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COMMUNICATION

Nocardia nova identification in a transtracheal wash of a horse with recurrent airway obstruction[#]

Identificación de *Nocardia nova* en lavado transtraqueal de un caballo con obstrucción recurrente de las vías aéreas

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RESUMEN

Un equino con enfermedad pulmonar recurrente presentó disnea, secreción nasal purulenta bilateral y sonidos anormales de los pulmones. El cultivo microbiológico, citológico e identificación molecular (16S rRNA secuencias de genes) se realizó con material obtenido del lavado transtraqueal, lo que permitió la identificación de *Nocardia nova*, un agente asociado a las alteraciones respiratorias inusuales en caballos.

Palabras clave: equinos, nocardiosis, neumonía, obstrucción recurrente de las vías aéreas.

SUMMARY

A horse with recurrent airway disease was presented with dyspnea, mucopurulent bilateral nasal discharge and abnormal lung sounds. Microbiological culture, cytological examination and molecular identification (16S rRNA gene sequence) were performed with the transtracheal wash material and allowed the identification of *Nocardia nova,* an uncommon agent associated with equine respiratory abnormalities.

Key words: equine, nocardiosis, pneumonia, recurrent airway obstruction.

INTRODUCTION

The members of the *Nocardia* species are gram-positive, aerobic, soil saprophyte, opportunistic, facultative intracellular bacteria. Recently, an increased number of species of the genus *Nocardia* has been recognised and re-classified using molecular methods (Kiska *et al* 2002, Brown-Elliott *et al* 2006). Briefly, the genus *Nocardia* was separated into groups representing one or more species of *Nocardia*. One of these groups named as *N. asteroides* was previously defined biochemically as those isolates of *Nocardia* that do not decompose xanthine, tyrosine, and casein. With the increase in the number of species identified through new molecular techniques it became evident that phenotypic taxonomical classification had become obsolete (Brown-Elliott *et al* 2006). Currently the taxonomical classification of this genus is highly complex, comprised of approximately 50 species of medical and veterinary medical interest. In domestic animals the main species described in case reports were members of the former *N. asteroides* complex, which currently includes *N. asteroides*, *N. nova, N. abscessus*, *N. cyriacigeorgica*, *N. farcinica*, and *N. transvalensis* (Kiska *et al* 2002, Brown-Elliott *et al* 2006, Radostits *et al* 2007).

Nocardiosis is an uncommon infectious disease in humans and animals. In livestock, the most common clinical forms of nocardiosis are oral lesions, mastitis and pyogranulomatous pneumonia, which are mainly caused by *N. asteroides*, *N. nova*, *N. farcinica* and *N. brasiliensis* (Beaman and Sugar 1983, Radostits *et al* 2007).

It is important to note that some reports of Nocardiosis in horses antedated molecular testing and usually only phenotypic tests were performed. Identification of Nocardia species without molecular methods should be considered with caution.

Equine nocardiosis is an uncommon disease, and its clinical forms can be localised or disseminated. They are typically caused by *N. farcinica* and *N. nova*, and less

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frequently by *N. brasiliensis* (Menéndez *et al* 1997, Arguedas 2007, Brown-Elliott *et al* 2006). Transmission occurs by aerosol inhalation and the transcutaneous inoculation of contaminated soil or organic material (Beaman and Sugar 1983, Deem and Harrington 1980). There are only a few reports of equine nocardiosis, which have described pneumonia, cutaneous and subcutaneous pyogranulomas, mycetomas, fistulous tracts in the mandible, hepatitis, and abortion (Deem and Harrington 1980, Biberstein *et al* 1985, Bolon *et al* 1989, Hong *et al* 1993, Arguedas 2007, Fernandes *et al* 2011, Motta *et al* 2011).

The clinical signs associated with pulmonary nocardiosis in horses include intermittent cough, an inability to perform exercises, fever, mucopurulent nasal discharge, and increased respiratory movements and effort (Beaman and Sugar 1983, Biberstein *et al* 1985). The clinical disease in horses occurs mainly in immunosuppressed individuals (Biberstein *et al* 1985, Arguedas 2007), or is associated with chronic bronchopulmonary disease (Provost *et al* 1997, Brown-Elliott *et al* 2006). The prognosis for horses with pulmonary or disseminated manifestations caused by genus Nocardia is uncertain or poor, due to the development of pyogranulomatous lesions, and resistance of the microorganism to conventional antimicrobials (Deem and Harrington 1980, Beaman and Sugar 1983, Biberstein *et al* 1985, Arguedas 2007, Erol *et al* 2012).

