Veterinary Dermatology

Vet Dermatol 2016; **27**: 188–e47 DOI: 10.1111/vde.12313

Antibacterial effect of *N*-acetylcysteine on common canine otitis externa isolates

Elizabeth R. May*, Katherine A. Conklin* and David A. Bemis†

*Departments of Small Animal Clinical Sciences, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA
†Biomedical and Diagnostic Sciences, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA

Correspondence: Elizabeth R. May, Department of Small Animal Clinical Sciences, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA. E-mail: ermay@utk.edu

Background – *N*-Acetylcysteine (NAC) has the potential to be a useful therapeutic agent for the treatment of otitis externa due to its antimicrobial and mucolytic properties, as well as its ability to disrupt bacterial biofilm.

Hypothesis/Objectives – To determine the antibacterial activity of NAC against common bacterial isolates associated with canine otitis externa.

Animals – Twenty two isolates from canine clinical cases of otitis externa were identified and tested, including five *Staphylococcus pseudintermedius*, six *Pseudomonas aeruginosa*, five *Corynebacterium* spp. and six β-*haemolytic Streptococcus* spp. isolates.

Methods – Each isolate was grown on blood agar for 24 h and transferred to Mueller Hinton Broth (MHB) to achieve a final concentration of 5×10^5 CFU/mL. NAC was diluted in MHB to a starting concentration of 160 mg/mL and serial two-fold microdilution assays were performed in triplicate with negative controls for all isolates tested. Concentrations of NAC tested ranged from 0.125 to 80 mg/mL. A 50 μL volume of bacterial suspension was used to inoculate each well.

Results – The minimum inhibitory concentration (MIC) of NAC for all isolates tested ranged from 5 to 20 mg/mL.

Conclusions and Clinical Relevance – *N*-Acetylcysteine inhibits clinically relevant and drug resistant bacteria *in vitro*, and has potential for use as a novel agent for treatment of otitis externa.

Introduction

Canine otitis externa is often a chronic and recurrent problem with limited options for treatment. It is estimated to occur frequently in the population that seeks veterinary care, being listed as the second most common condition for US canine health insurance claims.[1] Underlying allergic conditions, such as atopic dermatitis or cutaneous adverse reactions to food, contribute to the development of primary inflammation, with secondary bacterial otitis as a complicating, perpetuating factor. Bacterial pathogens frequently associated with canine otitis externa include *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa*, β-*haemolytic Streptococcus* spp. and *Proteus* spp. *Corynebacterium* spp. has also been recovered and is typically cultured in association with other bacterial species.[2]

Commercially available products with documented efficacy against commonly encountered otitis pathogens are available; however, they contain antibacterials from a limited number of drug classes. In addition, these topical treatments often contain potentially ototoxic agents, such as aminoglycosides, that can cause temporary or even permanent hearing loss in veterinary patients.[3,4] With limited choices available, it is desirable to identify novel, safe treatment options that are effective against organisms associated with canine otitis externa.

N-Acetylcysteine (NAC) is a readily available and commonly used mucolytic agent with both antibacterial and antioxidant capabilities.[5] It is utilized therapeutically in veterinary medicine for the treatment of acetaminophen toxicity, corneal ulcers and is nebulized for the adjunctive treatment of lower respiratory tract disease. In addition, it has been reported to be otoprotective and can prevent chemotherapy associated hearing loss.[6] Because of the established safety profile and other benefits described for this compound, NAC could potentially be of great benefit when applied topically for the treatment of canine otitis externa. The purpose of this study was to evaluate the antibacterial effect of NAC on common canine otitis externa bacterial isolates *in vitro*.

Materials and methods

Twenty two isolates from canine clinical cases of otitis externa evaluated by the Dermatology service and submitted to the Department of Biomedical and Diagnostic Sciences, University of Tennessee, for culture and susceptibility testing during the month of June 2014 were included. Speciation was by standard methods employed in the laboratory and antimicrobial susceptibility tests followed general guidelines for microbroth dilution testing established by the Clinical and Laboratory Standards Institute.[7] Five *Staphylococcus pseudintermedius*, six

Accepted 11 March 2016
Sources of funding: Supported with funding from the Department of Small Animal Clinical Sciences, University of Tennessee College of Veterinary Medicine.
Conflict of interest: None declared
Presented at the 29th Annual North American Veterinary Dermatology Forum (2015), Nashville, TN, USA. *Vet Dermatol* 2015; 26: 159 (abstract).

