ORIGINAL ARTICLE

Antimicrobial susceptibility profile of soil isolates of *Nocardia asteroides* from Kuwait

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Objectives To find the antimicrobial susceptibility profile of 42 soil isolates of *Nocardia asteroides* against 14 antimicrobial agents representing β-lactams, aminoglycosides, ciprofloxacin, minocycline, erythromycin and third generation cephalosporins.

Methods The antimicrobial susceptibility was determined by the disk diffusion method using Mueller–Hinton agar medium. A homogeneous suspension giving an inoculum of 10^6–10^8 CFU/mL was used to streak the plates. The zone of inhibition was read after 36–48 h of incubation at 37 °C.

Results All the soil isolates of *N. asteroides* were susceptible to amikacin, imipenem and tobramycin. Susceptibility to cephalosporins was quite variable; 86% of the isolates were susceptible to cefotaxime, 57% to ceftriaxone and 40% to cefamandole. Fifty-seven per cent of the isolates showed intermediate susceptibility to cefamandole, 33% to ceftriaxone and 5% to cefotaxime. Ninety-three per cent of the isolates were resistant to sulfamethoxazole alone or in combination with trimethoprim.

Conclusions The study reports a wide variation in the antimicrobial susceptibility profile of soil isolates of *N. asteroides* originating from a single geographical area. Of interest is the finding that over 90% of *N. asteroides* isolates were resistant to sulfamethoxazole without any previous exposure to this drug. This may have serious therapeutic implications as sulphonamides or the combination of trimethoprim–sulfamethoxazole is the therapy of choice for nocardiosis. Demonstration of resistance to β-lactam antibiotics may be attributed to the presence of β-lactamases which was detectable in >90% of the soil strains of *N. asteroides*. The study underscores the importance of antimicrobial susceptibility testing for clinical isolates of *Nocardia* since individual strains show considerable differences in their susceptibility patterns necessitating therapeutic adjustments.

Keywords *Nocardia asteroides,* susceptibility testing, sulphonamide resistance, nocardiae in soil, nocardia in Kuwait, β-lactamases

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INTRODUCTION

Among the actinomycetes, members of the genus *Nocardia* are, with the exception of mycobacteria, the most commonly implicated pathogens in human disease, mostly as opportunists [1]. The majority of infections are pulmonary in origin and the organisms can disseminate through the hematogenous route to any other organ/site [2,3]. With the introduction of sulphonamides as the drug of choice for nocardiosis, the fatality rate has been reduced substantially [4]. However, instances of in vitro resistance and poor patient response to treatment, including relapses, have been reported in recent years [3–5]. This has not only necessitated the use of alternative therapeutic regimens but also underscored the importance of *in vitro* susceptibility testing of nocardia species to improve the therapeutic outcome [3–7]. Moreover, the antimicrobial susceptibility pattern has also been used as a basis for presumptive identification of some of the nocardia species [8–10]. In this paper, we present data on the antimicrobial susceptibility of 42 soil isolates of *Nocardia asteroides* originating from a single geographical area.

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MATERIALS AND METHODS

Bacterial strains and inoculum preparation

Strains of *N. asteroides* included in the study were isolated from soil samples collected from different localities in Kuwait [11].

Their identity was confirmed by standard biochemical tests including their susceptibility to tobramycin and cefamandole to exclude *Nocardia farcinica* [10,12]. Each test isolate was checked for its purity and several colonies were emulsified into 50 mL of Mueller–Hinton broth (Sanofi Diagnostics Pasteur, Marnes-la-Conquette, France). The inoculated flasks were incubated at 37 °C for 24–48 h on a rotary shaker. Sterile glass beads (5 mm in diameter) were added to each flask to minimize the formation of clumps of nocardial growth during cultivation. Most of the test strains yielded a uniform suspension of growth. The growth so obtained was diluted 1 : 10 so as to give an approximate concentration of 10^6–10^8 CFU/mL in accordance with the recommendations of the National Committee of Clinical Laboratory Standards (NCCLS) [13]. Reference strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *N. asteroides* ATCC 19247 were used as controls to monitor the antimicrobial disk susceptibility test [13].

