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Brief Original Article

Virulence genes in *Moraxella* spp. isolates from infectious bovine keratoconjunctivitis cases

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Abstract

Introduction: Infectious bovine keratoconjunctivitis (IBK) is an important ocular disease which affects cattle worldwide. To advance towards IBK effective prevention and treatment strategies, it is important to define the distribution and genetic diversity of potential virulence factors present in *M. bovis* and *M. bovoculi*. The objective of this work was to identify and to analyze *Moraxella* spp. potential virulence factor genes in a collection of clinical isolates.

Methodology: The presence and diversity of virulence factors in a collection of *Moraxella* spp. strains isolated since 1983 to 2009 in Uruguay was analyzed by PCR using primers for partial amplification of *tolC*, *omp79*, *plb*, *fur* and *mbxA*. The selection criterion of these genes was based on the fact that they encode virulence factors which could be present and conserved within strains, an important issue for the development of vaccines.

Results: Differences in PCR amplification were observed within *tolC* (84%), *omp79* (80%), *plb* (76%) and *fur* (44%) in *M. bovis* strains, whereas *mbxA* was amplified in all *M. bovis* and *M. bovoculi* strains. Regarding genetic diversity, the *tolC* nucleotide sequences were the less diverse within all *M. bovis* and *mbxA* were the less diverse within all *M. bovis* and *M. bovoculi* strains.

Conclusions: PCR amplifications suggest the occurrence of differences between both *Moraxella* species, related to evaluated genes within *Moraxella* spp. strains and suggests that both species may have different pathogenic attributes. MbxA and the outer membrane protein TolC might be considered for future studies to develop new vaccines against IBK.

Key words: *Moraxella*; IBK; *tolC*; *mbxA*; vaccines.

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Introduction

Infectious bovine keratoconjunctivitis (IBK) is an important ocular disease in cattle worldwide [1]. Affected animals can exhibit corneal ulcers, corneal edema, photophobia, blepharospasm and lacrimation. Although several corneal ulcers associated with IBK heal with varying degrees of scarring, ocular damage may lead to permanent blindness [2]. It has been proved that an IBK infection condition has negative effects on animal welfare as well as having serious effects on animal production [3]. Although IBK is rarely fatal, the associated impaired vision, pain and stress result in decreased weight gain, decreased milk production, increased treatment costs and market depreciation [4,5].

The Gram negative bacterium *Moraxella bovis* has been considered the main etiological agent of IBK for a long time. However, more recently, other species belonging to the genus like *Moraxella bovoculi* have also been associated to IBK cases in USA, Brazil and Uruguay so far [1,6-8].

M. bovis exhibits several potential virulence factors that allow eye colonization and exert eye damage. Fimbriae (type IV pili) play a crucial role in adhesion and corneal colonization and two functionally distinct pili have been identified. The Q pilus is responsible for initial attachment while the I pilus allows local persistence and maintenance of an established infection [9]. It is thought that bacteria expressing Q pilus can convert into I pilus during the course of the disease due to a site-specific DNA inversion system [9].

M. bovis also produces a hemolysin which damages neutrophils that are recruited to the area of infection and can also release collagenolytic enzymes that lead to corneal liquefaction and ulceration [10]. On the other hand, nonhemolytic strains of *M. bovis* are considered as nonpathogenic [11]. Other potential virulence factors include phospholipases [12], outer

membrane proteins (OMPs), iron acquisition systems [13] and hydrolytic and proteolytic enzymes [14].

One of the main strategies to prevent IBK is vaccination. Currently, there are different available vaccines but they provide variable protection against clinical disease [15-16]. So far, commercial vaccines have been based on expression of fimbriae by *Moraxella* spp. cells, but a high antigenic diversity of these surface organelles has been observed [9,17-18].

The objective of this work was to identify and to characterize *Moraxella* spp. virulence factors distribution. Five genes encoding potential virulence factors (*tolC*, *omp79*, *plb*, *fur* and *mbxA*) were selected and amplified by PCR with specific primers. Selection criterion of these genes was based on the fact they encode antigens which may be located and conserved in the majority of the strains, an important issue for the development of vaccines to be used in prophylactic programs.