MATERIAL AND METHODS

CASE DESCRIPTION

The present report describes uncommon *N. nova* isolation in a horse with recurrent airway obstruction (RAO) diagnosed in the Veterinary Hospital of Univesidade Estadual Julio de Mesquita Filho-UNESP, Botucatu, São Paulo, Brazil. Species identification was based on phenotypic and molecular methods with collaboration of Medical Mycology Research Centre, Chiba, Japan.

A 13-year-old male horse of Mangalarga breed was admitted to the Veterinary Hospital with an one-month history of weariness, dyspnea, cough and bilateral mucopurulent nasal discharge. The animal was raised in a poorly ventilated stall and was let out in a coast-cross paddock for four hours each day. The horse received alfalfa hay and rations based on wheat bran and wheat corn.

RESULTS AND DISCUSSION

A clinical examination showed expiratory dyspnea, cough and mucopurulent nasal discharge. Bilateral crackling areas in the cranial lung lobes and bilateral wheezing in almost the entire lung were observed during auscultation. An ultrasound examination revealed irregular pleural thickening of areas measuring 0.22 to 0.32 cm located between the 11thand 13th intercostal spaces. A percutaneous transtracheal wash was performed, and cytological and microbiological exams were carried out. The cytology examination showed neutrophils (91%), macrophages (2%); including giant and epithelioid cells, and rare eosinophils and lymphocytes (7%). Gram staining of the sample revealed rod-shaped to coccoid forms and gram-positive branching filaments, suggestive of *Nocardia*. A modified Kinyoun method showed partially acid-fast organisms. In the last decades, traditionally the species identification of *Noacardia* have been based on phenotypic methods including growth characteristics, use of different substrates and antimicrobial susceptibility profile. However, diagnosis based exclusively on phenotypic methods is insufficient to distinguish some species of *Nocardia*. More recent studies in domestic animals and humans have revealed that speciation of *Nocardia* require confirmation using molecular methods, including analysis of 16S rRNA gene, 65-kDa heat shock protein gene (*hsp65*), essential secretory protein A (*secA1*), gyrase B (*gyrB*) or DNA-DNA hybridisation. The molecular methods have showed a reliable and rapid means of speciation, and provided a number of taxonomic changes as well inclusion of new species of *Nocardia* (Baio *et al* 2013, Condas *et al* 2013).

The tracheobronchial fluid was plated on defibrinated sheep blood agar and Sabouraud agar in aerobic conditions at 37ºC. After 48 hours, white, dry, cerebriform, adhered colonies with a powdery appearance were observed. Gram-stained smears revealed characteristic gram-positive, branching, filamentous organisms that were partially acid fast bacilli suggestive of genus *Nocardia*.

Although the broth microdilution method is the current CLSI recommended method for susceptibility testing, an *in vitro* antimicrobial disk diffusion susceptibility test was performed due to lack of availability of the CLSI preferred method in our laboratory (CSLI 2011). The inhibition zones were interpreted following methods and standards of Bauer *et al* (1966), Wallace (1988) and Ambaye *et al* (1997). The antimicrobials selected were the most frequently used in the treatment of large animals in Brazil: amikacin (30µg), amoxicillin/clavulanate (20/10µg), ceftiofur (30µg), cefoperazone sodium (75µg), ceftriaxone (30µg), clarithromycin (15g), gentamicin (10µg) and sulfamethoxazole-trimethoprim (25µg). The agar disk diffusion presented zones of inhibition to amoxicillin/clavulanate, amikacin, ceftiofur, cefoperazone, ceftriaxone, clarithromycin, gentamicin and sulfamethoxazole/trimethoprim (table 1). Note that ceftiofur and cefoperazone–cephalosporins applied in large animal treatment–were not submitted to such a comparison Bauer *et al* (1966), Wallace (1988) and Ambaye *et al* (1997).

