 and EV121/EV345, obtained from the same IBK outbreak, respectively. When the diversity was analyzed using the JWP1 primer, CANIIIA and CANIIIB also obtained from the same IBK outbreak, showed an identical bands pattern.

SJ01 and SJ02 were the only isolates that showed the same ERIC-PCR bands patterns. When BOX-

PCR was used, the only isolates the showed the same pattern were EV345 and EV366.

Genetic diversity

The genetic relationships among electrophoretic types were evaluated with the unweighted-pair group method using average linkages (UPGMA) were represented by a dendogram. The discriminatory index was calculated using the Rho coefficient (Figure 2). The results showed that exist a high degree of genotypic diversity among *Moraxella* spp. isolates recovered from IBK cases.

Antimicrobial resistance

Resistance to different antimicrobial agents was assessed using the agar disk diffusion technique (Table 2). As it was stated above, an isolate was considered resistant when growth was not at all inhibited around the disks containing the drug.

Most of the isolates were non resistant to the majority of the antibiotics used. However, significant differences were observed between *M. bovis* and *M. bovoculi* in the pattern of resistance to cloxacillin, cotrimoxazole and tetracycline ($P = 6.6x10^{-4}$, $P = 0.008$ and $P = 0.008$, respectively). In all cases, *M. bovis* showed the higher number of resistant isolates compared to *M. bovoculi*.

Discussion

The diversity of a wide group of *Moraxella* spp. strains collected since 1983 to 2009 from different geographical locations of Uruguay was analyzed by DNA fingerprint. Resistance against a series of commonly used antibiotics was also assessed.

When the genetic diversity of the isolates was analyzed using RAPD-PCR, a high heterogeneity of bands patterns was observed. This result was similar to that obtained by Conceição et al. [14] when a limited collection of clinical *M. bovis* isolates obtained from Argentina, Brazil and Uruguay was analyzed by the same technique.

In this study, we found that both ERIC- and BOX- like sequences are present in the genomes of *M. bovis* and *M. bovoculi*. When our collection of isolates was analyzed by ERIC-PCR, results were similar to those obtained by Prieto et al. [4] who evaluated intra-specific diversity within a limited number of Argentinian *M. bovis* strains using ERIC-PCR and obtaining a very high degree of intraspecific heterogeneity.

In our study, BOX-PCR was used for the first time for the typing of *M. bovis* and *M. bovoculi* strains. Our results indicated that BOX-PCR allowed the typing of the different isolates although intraspecific heterogeneity was remarkably high so it was not possible to correlate bands profiles and characteristic of isolates. This result was similar to those obtained in the cases of RAPD- and ERIC-PCR.

In general, our results indicated that there were no obvious relationships between geographical and/or temporal origin or even species of isolates and fingerprint profiles. It can be stated that in general, RAPD, ERIC and BOX-PCR produced complex DNA fingerprint patterns for all of the isolates tested showing a high discriminatory power for the typing of *Moraxella* spp. isolates.

This is the first report that describes the genotypic diversity of *M. bovoculi* strains associated to IBK using DNA fingerprints. In a recent study, we were able to differentiate *M. bovis* and *M. bovoculi* using the sequences of ribosomal RNA genes [3]. However, in the present study we were unable to differentiate both species at a genetic level through fingerprint patterns.

Results obtained in this study showed that most of the strains were susceptible to the antibiotics used in the treatment of IBK [20]. Another interesting finding was that the antibiotic resistance profile differed between *M. bovis* and *M. bovoculi*. *M. bovis* strains presented a significant higher resistance against cloxacillin, cotrimoxazole and tetracycline compared to *M. bovoculi*. Although Angelos et al. [21] found a 3.5% of resistant *M. bovoculi* to oxytetracycline, in our study all the isolates were sensitive against this antibiotic. The difference observed in resistant pattern against antibiotics between *M. bovis* and *M. bovoculi* may have implications in the pathogenicity and the treatment of IBK.

Conclusions

Results of this study confirm the high genotypic diversity within *M. bovis* and *M. bovoculi* isolates, which can be a serious problem for the design of effective vaccines against IBK. Additional research is required to evaluate the diversity within both species of *Moraxella* and its relevance for the control of IBK.

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