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Mannheimia varigena as the etiologic agent of lameness and coronary band lesion in cattle*

Mannheimia varigena como agente etiológico de claudicação e lesão na banda coronária em bovinos

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Abstract

Mannheimia varigena was identified as the etiologic agent of lameness and coronary band lesion in 30% of cattle in a farm located in Rio Grande do Sul, Brazil. Swab samples from the lesions were cultured in McConkey Agar and Blood Agar for microbiological identification. Culture growth was submitted to Gram staining and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) identification. Antimicrobial susceptibility test based on disc diffusion was performed for three antibiotics: ceftiofur, gentamicin and florfenicol. Furthermore, molecular characterization of 16S rDNA gene sequencing was performed and the data was used in a phylogenetic analysis. For that purpose, total DNA was extracted by thermo extraction directly from the bacterial colonies and the polymerase chain reaction (PCR) was performed. Gram-negative *Mannheimia varigena* strain LBV010/22 was identified as the causative of the lesions. The strain was susceptible to all antibiotics tested. The phylogenetic analysis demonstrated that the analyzed strain is closely related to *M. varigena* strains from pyelonephritis and respiratory tract. Overall, this is the first report of *M. varigena* as the causative agent of coronary band injury in bovine. Therefore, our findings show the importance of an accurate microbiological identification of infectious agent in lameness cases in order to prevent the occurrence and perform an appropriate treatment in the future.

Keywords: bovines, hoof, *Pasteurellacea*, unusual infection.

Resumo

Mannheimia varigena foi identificada como agente etiológico de claudicação e lesão de banda coronária em 30% dos bovinos de uma fazenda localizada no Rio Grande do Sul, Brasil. Amostras de *swab* das lesões foram cultivadas em Ágar McConkey e Ágar Sangue para identificação microbiológica. O crescimento da cultura foi submetido à coloração de Gram e identificação por Espectrometria de Massa de Ionização por Dessorção a Laser Assistida por Matriz (MALDI-TOF MS). O teste de suscetibilidade antimicrobiana baseado na difusão em disco foi realizado para três antibióticos: ceftiofur, gentamicina e florfenicol. Além disso, foi realizada a caracterização molecular do sequenciamento do gene 16S rDNA e o resultado utilizado para análise filogenética. Para tanto, o DNA total foi extraído por termoextração diretamente das colônias bacterianas e uma reação em cadeia da polimerase (PCR) foi realizada. Foi identificada como causadora das lesões a cepa gram-negativa de *Mannheimia varigena*_LBV010/22. Ela foi suscetível a todos os antibióticos testados. A análise filogenética demonstrou que a cepa analisada está intimamente relacionada às *M. varigena* presentes em pielonefrite e no trato respiratório. No geral, este é o primeiro relato de *M. varigena* como agente causador de lesão de banda coronária em bovinos. Portanto, nossos achados mostram a importância de uma identificação microbiológica precisa do agente infeccioso nos casos de claudicação, a fim de prevenir a ocorrência e realizar um tratamento adequado no futuro.

Palavras-chave: bovinos, casco, *Pasteurellacea*, infecção incomum.

Introduction

Lameness in cattle is a major concern due to the economic losses in bovine industry and animals' welfare issue, affecting longevity and productivity (Coetzee et al. 2017). The incidence of clinical lameness can exceed other clinical sign in many herds, although its detection remains a challenge (Shearer et al. 2012). The etiological cause of foot lesions that results in lameness can be either infectious or non-infectious. Non-infections causes such as white line disease, sole ulcer, sole hemorrhage, toe ulcer, corkscrew claw, horizontal fissure, vertical fissure, axial fissure, interdigital hyperplasia, and thin sole, are more commonly

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described in cattle (Correa-Valencia et al. 2019). Infectious causes are usually associated to digital dermatitis, interdigital dermatitis, and heel erosion (Cramer et al. 2009), with infectious agents as etiological agents.

