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Antimicrobial susceptibility testing and profiling of Nocardia species and other aerobic actinomycetes from South Africa: a comparative evaluation of broth microdilution versus the Etest

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Research Report for
a Master of Medicine in Microbiology

Warren Lowman
2010

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Warren Lowman

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Master of Medicine in Medical Microbiology

Declaration

I hereby declare that the contents of this submission are the sole work and views of the candidate, Warren Lowman.

Contribution to published paper by Warren Lowman

I, Naseema Aithma, hereby declare that Warren Lowman was the primary author of the paper Antimicrobial susceptibility testing and profiling of *Nocardia* species and other aerobic actinomycetes from South Africa: a comparative evaluation of broth microdilution versus the Etest, published in the Journal of Clinical Microbiology December 2010, Vol. 48 pages 4534-4540

He was actively involved in all aspects of the study and was solely responsible for design and analysis.

Signed: _N. Aithma_____

Date: 10/11/2010

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Antimicrobial susceptibility testing and profiling of *Nocardia* species and other aerobic actinomycetes from South Africa: a comparative evaluation of broth microdilution versus the Etest.

Warren Lowman

Naseema Aithma

Abstract

Nocardiosis is an under-recognized clinical entity in South Africa, for which interspecies epidemiological and clinical differences are poorly understood. The taxonomical state of flux and the lack of a simple antimicrobial susceptibility testing method are partly responsible. Definitive identification is molecular-based which further complicates the study of this ubiquitous organism, as this methodology is beyond the scope of most routine diagnostic laboratories. The Etest methodology has been proposed as an alternative to the reference broth microdilution method although there have been a limited number of comparative studies. We profiled fifty-one clinical isolates of aerobic actinomycetes, including thirty-nine *Nocardia* species, using sequence-based (16S rRNA) identification. Broth microdilution and Etests were done concurrently on all isolates. The overall level of categorical and essential agreement for broth microdilution and Etest for the *Nocardia* isolates ranged from 67.5 – 100% and 46.2 – 81.6%, respectively. Very major errors were seen with amikacin, amoxicillin-clavulanate, ciprofloxacin, clarithromycin and imipenem. For *Nocardia* species uniform susceptibility to cotrimoxazole, amikacin and linezolid was demonstrated with a 48.8% susceptibility rate to imipenem. *Nocardia farcinica* (20.5%) and *Nocardia cyriacigeorgica* (15.4%) were the most commonly identified species amongst the 82% of isolates identified to species level using 16S rRNA sequences. Furthermore drug susceptibility patterns demonstrated limited concordance with species identification. Our results suggest that, in a routine diagnostic setting, the Etest is not an acceptable alternative to the reference method of broth microdilution for antimicrobial susceptibility testing. Given the diversity and limited

understanding of this group of organisms further widespread evaluation of clinical isolates, from both a clinical and diagnostic perspective, is warranted.

Introduction

Nocardiosis is a disease of protean manifestations and a great mimicker of other infectious diseases. It is an under-diagnosed entity, especially in the South African setting where tuberculosis is so prolific (16). Nocardiosis is more common in the immunocompromised and commonly presents as disseminated disease in AIDS patients (10, 24). Sulfonamides have formed the basis of treatment since the 1940's, however, case reviews have shown poor outcomes for patients treated with sulfonamides alone. The mortality rate is high for patients with disseminated disease, reaching almost 50% in patients with central nervous system (CNS) infections (4). In a retrospective review of patients with AIDS, 90% of patients that were treated with sulfonamides did not respond and subsequently died (13). These findings, coupled with a general need for standardization, prompted a response from the Clinical and Laboratory Standards Institute (CLSI) to standardize and validate antimicrobial susceptibility testing (AST) of aerobic actinomycetes (17). CLSI advises routine AST of all significant aerobic actinomycete/ *Nocardia* isolates. This may be impractical for many laboratories considering broth microdilution (BMD) is the reference method. BMD is a resource intensive method limited by both availability of supplies and expertise. It is a technically demanding technique and requires considerable experience in both preparation and interpretation. An attractive alternative is the Etest, a simplified gradient-diffusion AST method that also derives a minimum inhibitory concentration (MIC). A limited number of comparative evaluations suggest that the Etest is a viable and practical alternative to BMD for AST of aerobic actinomycetes (1, 2, 23). A lack of standardized methodology has made it difficult to compare these studies. Etest methodology, in particular inoculum preparation has varied substantially. This is partly due to the discrepancy

between the manufacturers' recommendations and the findings of Biehle et al, who demonstrated a ~1$\log_{10}$ dilution lower inoculum size when using the recommended 0.5 MacFarland suspension (2). Major taxonomic revisions have occurred within the last 10 years further complicating the comparison of studies (7, 11, 12, 21). *Nocardia asteroides*, the most well known species of Nocardia is in fact obsolete, with molecular-based identification methods demonstrating that clinical isolates do not match the ATCC type strain of *N. asteroides* (4). With potentially important clinical and epidemiological differences between species it is important to speciate as accurately and rapidly as possible. Conventional phenotypic methods are unable to do this and consequently molecular-based identification, specifically sequence-based methodology is now the preferred means of identification (4, 6).

The influence of the HIV-TB co-epidemic on aerobic actinomycete infections is probably quite substantial and multifactorial in nature. From the widespread use of bactrim prophylaxis, which may undermine its use in the treatment of nocardial infections, to the misdiagnosis of nocardial pneumonia, and to the possible unquantified increase in incidence associated with AIDS, there are numerous research questions that remain unanswered. The last South African study evaluating nocardial infection in HIV patients was conducted over a 2 year period from 1995 – 1997 (10). This study identified *N. asteroides* as the most common isolate and pulmonary infection to be the commonest clinical presentation. Furthermore the authors attempted to compare the incidence of pulmonary nocardiosis with pulmonary tuberculosis given the similarity in clinical presentation. The ratio of one case of pulmonary nocardiosis to 90 cases of pulmonary tuberculosis contrasts with the thirty-three to one ratio determined in an autopsy study on miners by Murray et al (16). The rate of nocardiosis in this study was 0.66% and none

of the cases were diagnosed antemortem, with the majority diagnosed as pulmonary tuberculosis. It is thus conceivable that the 0.07% rate determined in the study by Jones et al is a gross underestimation and that many cases of pulmonary nocardiosis in HIV patients are erroneously diagnosed and treated as pulmonary tuberculosis.

The aim of this study is to characterize clinical isolates of South African aerobic actinomycetes, principally *Nocardia* isolates, using the current gold standards of sequence-based identification and broth microdilution AST. Furthermore we evaluate the Etest as an alternative to the reference AST method in an attempt to establish its feasibility for routine diagnostic testing. This will hopefully increase awareness and encourage further study of this poorly understood group of organisms. To better understand the clinical nuances and to optimize management approaches requires a sound pathological background and consequently laboratory diagnostics must be improved.

Methods

Isolates: A total of 55 aerobic actinomycetes strains were included in the study. This included 51 clinical isolates (39 *Nocardia* spp.; 12 other aerobic actinomycetes) and 4 reference/ type strains (*N. asteroides* sensu stricto ATCC 19247, *N. otitidiscaviarum* ATCC 14629, *N. asteroides* national reference strain Nocas001, *Gordonia amarae* ATCC 27808T). All isolates were stored at -70^{o}C and subcultured twice prior to phenotypic characterization and AST.

Phenotypic characterization: Isolates were cultured on blood agar plates at 35^{o}C until sufficient growth was obtained (usually 48-72 hours). Colonial morphology (including presence or absence of aerial hyphae), Gram stain, partial acid-fastness (modified Kinyoun), urea hydrolysis, citrate utilization, esculin hydrolysis and resistance to lysozyme were determined for all isolates. A subset of isolates (28 isolates) was examined for tyrosine, xanthine, casein and hypoxanthine hydrolysis. All phenotypic testing was done according to standard laboratory protocols (3, 8). The results of AST were also used to phenotypically categorise *Nocardia* isolates according to the 6 drug patterns originally described by Wallace et al (4, 25).

Inoculum preparation: The inoculum was prepared according to the CLSI (formerly NCCLS) guidelines for rapidly growing mycobacteria and aerobic actinomycetes (17). Briefly isolates were swabbed from a blood agar plate (48-72 hour), using a sterile swab, and transferred to 4.5ml sterile water. Five to seven 5mm sterile beads (Sigma-Aldrich®) were added to assist in breaking up clumps. The inoculum was then vortexed for 15-20 seconds and any remaining clumps allowed to settle. An optical density of a 0.5 McFarland was achieved in this manner. For BMD 0.5ml of the 0.5 McFarland suspension was added to 4.5ml sterile water or saline

(1:10 dilution), resulting in ~10^7 CFU/ml. For the Etest the 0.5 McFarland suspensions were used to inoculate the plates. Colony counts were done on random samples.

Broth microdilution (BMD): Performed in accordance with the CLSI reference method (5, 17). Microtiter plates (96-well) were prepared prior to testing, stored at -70^{o}C and thawed immediately before use. The antimicrobial agents tested included amikacin (64µg/ml); amoxicillin-clavulanate (64/32 µg/ml); ceftriaxone (256 µg/ml); ciprofloxacin (64 µg/ml); clarithromycin (64 µg/ml); imipenem (64 µg/ml); cotrimoxazole [trimethoprim/sulfamethoxazole] (8/152 µg/ml) and linezolid (64 µg/ml). All antibiotic powders were diagnostic reference powders (Abtek Biologicals Ltd, Liverpool, UK) and were quality controlled with the following organisms: *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922 and ATCC 35218; *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212. Five microlitres of the aforementioned inoculum was added to each well containing 100µl of Mueller Hinton broth/antibiotic (1:100 dilution), resulting in a final concentration of ~10^5 CFU/ml or ~10^4 CFU/well. Microtiter plates were sealed in plastic bags and incubated at 35^{o}C in ambient air. The MICs were read at 24, 48 and 72 hours. If required, due to poor growth, plates were reincubated for a further 48 hours and a final MIC reading was done on day 5 (120 hours). The MIC was defined as the lowest concentration of antimicrobial agent to inhibit visible growth, except for cotrimoxazole where 80% inhibition was used.

Etest: Mueller-Hinton agar (MHA) plates (90mm in size) were inoculated by confluent swabbing of the surface with a 0.5 McFarland organism suspension, prepared as described above. Two Etest strips were applied to each plate and the plates were incubated at 35 ^{o}C in ambient air.