Antimicrobial susceptibility testing

The soil isolates of *N. asteroides* were tested for their susceptibility to 14 antimicrobial agents by the disk diffusion method [13]. The inoculum as prepared above, was spread with a cotton swab on the surface of a square Petri dish (measuring 120 mm on each side) containing 50 mL of Mueller–Hinton agar (MHA). Filter paper disks containing the NCCLS recommended concentrations of the antibiotics were used. Only four antimicrobials were tested on each plate. The plates were incubated at 37 °C after which the diameter of the clear zone of inhibition including the size of the 6 mm disk was measured in millimetres and recorded at 24 and 36–48 h. Occasionally, readings were taken after 72 h for some strains in order to confirm the results previously obtained.

Detection of β-lactamase activity

The β-lactamase activity of the isolates was determined by Intralactam (ETo1) strips (Mast Diagnostics, Merseyside, United Kingdom) following the directions of the manufacturer. These strips had been impregnated with benzyl penicillin and bromocresol purple in appropriate concentrations [14] and thus were different from the nitrocefin (chromogenic cephalosporin) method. Briefly, the strips were moistened with sterile distilled water and growth from several well-isolated colonies from MHA plates was smeared over them. The readings for color change from purple to yellow were taken up to 1 h and recorded as positive (+) and negative (−).

Interpretation of susceptibility results

The response of each *N. asteroides* isolate to the antimicrobial agent was graded as susceptible, intermediate susceptible and resistant on the basis of the criteria recommended by the Antibiogram Committee of the French Society for Microbiology [15].

RESULTS

Antimicrobial susceptibility of soil isolates

The activity of the 14 antimicrobial agents against 42 isolates of *N. asteroides* is presented in Table 1. All the soil isolates of *N. asteroides* were susceptible to amikacin, imipenem and tobramycin. With the exception of imipenem, susceptibility to other β-lactams was quite variable; 86% of the isolates were susceptible to cefotaxime, 57% to ceftriaxone, 41% to cefamandole, 21% to ampicillin and 5% to carbenicillin. Fifty-seven per cent of isolates showed intermediate susceptibility to cefamandole, 33% to ceftriaxone and 5% to cefotaxime. Ninety-three per cent of the isolates were resistant to sulfamethoxazole alone or in combination with trimethoprim (TMP-SMX). The other antimicrobials which showed low activity against *N. asteroides* strains included erythromycin (93% resistant), ciprofloxacin (86%) and minocycline (55%).

β-lactamase activity

All the isolates demonstrated β-lactamase activity by Intralactam strips. Twenty-eight strains showed positive reaction (+) within 5 min and 10 isolates showed a positive reaction gradually over a period of 1 h. The four isolates that were initially negative for β-lactamase activity became positive when they were subcultured on MHA plates with amoxicillin-clavulanic disks and colonies taken from near the zone of inhibition were retested for β-lactamase activity.

DISCUSSION

The present study has two noteworthy features. Firstly, it reports the antimicrobial susceptibility profile of 42 soil isolates of *N. asteroides* from Kuwait, which is by far the largest number of strains investigated from a saprobic source. Secondly, it reports an unusually high prevalence of resistance to sulfamethoxazole which is the drug of choice for the treatment of nocardiosis [6,7]. The presence of *in vitro* resistance to sulfamethoxazole in over 90% of naturally occurring isolates of *N. asteroides* may have serious therapeutic implications as the infection is acquired exogenously from soil [7]. Furthermore, these isolates were not previously exposed to sulfamethoxazole or to any other folate pathway antagonists thus excluding the possibility of development of acquired resistance. However, to what extent *in vitro* susceptibility data can be depended upon in predicting therapeutic benefit is a controversial issue. Perhaps

Table 1 Summary of antimicrobial susceptibility results of soil isolates of *N. asteroides*