Methodology

Bacterial strains, media and growth conditions

Fifty four *Moraxella* spp. strains comprising forty five Uruguayan field isolates (21 *M. bovis* 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 detected in Kansas, USA, were evaluated. The latter were kindly provided by Dr. Brad Fenwick (Kansas State University, USA). Field isolates were obtained from several Uruguayan provinces between 1983 and 2009. All isolates had been previously characterized and extensively identified in our laboratory. All of them exhibited β-hemolytic activity when grown on blood agar plates [7,19].

DNA amplification by PCR

Genomic DNA of the different isolates was used as a template for PCR analysis. In this study, primers for virulence genes amplification were designed using previously published *M. bovis* genes sequences. Selected genes included *tolC* (encodes an OMP that plays a role in the movement of molecules across bacterial membranes and periplasm into the environment, GenBank accession number: AF205359.3), *omp79* (OMP of 79kDa, involved in iron acquisition, GenBank accession number: AB106521.1), *fur* (regulator gene involved in iron acquisition, GenBank accession number: AB079375.1), *plb* (encodes a lipase/phospholipase, GenBank accession number: AY032849.1) and *mbxA* (structual cytotoxic hemolysin, GenBank accession number: EF436235.1). Primers for *mbxA* amplification were designed according to previously reported sequences [20]; *tol*C, *omp79*, *plb* and *fur* primers were designed during this study. All primers were synthesized by IDT (USA). Primers sequences and PCR programs used in this study are described in Table 1.

For DNA extraction, bacterial isolates were grown in brain heart infusion (BHI) supplemented with 1.5% agar and incubated at 37ºC for 24 hours. Genomic DNA from each strain was obtained using a DNA extraction kit (GenElute Bacterial Genomic DNA Kit, Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. Reactions were performed using a DNA thermal cycler (T1, Biometra, Göttingen, Germany). Following PCR, 8 µl of the reaction mixture were analyzed by gel electrophoresis in a 0.8% agarose gel and stained with ethidium bromide. A 1 kb DNA ladder (Fermentas, Waltham, USA) was used to determine molecular size. The gels were photographed under UV light after electrophoresis to record results. DNA samples were considered negative for the presence of each gene after three negative independent PCR assays where the positive control

Table 1. Primers and reaction programs.

Gene	Primer		Reaction program	Reference
	Name	Sequence (5´-3´)		
*mbx*A	P-hemstart	ATHGAYTGGATHGCNCCNTTYGGNGAY	95ºC 1', 30 cycles of 95ºC 1', 56ºC 30'', 72ºC 2' and 72ºC for 5'	[21]
	NewBgeneup	ACCTTATCCATCACAACCTGAAAAAAC		
fur	Mbfur_fwd	GTTTTGGTATTTTAGATGG	94ºC 3', 30 cycle of 94ºC 1', 40ºC 1', 72ºC by 1' and 72ºC for 5'.	This work
	Mbfur_rev	AGTGCCTTATACACGTCT		
*tol*C	MbTolC_fwd	CTGCACAGCAACATTCTATTG		
	MbTolC_rev	TTTAGCGATTCACGCATC		
*omp*79	Mbomp79_fwd	GATTTGCTAAAACACACTGA		
	Mbomp79_rev	TGGCTTGTTCTGCTTTTG		
plb	Mbplb_fwd	CGCAAATCACCGACCATTT		
	Mbplb_rev	AAACATCAAGCGAACCCCAA		

(*M. bovis* ATCC Epp63) was correctly amplified.

Analysis of genetic diversity

tolC, *omp79*, *plb*, *fur* and *mbxA* sequences were compared using the MEGA software [22]. Phylogeny trees were generated using the Maximum Likelihood method and the Kimura two-tailed model [23].

Results

tolC, *omp79*, *plb*, and *fur* were amplified in variable proportions within the collection of *M. bovis* strains (84%, 80%, 76% and 44% of the total of *M. bovis* strains, respectively). However, no amplification products were observed in any case when *M. bovoculi* genomic DNA was used.

It is important to consider that primers used in PCR reactions were designed according to *M. bovis* available sequences.

Partial DNA sequences of *M. bovoculi tolC* and *fur* are known and published (GenBank: DQ155435.1 and AOMT01000028.1, respectively) although in this study these genes were not amplified. However, presence of *plb* and *omp79* has not been reported in *M. bovoculi* so far.