The same antimicrobial agents as used for BMD were tested. Etests were done concurrently with BMD and read at the same time intervals. MICs were determined by examining the plates for complete inhibition within the elliptical zone, the only exception being cotrimoxazole for which an 80% zone of inhibition was read.

Additional AST: Certain isolates consistently failed to grow in Mueller-Hinton Broth (MHB) and on MHA plates. These isolates were then tested by BMD using MHB supplemented with 5% lysed horse blood and by Etest using MHA + 5% sheep blood plates. Inoculum preparation, incubation and reading intervals were otherwise unchanged.

Interpretation of susceptibility results: Endpoints for both BMD and Etest were read independently by both authors, discrepancies being resolved by re-evaluation and a consensus decision. All MICs were interpreted according to the NCCLS breakpoints for aerobic actinomycetes (17) and error rates were assessed. A very major error (VME) constituted a resistant isolate by BMD designated susceptible by Etest. A major error (ME) constituted a susceptible isolate by BMD designated resistant by Etest. Minor errors (mE) were designated by intermediate susceptibility according to BMD and as either sensitive or resistant by Etest. Frequencies of very major errors (# VME/total # resistant), major errors (# ME/total # sensitive) and minor errors (# mE/ total #) were calculated. Categorical (breakpoint determination) and essential (within 1 two-fold dilution) agreement between the two methods was assessed.

Sequence-based identification: Partial 16S rRNA gene sequences of all strains were determined using the domain *Bacteria*-specific 27F forward primer (20). Sequences greater than 500 base pairs in length were evaluated. The unknown sequences were compared to all sequences

deposited in the GenBank database (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Sequencing Tool for nucleotide sequence queries (BLASTN). The GenBank query type strain or culture collection strain with the highest score was then downloaded, trimmed and compared to the subject sequence, with re-evaluation of the electropherogram to reconcile any ambiguous or conflicting base assignments. The percentage similarity was then determined for each strain. Isolates for which there was no distinct aligned type or culture-collection strain were identified to genus level, with a species identification made only if a characteristic phenotypic trait, that reinforced the sequencing results, was present. The percentage homology was stratified into 4 groups: 100%; >99.5%; >99% and <99%. Strains with a greater than 99.5% homology to a single type strain were considered definitive for speciation purposes. Typical phenotypic characteristics were sought for isolates with less than 99.5% homology to a type strain, which if present were used to speciate organisms and if not a genus level identification was made.

Results

Phenotypic characterization: Thirty nine clinical isolates were phenotypically characterized as *Nocardia* species based on colony morphology, presence of aerial hyphae, typical Gram stain morphology, partial acid-fastness and resistance to lysozyme. The twelve aerobic actinomycetes were comfortably distinguished from the *Nocardia* genus but, with the exception of *Streptomyces* species which had a very typical colonial morphology, were not easily differentiated from each other based on the available phenotypic data.

Genotypic characterization: The 16S rRNA sequences of 25 phenotypic *Nocardia* clinical isolates demonstrated a similarity rate of 99.5 - 100% with a distinct culture-collection/ type-strain *Nocardia* species isolate deposited in the GenBank database. For the non-nocardial aerobic actinomycetes a greater than 99.5% similarity identity was achieved for four isolates. Nine isolates were identified to species level using genotypic sequence identity and corroborating phenotypic traits. For the remaining eleven isolates the sequencing result did not adequately discriminate between species, and a genus level identification was made. For three of the eleven isolates we failed to generate sequences of greater than 500bp. The reference strains all demonstrated >99.5% identity with the deposited type strain sequence. Interestingly the national reference strain Nocas001, designated as *Nocardia asteroides* is in fact a *Nocardia farcinica*. To our knowledge this is the first time that this strain has been characterized genotypically, and the phenotypic data corroborates this identification. *Nocardia farcinica* (8 isolates) and *Nocardia cyriacigeorgica* (6 isolates) were the most commonly identified clinical isolates. Table 1 highlights the species distribution and sequence identity. The twelve aerobic actinomycetes isolates comprised *Rhodococcus* species (1), *Rhodococcus equi* (1), *Gordonia*

bronchialis (2), *Streptomyces* species (5), *Tsukumurella pulmonis* (1), *Tsukumurella tyrosinosolvens* (1) and *Actinomadura* species (1).

Antimicrobial Susceptibility Testing: All *Nocardia* isolates demonstrated 100% susceptibility to cotrimoxazole, amikacin and linezolid. Furthermore only these 3 antimicrobial agents showed a categorical agreement concordance rate between BMD and Etest of 100%. All other agents varied substantially, both in susceptibility rates (20.9 – 61.9%) and categorical agreement (67.5 – 90.2%) [Table 2]. Essential agreement for the *Nocardia* isolates ranged from 46.2% to 81.6% and very major errors (VME) were seen with amoxicillin-clavulanate (11.8%), ciprofloxacin (3.3%), clarithromycin (3.7%) and imipenem (5.6%). It should be noted that for the 3 most common isolates (*N. farcinica, N. cyriacigeorgica* and *N. otitidiscaviarum),* a total of 20 comparative evaluations showed no VME and a single ME, however agreement remained low (Table 3). The non-nocardial aerobic actinomycetes demonstrated an essential agreement rate of 50% to 91.7%, with VME seen with amikacin (100%), amoxicillin-clavulanate (50%) and clarithromycin (100%) [Table 4].

The concordance rate between species identification and antimicrobial drug pattern was also evaluated (Table 5). The reference strain ATCC 19247, which correlates with drug pattern VI, was tested on two separate occasions and was included with the *N. cyriacigeorgica* for this evaluation. No corresponding drug pattern was consistently seen amongst all isolates of a particular species, however using intermediate susceptibility results improved the concordance rates. Of interest all *N. otitidiscaviarum* isolates were resistant to ciprofloxacin whereas 44.4% of *N. farcinica* isolates were susceptible to ceftriaxone.

The results of repeat susceptibility testing showed no interpretive changes (categorical agreement = 100%) with 85.4% of MICs (79.2% for Etest and 91.7% for BMD) within a two-fold dilution of the original result (data not shown). Three isolates (*Nocardia* spp; *Nocardia exalbida; Gordonia amarae*) consistently failed to grow and required blood-supplemented media for AST. For the *Nocardia* spp. isolate a single very major error (clarithromycin) and a single major error (amoxicillin-clavulanate) was noted, but for the remaining two isolates there was 100% categorical agreement for all antimicrobials.

Discussion

This study represents the largest sample of aerobic actinomycete isolates from South Africa. This is important given that South Africa has an exceptionally high HIV/AIDS burden, an established risk factor for nocardiosis (10, 24). Furthermore TB incidence rates in South Africa are 948 per 100 000 population per year (26), and given the ability of *Nocardia* spp to mimic pulmonary tuberculosis it is important to consider it in the differential diagnosis, particularly in patients who do not respond to first-line TB treatment. The majority of isolates in this study, for which clinical details were known, were obtained from AIDS patients presenting with extrapulmonary disease. In South Africa very little is known about the distribution of *Nocardia* species and the most widely known species amongst clinicians is still the now defunct *N. asteroides*. Our study suggests that *N. farcinica* and *N. cyriacigeorgica* are the 2 most commonly isolated species, similar to that published elsewhere (14, 15). Our results highlight the now well accepted fact that phenotypic characteristics do not adequately speciate the aerobic actinomycetes. However 16S rRNA sequencing as an identification tool also has its limitations (18) and our study supports this in that we were unable to speciate 6 out of 39 (15%) clinical nocardial isolates and 7 out of 12 (58%) non-nocardial aerobic actinomycetes isolates. This is a limitation of our study as the lack of significant homology with a type strain may mean that some of our identifications, which were made with corroborating phenotypic characteristics, are possibly inaccurate. However only three *Nocardia* species (a total of 7 isolates) demonstrated less than 99.5% homology and it is well accepted that genotypic data should be interpreted in the context of phenotypic data. Furthermore this does not detract from the results of our comparison of AST methods for this group of organisms. The

epidemiological and clinical differences that are beginning to be unraveled with accurate species identification highlights the importance of speciation, and in the setting of a high HIV/AIDS incidence these differences still remain to be elucidated.

Antimicrobial susceptibility testing of aerobic actinomycetes is not widely offered in South Africa, primarily due to lack of a simple and practical standardized method. The last South African study to evaluate the antimicrobial susceptibility profile of *Nocardia* spp. isolates was by Jones et al over a 2 year period from 1995 – 1997, in a series of ten patients presenting to the Chris Hani Baragwanath hospital (10). The AST method used was not standardized and speciation was done using phenotypic methods only. Of the 10 isolates, 7 were identified as *N. asteroides*, although based on the drug susceptibility patterns six of these isolates conformed to drug pattern type VI (*N. cyriacigeorgica*). Furthermore resistance to cotrimoxazole was demonstrated in five isolates, an unusual finding and in distinct contrast to our findings.

The broth microdilution reference method for AST developed by the CLSI has provided a standardized method however it remains beyond the scope of most routine microbiology laboratories. The Etest method is a viable alternative although the data comparing it with BMD is limited. Of interest is the variation in methodology between studies, probably due to the lack of a standardized method at the time that most studies were performed. This study, to our knowledge, is the first that compares the Etest to BMD using the CLSI standard and thus represents an evaluation with a recognized reference method.

We sought to evaluate whether the Etest is a feasible and reliable option such that laboratories may be able to provide AST without referral to a reference centre. For *Nocardia* species, with

the exception of amikacin, linezolid and cotrimoxazole, the essential and categorical agreement rates fell short of the accepted international standard of greater than 90% concordance and <3% ME/ no VME, respectively. However these pooled results did not reflect the acceptable error rates seen between the most commonly isolated species, *N. farcinica, N. cyriacigeorgica* and *N. otitidiscaviarum.* For these 3 species categorical agreement ranged from 60 – 100% and essential agreement from 12.5 – 100%, still reflective of an overall poor level of agreement. The uniform susceptibility of all isolates to amikacin, linezolid and cotrimoxazole precludes any definitive conclusion in comparing the two methods. Ciprofloxacin was the only other agent that showed greater than 90% categorical agreement.