A nearly complete 16S rRNA gene (rDNA) was sequenced as described previously for *Nocardia* spp. (Kageyama *et al* 2004). Briefly, genomic DNA was extracted from the strain, and the 16S rRNA was amplified using prokaryotic 16S rDNA universal primer pairs 8F and 691R, 520F and 1100R, and 926F and 1542R (Thermal Cycler TaKaRa Bio Inc., Japan). The DNA sequences were determined with an automatic sequence analyzer

Table 1. Antimicrobial standard inhibition zone diameter and growth inhibition zone diameter of *N. nova* isolated from horse with RAO (UNESP, Botucatu/SP).

Diámetro estándar de la zona de inhibición antimicrobiana y diámetro de zona de crecimiento de *N. nova* aislada de un caballo con ORVA (UNESP, Botucatu/SP).

Antimicrobial	Zone diameter (mm)[a]			N. nova inhibition zone (mm)
	R	I	S	
Amikacin	≤14	15-16	≥17	>30
Amoxicillin/clavulanate	≤13	14-17	≤13	>30
Cefoperazone	≤15	16-20	≥21	>30
Ceftiofur	≤17	18-20	≥21	>30
Ceftriaxone	≤13	14-20	≥21	>30
Cefuroxime	≤14	15-17	≥18	>30
Clarithromycin	≤14	15-17	≥18	>30
Gentamicin	≤10	11-14	≥15	>30
Sulfamethoxazole/trimethoprim	≤10	11-15	≥16	>30

[a] Zone of inhibition diameter (mm) by disk diffusion method susceptibility interpretative guidelines based on Ambaye et al. (1997) and Bauer *et al* (1966). In the table: R= resistant, I= intermediate, S= susceptible.

(ABI Prism 3130; Applied Biosystems) using a dye terminator cycle sequencing kit (Applied Biosystems). The sequence of the 16S rRNA gene was compared against database sequences (DDBJ/GenBank/EMBL) using BLAST. Phylogenetic trees were built using the neighbor-joining method. A sequencing analysis of the 16S rRNA segments identified the organism as *Nocardia nova*, based on its 100% sequence (1494bp) similarity to the reference sequence (GenBank accession # AB671775.1, strain IFM 11292). Also, the similarity to other *Nocardia* species was calculated using the link www.bacterio.cict.fr/n/nocardia.html,in which *N. nova* (IFM 11292) / (*N. nova* AF430028) showed 99.9%, *N. nova* (IFM 11292) / *N. africana* (AF430054) was 98.3% similar; *N. nova* (IFM 11292) / *N. elegans* (AJ854057) was 98.1% similar; *N. nova* (IFM 11292) / *N. kruczakiae* (AY441974) was 98.1%; and *N. nova* (IFM 11292) / *N. veterana* (AF430055) was 98.0% similar.

A bronchodilator (clenbuterol, 1.1mcg/kg, PO, BID) and sulfadoxina potentiated by trimethoprim (20.0 mg/kg, IV, SID) were used for 30 days. Good handling practices were also recommended in place to minimize respiratory signs associated with recurrent airway obstruction. The animal showed a good response to the treatment, and the clinical signs went into remission after one month.

Recurrent airway obstruction is characterised by a hypersensitivity reaction to inhaled allergens that can affect genetically predisposed horses confined for long periods in stalls without proper ventilation and fed hay or other products with excessive chaff (Woods *et al* 1993, Ainsworth *et al* 2003, Radostits *et al* 2007, Gerber *et al* 2009). The exposure of susceptible horses to hay and straw containing dust can initiate inflammation in the lower airways (Fairbairn *et al* 1993). Tracheal aspirates contain a large number of immune cells, particularly neutrophils, macrophages and lymphocytes (Hoffman 1999, Mair and Derksen 2000). In the current report, recurrent airway obstruction was diagnosed due to the respiratory clinical signs (Snapper 1986, Mair and Derksen 2000), and may predispose the horse to *N. nova* infection. Although human asthma and recurrent airway obstruction in horses do not share the same characteristics, the former is considered one of the main factors predisposing humans to pulmonary nocardiosis (Beaman and Beaman 1994).

In humans, chronic obstructive pulmonary disease (COPD), neoplastic disease and human immunodeficiency virus (HIV) infection were the most frequent predisposing factors of nocardiosis (Menéndez *et al* 1997). Likewise, equine nocardiosis was also described in animals with immunosuppression by pituitary hyperadrenocorticism secondary to adrenocorticotropic hormone (ACTH) secreting pituitary tumors, and in cases of Arabian foal immunodeficiency (Biberstein *et al* 1985). The horse evaluated in our study did not show any clinical signs that could suggest pituitary hyperadrenocorticism or immunosuppression. In fact, the absence of differential diagnosis was corroborated by the good response to the prescribed therapy based only in bronchodilator and antimicrobial drugs.