Confirming the differences detected between *M. bovis* and *M. bovoculi* PCR amplifications, published sequences of *M. bovoculi tolC* and *fur* grouped with *M. ovis* and *M. catarrhalis* sequences respectively, whereas *M. bovis* sequences grouped in different clusters (Figure1 a, c).

Regarding genetic diversity of the different genes, *tolC* nucleotide (Figure 1a) sequences were the least diverse within all *M. bovis* strains and *plb* sequences the most diverse (Figure 1d).

A different situation was observed when *mbxA* was amplified within the collection of *Moraxella* spp. strains. In this case, 100% of *M. bovis* and *M. bovoculi* strains showed *mbxA* amplification. This result was confirmed by hemolysis of ovine blood cells on Blood Agar plates, which showed that all strains were β-hemolytic. In addition, when nucleotide and translated *mbxA* sequences were analyzed, isolates of *Moraxella* spp. grouped according to the species they belonged (Figure 2 a, b).

Discussion

M. bovis has been considered the main aetiological agent of IBK for decades [24]. Recently an increasing number of studies have demonstrated the presence of *M. bovoculi* associated to IBK cases [6-8]. It has been established that this is the *Moraxella* spp. most prevalent and the most frequent species isolated from IBK cases [25], although its causal role in IBK is still under debate [1,18,26].

Figure 1. Phylogeny of *tolC* (a), *omp79* (b), *fur* (c) and *plb* (d) genes using the Maximum Likelihood method and the Kimura two-tailed model. Boostrap values are product of 500 replicates.

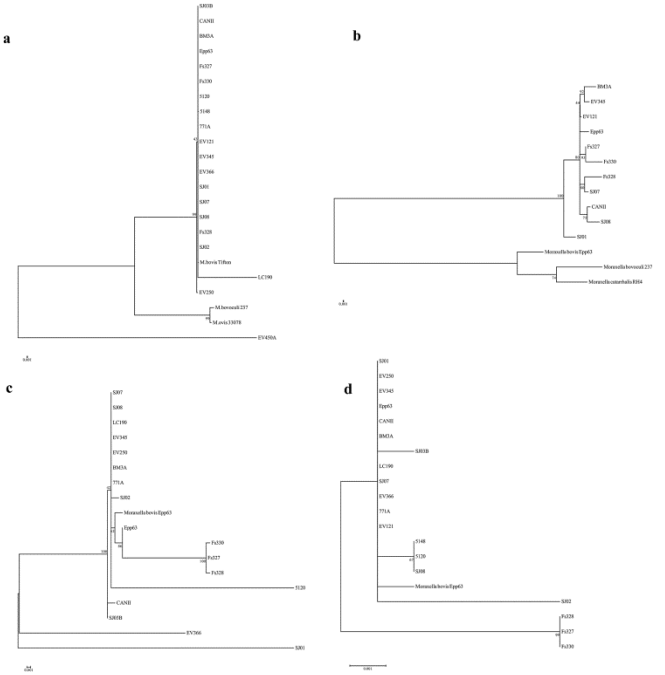
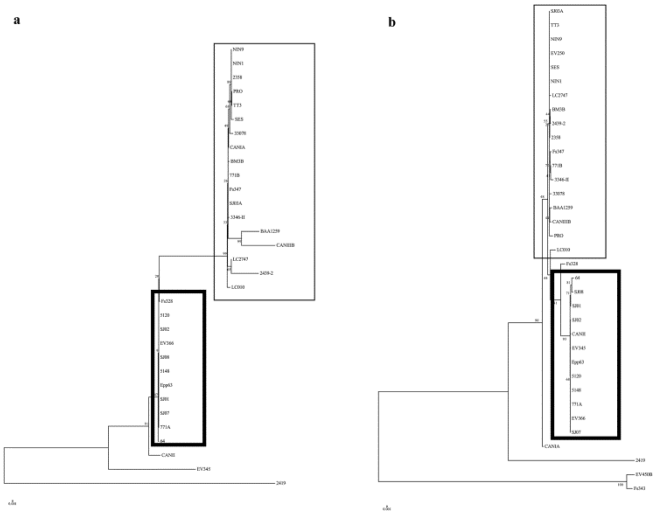


Figure 2. Phylogeny of 5`(a) and 3`(b) nucleotide sequences ends of *mbxA* gene using the Maximum Likelihood method and the Kimura two-tailed models. Boostrap values are product of 500 replicates. Dark squares: *M. bovis* strains. Light squares: *M. bovoculi* strains.