The single *N. transvalensis* isolate was identified genotypically (100% homology, 764bp) and demonstrated an elevated MIC to amikacin (BMD MIC of 2µg/ml and an Etest MIC of 6µg/ml). Despite the elevated MIC it still fell within the susceptible breakpoint of 8µg/ml, an unusual finding for an organism that is characteristically resistant to amikacin. This may reflect geographical variation and a peculiarity of South African isolates, or may simply be an anomaly. Further testing of a greater number of isolates is warranted, although in our experience it is a rarely isolated species.

Etests were read independently by 2 observers and discrepant results were resolved by consensus. Determination of zones of inhibition by Etest was variable. We found it easy to determine points of intersection for amikacin, imipenem and cotrimoxazole at 72 hours. Determination of cotrimoxazole zones is assisted by the 80% inhibition which eliminates the effect of haziness, an aspect we found hampers Etest readings, particularly for linezolid and

ceftriaxone. Etest readings were generally easier after 72 hours and we feel that an earlier reading, apart from being technically difficult to interpret may result in a falsely lower MIC reading.

Our results contrast significantly with those of other published studies. Biehle et al (2) found an overall agreement of 96.2% and Ambaye et al (1) an overall agreement of 96.6% between the 2 methods . Very major errors were noted for amoxicillin-clavulanate and ceftriaxone only. The reasons for the discrepancies are numerous. Firstly our inoculum preparation strictly followed that outlined in the NCCLS guideline (17). Random colony counts highlighted variation in actual CFU/ml despite close attention to inoculum preparation. Colony counts, when not within the specified ~10^5 CFU/ml range, were ~1log10 dilution lower, in accordance with the findings of Biehle et al. The issues surrounding the initial inoculum have been extensively discussed previously (2, 23) although we do not feel that the issue has been resolved. In a small subset of isolates within our collection (data not shown) we used a non-diluted 0.5 McFarland inoculum for inoculation of the BMD microtitre plates. Interestingly there was only one discordant categorical result (a minor error). Achieving a homogenous suspension of organisms is difficult, despite the addition of glass beads, and we feel that this may hamper the performance of the Etest in particular. Furthermore a 0.5 McFarland standard should result in ~10^8 CFU/ml, as specified by the CLSI guideline, however this has been shown to not always be the case (2) and our experience is similar. The effect of inoculum size is worthy of further study. Secondly the interpretive breakpoints used in this study are specific to the aerobic actinomycetes, breakpoints which were only established in 2003 and thus have not been used before in a comparative evaluation. Thirdly the range of isolates tested appears to have differed

substantially, not entirely unexpected given the recent advances in *Nocardia* taxonomy and our use of genotypic identification. Lastly the antimicrobial agents tested differed between studies, although this would only impact on overall level of agreement. Our results of repeat testing showed no discordance in categorical agreement, although the overall poor essential agreement of the repeat Etests (79.2%) is noteworthy and may possibly reflect the inherent limitations of the Etest methodology for AST of this group of organisms. It is worth mentioning that our ATCC 19247 strain, a strain that has been proposed as a quality control strain (23) and which was used as such, gave reproducible results that consistently fell within the proposed reference range at 72 hours.

The limited number of other aerobic actinomycetes evaluated precludes any definitive conclusions regarding the Etest as an alternative method, however from our experience the same issues outlined above are applicable. For *Streptomyces* species it is especially difficult to obtain a homogenous suspension, particularly if aerial hyphae do not develop. The high rates of very major errors for amikacin and clarithromycin are due to the paucity of resistant strains and should thus be treated with reserve.

The value of the drug susceptibility pattern as a tool to aid in species identification was somewhat limited, although we acknowledge that the number of species may not have been sufficient. Interestingly only two thirds of *N. farcinica* isolates were resistant to ceftriaxone and all *N. otitidiscaviarum* isolates were resistant to ciprofloxacin. Species distribution and drug susceptibility profiles appear to vary by geographical region and patient population (9, 14, 19, 22), thus it is not surprising that isolates do not always conform to specific drug patterns. Of

interest is that only 48.8% of all *Nocardia* isolates were susceptible to imipenem with an MIC_{90} > 64µg/ml, whereas susceptibility to cotrimoxazole was 100%, with an MIC_{90} of 0.5 µg/ml. In South Africa imipenem is often advocated for the treatment of severe nocardiosis, is usually started empirically (together with amikacin and cotrimoxazole) and often continued in the absence of AST. This practice highlights the need for local AST and further epidemiological investigation. Pulmonary nocardiosis is under recognized in South Africa where an autopsy study showed that 38% of cases were diagnosed antemortem as pulmonary tuberculosis (16). This series highlighted the high HIV rate (76%) and co-pathology (50%) in patients with pulmonary nocardiosis. Despite it being under diagnosed it is still a relatively uncommon disease in South Africa, an enigma to many when one considers that immunosuppression is a primary risk factor. The majority of cases seen are the severe extrapulmonary forms of disease and the question why nocardiosis is not seen more commonly in South Africa remains a pertinent one. The potential reasons are numerous including misdiagnosis, difficulty isolating the slow growing organism and use of cotrimoxazole as prophylaxis. In transplant patients it has been shown that bactrim prophylaxis is not protective against nocardia infection (19). Given the widespread prophylactic use of this agent, it is remarkable that it remains highly active *in vitro*. Further evaluation of its clinical efficacy is warranted given its prescription as the drug of choice for treatment of nocardiosis.

In conclusion we have profiled 51 South African isolates of aerobic actinomycetes, including 39 *Nocardia* species, and compared the Etest with the BMD reference method. We demonstrated varied susceptibility patterns amongst different isolates of the same species, uniform susceptibility to amikacin, linezolid and cotrimoxazole for *Nocardia* species, and a high level of

resistance to imipenem. Previous comparative evaluations between the two methods were done in the absence of a standardized method. We demonstrated a significant lack of agreement, both categorical and essential, between the two methods. Inoculum preparation, reading and interpretation of Etest AST results requires considerable familiarity and experience. Considering the AST issues highlighted by our study, the dearth of data comparing the Etest to the reference method and the need for further epidemiological evaluation of aerobic actinomycetes we would suggest that the BMD reference method be used as the primary method of AST in future studies. Until such time that the accumulated body of knowledge addresses many of these unresolved issues we would not recommend that the Etest be offered in the routine microbiology laboratory for the occasional aerobic actinomycete isolate.

References

1. **Ambaye, A.**, **P. C. Kohner, P. C. Wollan, K. L. Roberts, G. D. Roberts, and F. R. Cockerill III.** 1997. Comparison of agar dilution, broth microdilution, disk diffusion, E-test, and BACTEC radiometric methods for antimicrobial susceptibility testing of clinical isolates of the *Nocardia asteroides* complex. J. Clin. Microbiol. **35:**847-852.
2. **Biehle, J.R., S. J. Cavalieri, M. A. Saubolle, and L. J. Getsinger.** 1994. Comparative evaluation of the Etest for susceptibility testing of *Nocardia* species. Diagn. Microbiol. Infect. Dis. **19:**101-110.
3. **Brown, J. M., and M. M. McNeil** 2004. Media and methods used for the identification of the aerobic actinomycetes, p. 6.2.1-6.2.11. *In* H. D. Isenberg (2nd ed.), Clinical microbiology procedures handbook, vol. 2. American Society for Microbiology, Washington, DC.
4. **Brown-Elliot, B. A., J. M. Brown, P. S. Conville, and R. J. Jr. Wallace.** 2006. Clinical and laboratory features of the *Nocardia* spp. based on current molecular taxonomy. Clin.Micro. Rev. **19:**259-282.
5. **Clinical Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard- seventh edition. CLSI document M7-A7, 2006.
6. **Cloud, J. L., P. S. Conville, A. Croft, D. Harmsen, F. G. Witebsky, and K. C. Carroll.** 2004. Evaluation of partial 16S ribosomal DNA sequencing for identification of *Nocardia* species by using the MicroSeq 500 system with an expanded database. J. Clin. Microbiol. **42:**578-584.

7. **Conville, P. S. and F. G. Witebsky.** 2007. Organisms designated as *Nocardia asteroides* drug pattern type VI are members of the species *Nocardia cyriacigeorgica*. J. Clin. Microbiol. **45:**2257-2259.
8. **Conville, P. S. and F. G. Witebsky.** 2007. *Nocardia, Rhodococcus, Gordonia, Actinomadura, Streptomyces*, and other aerobic actinomycetes. In Manual of Clinical Microbiology, 9th ed.,515-542. ASM, Washington, DC.
9. **Glupczynski, Y., C. Berhin, M. Janssens, and G. Wauters.** 2006. Determination of antimicrobial susceptibility patterns of *Nocardia* spp. from clinical specimens by Etest. Clin Microbiol Infect. **12:** 905-912.
10. **Jones, N., M. Khoosal, M. Louw, and A. Karstaedt.** 2000. Nocardial infection as a complication of HIV in South Africa. J. Infect. **41**:232-239.
11. **Kageyama, A., K. Yazawa, A. Mukai, T. Kohara, K. Nishimura, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia aroensis* sp. nov. and *Nocardia pneumonia* sp. nov., isolated from patients in Japan. Int. J. Syst. Evol. Microbiol. **54:**2025-2029.
12. **Kageyama, A., N. Poonwan, K. Yazawa, Y. Mikami, and K. Nishimura.** 2004. *Nocardia beijingensis*, is a pathogenic bacterium to humans: the first infectious cases in Thailand and Japan. Mycopathologia. **157:**155-161.
13. **Long, P. F**. 1994. A retrospective study of *Nocardia* infections associated with the acquired immune deficiency syndrome (AIDS). Infection. **22:**362-364.
14. **Minero, M. V., M. Marin, E. Cercenado, P. M. Rabadán, E. Bouza, and P. Muñoz.** 2009. Nocardiosis at the turn of the century. Medicine. **88:**250-261

15. **Muñoz, J., B. Mirelis, L. M. Aragón, N. Gutiérrez, F. Sánchez, M. Español, O. Esparcia, M. Gurguí, P. Domingo, and P. Coll.** 2007. Clinical and microbiological features of nocardiosis 1997 – 2003. J. Med. Microbiol. **56:**545-550.

16. **Murray, J., P. Back, and G. Nelson.** 2004. Pulmonary nocardiosis in autopsies of South African miners. South Afr. J. Epidemiol. Infect. **19(2)**:60-62.