Pseudomonas aeruginosa, five *Corynebacterium* spp. and six β-*haemolytic Streptococcus* spp. isolates were evaluated.

Preliminary testing was performed on reference isolates which were also included as controls when testing clinical isolates. American Type Culture Collection (ATCC) control strain *S. aureus* 29213 and ATCC control strain *P. aeruginosa* 27853 served as the control strains and were included. Each isolate was inoculated onto Columbia Agar (BBL, BD Diagnostic Systems; Sparks, MD, USA) with 5% sheep blood (Hemostat Laboratories; Dixon, CA, USA) and incubated for 24 hours at 35°C. Isolates were transferred to Mueller Hinton Broth (MHB) for a final inoculum concentration of 5×10^5 CFU/mL.[7]

A commercially available 20% NAC solution was obtained from APP Pharmaceuticals (Schaumburg, IL, USA) and diluted in MHB to a starting concentration of 160 mg/mL. For comparison, pharmaceutical grade NAC was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was diluted in MHB to a starting concentration of 160 mg/mL. Both NAC formulations underwent serial two-fold microdilution assays,[7] with concentrations of NAC tested ranging from 0.125 to 80 mg/mL. A 50 μL volume of bacterial suspension was added to each well of a Polystyrene 96 well plate (Fisher Scientific, 12-565-500; Hanover Park, IL, USA) and each assay was performed in triplicate for each bacterial isolate. Concentrations tested were based upon previous work demonstrating that NAC, at a concentration of 80 mg/mL, was consistently bactericidal against meticillin-sensitive and -resistant *Staphylococcus aureus, S. epidermidis,* vancomycin-resistant *Enterococcus faecalis, Pseudomonas aeruginosa, Enterobacter cloacae* and *Klebsiella pneumoniae*.[8] Growth controls containing untreated bacteria were included for all isolates tested, including the control strains. The minimum inhibitory concentration (MIC) was defined as the lowest concentration with complete growth inhibition, as determined visually. The highest MIC value for each triplicate run was reported as the representative MIC for each organism tested, in order to reflect a concentration predicted to consistently inhibit growth.

Results

Consistent bacterial growth was evident in all control samples, as expected. The NAC minimum inhibitory concentration for all isolates tested ranged from 5 to 20 mg/mL (Table 1). Seven of the 22 isolates tested (31.8%) had NAC MICs of 5 mg/mL or less, 95.5% of the isolates tested had NAC MICs of 10 mg/mL or less, and all isolates had NAC MICs of 20 mg/mL or less. (Table 2).

Table 1. Range of minimum inhibitory concentrations (MIC) of *N*-Acetylcysteine for each bacterial species tested

Quality control	MIC range
Staphylococcus aureus (ATCC 29213)	10 mg/mL
Pseudomonas aeruginosa (ATCC 27853)	10 mg/mL
Clinical isolates	
Staphylococcus pseudintermedius ($n = 5$)	5–10 mg/mL
Pseudomonas aeruginosa ($n = 6$)	5–20 mg/mL
Corynebacterium spp. ($n = 5$)	5–10 mg/mL
β-haemolytic *Streptococcus* ssp. ($n = 6$)	5–10 mg/mL

Table 2. Distribution of *N*-Acetylcysteine minimum inhibitory concentrations among tested bacteria

N-Acetylcysteine concentration tested Organism tested	5 mg/mL	10 mg/mL	20 mg/mL
Staphylococcus pseudintermedius $n = 5$	1*	5	5
Pseudomonas aeruginosa $n = 6$	1	5	6
Corynebacterium spp. $n = 5$	2	5	5
β-*haemolytic Streptococcus* spp. $n = 6$	3	6	6

*Number of isolates with MICs equal to or less than the concentration tested.

Discussion

The findings of this *in vitro* study indicate that NAC has inhibitory activity against common pathogens associated with canine otitis externa. This is in agreement with previous studies demonstrating inhibition of both Gram-positive and Gram-negative bacteria, with the growth of *P. aeruginosa* inhibited at low concentrations.[5] We found that inhibitory activity occurred most frequently at 10 mg/mL of NAC, a concentration that is approximately 5% of the concentration of the commercially available product already in clinical use (Mucomyst, 20%, 200 mg/mL NAC, Bristol-Myers Squibb; Princeton, NJ, USA). This concentration would be expected to be safe and well tolerated clinically. Middle ear mucosal inflammation has been reported at concentrations greater than 20 mg/mL,[9] and external auditory canal and middle ear inflammation, resulting in conductive hearing loss, have been reported at a concentration of 40 mg/mL.[10] Concentrations of 20 mg/mL were not associated with an inflammatory response;[9] therefore, the results reported in our study are expected to be inhibitory without being inflammatory.