In this study, results of distribution and genetic diversity of different virulence genes between *M. bovis and M. bovoculi* suggest that both species have

different pathogenic attributes. This is in accordance with the recent *M. bovoculi* (ATCC BAA1259) genome sequencing, which demonstrated the occurrence of important differences in the pilin nucleotide sequence of *M. bovoculi* compared to *M. bovis* and *Moraxella lacunata*. Moreover, authors propose that lateral gene transfer to *M. bovoculi* led to clear differences with *M. bovis* (ATCC Epp63) and *Moraxella ovis* type strain [27].

Considering the analysis of the different genes, it is interesting to observe that *tolC* was amplified in a very high proportion of *M. bovis* isolates. In addition, sequences of this gene were rather conserved among the different *M. bovis* isolates. As it was explained above, *tolC* encodes for an OMP that has a role in molecules transport across bacterial membranes and periplasm, including MbxA hemolysin, and it has already been detected also in *M. bovoculi* [24]. Previous studies have evaluated the use of OMPs as candidates for the development of new vaccines, and have proposed the use of TolC for vaccines against other pathogens [28]. Therefore, we consider this OMP could be taken into account for the design of alternative vaccines, although further studies are needed.

Conversely, *mbxA* was amplified within the whole strains collection (*M. bovis* and *M. bovoculi*) when primers designed according to *M. bovis* sequences were used. Moreover, these results are in accordance with our previous phenotypic analyses which reported that all these strains were β-hemolytic [7]. According to these results and to previous reports, *Moraxella* spp. hemolysin could be considered a potential antigen for vaccines against IBK [20].

Vaccines have been generally based on *Moraxella* spp. fimbriae expression, but a high antigenic diversity of these surface organelles may limit the protection conferred [29].

The use of antigens other than fimbriae for IBK vaccines appears as a possible strategy for the design of suitable prophylactic practices. Besides, differences between *M. bovis* and *M. bovoculi* virulence genetic bases will have to be considered.

Findings reported in this study may provide useful information about local transmitted strains for the development of new vaccination strategies to prevent IBK.

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References

1. O'Connor AM, Shen HG, Wang C, Opriessnig T (2012) Descriptive epidemiology of *Moraxella bovis*, *Moraxella bovoculi* and *Moraxella ovis* in beef calves with naturally occurring infectious bovine keratoconjunctivitis (Pinkeye). Vet Microbiol 155: 374-380.
2. Chandler RL, Smith K, Turfrey BA (1985) Exposure of bovine cornea to different strains of *Moraxella bovis* and to other bacterial species *in vitro*. J Comp Pathol 95: 415-423.
3. Williams DL (2010) Welfare issues in farm animal ophthalmology. Vet Clin Food Anim 26: 427-435.
4. Thrift FA, Overfield JR (1974) Impact of pinkeye (infectious bovine kerato-conjunctivitis) on weaning and postweaning performance of Hereford calves. J Anim Sci 38: 1179-1184.
5. Slatter DH, Edwards ME, Hawkins CD, Wilcox GE (1982) A national survey of the occurrence of infectious bovine keratoconjunctivitis. Aust Vet J 59: 65-68.
6. Angelos JA, Spinks PQ, Ball LM, George LW (2007) *Moraxella bovoculi* sp. nov., isolated from calves with infectious bovine keratoconjunctivitis. Int J Syst Evol Microbiol 57: 789-795.
7. Sosa V, Zunino P (2012) Molecular and phenotypic analysis of *Moraxella* spp. associated to infectious bovine keratoconjunctivitis. Vet J 193: 595-597.
8. Libardoni F, Scherer CFC, Farias L, Vielmo A, Balzan C, de Vargas AC (2012) *Moraxella bovoculi* in cases of infectious bovine keratoconjunctivitis in Rio Grande do Sul, Brazil. Pesq Vet Bras 32: 743-746.
9. Ruehl WW, Marrs C, Beard MK, Shokooki V, Hinojoza JR, Banks S (1993) Q pili enhance the attachment of *Moraxella bovis* to bovine corneas *in vitro*. Mol Microbiol 7: 285-288.
10. Beard MK, Moore LJ (1994) Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of *Moraxella bovis*. Vet Microbiol 42: 15-33.
11. Postma GC, Carfagnini JC, Minatel L (2008) *Moraxella bovis* pathogenicity: An update. Comp Immunol Microbiol Infect Dis 29: 449-458.
12. Farn JL, Strugnell RA, Hoyne PA, Michalski WP, Tennent JM (2001) Molecular characterization of a secreted enzyme with phospholipase B activity from *Moraxella bovis*. J Bacteriol 183: 6717-6720.
13. Fenwick B, Rider M, Liang J, Brightman A (1996) Iron repressible outer membrane proteins of *Moraxella bovis* and demonstration of siderophore-like activity. Vet Microbiol 48: 315-324.
14. Frank SK, Gerber JD (1981) Hydrolytic enzymes of *Moraxella bovis*. J Clin Microbiol 13: 269-371.
15. Burns MJ, O´Connor AM (2008) Assessment of methodological quality and sources of variation in the magnitude of vaccine efficacy: A systematic review of studies from 1960 to 2005 reporting immunization with *Moraxella bovis* vaccines in young cattle. Vaccine 26: 144-152.
16. Funk L, O'Connor AM, Maroney M, Engelken T, Cooper VL, Kinyon J, Plummer P (2009) A randomized and blinded field trial to assess the efficacy of an autogenous vaccine to prevent naturally occurring infectious bovine keratoconjunctivis (IBK) in beef calves. Vaccine 27: 4585-4590.