17. **NCCLS.** 2003. Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes. Approved standard. NCCLS document M-24A. NCCLS, Wayne, Pa.

18. **Patel, J. B., R. J. Wallace, Jr., B. A. Brown-Elliot, T. Taylor, C. Imperatrice, D. G. B. Leonard, R. W. Wilson, L. Mann, K. C. Jost, and I, Nachamkin.** 2004. Sequence-based identification of aerobic actinomycetes. J. Clin. Microbiol. **42:**2530-2540.

19. **Peleg, A. Y., S. Husain, Z. A. Qureshi, F. P. Silveira, M. Sarumi, K. A. Shutt, E. J. Kwak, and D. L. Paterson.** 2007. Risk factors, clinical characteristics and outcome of *Nocardia* infection in organ transplant recipients: A matched case-control study. Clin. Infect. Dis. **44:**1307-1314.

20. **Reysenbach, A. L., K. Longnecker, and J. Kirschstein.** 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a mid-atlantic ridge hypothermal vent. Appl. Environ. Microbiol. **66:**3798-3806.

21. **Roth, A., S. Andrees, R. M. Kroppenstedt, D. Harmsen, and H. Mauch.** 2003. Phylogeny of the genus *Nocardia* based on reassessed 16S rRNA gene sequences reveals underspeciation and division of strains classified as *Nocardia asteroides* into three established species and two unnamed taxons. J. Clin. Microbiol. **41:**851-856.

22. **Tan, C. K., C. C. Lai, S. H. Lin, C. H. Liao, C. H. Chou, H. L. Hsu, Y. T. Huang, and P. R. Hsueh.** 2009. Clinical and microbiological characteristics of nocardiosis including those caused by emerging *Nocardia* species in Taiwan, 1998 – 2008. Clin Microbiol Infect. doi: 10.1111/j.1469-0691.2009.02950.x

23. **Tomlin, P., C. Sand, and R. P. Rennie.** 2001. Evaluation of Etest, disk diffusion and broth microdilution to establish tentative quality control limits and review susceptibility breakpoints for two aerobic actinomycetes. Diagn Microbiol Infect Dis. **40:**179-186.

24. **Uttamchandani, R. B., G. L. Daikos, R. R. Reyes, M. A. Fischl, G. M. Dickinson, E. Yamguchi, and M. R. Kramer.** 1994. Nocardiosis in 30 patients with advanced human immunodeficiency virus infection: clinical features and outcome. Clin. Infect. Dis. **18:**348-353.

25. **Wallace, R. J., L. C. Steele, G. Sumter, and J. M. Smith.** 1988. Antimicrobial susceptibility patterns of *Nocardia asteroides.* Antimicrob. Agents Chemother. **32:**1776-1779.

26. **World Health Organization Report 2009. Global tuberculosis control. South Africa 2007.** http://apps.who.int/globalatlas/predefinedReports/TB/PDF_Files /zaf.pdf. Last accessed 20 May 2010.

Appendices

Appendix 1: Reprint of published manuscript

Appendix 2: External reviewers comments and author's rebuttal

Appendix 3: Ethics clearance

Appendix 1

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Antimicrobial Susceptibility Testing and Profiling of *Nocardia* Species and Other Aerobic Actinomycetes from South Africa: Comparative Evaluation of Broth Microdilution versus the Etest[▽]§

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Nocardiosis is an underrecognized clinical entity in South Africa, for which interspecies epidemiological and clinical differences are poorly understood. The taxonomical state of flux and the lack of a simple antimicrobial susceptibility testing method are partly responsible. Definitive identification is molecularly based, which further complicates the study of this ubiquitous organism, as this methodology is beyond the scope of most routine diagnostic laboratories. The Etest methodology has been proposed as an alternative to the reference broth microdilution method, although there have been a limited number of comparative studies. We profiled 51 clinical isolates of aerobic actinomycetes, including 39 *Nocardia* species, using sequence-based (16S rRNA) identification. Broth microdilution and Etests were done concurrently on all isolates. The overall level of categorical and essential agreement for broth microdilution and Etest for the *Nocardia* isolates ranged from 67.5 to 100% and 46.2 to 81.6%, respectively. Very major errors were seen with amikacin, amoxicillin-clavulanate, ciprofloxacin, clarithromycin, and imipenem. For *Nocardia* species, uniform susceptibility to co-trimoxazole, amikacin, and linezolid was demonstrated, with a 48.8% susceptibility rate to imipenem. *Nocardia farcinica* (20.5%) and *Nocardia cyriacigeorgica* (15.4%) were the most commonly identified species among the 82% of isolates identified to species level using 16S rRNA sequences. Furthermore, drug susceptibility patterns demonstrated limited concordance with species identification. Our results suggest that, in a routine diagnostic setting, the Etest is not an acceptable alternative to the reference method of broth microdilution for antimicrobial susceptibility testing. Given the diversity and limited understanding of this group of organisms, further widespread evaluation of clinical isolates, from both clinical and diagnostic perspectives, is warranted.

Nocardiosis is a disease of protean manifestations and a great mimicker of other infectious diseases. It is an underdiagnosed entity, especially in the South African setting, where tuberculosis (TB) is so prolific (16). Nocardiosis is more common in the immunocompromised and commonly presents as disseminated disease in AIDS patients (10, 24). Sulfonamides have formed the basis of treatment since the 1940s; however, case reviews have shown poor outcomes for patients treated with sulfonamides alone. The mortality rate is high for patients with disseminated disease, reaching almost 50% in patients with central nervous system (CNS) infections (4). In a retrospective review of patients with AIDS, 90% of patients who were treated with sulfonamides did not respond and subsequently died (13). These findings, coupled with a general need for standardization, prompted a response from the Clinical and Laboratory Standards Institute (CLSI) to standardize and validate antimicrobial susceptibility testing (AST) of aerobic actinomycetes (17). CLSI advises routine AST of all significant aerobic actinomycete/*Nocardia* isolates. This may be impractical for many laboratories, considering that broth microdilution (BMD) is the reference method. BMD is a resource-intensive method limited by both availability of supplies and expertise. A limited number of comparative evaluations suggest that the Etest is a viable and practical alternative to BMD (1, 2, 23). A lack of standardized methodology has made it difficult to compare these studies. Etest methodology, in particular inoculum preparation, has varied substantially. This is partly due to the discrepancy between the manufacturers' recommendations and the findings of Biehle et al., who demonstrated an ~1-$\log_{10}$-dilution-lower inoculum size when using the recommended 0.5 MacFarland suspension (2). Major taxonomic revisions have occurred within the last 10 years, further complicating the comparison of studies (7, 11, 12, 21). With potentially important clinical and epidemiological differences between species, it is important to identify isolates to the species level as accurately and rapidly as possible. Conventional phenotypic methods are unable to do this, and consequently molecularly based identification, specifically sequence-based methodology, is now the preferred means of identification (4, 6).

The influence of the HIV-TB coepidemic on aerobic actinomycete infections is probably quite substantial and multifactorial in nature. From the widespread use of bactrim prophy-

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laxis, which may undermine its use in the treatment of nocardial infections, to the misdiagnosis of nocardial pneumonia and to the possible unquantified increase in incidence associated with AIDS, there are numerous research questions that remain unanswered. The aim of this study is to characterize clinical isolates of South African aerobic actinomycetes, principally *Nocardia* isolates, using the current gold standards of sequence-based identification and broth microdilution AST. Furthermore we evaluate the Etest as an alternative to the reference AST method in an attempt to establish its feasibility for routine diagnostic testing.

MATERIALS AND METHODS

Isolates. A total of 55 aerobic actinomycete strains were included in the study. This included 51 clinical isolates (39 *Nocardia* spp.; 12 other aerobic actinomycetes) and 4 reference/type strains (*N. asteroides* sensu stricto ATCC 19247, *N. otitidiscaviarum* ATCC 14629, *N. asteroides* national reference strain Nocas001, *Gordonia amarae* ATCC 27808^T). All isolates were stored at −70°C and subcultured twice prior to phenotypic characterization and AST.

Phenotypic characterization. Isolates were cultured on blood agar plates at 35°C until sufficient growth was obtained (usually 48 to 72 h). Colonial morphology (including presence or absence of aerial hyphae), Gram stain, partial acid fastness (modified Kinyoun), urea hydrolysis, citrate utilization, esculin hydrolysis, and resistance to lysozyme were determined for all isolates. A subset of isolates (28 isolates) was examined for tyrosine, xanthine, casein, and hypoxanthine hydrolysis. All phenotypic testing was done according to standard laboratory protocols (3, 8). The results of AST were also used to phenotypically categorize *Nocardia* isolates according to the six drug patterns originally described by Wallace et al. (4, 25).

Inoculum preparation. The inoculum was prepared according to the CLSI (formerly NCCLS) guidelines for rapidly growing mycobacteria and aerobic actinomycetes (17). Briefly, isolates were swabbed from a blood agar plate (48 to 72 h), using a sterile swab, and transferred to 4.5 ml sterile water. Five to seven 5-mm sterile beads (Sigma-Aldrich) were added to assist in breaking up clumps. The inoculum was then vortexed for 15 to 20 s, and any remaining clumps were allowed to settle. An optical density of a 0.5 McFarland standard was achieved in this manner. For BMD, 0.5 ml of the 0.5 McFarland suspension was added to 4.5-ml sterile water or saline (1:10 dilution), resulting in $\sim 10^7$ CFU/ml. For the Etest, the 0.5 McFarland suspensions were used to inoculate the plates. Colony counts were done on random samples.

BMD. Broth microdilution (BMD) was performed in accordance with the CLSI reference method (5, 17). Microtiter plates (96 well) were prepared prior to testing, stored at −70°C, and thawed immediately before use. The antimicrobial agents tested included amikacin (64 μg/ml), amoxicillin-clavulanate (64/32 μg/ml), ceftriaxone (256 μg/ml), ciprofloxacin (64 μg/ml), clarithromycin (64 μg/ml), imipenem (64 μg/ml), co-trimoxazole (trimethoprim-sulfamethoxazole) (8/152 μg/ml), and linezolid (64 μg/ml). All antibiotic powders were diagnostic reference powders (Abtek Biologicals Ltd., Liverpool, United Kingdom) and were quality controlled with the following organisms: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212. Five microliters of the aforementioned inoculum was added to each well containing 100 μl of Mueller-Hinton broth (MHB)/antibiotic (1:100 dilution), resulting in a final concentration of $\sim 10^5$ CFU/ml or $\sim 10^4$ CFU/well. Microtiter plates were sealed in plastic bags and incubated at 35°C in ambient air. The MICs were read at 24, 48, and 72 h. If required due to poor growth, plates were reincubated for a further 48 h and a final MIC reading was done on day 5 (120 h). The MIC was defined as the lowest concentration of antimicrobial agent to inhibit visible growth, except for co-trimoxazole, where 80% inhibition was used.