Mannheimia genera is a Gram-negative coccobacilli bacterium that belongs to the *Pasteurellaceae* family. *Mannheimia* spp. virulence is related to the production of proteins with toxic activity such as the RTX toxin (Woolums 2013). The current literature demonstrated that *Mannheimia* species have become increasingly resistant to penicillins, tetracyclines, and sulfonamides (Woolums 2013). The main pathogenic species in *Mannheimia* genus is *Mannheimia haemolytica,* which is often associated to respiratory disease complex in bovine (Snyder and Credille 2020). In contrast, few reports about infections caused by *Mannheimia varigena* have been published so far. *Mannheimia varigena* have been previously described in respiratory infections and bacteremia in ruminants, and mastitis in cattle (Woolums 2013), meningitis in calf (Catry et al. 2004) and pyelonephritis in calf (Komatsu et al. 2019). Furthermore, *M. varigena* could be an opportunistic pathogen and it is isolated from cattle with shipping fever (Harhay et al. 2014). Therefore, the aim of the present case report was to describe an unusual case of lameness in cattle caused by a *M. varigena*.

Case Report

In a farm located in Rio Grande do Sul (30°04'20.1"S, 51°05'49.5"W), southern Brazil, 30 out of 100 bovines presented lameness with coronary band lesion for over 30 days. In February 2022, after numerous attempts to contain the illness, the veterinarian responsible for caring the animals have performed two sample collection, by swabs, from foot lesions of a calve and a cows from the same feedlot. The collected samples were sent to microbiological analysis in the Laboratory of Bacteriology of the Federal University of Rio Grande do Sul (LaBacVet).

Swab samples were cultured in Blood Agar Base supplemented with 5% sheep blood (Kasvi, São José do Pinhais, PR, Brazil) and MacConkey agar (Kasvi, São José do Pinhais, PR, Brazil), followed by incubation at 37 °C for 24 h in aerobic and microaerophilic conditions. After incubation in aerobic and microaerophilic atmosphere, in both samples, pure small growth of grayish pigmented colonies with hemolysis were observed on 5% sheep blood agar. Gram-stained revealed Gram-negative coccobacillus. No growth was observed on MacConkey agar. As morphological analysis and phenotypic tests demonstrated that the same bacteria were involved in both samples, a sample was selected for the following analyses.

The isolated bacterium was subjected to identification in Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), using Microflex LT instrument and MALDI Biotyper 3.1 software (Bruker Daltonik, Bremen, Germany). According to MALDI-TOF analysis, the strain was identified as *Mannheimia varigena* (score 1.881). We have named the strains as *Mannheimia varigena*_LBV010/22.

Antimicrobial susceptibility test was performed using Kirby & Bauer method (CLSI 2015). The antibiotics discs used were: ceftiofur (30µg), gentamicin (10µg), and florfenicol (30µg). The antimicrobial susceptibility test determined that the *M. varigena*_ LBV010/22 was susceptible to all antibiotics.

*Mannheimia varigena*_LBV010/22 was then subjected to a discriminatory identification based on 16S rDNA gene sequence analysis. For that purpose, total DNA was extracted by thermo extraction directly from the bacterial colonies. In summary, three colonies were resuspended in 50 l of ultrapure water and incubated at -20 °C for 30 min, followed by incubation at 100 °C for 7 min. Then, the sample was centrifuged for 3 min at 14,000 rpm, and the supernatant containing the DNA was transferred to a new microtube. The DNA was stored at -20 °C up to the following analysis.

For partial 16S rDNA amplification, prokaryotic universal oligonucleotides 27F (5' -AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5' -GGTTACCTTGTTACGACTT- 3') was used. The polymerase chain reaction (PCR) was performed in 25 µL volume, containing 0.5 U of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), 10 X reaction buffer, 1.5 mM of $MgCl_2$, 0.4 µM of each primer, 0.2 mM of deoxynucleotides triphosphate (dNTPs), and 10 ng of DNA template. Cycling conditions were as follows: 1 cycle at 94 ºC for 5 min followed by 35 cycles of 94 ºC for 30 s; 52 ºC for 20 s and 72 ºC for 2 min. The final extension step was at 72 ºC for 10 min. The negative control was prepared in parallel, differing from the other reactions by the absence of genomic DNA. Reaction products were analyzed in 1% agarose gel stain with Unisafe Dye (Uniscience, Miami Lakes, FL, USA).

