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Short communication

Phylogenetic analysis and genetic diversity of 3′ region of *rtxA* gene from geographically diverse strains of *Moraxella bovis, Moraxella bovoculi* and *Moraxella ovis*

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ABSTRACT

The cytotoxin A (MbxA) is one of the main virulence factors of *Moraxella bovis* involved in the pathogenesis of infectious bovine keratoconjunctivitis (IBK). *Moraxella ovis* and *Moraxella bovoculi*, suspected to be associated with infectious keratitis in sheep and cattle respectively, also have a gene that encodes the cytotoxin A (*movA* and *mbvA*, respectively). The aim of this study was to determine the molecular sequence of the 3' region of the cytotoxin gene of *Moraxella* spp. strains isolated from clinical cases to establish phylogenetic and evolutionary comparisons. PCR amplification, nucleotide sequencing (nt) and amino acid (aa) sequence prediction were performed, followed by the sequences comparison, identity level calculation and selective pressure analysis. The phylogenetic reconstruction based on nt and aa sequences clearly differentiate *M. bovis* ($n = 15$), *M. bovoculi* ($n = 11$) and *M. ovis* ($n = 7$) and their respective reference strains. An alignment of 843 nt revealed high similarity within bacterial species (*MbxA* = 99.9% nt and aa; *MbvA* = 99.3% nt and 98.8% aa; *MovA* = 99.5% nt and 99.3% aa). The similarity of partial sequences (nt 1807–2649) of *MbxA* in relation to *MbvA* and *MovA* ranged from 76.3 to 78.5%; similarity between *MbvA* and *MovA* ranged from 95.7 to 97.5%. A negative selection on *mbvA* and *movA* sequences was revealed by the molecular evolution analysis. The phylogenetic analysis of *movA* and *mbvA* allowed different strains of *Moraxella* spp. to be grouped according to the period of isolation. Sequence analysis of cytotoxin may provide insights into genetic and evolutionary relationships and into the genetic/molecular basis of *Moraxella* spp.

1. Introduction

MbxA, a cytotoxin member of the RTX (repeats in the structural toxin) family of bacterial proteins, is thought to be a major *Moraxella bovis* virulence factor associated with infectious bovine keratoconjunctivitis (IBK) (Angelos et al., 2001). There are evidences that MbxA may play a regulatory role in the development and progression of infectious keratoconjunctivitis (IK) (Beard and Moore, 1994), moreover, vaccine trials have demonstrated that MbxA has immunogenic properties (Angelos et al., 2004; Angelos et al., 2007c).

Limited effectiveness of commercially available vaccines containing *M. bovis* and presence of other immunogenic microorganisms in IK lesions suggest that other *Moraxella* species may be also involved in the IK pathogenicity (Burns and O'Connor, 2008; Angelos, 2010). Although early investigations proposed *M. ovis*, bacterium involved in sheep eye disease, might be the case (Elad et al., 1988), more recent investigations isolated and identified *M. bovoculi* in cattle lesions (Angelos et al., 2007a; Libardoni et al., 2012; Sosa and Zunino, 2013).

Both *M. ovis* and *M. bovoculi* contain cytotoxin A encoding genes called *movA* and *mbvA*, respectively (Angelos et al., 2007b), as well as complete RTX operon, which was identified in pathogenic

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strains of *M. bovis* (Angelos et al., 2003). The 3′ region of cytotoxin A is responsible for the host cell membrane recognition (Frey, 2011).

The present study examined the 3′ region of the cytotoxin A gene of 35 *Moraxella* spp. strains isolated between 1986 and 2013 from cattle and sheep, in order to provide further insights about similarity levels and phylogenetic relationships between species of *M. bovis, M. bovoculi* and *M. ovis*.

2. Materials and methods

2.1. Bacterial characterization

The characterization analysis included 33 bacterial strains from 32 ocular secretion samples collected from cattle ($n = 25$) and sheep ($n = 7$) displaying clinical signs of IK. Samples were collected between 1986 and 2013 from 31 different outbreaks in farms located in 19 counties in southern Brazil, and one county in Argentina (Table 1). Bacterial strains were characterized using previously established criteria for Moraxella spp. (Angelos and Ball, 2007b).

2.2. DNA isolation, PCR amplification and sequencing

Strains were treated with proteinase K (20 mg/ml) for 60 min at 37 °C and DNA was isolated using CTAB-based (cetyltrimethyl ammonium bromide) method. Subsequently, PCR with primers targeting the 3′ region of the cytotoxin gene was performed. Primers were designed targeting the *mbxA* gene (F: 5′-GCAAAAGCTGGCAATGACGA-3′ and R: 5′-GTGCCATTGACCCAAC-TAGC-3′) of *M. bovis*, and *mbvA/movA* gene (F: 5′-AATGCTGGTGCTGGTAACGA-3′ and R: 5′-TGGTTGCAGGGTATTG-GAGC-3′) of *M. bovoculi* and *M. ovis*. Both PCR conditions were an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 50 s, 65 °C for 50 s, and 72 °C for 1 min.. PCR products were sequenced by ACTGene Molecular Analysis LTDA (Biotechnology Center, UFRGS, Porto Alegre, RS, Brazil).

Table 1
Moraxella bovoculi,*Moraxella ovis* and *Moraxella bovis* strains from IK outbreaks and the reference strains.