Etest. Mueller-Hinton agar (MHA) plates (90 mm in size) were inoculated by confluent swabbing of the surface with a 0.5 McFarland standard organism suspension, prepared as described above. Two Etest strips were applied to each plate, and the plates were incubated at 35°C in ambient air. The same antimicrobial agents used for BMD were tested. Etests were done concurrently with BMD and read at the same time intervals. MICs were determined by examining the plates for complete inhibition within the elliptical zone, the only exception being co-trimoxazole, for which an 80% zone of inhibition was read.

Additional AST. Certain isolates consistently failed to grow in MHB and on MHA plates. These isolates were then tested by BMD using MHB supplemented with 5% lysed horse blood and by Etest using MHA-5% sheep blood plates. Inoculum preparation, incubation, and reading intervals were otherwise unchanged.

Interpretation of susceptibility results. Endpoints for both BMD and Etest were read independently by both authors, discrepancies being resolved by reevaluation and a consensus decision. All MICs were interpreted according to the NCCLS breakpoints for aerobic actinomycetes (17), and error rates were assessed. A very major error (VME) was defined as a resistant isolate by BMD being designated susceptible by Etest. A major error (ME) was defined as a susceptible isolate by BMD being designated resistant by Etest. Isolates that were intermediately susceptible according to BMD and either sensitive or resistant by Etest were designated as having minor errors (mE). Frequencies of very major errors (number VME/total number resistant), major errors (number ME/total number sensitive), and minor errors (number mE/total number) were calculated. Categorical (breakpoint determination) and essential (within one 2-fold dilution) agreement between the two methods was assessed.

Sequence-based identification. Partial 16S rRNA gene sequences of all strains were determined using the domain *Bacteria*-specific 27F forward primer (20). Sequences greater than 500 bp in length were evaluated. The unknown sequences were compared to all sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Sequencing Tool for nucleotide sequence queries (BLASTN). The GenBank query type strain or culture collection strain with the highest score was then downloaded, trimmed, and compared to the subject sequence, with reevaluation of the electropherogram to reconcile any ambiguous or conflicting base assignments. The percentage similarity was then determined for each strain. Isolates for which there was no distinct aligned type or culture collection strain were identified to genus level, with a species identification made only if a characteristic phenotypic trait that reinforced the sequencing results was present. The percentage homology was stratified into four groups: 100%, >99.5%, >99%, and <99%. Strains with a greater than 99.5% homology to a single type strain were considered definitive for purposes of identification to the species level. Typical phenotypic characteristics were sought for isolates with less than 99.5% homology to a type strain, which if present were used to identify organisms to the species level; if not, a genus-level identification was made.

Nucleotide sequence accession numbers. Sequences derived from this study were submitted to the GenBank database under the following accession numbers: HQ005380, HQ005381, HQ005382, HQ005383, HQ005384, HQ005385, HQ005386, HQ005387, HQ005388, HQ005389, HQ005390, HQ005391, HQ005392, HQ005393, HQ005394, HQ005395, HQ005396, HQ005397, HQ005398, HQ005399, HQ005400, HQ005401, HQ005402, HQ005403.

RESULTS

Phenotypic characterization. Thirty-nine clinical isolates were phenotypically characterized as *Nocardia* species based on colony morphology, presence of aerial hyphae, typical Gram stain morphology, partial acid fastness, and resistance to lysozyme. The 12 aerobic actinomycetes were comfortably distinguished from the *Nocardia* genus but, with the exception of *Streptomyces* species, which had a very typical colonial morphology, were not easily differentiated from each other based on the available phenotypic data.

Genotypic characterization. The 16S rRNA sequences of 25 phenotypic *Nocardia* clinical isolates demonstrated a similarity rate of 99.5 to 100% with a distinct culture collection/type strain *Nocardia* species isolate deposited in the GenBank database. For the nonnocardial aerobic actinomycetes, a greater than 99.5% similarity identity was achieved for four isolates. Nine isolates were identified to species level using genotypic sequence identity and corroborating phenotypic traits. For the remaining 11 isolates, the sequencing result did not adequately discriminate between species, and a genus-level identification was made. For three of the 11 isolates we failed to generate sequences of greater than 500 bp. The reference strains all demonstrated >99.5% identity with the deposited type strain sequence. Interestingly the national reference strain Nocas001,

TABLE 1. Distribution of clinical[a] isolates of *Nocardia* species and other aerobic actinomycetes based on 16S rRNA sequencing data

Species	No. of isolates	Frequency (%) of *Nocardia* isolates	No. of isolates with % homology with type/culture collection strain in GenBank database			
			100%	>99.5%	>99%	<99%
Nocardia isolates						
N. farcinica	8	20.5	1	3	3	1
N. cyriacigeorgica	6	15.4	5	1	0	0
N. otitidiscaviarum	5	12.8	3	2	0	0
N. brasiliensis	3	7.7	1	2	0	0
N. nova complex	3	7.7	0	1	1	1
N. abscessus	2	5.1	1	1	0	0
N. exalbida	3	7.7	1	1	1	0
N. transvalensis	1	2.6	1	0	0	0
N. pseudobrasiliensis	1	2.6	1	0	0	0
Nocardia spp.	7	17.9	NA[b]	NA	NA	NA
Total	39		14	11	5	2
Other aerobic actinomycetes						
Rhodococcus spp.	1		NA	NA	NA	NA
Rhodococcus equi	1		1	0	0	0
Gordonia bronchialis	2		1	0	1	0
Tsukumurella tyrosinosolvens	1		0	1	0	0
Tsukumurella pulmonis	1		1	0	0	0
Streptomyces spp.	5		NA	NA	NA	NA
Actinomadura spp.	1		NA	NA	NA	NA
Total	12		3	1	1	0

[a] Excluding reference/type strains.
[b] NA, not available.

designated *Nocardia asteroides*, is in fact a *Nocardia farcinica*. To our knowledge this is the first time that this strain has been characterized genotypically, and the phenotypic data corroborate this identification. *Nocardia farcinica* (eight isolates) and *Nocardia cyriacigeorgica* (six isolates) were the most commonly identified clinical isolates. Table 1 highlights the species distribution and sequence identity. The 12 aerobic actinomycete isolates comprised *Rhodococcus* species (1 isolate), *Rhodococcus equi* (1 isolate), *Gordonia bronchialis* (2 isolates), *Streptomyces* species (5 isolates), *Tsukumurella pulmonis* (1 isolate), *Tsukumurella tyrosinosolvens* (1 isolate), and *Actinomadura* species (1 isolate).

Antimicrobial susceptibility testing. All *Nocardia* isolates demonstrated 100% susceptibility to co-trimoxazole, amikacin, and linezolid. Furthermore only these three antimicrobial agents showed a categorical agreement concordance rate between BMD and Etest of 100%. All other agents varied substantially, in both susceptibility rates (20.9 to 61.9%) and categorical agreement (67.5 to 90.2%) (Table 2). Essential agreement for the *Nocardia* isolates ranged from 46.2% to

TABLE 2. MIC_{50}, MIC_{90}, and range of MICs determined by broth microdilution, and comparative evaluation with the Etest method for the 42[a] *Nocardia* isolates

Antimicrobial agent	Broth microdilution method (BMD)				BMD vs Etest					
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)	% Susceptible[b]	% Categorical agreement	% Essential agreement	Correlation coefficient	% of errors		
								Very major	Major	Minor
Amikacin[c]	0.25	0.5	<0.12–2	100.0	100.0	65.9	0.79	0.0	0.0	0.0
Amoxicillin-clavulanate[d]	16	>64	1–>64	45.0	79.5	46.2	0.81	11.8	5.9	12.8
Ceftriaxone[e]	4	256	<0.5–>256	61.9	67.5	60.0	0.64	0.0	12.0	25.0
Ciprofloxacin[c]	4	64	0.25–>64	20.9	90.2	46.3	0.72	3.3	0.0	7.3
Clarithromycin[c]	32	>64	<0.12–>64	30.2	73.2	61.0	0.25	3.7	33.3	14.6
Imipenem[c]	8	>64	<0.12–>64	48.8	82.9	61.0	0.97	5.6	0.0	14.6
Trimethoprim-sulfamethoxazole[c]	0.12	0.5	<0.015–1	100.0	100.0	53.7	0.66	0.0	0.0	0.0
Linezolid[c]	2	2	0.25–4	100.0	100.0	81.6	0.51	0.0	0.0	0.0

[a] Includes reference strains.
[b] Susceptibility based on NCCLS interpretive criteria (17).
[c] 43 MICs evaluated; ATCC 19247 tested twice on separate occasions. No influence on MIC_{50}, MIC_{90}, or range.
[d] 40 MICs evaluated.
[e] 42 MICs evaluated.

TABLE 3. Categorical and essential agreement for the three most common *Nocardia* isolates

Antimicrobial agent	Result (%) for species (no. of isolates)														
	N. farcinica (8)					*N. cyriacigeorgica* (6)					*N. otitidiscaviarum* (6)				
	Categorical agreement	Essential agreement	VME	ME	mE	Categorical agreement	Essential agreement	VME	ME	mE	Categorical agreement	Essential agreement	VME	ME	mE
Amikacin	100.00	87.50	0.00	0.00	0.00	100.00	33.30	0.00	0.00	0.00	100.00	33.30	0.00	0.00	0.00
Amoxicillin-clavulanate	75.00	12.50	0.00	14.30	12.50	60.00	40.00	0.00	0.00	40.00	83.30	83.30	0.00	0.00	16.70
Ceftriaxone	75.00	50.00	0.00	0.00	25.00	83.30	83.30	0.00	0.00	16.70	66.70	83.30	0.00	0.00	33.30
Ciprofloxacin	75.00	62.50	0.00	0.00	25.00	100.00	33.30	0.00	0.00	0.00	83.30	0.00	0.00	0.00	16.70
Clarithromycin	100.00	100.00	0.00	0.00	0.00	100.00	66.70	0.00	0.00	0.00	66.70	83.30	0.00	0.00	33.30
Imipenem	75.00	25.00	0.00	0.00	25.00	83.30	50.00	0.00	0.00	16.70	66.70	66.70	0.00	0.00	33.30
Co-trimoxazole	100.00	37.50	0.00	0.00	0.00	100.00	83.30	0.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00
Linezolid	100.00	100.00	0.00	0.00	0.00	100.00	80.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00

81.6%, and very major errors (VME) were seen with amoxicillin-clavulanate (11.8%), ciprofloxacin (3.3%), clarithromycin (3.7%), and imipenem (5.6%). It should be noted that for the 3 most common isolates (*N. farcinica*, *N. cyriacigeorgica*, and *N. otitidiscaviarum*), a total of 20 comparative evaluations showed no VME and a single ME; however, agreement remained low (Table 3). The nonnocardial aerobic actinomycetes demonstrated an essential agreement rate of 50% to 91.7%, with VME seen with amikacin (100%), amoxicillin-clavulanate (50%) and clarithromycin (100%) (Table 4).