The PCR product was purified using Invitrogen PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Both strands of the amplicon were sequenced by the automatic sequencer ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems Inc., Norwalk, CT, USA). After sequencing, DNA fragments were inspected, trimmed, and assembled (forward and reverse) on Geneious v.11.1.5 (Biomatters Ltd., Auckland, New Zealand). The generated consensus sequence was subjected to BlastN (NCBI database), which aligns with 99.3% of identity to *M. varigena* available sequences. *M. varigena*_LBV010/22 16S rDNA gene sequence was deposited in the GenBank database (available by accession number: ON109662).

For the phylogenetic analyses, 16S rDNA sequences of *M. varigena*_LBV010/22, and clinical strains of *M. varigena* and other *Mannheimia* spp. that are currently available on GenBank (March, 2022) were retrieved and used to build a phylogenetic analysis. In detail, the phylogenetic relationship of *M. varigena*_ LBV010/22 with *Mannheimia* spp. was built using 16 partial 16S rDNA sequences (Table 1).

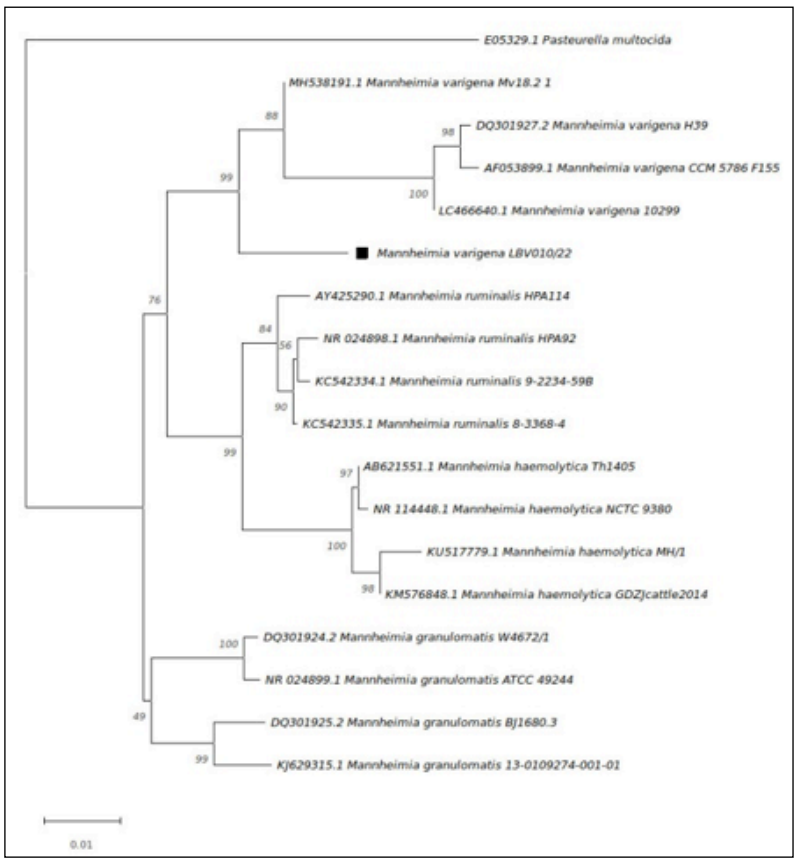
Nucleotide alignment was performed on MEGA v.11.0.9 (Tamura et al. 2021), where short sequences (< 1,300 bp) were excluded. Finally, a phylogenetic tree was built using the Neighbor-joining method, and evolutionary distances were calculated by Tajima-Nei model. The robustness of the hypothesis was tested in 1,000 non-parametric bootstrap analyses. According to the phylogenetic tree, *Mannheimia* spp. were clustered supporting the species identity and the homology (Figure 1). *M. varigena*_ LBV010/22 showed to be closely related to *M. varigena* strains from bovine pyelonephritis and respiratory tract.

Table 1: *Mannheimia* spp. 16S rDNA nucleotide sequence information.

