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 (structural cytotoxic hemolysin, GenBank accession number: EF436235.1). Primers for mbxA amplification were designed according to previously reported sequences [20]; tolC, 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 37°C 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

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<tr>
<th>Gene</th>
<th>Reference</th>
<th>Reaction program</th>
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<tr>
<td>mbxA</td>
<td>[21]</td>
<td>95°C 1’, 30 cycles of 95°C 1’, 56°C 30’, 72°C 2’ and 72°C for 5’</td>
</tr>
<tr>
<td>fur</td>
<td></td>
<td>94°C 3’, 30 cycle of 94°C 1’, 40°C 1’, 72°C by 1’ and 72°C for 5’</td>
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<tr>
<td>tolC</td>
<td></td>
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<td>omp79</td>
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<td>plb</td>
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Table 1. Primers and reaction programs.
(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 (Figure 1a, 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 2a, 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].

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**


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