The concordance rate between species identification and antimicrobial drug pattern was also evaluated (see Table S1 in the supplemental material). The reference strain ATCC 19247, which correlates with drug pattern VI, was tested on two separate occasions and was included with the *N. cyriacigeorgica* for this evaluation. No corresponding drug pattern was consistently seen among all isolates of a particular species; however, using intermediate susceptibility results improved the concordance rates. Of interest, all *N. otitidiscaviarum* isolates were resistant to ciprofloxacin whereas 44.4% of *N. farcinica* isolates were susceptible to ceftriaxone.

The results of repeat susceptibility testing showed no interpretive changes (categorical agreement, 100%) with 85.4% of MICs (79.2% for Etest and 91.7% for BMD) within a 2-fold dilution of the original result (data not shown). Three isolates (*Nocardia* spp., *Nocardia exalbida*, and *Gordonia amarae*) consistently failed to grow and required blood-supplemented media for AST. For the *Nocardia* species isolate a single very major error (clarithromycin) and a single major error (amoxicillin-clavulanate) were noted, but for the remaining two isolates there was 100% categorical agreement for all antimicrobials.

DISCUSSION

This study represents the largest sample of aerobic actinomycete isolates from South Africa. This is important given that South Africa has an exceptionally high HIV/AIDS burden, an established risk factor for nocardiosis (10, 24). Furthermore, TB incidence rates in South Africa are 948 per 100,000 population per year (26), and given the ability of *Nocardia* spp to mimic pulmonary tuberculosis it is important to consider it in the differential diagnosis, particularly in patients who do not respond to first-line TB treatment. The majority of isolates in this study for which clinical details were known were obtained from AIDS patients presenting with extrapulmonary disease. In South Africa very little is known about the distribution of *Nocardia* species and the most widely known species among clinicians is still the now-defunct *N. asteroides*. Our study suggests that *N. farcinica* and *N. cyriacigeorgica* are the two most commonly isolated species, similar to results published elsewhere (14, 15). Our results highlight the now-well-accepted

TABLE 4. MIC_{50}, MIC_{90}, and range of MICs determined by broth microdilution and comparative evaluation with the Etest method for 13 aerobic actinomycetes[a]

Antimicrobial agent	Broth microdilution method (BMD)				BMD vs Etest				
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)	% Susceptible[b]	% Categorical agreement	% Essential agreement	% of errors		
							Very major	Major	Minor
Amikacin	0.5	2	<0.12–32	92.3	91.7	91.7	100.0	0.0	0.0
Amoxicillin-clavulanate	32	>64	<0.12–>64	41.7	63.6	54.5	50.0	0.0	9.1
Ceftriaxone	8	256	<0.5–>256	61.5	91.7	83.3	0.0	0.0	9.1
Ciprofloxacin	1	2	<0.12–>64	76.9	75.0	83.3	0.0	0.0	27.3
Clarithromycin	1	2	<0.12–16	92.3	75.0	66.7	100.0	10.0	9.1
Imipenem	0.5	8	<0.12–16	84.6	91.7	66.7	0.0	11.1	0.0
Trimethoprim-sulfamethoxazole	0.25	4	<0.015–>8	84.6	91.7	50.0	0.0	11.1	0.0
Linezolid	2	4	1–4	100.0	100.0	50.0	0.0	0.0	0.0

[a] Including *Rhodococcus equi* (1), *Rhodococcus* spp. (1), *Gordonia bronchialis* (2), *Gordonia amarae* 27808^T, *Streptomyces* spp. (5), *Tsukamurella pulmonis* (1), *Tsukamurella tyrosinosolvens* (1), and *Actinomadura* spp. (1).

[b] Susceptibility based on NCCLS interpretive criteria (17).

fact that phenotypic characteristics do not adequately identify the aerobic actinomycete isolates to the species level. However, 16S rRNA sequencing as an identification tool also has its limitations (18) and our study supports this in that we were unable to identify to the species level 6 out of 39 (15%) clinical nocardial isolates and 7 out of 12 (58%) nonnocardial aerobic actinomycete isolates. This is a limitation of our study, as the lack of significant homology with a type strain may mean that some of our identifications, which were made with corroborating phenotypic characteristics, are possibly inaccurate. However, only three *Nocardia* species (a total of seven isolates) demonstrated less than 99.5% homology and it is well accepted that genotypic data should be interpreted in the context of phenotypic data. Furthermore this does not detract from the results of our comparison of AST methods for this group of organisms. The epidemiological and clinical differences that are beginning to be unraveled with accurate species identification highlight the importance of identification to the species level, and in the setting of a high HIV/AIDS incidence these differences still remain to be elucidated.

Antimicrobial susceptibility testing of aerobic actinomycetes is not widely offered in South Africa, primarily due to lack of a simple and practical standardized method. The last South African study to evaluate the antimicrobial susceptibility profile of *Nocardia* species isolates was by Jones et al. over a 2-year period from 1995 to 1997, in a series of 10 patients presenting to the Chris Hani Baragwaneth hospital (10). The AST method used was not standardized, and identification to the species level was done using phenotypic methods only. Of the 10 isolates, 7 were identified as *N. asteroides*, although based on the drug susceptibility patterns 6 of these isolates conformed to drug pattern type VI (*N. cyriacigeorgica*). Furthermore, resistance to co-trimoxazole was demonstrated in five isolates, an unusual finding and in distinct contrast to our findings.

The broth microdilution reference method for AST developed by the CLSI has provided a standardized method; however, it remains beyond the scope of most routine microbiology laboratories. The Etest method is a viable alternative, although the data comparing it with BMD are limited. Of interest is the variation in methodology between studies, probably due to the lack of a standardized method at the time that most studies were performed. This study, to our knowledge, is the first that compares the Etest to BMD using the CLSI standard and thus represents an evaluation with a recognized reference method.

We sought to evaluate whether the Etest is a feasible and reliable option such that laboratories may be able to provide AST without referral to a reference center. For *Nocardia* species, with the exception of amikacin, linezolid, and co-trimoxazole, the essential and categorical agreement rates fell short of the accepted international standard of greater than 90% concordance and <3% ME/no VME, respectively. However, these pooled results did not reflect the acceptable error rates seen between the most commonly isolated species, *N. farcinica*, *N. cyriacigeorgica*, and *N. otitidiscaviarum.* For these three species, categorical agreement ranged from 60 to 100% and essential agreement from 12.5 to 100%, still reflective of an overall poor level of agreement. The uniform susceptibility of all isolates to amikacin, linezolid, and co-trimoxazole precludes any definitive conclusion in comparing the two methods. Ciprofloxacin was the only other agent that showed greater than 90% categorical agreement.

The single *N. transvalensis* isolate was identified genotypically (100% homology, 764 bp) and demonstrated an elevated MIC to amikacin (BMD MIC of 2 μg/ml and an Etest MIC of 6 μg/ml). Despite the elevated MIC it still fell within the susceptible breakpoint of 8 μg/ml, an unusual finding for an organism that is characteristically resistant to amikacin. This may reflect geographical variation and a peculiarity of South African isolates or may simply be an anomaly. Further testing of a greater number of isolates is warranted, although in our experience it is a rarely isolated species.

Etests were read independently by two observers, and discrepant results were resolved by consensus. Determination of zones of inhibition by Etest was variable. We found it easy to determine points of intersection for amikacin, imipenem, and co-trimoxazole at 72 h. Determination of co-trimoxazole zones is assisted by the 80% inhibition which eliminates the effect of haziness, an aspect that we found hampers Etest readings, particularly for linezolid and ceftriaxone. Etest readings were generally easier after 72 h, and we feel that an earlier reading, apart from being technically difficult to interpret, may result in a falsely lower MIC reading.

Our results contrast significantly with those of other published studies. Biehle et al. (2) found an overall agreement of 96.2% and Ambaye et al. (1) an overall agreement of 96.6% between the two methods. Very major errors were noted for amoxicillin-clavulanate and ceftriaxone only. The reasons for the discrepancies are numerous. First, our inoculum preparation strictly followed that outlined in the NCCLS guideline (17). Random colony counts highlighted variation in actual CFU/ml despite close attention to inoculum preparation. Colony counts, when not within the specified $\sim 10^5$-CFU/ml range, were ~ 1 $\log_{10}$ dilution lower, in accordance with the findings of Biehle et al. The issues surrounding the initial inoculum have been extensively discussed previously (2, 23), although we do not feel that the issue has been resolved. In a small subset of isolates within our collection (data not shown) we used a nondiluted 0.5 McFarland inoculum for inoculation of the BMD microtiter plates. Interestingly, there was only one discordant categorical result (a minor error). Achieving a homogenous suspension of organisms is difficult, despite the addition of glass beads, and we feel that this may hamper the performance of the Etest in particular. Furthermore, a 0.5 McFarland standard should result in $\sim 10^8$ CFU/ml, as specified by the CLSI guideline; however, this has been shown to not always be the case (2) and our experience is similar. The effect of inoculum size is worthy of further study. Second, the interpretive breakpoints used in this study are specific to the aerobic actinomycetes, breakpoints which were established only in 2003 and thus have not been used before in a comparative evaluation. Third, the range of isolates tested appears to have differed substantially, not entirely unexpected given the recent advances in *Nocardia* taxonomy and our use of genotypic identification. Lastly, the antimicrobial agents tested differed between studies, although this would only impact on overall level of agreement. Our results of repeat testing showed no discordance in categorical agreement, although the overall poor essential agreement of the repeat Etests (79.2%) is noteworthy and may possibly reflect the inherent limitations of the Etest

methodology for AST of this group of organisms. It is worth mentioning that our ATCC 19247 strain, a strain that has been proposed as a quality control strain (23) and that was used as such, gave reproducible results that consistently fell within the proposed reference range at 72 h.