Strain	Classification	Origin	Year	Host	Alleles	GenBank ID
237^T	*M. bovoculi*	US	2002	Bovine	1bv	AOMT00000000
371	*M. bovoculi*	US	2002	Bovine	2bv	DQ155439
GR9	*M. bovoculi*	Br	1986	Bovine	3bv	KP410763
R2	*M. bovoculi*	Br	1989	Bovine	3bv	KP410764
R7	*M. bovoculi*	Br	1989	Bovine	3bv	KP410765
SB273	*M. bovoculi*	Br	1996	Bovine	4bv	KP410767
186V	*M. bovoculi*	Ar	1999	Bovine	1bv	KP410766
SB150a	*M. bovoculi*	Br	2002	Bovine	2bv	KP410768
SB163 N2	*M. bovoculi*	Br	2003	Bovine	2bv	KP410769
SB163 N5	*M. bovoculi*	Br	2003	Bovine	1bv	KP410770
SB296a	*M. bovoculi*	Br	2007	Ovine	5bv	KP410771
SB139	*M. bovoculi*	Br	2010	Bovine	6bv	KP410772
SB150b	*M. bovoculi*	Br	2010	Bovine	7bv	KP410773
ATCC33078	*M. ovis*	No	1960	Ovine	1ov	DQ155443
Nunes	*M. ovis*	Br	1998	Ovine	2ov	KP410774
SB567	*M. ovis*	Br	2005	Ovine	3ov	KP410775
SB296b	*M. ovis*	Br	2007	Ovine	4ov	KP410776
SB326	*M. ovis*	Br	2007	Ovine	5ov	KP410777
SB06	*M. ovis*	Br	2008	Ovine	6ov	KP410778
SB07a	*M. ovis*	Br	2008	Ovine	5ov	KP410779
SBP07b	*M. ovis*	Br	2013	Ovine	5ov	KP410780
Epp63	*M. bovis*	US	<1985	Bovine	1bx	EF436235
SFS9a	*M. bovis*	US	2007	Bovine	2bx	EF436243
SB24	*M. bovis*	Br	1990	Bovine	1bx	KP402166
SB234	*M. bovis*	Br	1993	Bovine	1bx	KP402167
PH02	*M. bovis*	Br	1995	Bovine	1bx	KP402168
PH05	*M. bovis*	Br	1996	Bovine	1bx	KP402169
PH06	*M. bovis*	Br	1997	Bovine	1bx	KP402170
PH07	*M. bovis*	Br	1998	Bovine	1bx	KP402171
PH08	*M. bovis*	Br	1998	Bovine	1bx	KP402172
PH09	*M. bovis*	Br	1999	Bovine	1bx	KP402174
SB548	*M. bovis*	Br	1999	Bovine	1bx	KP402173
PH10	*M. bovis*	Br	2000	Bovine	1bx	KP402175
Torres	*M. bovis*	Br	2000	Bovine	1bx	KP402176
SB246	*M. bovis*	Br	2004	Bovine	1bx	KP402177
SB21	*M. bovis*	Br	2008	Bovine	1bx	KP402178
SB207C21	*M. bovis*	Br	2009	Bovine	1bx	KP402179
SB111	*M. bovis*	Br	2012	Bovine	1bx	KP402180

US, United States of America; Br, Brazil; Ar Argentina; No, Norway.

2.3. Nucleotide sequencing and analysis

The quality of the DNA sequences were analyzed by GAP software implemented in Staden Package (Staden, 1996) and the overlap of the fragments of each sequence was used to perform a consensus sequence. Based on the nt sequence, the amino acid (aa) sequences corresponding to 3′ region of the cytotoxin A were translated in reading frame 1 on the direct strand by The Sequence Manipulation Suite software version 2 (http://www.bioinformatics.org/sms2/).

The sequences of translated aa were submitted to comparison using protein homology/analogy recognition engine (Phyre 2) server (Kelley and Sternberg, 2009). The alignment of the sequences (nt and deduced aa) were made using Clustal W software implemented in MEGA 6. The identity matrix was obtained by the BioEdit software and SWAAP 1.0.3 software (http://www.bacteriamuseum.org/SWAAP/SwaapPage.htm). The MODELTEST software was used to determine the best evolutionary model for substitution of nucleotides (http://www.hiv.lanl.gov) and MEGA 6 was used to determine the best evolutionary model for aa replacement in the analyzed group of sequences.

2.4. Phylogenetic analysis

The nt sequences of cytotoxin of *M. bovis* (GenBank ID EF436235), *M. bovoculi* (GenBank ID AOMT00000000), *M. ovis* (GenBank ID DQ155443), as well as the translations used as reference sequences were obtained from GenBank. Additionally, sequences of *mbxA* gene from *M. bovis* SFS9a strain (GenBank ID EF436243) and *mbvA* gene from *M. bovoculi* 371 strain (GenBank ID DQ155439) was used, as well as the 3' region of *LktA* gene from *Mannheimia haemolytica* (GenBank ID P0C085), which was applied as an outgroup.

2.5. Selective pressure analysis

The selective pressure analysis on cytotoxin A genes was determined using synonymous and nonsynonymous substitutions, and the distance between them was calculated based on the Nei–Gojobori method (Nei and Gojobori, 1986). The rates of dN/dS and the rates of transition and transversion were calculated based on Kimura 2-Parameter, via SNAP software (*synonymous and non-synonymous analysis program*) (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) and the DnaSP 5.10 software (*sequence polymorphism*) (Librado and Rozas, 2009). The DnaSP 5.10 software was also used to divide sequences in alleles according to the amount of alternative forms of the same gene from each species of *Moraxella* spp.. Alleles from *M. bovis* were named 'bx', *M. bovoculi* 'bv' and *M. ovis* 'ov'.

3. Results

The 33 bacterial strains were classified as: 15 *Moraxella bovis*, 11 *Moraxella bovoculi* and seven *Moraxella ovis* (Table 1).

3.1. Nucleotide sequencing and deduced amino acid sequence analyis

Cytotoxin-encoding gene from *M. bovis, M. bovoculi* and *M. ovis* strains were partially sequenced. The sequenced fragment covered