None of the isolates tested in our study were resistant to inhibition by NAC at the concentrations tested. The inhibitory activity of NAC against bacterial pathogens is known to be both inoculum size and dose dependent;[5] however, the precise mechanism of action is unknown. There are several proposed mechanisms for the antibacterial properties of NAC, focusing predominantly on the inhibition of amino acid utilization in bacterial cells, such as competing with cysteine, or possibly interaction between proteins normally found in the bacterial cell and the sulfhydryl group of NAC.[11,12]

Minimum inhibitory concentration values varied slightly among the bacterial species as well as among the individual isolates examined, which was not unexpected. With the exception of the control reference strains, that were shown to be within acceptable QC range, antimicrobial susceptibility profiles of the organisms included were not evaluated prior to inclusion in the study, thus different strains may possess traits that require increased concentration of NAC to inhibit growth. Despite the slight MIC concentration variability, growth inhibition was either similar or within one dilution for all bacterial isolates tested.

Biofilm formation produced by *P. aeruginosa* has been associated with respiratory tract infections that are chronic and difficult to treat in humans.[13] *N*-Acetylcysteine has been shown to contribute to the detachment of biofilms associated with *P. aeruginosa*[11] and is used clinically as a mucolytic agent. Although it is currently unknown whether or not biofilm formation contributes to treatment failure in cases of canine otitis, the formation of biofilm has been documented in association with isolates of *P. aeruginosa* from dogs with otitis externa and was reported in 40% of the isolates tested in one study.[14]

If biofilm formation is an obstacle to treatment, NAC could potentially be utilized clinically to either prevent or detach biofilms, allowing for a more successful treatment outcome via access to the bacteria. This would be expected to result in reduction of the MIC value of the pathogenic organism.

As a mucolytic and antioxidant agent with the ability to disrupt the formation of bacterial biofilm, the use of NAC is of interest in veterinary medicine. The results reported here provide information that could translate into potential new treatment options for canine otitis externa. *N*-Acetylcysteine actively inhibits clinically relevant and drug-resistant bacteria *in vitro,* and has potential for use as a novel agent for treatment of otitis externa.

Acknowledgment

The authors thank Mary Jean Bryant and Rebekah Jones for technical assistance.

References

1. Top ten most common medical conditions for dogs and cats. Veterinary Pet Insurance 2015(USA). http://press.petinsurance.-com/pressroom/448.aspx#primaryNav. Accessed 22/03/2016.
2. Miller WH, Griffin CE, Campbell KL. Diseases of eyelids, claws, anal sacs, and ears. *Muller and Kirk's Small Animal Dermatology*, 7th edition. St. Louis, MO: Elsevier, 2013; 724–773.
3. Forge A, Schacht J. Aminoglycoside antibiotics. *Audiol Neurootol* 2000; 5: 3–223.
4. Oishi N, Talaska AE, Schacht J. Ototoxicity in dogs and cats. *Vet Clin North Am Small Anim Pract* 2012; 42: 1259–1271.
5. Parry MF, Neu HC. Effect of *N*-Acetylcysteine on antibiotic activity and bacterial growth *in vitro*. *J Clin Microbiol* 1977; 5: 58–61.
6. Feghali JG, Liu W, Van De Water TR. L-N-Acetyl-Cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity. *Laryngoscope* 2001; 111: 1147–1155.
7. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standards – 4th edition. CLSI document VET01-A4. Wayne, PA: CLSI; 2013.
8. Aslam S, Darouiche RO. Role of antibiofilm-antimicrobial agents in control of device-related infections. *Int J Artif Organs* 2011; 34: 752–758.
9. Choe WT, Chinosornvatana N, Chang KW. Prevention of cisplatin ototoxicity using transtympanic *N*-Acetylcysteine and lactate. *Otol Neurotol* 2004; 25: 910–915.
10. Saliba I, Fouad EF, Ouelette V et al. Are intratympanic injections of N-acetylcysteine and methylprednisolone protective against cisplatin induced ototoxicity? *J Otolaryngol Head Neck Surg* 2010; 39: 236–243.
11. Zhao T, Liu Y. N-acetylcysteine inhibits biofilms produced by *Pseudomonas aeruginosa*. *BMC Microbiol* 2010; 10: 140.
12. Moon JH, Jang EY, Shim KS et al. *In vitro* effects of *N*-acetyl cysteine alone and in combination with antibiotics on *Prevotella intermedia*. *J Microbiol* 2015; 53: 321–329.
13. Moreau-Marquis S, Stanton BA, O'Toole G. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. A short review. *Pulm Pharmacol Ther* 2008; 21: 595–599.
14. Pye CC, Yu AA, Weese JS. Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vitro*. *Vet Dermatol* 2013; 24: 446–449.