Specie	Strain ID	Acession Number	Host	Source
*M. varigena**	LBV010/22	ON109662	Cattle	Coronary band lesion
M. varigena	Mv18.2	MH538191	Bovine	Respiratory tract
M. varigena	H39	DQ301927	NI	NI
M. varigena	CCM 5786	AF053899	NI	NI
M. varigena	10299	LC466640	Bovine	Pyelonephritis
M. ruminalis	HPA114	AY425290	NI	NI
M. ruminalis	HPA92	NR_024898	NI	NI
M. ruminalis	9-2234-59B	KC542334	Bighorn sheep	NI
M. ruminalis	8-3368-4	KC542335	Bighorn sheep	NI
M. haemolytica	Th1405	AB621551	*Bos taurus*	NI
M. haemolytica	NCTC 9380	NR_114448	NI	NI
M. haemolytica	MH/1	KU517779	*Ovis aries*	Nasal sample
M. haemolytica	GDZJcattle2014	KM576848	Cattle	NI
M. granulomatis	13-0109274-001-01	KJ629315	*Dama dama*	Tongue
M. granulomatis	W4672/1	DQ301924	NI	NI
M. granulomatis	BJ1680.3	DQ301925	NI	NI
M. granulomatis	ATCC 49244	NR_024899	NI	NI

NI: Not informed by authors. *: Strain of the present study. Data collection: March, 2022.

Figure 1: Phylogenetic relationship of *M. varigena*_LBV010/22 to other *Mannheimia* species



Neighbor-joining analysis constructed by MEGA v.11 software using the Tajima-Nei model. Bootstraps (1,000 replicates) values are indicated at the internal nodes. Black square: *M. varigena* strain of this present study (*M. varigena*_LBV010/22). *Pasteurella multocida* was the outgroup. The accession numbers of the 16S rDNA sequences were listed in Table 1.

Discussion

The present case report described a case of lameness and coronary band lesion in cattle caused by *M. varigena*. *Mannheimia varigena* had not been described as involved in hoof injuries in cattle until yet. The current diagnostic was based on the clinical history, microbiological characterization, MALDI-TOF identification, and 16S rDNA analysis. Altogether these results demonstrated that the use of molecular tools in veterinarian labs can contribute to increased sensitivity and specificity in diagnostics (Daniels 2013; Middleton et al. 2021).

Mannheimia spp. are commonly carried in the oropharyngeal region of susceptible host species. Infection is by inhalation, ingestion, or bites and scratch wounds and environmental contamination can contribute to indirect transmission (Woolums 2013). Moreover, feedlot management practices and therapeutic intervention could influence the behavior of the *M. varigena*, as occur in cases involving *M. haemolytica*. For example, carefully plan about when and how calves are weaned, sold and transported will have an impact on the prevalence (Rice et al. 2007).

In the present study, *M. varigena* strain_LBV010/22 showed to be susceptible to all tested antibiotics. Similarly, *Mannheimia* especies have also been described as susceptible to a variety of antimicrobials effective against gram-negative bacteria (Woolums 2013). However, *M. varigena* antimicrobial resistance could emerge depending on treatment, as occur with *M. haemolytica* isolates from cattle when they received three or more antimicrobial treatments (Magstadt et al. 2018).

The phylogenetic analysis demonstrated that there are two major clades in *Mannheimia* genera (Figure 1). *Mannheimia varigena* are in a different ramification confirming what previous study have already showed (Suástegui-Urquijo et al. 2015), which could indicate the probability of an older common ancestor and genetic variability among *M. varigena* strains. Since 1999 there were many changes in the taxonomy of the Pasteurellaceae family, including the emergence of the genera *Mannheimia* (Woolums 2013). The clusterization of the isolates from the same species, corroborating the current literature and taxonomic attributions. Interestingly, *M. varigena*_LBV010/22 was clustered in the clade that encompasses the other strains of the specie, but it was in another branch, indicating that *M. varigena*_LBV010/22 has phylogenetical differences from the others. This divergence may be due to the isolation site, since *M. varigena*_LBV010/22 is a hoof isolate not yet reported in the literature.

Conclusions

We have reported the first case of lameness and coronary band lesion caused by *M. varigena* in cattle. Modern methods such as MALDI-TOF MS and analysis of the 16S rDNA gene sequence allowed the correct identification of the etiologic agent of the disease. The present study demonstrated the importance of investigating infectious cases of lameness in cattle to provide the implementation of adequate management practices and treatment.

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