The limited number of other aerobic actinomycetes evaluated precludes any definitive conclusions regarding the Etest as an alternative method; however, from our experience the same issues outlined above are applicable. For *Streptomyces* species it is especially difficult to obtain a homogenous suspension, particularly if aerial hyphae do not develop. The high rates of very major errors for amikacin and clarithromycin are due to the paucity of resistant strains and should thus be treated with caution.

The value of the drug susceptibility pattern as a tool to aid in species identification was somewhat limited, although we acknowledge that the number of species may not have been sufficient. Interestingly only two thirds of *N. farcinica* isolates were resistant to ceftriaxone and all *N. otitidiscaviarum* isolates were resistant to ciprofloxacin. Species distribution and drug susceptibility profiles appear to vary by geographical region and patient population (9, 14, 19, 22); thus, it is not surprising that isolates do not always conform to specific drug patterns. Of interest is that only 48.8% of all *Nocardia* isolates were susceptible to imipenem with an MIC_{90} of >64 μg/ml, whereas susceptibility to co-trimoxazole was 100% with an MIC_{90} of 0.5 μg/ml. In South Africa imipenem is often advocated for the treatment of severe nocardiosis, is usually started empirically (together with amikacin and co-trimoxazole), and is often continued in the absence of AST. This practice highlights the need for local AST and further epidemiological investigation. Pulmonary nocardiosis is underrecognized in South Africa, where an autopsy study showed that 38% of cases were diagnosed antemortem as pulmonary tuberculosis (16). This series highlighted the high HIV rate (76%) and copathology (50%) in patients with pulmonary nocardiosis. Despite its being underdiagnosed it is still a relatively uncommon disease in South Africa, an enigma to many when one considers that immunosuppression is a primary risk factor. The majority of cases seen are the severe extrapulmonary forms of disease, and the question why nocardiosis is not seen more commonly in South Africa remains a pertinent one. The potential reasons are numerous, including misdiagnosis, difficulty isolating the slow-growing organism, and the use of co-trimoxazole as prophylaxis. In transplant patients it has been shown that bactrim prophylaxis is not protective against nocardia infection (19). Given the widespread prophylactic use of this agent, it is remarkable that it remains highly active *in vitro*. Further evaluation of its clinical efficacy is warranted given its prescription as the drug of choice for treatment of nocardiosis.

In conclusion, we have profiled 51 South African isolates of aerobic actinomycetes, including 39 *Nocardia* species, and compared the Etest with the BMD reference method. We demonstrated varied susceptibility patterns among different isolates of the same species, uniform susceptibility to amikacin, linezolid, and co-trimoxazole for *Nocardia* species, and a high level of resistance to imipenem. Previous comparative evaluations between the two methods were done in the absence of a standardized method. We demonstrated a significant lack of agreement, both categorical and essential, between the two methods. Inoculum preparation and reading and interpretation of Etest AST results require considerable familiarity and experience. Considering the AST issues highlighted by our study, the dearth of data comparing the Etest to the reference method, and the need for further epidemiological evaluation of aerobic actinomycetes, we would suggest that the BMD reference method be used as the primary method of AST in future studies. Until such time that the accumulated body of knowledge addresses many of these unresolved issues, we would not recommend that the Etest be offered in the routine microbiology laboratory for the occasional aerobic actinomycete isolate.

ACKNOWLEDGMENTS

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We thank Lorna Jenkin for her excellent critical review of the manuscript.

REFERENCES

1. **Ambaye, A., P. C. Kohner, P. C. Wollan, K. L. Roberts, G. D. Roberts, and F. R. Cockerill III.** 1997. Comparison of agar dilution, broth microdilution, disk diffusion, E-test, and BACTEC radiometric methods for antimicrobial susceptibility testing of clinical isolates of the *Nocardia asteroides* complex. J. Clin. Microbiol. **35:**847–852.
2. **Biehle, J. R., S. J. Cavalieri, M. A. Saubolle, and L. J. Getsinger.** 1994. Comparative evaluation of the Etest for susceptibility testing of *Nocardia* species. Diagn. Microbiol. Infect. Dis. **19:**101–110.
3. **Brown, J. M., and M. M. McNeil.** 2004. Media and methods used for the identification of the aerobic actinomycetes, p. 6.2.1–6.2.11. *In* H. D. Isenberg (ed.), Clinical microbiology procedures handbook, 2nd ed., vol. 2. ASM Press, Washington, DC.
4. **Brown-Elliot, B. A., J. M. Brown, P. S. Conville, and R. J. Wallace, Jr.** 2006. Clinical and laboratory features of the *Nocardia* spp. based on current molecular taxonomy. Clin. Microbiol. Rev. **19:**259–282.
5. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, seventh edition. CLSI document M7–A7. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Cloud, J. L., P. S. Conville, A. Croft, D. Harmsen, F. G. Witebsky, and K. C. Carroll.** 2004. Evaluation of partial 16S ribosomal DNA sequencing for identification of *Nocardia* species by using the MicroSeq 500 system with an expanded database. J. Clin. Microbiol. **42:**578–584.
7. **Conville, P. S., and F. G. Witebsky.** 2007. Organisms designated as *Nocardia asteroides* drug pattern type VI are members of the species *Nocardia cyriacigeorgica*. J. Clin. Microbiol. **45:**2257–2259.
8. **Conville, P. S., and F. G. Witebsky.** 2007. *Nocardia*, *Rhodococcus*, *Gordonia*, *Actinomadura*, *Streptomyces*, and other aerobic actinomycetes, p. 515–542. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of clinical microbiology, 9th ed., vol. 1. ASM Press, Washington, DC.
9. **Glupczynski, Y., C. Berhin, M. Janssens, and G. Wauters.** 2006. Determination of antimicrobial susceptibility patterns of *Nocardia* spp. from clinical specimens by Etest. Clin. Microbiol. Infect. **12:**905–912.
10. **Jones, N., M. Khoosal, M. Louw, and A. Karstaedt.** 2000. Nocardial infection as a complication of HIV in South Africa. J. Infect. **41:**232–239.
11. **Kageyama, A., K. Yazawa, A. Mukai, T. Kohara, K. Nishimura, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia aroensis* sp. nov. and *Nocardia pneumonia* sp. nov., isolated from patients in Japan. Int. J. Syst. Evol. Microbiol. **54:**2025–2029.
12. **Kageyama, A., N. Poonwan, K. Yazawa, Y. Mikami, and K. Nishimura.** 2004. *Nocardia beijingensis* is a pathogenic bacterium to humans: the first infectious cases in Thailand and Japan. Mycopathologia **157:**155–161.
13. **Long, P. F.** 1994. A retrospective study of *Nocardia* infections associated with the acquired immune deficiency syndrome (AIDS). Infection **22:**362–364.
14. **Minero, M. V., M. Marin, E. Cercenado, P. M. Rabadán, E. Bouza, and P. Muñoz.** 2009. Nocardiosis at the turn of the century. Medicine. **88:**250–261.
15. **Muñoz, J., B. Mirelis, L. M. Aragón, N. Gutiérrez, F. Sánchez, M. Español, O. Esparcia, M. Gurguí, P. Domingo, and P. Coll.** 2007. Clinical and microbiological features of nocardiosis 1997–2003. J. Med. Microbiol. **56:**545–550.
16. **Murray, J., P. Back, and G. Nelson.** 2004. Pulmonary nocardiosis in autopsies of South African miners. S. Afr. J. Epidemiol. Infect. **19**(2)**:**60–62.
17. **NCCLS.** 2003. Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes. Approved standard. NCCLS document M-24A. NCCLS, Wayne, PA.

18. **Patel, J. B., R. J. Wallace, Jr., B. A. Brown-Elliot, T. Taylor, C. Imperatrice, D. G. B. Leonard, R. W. Wilson, L. Mann, K. C. Jost, and I. Nachamkin.** 2004. Sequence-based identification of aerobic actinomycetes. J. Clin. Microbiol. **42:**2530–2540.
19. **Peleg, A. Y., S. Husain, Z. A. Qureshi, F. P. Silveira, M. Sarumi, K. A. Shutt, E. J. Kwak, and D. L. Paterson.** 2007. Risk factors, clinical characteristics and outcome of *Nocardia* infection in organ transplant recipients: a matched case-control study. Clin. Infect. Dis. **44:**1307–1314.
20. **Reysenbach, A. L., K. Longnecker, and J. Kirschstein.** 2000. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hypothermal vent. Appl. Environ. Microbiol. **66:**3798–3806.
21. **Roth, A., S. Andrees, R. M. Kroppenstedt, D. Harmsen, and H. Mauch.** 2003. Phylogeny of the genus *Nocardia* based on reassessed 16S rRNA gene sequences reveals underspeciation and division of strains classified as *Nocardia asteroides* into three established species and two unnamed taxons. J. Clin. Microbiol. **41:**851–856.
22. **Tan, C. K., C. C. Lai, S. H. Lin, C. H. Liao, C. H. Chou, H. L. Hsu, Y. T. Huang, and P. R. Hsueh.** 2009. Clinical and microbiological characteristics of nocardiosis including those caused by emerging *Nocardia* species in Taiwan, 1998–2008. Clin. Microbiol. Infect. doi:10.1111/j.1469–0691.2009.02950.x.
23. **Tomlin, P., C. Sand, and R. P. Rennie.** 2001. Evaluation of Etest, disk diffusion and broth microdilution to establish tentative quality control limits and review susceptibility breakpoints for two aerobic actinomycetes. Diagn. Microbiol. Infect. Dis. **40:**179–186.
24. **Uttamchandani, R. B., G. L. Daikos, R. R. Reyes, M. A. Fischl, G. M. Dickinson, E. Yamguchi, and M. R. Kramer.** 1994. Nocardiosis in 30 patients with advanced human immunodeficiency virus infection: clinical features and outcome. Clin. Infect. Dis. **18:**348–353.
25. **Wallace, R. J., L. C. Steele, G. Sumter, and J. M. Smith.** 1988. Antimicrobial susceptibility patterns of *Nocardia asteroides*. Antimicrob. Agents Chemother. **32:**1776–1779.
26. **World Health Organization Report.** 2009. Global tuberculosis control. South Africa 2007. http://apps.who.int/globalatlas/predefinedReports/TB/PDF_Files/zaf.pdf. Last accessed 20 May 2010.