Résumé

Contexte – La N-Acétylcystéine (NAC) a le potentiel d'être un agent thérapeutique utile dans le traitement des otites externes en raison de ses propriétés antimicrobiennes et mucolytiques ainsi que ses capacités d'altérer le biofilm bactérien.

Hypothèses/Objectifs – Déterminer l'activité antibactérienne du NAC contre les souches bactériennes fréquemment associées aux otites externes canines.

Sujets – Vingt-deux souches d'otites externes canines ont été identifiées et testées, incluant cinq souches de *Staphylococcus pseudintermedius*, six *Pseudomonas aeruginosa*, cinq *Corynebacterium* spp. et six β-*haemolytic Streptococcus* spp.

Méthodes – Chaque souche a été mise en culture sur blood-agar pendant 24h et transférée sur MHB (Mueller Hinton broth) pour atteindre une concentration finale de 5 9 105 CFU/mL. NAC a été dilué dans MHB à une concentration initiale de 160 mg/mL et des tests en série de double microdilution ont été réalisés en triple exemplaire avec des contrôles négatifs pour tous les isolats testés. Les concentrations de NAC testées étaient de 0.125 à 80 mg/mL. Un volume de suspension bactérienne de 50 lL a été utilisé pour inoculé chaque isolat.

Résultats – La concentration minimale inhibitrice (MIC) de NAC pour tous les isolats testés allaient de 5 à 20 mg/ml.

Conclusions et importance clinique – La N-Acétylcystéine inhibe les bactéries cliniquement pertinentes et les bactéries résistantes *in vitro* et a le potentiel d'être utilisée comme un nouvel agent de traitement des otites externes.

Resumen

Introducción – la N-acetil cisteína (NAC) tiene el potencial de ser un agente terapéutico útil para el tratamiento de la otitis externa debido a sus propiedades antimicrobiana es y mucolíticas, así como la habilidad de distorsionar las películas bacterianas.

Hipótesis/Objetivos – determinar la actividad antibacteriana de NAC frente a aislados bacterianos comunes asociados con la otitis canina externa

Animales – 22 aislados de casos clínicos caninos de otitis externa se identificaron y probaron, incluyendo cinco de *Staphylococcus pseudintermedius*, seis de *Pseudomonas aeruginosa*, cinco de *Corynebacterium spp.* y seis de *Streptococcus* beta hemolítico.

Métodos – cada aislado se creció en agar de sangre durante 24 horas y se transfirió a un caldo de Mueller-Hinton (MHB) para obtener una concentración final de 5,9 × 10^5 CFU/mL. NAC se diluyó en MHB a una concentración inicial de 160 mg/mL y se realizaron ensayos con micro diluciones dobladas por triplicado con controles negativos para todos los aislados probados. Las concentraciones de NAC probadas variaron entre 0,125 a 80 mg/mL. Un volumen de 50 μl de suspensión bacteriana se utilizó para inocular cada pocillo.
Resultados – la concentración mínima inhibitoria (MIC) de N AC para los aislados probados varió entre 5 a 20 mg/mL.
Conclusión e importancia clínica – la N-acetil- cisteína inhibe bacterias clínicamente relevantes y resistentes a otros fármacos *in vitro*, y tiene el potencial de ser utilizado como un agente novedoso para el tratamiento de la otitis externa canina.