17. Ruehl WW, Marrs C, Fernández R, Falkow S, Schoolnik GK (1988) Purification, characterization and pathogenicity of *Moraxella bovis* pili. J Exp Med 168: 983-1002.
18. Angelos JA (2010) *Moraxella bovoculi* and infectious bovine keratoconjunctivitis: cause or coincidence? Vet Clin North Am Food Anim Pract 26: 73-78.
19. Sosa V, Zunino P (2013) Diversity of *Moraxella* spp. strains recovered from infectious bovine keratoconjunctivitis cases in Uruguay. J Infect Dev Ctries 7: 819-824.
20. Angelos JA, Bonifacio RG, Ball LM, Hess JF (2007) Prevention of naturally occurring infectious bovine keratoconjunctivitis with a recombinant *Moraxella bovis* pilin-*Moraxella bovis* cytotoxin - ISCOM matrix adjuvanted vaccine. Vet Microbiol 125: 274-283.
21. Angelos JA, Ball LM, Hess JF (2007) Identification and characterization of complete RTX operons in *Moraxella bovoculi* and *Moraxella ovis*. Vet Microbiol 125: 73-79.
22. Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17: 1244-1245.
23. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120.
24. Rogers DG, Cheville NF, Pugh GW (1987) Conjunctival lesions caused by *Moraxella bovis* in gnotobiotic calves. Vet Pathol 24: 554-559.
25. Loy DJ, Brodersen BW (2014) *Moraxella* spp. isolated from field outbreaks of infectious bovine keratoconjunctivitis: a retrospective study of case submissions from 2010 to 2013. J Vet Diagn Invest 26: 761-768.
26. Gould S, Dewell R, Tofflemire K, Whitley RD, Millman ST, Opriessnig T, Rosenbusch R, Trujillo J, O'Connor AM (2013) Randomized blinded challenge study to assess association between *Moraxella bovoculi* and Infectious Bovine Keratoconjunctivitis in dairy calves. Vet Microbiol 164: 108-115.
27. Calcutt MJ, Foecking MF, Martin NT, Mhlanga-Mutangadura T, Reilly TJ (2014) Draft Genome Sequence of *Moraxella bovoculi* Strain 237T (ATCC BAA-1259T) isolated from a calf with Infectious Bovine Keratoconjunctivitis. Genome Announc 26: 1-2.
28. Yang TC, Ma XC, Liu F, Lin LR, Liu LL, Liu GL, Tong ML, Fu ZG, Zhou L (2012) Screening of the *Salmonella paratyphi* A CMCC 50973 strain outer membrane proteins for the identification of potential vaccine targets. Mol Med Rep 5: 78-83.
29. Lepper AW, Atwell JL, Lehrbach PR, Schwartzkoff CL, Egerton JR, Tennent JM (1995) The protective efficacy of cloned *Moraxella bovis* pili in monovalent and multivalent vaccine formulations against experimentally induced infectious bovine keratoconjunctivitis (IBK). Vet Microbiol 45: 129-138.

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