Antimicrobial agents	Disk potency	No. of isolates (%) in susceptibility range		
		Susceptible	Intermediate	Resistant
Ampicillin	10 μg	9 (21.4)**	14 (33.3)	19 (45.2)
Carbenicillin	10 μg	2 (4.8)	1 (2.4)	39 (92.9)
Ciprofloxacin	15 μg	5 (11.9)	1 (2.4)	36 (85.7)
Ceftriaxone	30 μg	24 (57.1)	14 (33.3)	4 (9.5)
Cefotaxime	30 μg	36 (85.7)	2 (4.8)	4 (9.5)
Imipenem	10 μg	42 (100)	0	0
Gentamicin	10 μg	13 (31)	21 (50)	8 (19.1)
Amikacin	30 μg	42 (100)	0	0
Minocycline	30 μg	13 (31)	6 (14.2)	23 (54.8)
Sulfamethoxazole	25 μg	3 (7.1)	0	39 (92.9)
TMP-SMX	1.25 + 23.75 μg	3 (7.1)	0	39 (92.9)
Erythromycin	15 μg	0	3 (7.1)	39 (92.9)
Tobramycin	10 μg	42 (100)	0	0
Cefamandole	30 μg	17 (40.5)	24 (57.1)	1 (2.4)

the state of the immune status of the host is crucial in determining the final outcome of the therapy.

A comparison of published reports on antimicrobial susceptibility profiles of *N. asteroides* revealed considerable differences. These differences were particularly more marked with respect to sulfamethoxazole and β-lactam antibiotics (Table 2) [16–19]. Here it may be mentioned that most of the published data on antimicrobial susceptibility of *Nocardia* are based on clinical isolates received in reference laboratories from diverse geographical regions. Some of these referral isolates may have originated from patients who were unresponsive to or intolerant of therapy. Moreover, the possibility that some of the strains of *N. asteroides* included in early studies might have belonged to *N. farcinica* or *N. nova*, cannot be excluded.

It is difficult to compare our *in vitro* susceptibility results with data reported by other investigators because of different in

Table 2 Comparative data on antimicrobial susceptibility profile of the soil and clinical isolates of *Nocardia asteroides*

Antifungal agents	% resistance						
	Present study		Other studies on clinical isolates*				
	Soil isolates *n* = 42	Clinical isolates *n* = 3	[16][a] *n* = 78	[17][b] *n* = 98	[18][b] *n* = 49	[19][a] *n* = 40	Resistance range (%)
Ampicillin	45	66	60	82	73	60	45–82
Carbenicillin	93	100	72	NA	NA	70	70–100
Ciprofloxacin	86	100	71	69	62	NA	62–100
Cefriaxone	10	0	18	16	2	0	0–18
Cefotaxime	10	0	18	21	2	0	0–21
Imipenem	0	33	12	11	23	8	0–33
Gentamicin	19	0	33	NA	NA	30	0–33
Amikacin	0	0	5	NA	0	0	0–5
Minocyclin	55	66	0	6	6	23	0–66
Sulphamethoxazole	93	100	0	9	0	93	0–100
TMP-SMX	93	33	NA	9	0	93	0–93
Erythromycin	93	66	78	65	40	78	40–93
Tobramycin	0	0	NA	NA	33	NA	0–33
Cefamandole	2	0	NA	NA	37	60	0–60

[a], Based on disk diffusion method; [b], Based on minimum inhibitory concentration; NA, not available.

* References: [16] isolates submitted for susceptibility testing to Department of Microbiology, University of Texas Health Science Center; [17, 18] isolates referred to Actinomycete Laboratory, Division of Mycotic diseases, Centers for Disease Control, Atlanta; [19] isolates referred to the National Reference Center for Mycoses and Antifungal agents, Institut Pasteur, Paris.

methodology, such as lack of interlaboratory standardization of inocula, or differences in interpretation criteria for resistance. Moreover a strain may br inherently distinctive due to differences in the source of origin (clinical, soil) or in the geographical origin.

The marked differences in the activity of sulfamethoxazole and TMP-SMX which are may be attributed to three factors, Firstly thymidine concentration of the medium. We used Mueller-Hinton medium which is considered ideal for this purpose. Secondly the inoculum size; we used an inoculum size of 10^6–10^8 CFU/mL as per the NCCLS recommendations. Thirdly, differences in the interpretative criteria of zone inhibition. We have considered a strain susceptible to sulfamethoxazole when clear zone of inhibition is observed around the disk. This is in contrast to criterion used by other investigators who have used 80% inhibition criteria for the interpretation of TMP-SMX susceptibility results. Most of the published data on antimicrobial testing are based on clinical isolates received in reference laboratories. Since some of these isolates may have originated from patients unresponsive to or intolerant of therapy, there is a possibility of selection bias while comparing susceptibility results.