Zusammenfassung
Hintergrund – *N*-Acetylcystein (NAC) hat aufgrund seiner antimikrobiellen und mucolytischen Eigenschaften Potential als sinnvoller therapeutischer Wirkstoff für die Behandlung einer Otitis externa, sowie die Fähigkeit einen bakteriellen Biofilm zu zerstören.
Hypothese/Ziele – Die Bestimmung der antibakteriellen Wirksamkeit von NAC gegenüber gängigen bakteriellen Isolaten im Zusammenhang mit Otitis externa beim Hund.
Tiere – Zweiundzwanzig Isolate von klinischen Fällen von Hunden mit Otitis externa wurden identifiziert und untersucht. Die Isolate bestanden aus fünf *Staphylococcus pseudintermedius*, sechs *Pseudomonas aeruginosa*, fünf *Corynebacterium* spp. und sechs β-*hämolytischen Streptococcus* spp. Isolaten.
Methoden – Jedes Isolat wurde auf Blutagar für 24h angezüchtet und auf Mueller Hinton Bouillon (MHB) transferiert um eine Endkonzentration von 5 9 105 CFU/ml zu erreichen. NAC wurde in MHB zu einer Startkonzentration von 160 mg/ml verdünnt und im Microverdünnungsassay wurde eine Serie von jeweils zweifacher Verdünnung mit den Proben im Triplikat mit Negativkontrollen für alle getesteten Isolate durchgeführt. Die getesteten Konzentrationen von NAC reichten von 0,125 bis 80 mg/ml. Ein 50 Il Volumen einer bakteriellen Suspension wurde zur Inokulation eines jeden Röhrchens verwendet.
Ergebnisse – Die minimale inhibitorische Konzentration (MIC) von NAC für alle Isolate reichte von 5 bis 20 mg/ml.
Schlussfolgerungen und klinische Bedeutung – *N*-Acetylcystein hemmt klinisch relevante und Medikamenten-resistente Bakterien *in vitro*, und hat Potential als neuer Wirkstoff für die Behandlung einer Otitis externa.

要約
背景 — N−アセチルシステイン(NAC)はその抗菌性および粘液溶解性だけでなく、細菌のバイオフィルムの破壊能力によって、耳炎のための治療薬となりうる。
仮説/目的 — イヌの外耳炎に関連する一般的な分離細菌に対するNACの抗菌活性を究明すること。
供与動物 — イヌの外耳炎の臨床症例から、5つのStaphylococcus pseudintermedius、6つのPseudomonas aeruginosa 、5つのCorynebacterium spp.ならびに6つのb-溶血性 Streptococcus spp.を含む22の分離株を同定し検査した。
方法 — それぞれの分離株を24時間血液寒天培地で培養し、5 9 105 CFU/mLの最終濃度を得るためにミューラーヒントン培地(MHB)に継代した。検査した全ての分離株に対して、NACはMHBで160 mg/mLの開始濃度で希釈し、系列2倍微量希釈分析と陰性コントロールの3つの検査を一組として分析を行った。検査したNAC濃度は0.125から80 mg/mLの範囲であった。それぞれのウェルを培養するために50IL量の細菌希釈液を使用した。
結果 — 検査したすべての分離株に対するNACの最小阻止濃度(MIC)は5〜30mg/mLの範囲であった。
結論および臨床的な関連性 — N-アセチルシステインは、in vitroで臨床的に関連する細菌や薬物耐性菌を抑制し、さらに外耳炎の治療のための新規物質として使用できる可能性がある。

摘要
背景 — N-乙酰半胱氨酸(NAC)具有抗菌性、粘液溶解力以及破坏菌膜的能力,因此可做为外耳炎的治疗药物。
假设/目的 — 确定NAC对犬外耳炎常见菌的抗菌活性。
动物 — 鉴定和检测22个犬外耳炎病例的分离菌株,包括五株假中间型葡萄球菌、六株假单胞菌、五株棒状杆菌以及六株b-溶血性链球菌。
方法 — 每株细菌均在血琼脂中培养24h,并转移到米勒赫顿肉汤(MHB)中,使得最后的浓度为5 9 105 CFU/mL。NAC在MHB中稀释,起始浓度为160 mg/mL,在阴性对照和试验组应用连续双倍微稀释法。试验NAC浓度为0.125到80 mg/mL。每组接种50 IL细菌悬浮液。
结果 — NAC对所有试验菌株最小抑菌浓度(MIC)为5到20 mg/mL。
总结与临床意义 — N-乙酰半胱氨酸在体外实验中可抑制临床相关且耐药的细菌,有做为外耳炎治疗用药的潜力。