N. nova affects several species, such as birds, wild mammals, and domestic species including cattle, dogs and cats (Confer *et al* 1981, Bacciarini *et al* 1999, Malik *et al* 2006, Ribeiro *et al* 2008). A tracheobronchial lavage allowed cytological identification of a fungal or actinomycete organism. The microbiological culture and molecular identification of this microorganism reinforce the importance of using methods association to increase the confirmation of equine nocardiosis (Fernandes *et al* 2000, Arguedas 2007). Furthermore, with the advent of molecular analyses, an identification method based on the almost complete 16S rRNA gene (1491bp) represents

a decreased turn-around time, improved accuracy, and taxonomical meaningfulness (Roth *et al* 2003).

Previous reports of equine nocardiosis have classified isolates as belongin to the former *Nocardia asteroides* complex (Biberstein *et al* 1985) and *Nocardia brasiliensis* (Deem and Harrington 1980) based on phenotypic criteria again prior to the molecular era of identification. In a report by Biberstein *et al* (1985), *N. asteroids* was detected in 16 horses, but a genotypic identification of the isolates was not performed, suggesting that *N. nova* could have been associated with some of the cases.

Successful nocardiosis therapy in equines is obtained with antimicrobial drug therapy and surgical drainage (Arguedas 2007). However, a few drugs reach therapeutic concentrations within the pyogranulomatous focus induced by nocardial infections. The intracellular location of the pathogen makes the therapeutic efficacy of conventional drugs and clinical signs of remission more difficult to determine (Deem and Harrington 1980, Biberstein *et al* 1985, Brown-Elliott *et al* 2006, Arguedas 2007).

The appropriate identification of *N. nova* by molecular techniques and *in vitro* susceptibility tests allows the selection of antimicrobials with good activity against the particular infecting species (Malik *et al* 2006). *N. nova* is generally susceptible *in vitro* to sulfonamides, tetracyclines, macrolides, carbapenems, cephalosporins 3rd generation injectables and ampicillin, but it is resistant to amoxicillin/clavulanate (Ambaye *et al* 1997, Brown-Elliott *et al* 2006, Cercenado *et al* 2007). Other studies with agar disk diffusion, which antedate the current CLSI guidelines, have shown good correlation to most of the drugs tested such as amoxicillin clavulanate, amikacin, ceftriaxone, clarithromycin and sulfamethoxazole/thrimetoprim (Saubolle and Sussland 2003, Ambaye *et al* 1997). These drugs, alone and in combination, must be administered for long periods in infected animals (Edwards 2006, Malik *et al* 2006). In the present report, therapeutic success was obtained using measures to control the recurrent airway obstruction and a 30-day treatment course of sulfonamide (Deem and Harrington 1980, Workman *et al* 1998). Clarithromycin is a possible alternative therapeutic option since *N. nova* is usually susceptible *in vitro* to this antibiotic (Brown-Elliott *et al* 2006) and have comproved efficacy in pulmonary treatment of rhodococcosis in horses (Giguère *et al* 2004). Unfortunately, other antimicrobials such as imipenem and linezolid, used in humans, have a presentation that does not favor their administration in horses or are extremely expensive to be applied in animal treatment. The absence of lung abscess or purulent content in the thoracic cavity as evidenced by ultrasound may have contributed to the good therapeutic response.

The adequate handling of the patient (RAO control) and the correct antimicrobial therapy contributed to a good therapeutic response, with the remission of clinical signs and performance improvement. Despite the presence of Nocardia in the samples, we cannot state that this organism was the solely responsible for the aetiopathogenesis of the clinical picture i.e., this finding may simply reflect to presence of the organism on inhaled material. However considering that this was the only agent identified in the transtracheal wash we suggest that if Nocardia was unrecognized and untreated, the outcome could be fatal.

This report describes the unusual identification of *N. nova* in transtracheal wash of a horse with chronic airway obstruction, and highlights the value of the molecular techniques as useful tools for the identification of opportunist pathogens.

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