In an early paper on the determination of sulphonamide susceptibility for bacterial pathogens, Bauer and Sherris [20] recommended that the point at which growth stops entirely or is markedly decreased (80%) can be taken as the edge of the inhibition zone. The growth within the zone is ignored. However, *Nocardia* was not included in this study. In subsequent papers, Wallace et al. [6,21] followed this criterion for susceptibility testing and found that most of their nocardia strains appeared susceptible to sulphonamides. In a recent publication, Biehle et al. [22] compared E-test, microbroth dilution and the disk-diffusion method for susceptibility testing of 52 clinical isolates of *Nocardia* using 80% inhibition criterion for the interpretation of TMP-SMX results. Eight per cent of the isolates were found resistant to this drug. Concerning other antimicrobials, amikacin, imipenem and cephalosporins have been found to have maximum activity against *N. asteroides* with resistant percentages not exceeding 15, 25 and 35, respectively. Consistent with these observations, none of our soil isolates were resistant to amikacin, imipenem, tobramycin, and resistance to cephalosporins was less than 10%. More recently, Ambaye et al. [23] compared five *in vitro* susceptibility methods which included agar dilution, broth microdilution, disk diffusion, E-test and the BACTEC Radiometric method. When the results were combined for all antimicrobial agents tested against all nocardia isolates by all methods and a standard result for each *Nocardia* isolate was established by a consensus, the BACTEC radiometric method produced the highest level of agreement (97.9%). For TMP-SMX, the agar dilution method showed 8% resistance, whereas all the other four methods showed all the 26 isolates of *Nocardia* as susceptible.

Demonstration of high resistance to β-lactam antibiotics in nocardia species has been attributed to the presence of β-lactamases [6] which were detectable in all the soil isolates by Intralactam strips. Extrinsic or acquired resistance to β-lactam antibiotics has been shown in $>90\%$ of the clinical isolates of *N. asteroides* [6,24] and the isolate that showed lowest resistance had no detectable β-lactamase activity [24]. Activity for β-lactamases has been reported in several *Nocardia* species including *N. asteroides* [24,25], *N. brasiliensis* [26,27] and *N. farcinica* [28]. β-Lactamases of *Nocardia* species have been reported to be either inducible [24] or noninducible [28] enzymes which may be released into culture filtrate during growth or may be obtained after cell disruption. Recently, Scopetti et al. [29] reported a noninducible, mainly cell-associated β-lactamase in a soil isolate of *N. asteroides* which was particularly abundant in the stationary phase of the growth, and produced evidence of its involvement in resistance to β-lactam antibiotics. It is apparent that within *Nocardia* species/strains, there are differences in the spectrum and kinetics of β-lactamases produced. Further studies are needed to clarify whether differences in β-lactamases could be correlated with variations in resistance to β-lactams [30,31].

It may be emphasized here that in vitro antimicrobial susceptibility testing for nocardia species needs standardization, both in terms of methodology and correlation with *in vivo* therapeutic response [3,32,33]. The NCCLS is currently pursuing antimicrobial susceptibility testing methods for aerobic actinomycetes which include modified disk diffusion, agar dilution, broth microdilution, and radiometric growth index [4,32]. It has been observed that media composition and inoculum size considerably influence the *in vitro* results. Each test technique requires a uniform homogeneous suspension but this may not be achievable with all strains, since nocardiae grow as long branching filaments that fragment and clump. Moreover, some nocardia isolates do not show good growth on MHA [4]. Considering the inherent problems associated with antimicrobial susceptibility testing of *Nocardia* and the wide variations which individual isolates exhibit, it is suggested that susceptibility testing should be carried out by reference laboratories. Moreover, there is need to develop a more reliable methodology and interpretive criteria for susceptibility testing of *Nocardia* species to minimize interlaboratory discrepancies.

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