Table S1. *Nocardia* species delineation according to drug pattern[a] & % concordance of test isolates

Species	# Isolates	Drug Pattern	Amikacin	Amoxicillin-clavulanate	Ceftriaxone	Cipro-floxacin	Clarithro-mycin	Imipenem	Trimethoprim-sulfamethoxazole	Linezolid
N. farcinica	9	V	S	NA	R	S	R	S		S
		% Concordance	100.0%		55.6%	66.7%	100.0%	88.9%		100.0%
		Intermediate included[b]	100%		66.7%	88.9%	100%	100.0%		100%
N. cyriacigeorgica	6	VI	S	R	S	R	R	S		S
		% Concordance	100.0%	83.3%	83.3%	100.0%	83.3%	66.7%		100.0%
		Intermediate included	100.0%	100.0%	83.3%	100.0%	83.3%	66.7%		100.0%
N. otitidiscaviarum	6	NA	S	R	R	S		R		S
		% Concordance	100.0%	83.3%	66.7%	0.0%		66.7%		100.0%
		Intermediate included	100.0%	100.0%	100.0%	16.7%		100.0%		100.0%
N. brasiliensis	3	NA	S	S		R	R		S	S
		% Concordance	100.0%	100.0%		100.0%	33.3%		100.0%	100.0%
		Intermediate included	100.0%	100.0%		100.0%	66.7%		100.0%	100.0%
N. nova complex	3	III	S	R	S		S	S		S
		% Concordance	100.0%	33.3%	100.0%		100.0%	100.0%		100.0%
		Intermediate included	100.0%	33.3%	100.0%		100.0%	100.0%		100.0%
N. abscessus	2	I	S	S	S	R	R	R		S
		% Concordance	100.0%	100.0%	0.0%	100.0%	100.0%	100.0%		100.0%
		Intermediate included			50.0%					
N. transvalensis	1	IV	R		S	S	R	S		S
		% Concordance	0.0%		100.0%	0.0%	0.0%	0.0%		100.0%

[a] according to reference 4

[b] intermediate susceptibility results included in the determination of concordance i.e. I = S or R

S = sensitive

R = resistant

Appendix 2

-----Original Message-----
From: pateljcm@mayo.edu [mailto:pateljcm@mayo.edu]
Sent: 12 August 2010 04:51 PM
To: rebel@mweb.co.za
Cc: rebel@mweb.co.za; Naseema Aithma
Subject: Decision on manuscript JCM01073-10 Version 1

Dr Warren Lowman
University of the Witwatersrand & National Health Laboratory Services, South Africa
Clinical Microbiology & Infectious Diseases
Wits Medical School 7 York Road
Parktown
Johannesburg, Gauteng
South Africa

Re: Antimicrobial susceptibility testing and profiling of Nocardia species and other aerobic actinomycetes from South Africa: a comparative evaluation of broth microdilution versus the Etest. (JCM01073-10 Version 1)

Dear Dr. Lowman:

Thank you for submitting your article to the Journal of Clinical Microbiology. Your article has been reviewed by two reviewers and myself. Your article may be acceptable for publication; however, please address the reviewers' comments with a point-by-point response and submit a revised manuscript.

Please return your modified manuscript within 60 days; if you cannot complete the modification within this time period, please contact me. If you decide that you do not want to modify the manuscript and wish to submit it to another journal, please notify me of your decision immediately so that the manuscript can be formally withdrawn.

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corrections/changes are NOT accepted. Accordingly, there may be differences between the JCM Accepts version and the final, typeset version. The manuscripts remain listed on the JCM Accepts page until the final, typeset versions are posted, at which point they are removed from the JCM Accepts page. Any supplemental material intended, and accepted, for publication is not posted until publication of the final, typeset article.

Thank you for submitting your paper to JCM.

Sincerely,
Robin Patel
Editor, Journal of Clinical Microbiology (JCM)

REVIEWER 1:
Comments:
To the authors:
This manuscript describes a study comparing the susceptibility results obtained using the CLSI approved method, broth microdilution, with the Etest for Nocardia species and a limited number of other aerobic actinomycetes. This is an important topic because the broth microdilution test may be difficult to perform in some geographic area and can be difficult to interpret by those with limited experience in endpoint interpretation. A relatively easy-to-perform test such as Etest might be tempting for laboratories to use instead of broth microdilution; it is important to discuss the value of results obtained by this method in comparison to the approved method. The susceptibility data appears to be nicely interpreted.

My main concern with this manuscript is the identification of Nocardia species. First, the authors do not state the specifics of their identification process, namely, the number of bases sequenced and the criteria they used for identification. I question the final identification of some of the isolates based on the information presented in Table 1. I assume that in table one, information under the heading >99% includes isolates whose sequences were 99.0 to 99.5% similar to the type strain of the organism, and that the heading <99% includes isolates whose sequences were <99.0% similar to that of the type strain. If this is true, then it is quite possible that some of the isolates that they identified as a particular species are not really members of that species, as in the literature, some Nocardia of different species are 99.8% similar by 16S sequence. I would certainly say that a Nocardia isolate that is <99.0% similar to a type strain cannot be called a member of that species. This is important because it affects the parts of this manuscript that address the susceptibility patterns of particular species, including Table 3 and Table 5, page 9 line 167, page 9 lines 172 to 176, page 10 line 189, page 11 line 220.

I also question the identification of the "N. transvalensis" isolate, as members of this species are characteristically resistant to amikacin. This should be addressed in the discussion if it is true.

It would also be useful for the authors to comment on how endpoints were determined, that is, was the person reading the results experienced with this technique, and were the results verified by another reader?

Other comments:
p 2 line 20 This sentence could be interpreted as meaning that there is no standardized testing method for aerobic actinomycetes. I think the authors intend to state that the method is not simple, so the sentence should be rephrased.
p 2 line 21 Molecular identification techniques make the study of these organisms easier, in that the exact identity can be determined in many cases. Do the authors think the study is complicated by molecular-based methods because they are not available to many laboratories? If so, then it should be stated as such.
p 2 line 27 "...and essential agreement for broth microdilution and Etest for the Nocardia isolates.."
p 2 line 28 "For all isolates tested" sounds like very major errors were found for every isolate with the listed drugs. This should be rephrased.
p 2 lines 31 to 34 see above
p 2 line 36 To what does "geographical differences" refer, susceptibility patterns or species prevalence. This topic is not addressed in the study design or in the results.

p 3 line 48 Was it the factors you stated that prompted the standardization of aerobic actinomycete susceptibility testing, or a general need for standardization of this method?
p 3 line 51 Mention the reasons the broth microdilution method may be impractical - such as difficulty obtaining panels, cost, interpretation difficulties, etc.
p 4 line 74 give more information about clinical isolates - how many isolates of Nocardia, how many of other genera.
p 5 lines 82-84 Phenotypic testing is not useful for the identification of these organisms. This should be deleted as you did identify isolates using 16S rRNA gene sequence.
p 5 line 94 Did you check the inoculum concentration by dilution and colony counts? This would be important since you do specifically state in the discussion that your inoculum was 104 CFU/well or 105 CFU/ml.
p 5 line 96 Did you use the procedure for aerobic bacteria (reference 5) or the procedure for aerobic actinomycetes (reference 17)? If you used both, please state why.
p 5 line 100 state that cotrimoxazole is the same as trimethoprim/sulfamethoxazole.
p 6 line 119 Which isolates were tested using supplemented media? Were there any discrepancies in the results obtained when these supplements were used?
p 7 lines 123-126 Define very major errors, major errors, minor errors, categorical and essential agreement.
p 7 line 139 No accession numbers were listed for the gene sequences. Please make sure that the identifications are correct before sequences are submitted. Unless the sequences you plan to submit are somehow different from the sequences already present in GenBank for the species you sequenced, there is really no need for them to be submitted.
p 8 line 147 I count 25 isolates with 16S sequences between 99.5 and 100% similar to a type strain, your text says 27.
p 8 line 160 "All Nocardia isolates demonstrated..."
p 12 line 235 see above about inoculum colony counts
Table 1 Include the non-Nocardia isolates in this table.
Table 2 Modify the title to reflect that the information is for various species of Nocardia; it is a little confusing because you also have an identification category of Nocardia sp.

Table 4 It is unclear what species were tested.

REVIEWER 2:
Comments:
The authors compared susceptibility testing of aerobic actinomycetes, including Nocardia spp., using broth microdilution and the E-test for 8 drugs. The manuscript provides data on actinomycete susceptibility patterns in South Africa and the author's results suggest that E-test does not compare equivalently to the CLSI broth microdilution method. This is in contrast to previous reports from the 1990's which was a period in time when broth dilution methods were not yet standardized.

Major concern
1. The abstract indicates that 41 clinical isolates of aerobic actinomycetes were tested including 39 Nocardia species, suggesting that 2 non-Nocardia actinomycetes were tested. The Materials and Methods section (pg 4, line 75) indicates that 51 clinical isolates were tested but doesn't breakdown how many were Nocardia spp. vs non-Nocardia spp. Table 1 indicates 42 Nocardia spp. isolates were tested. Table 4 provides broth dilution and E-test results for the non-Nocardia aerobic actinomycetes but doesn't indicated what the total number tested was. The data in the table would suggest it was more than 2 isolates. So the numbers aren't adding up throughout the manuscript. The authors should indicate in the Materials and Methods section and in the Table, how many Nocardia and non-Nocardia aerobic actinomycetes were tested and should list the identies of these isolates in the Methods section (eg., Streptomyces spp. (n=2), Gordonia sputi (n=3), etc.). They should also reconcile the non-concordant numbers throughout the manuscript.
2. All accession numbers are missing from the Methods section (pg 7, line 139).

Minor concerns
1. Tables 2, 3 and 4 could be consolidated into a single table which would make comparisons easier for the reader. My suggestion is to list drugs across the top and have rows listing all Nocardia spp (currently Table 2), the 3 most common species broken out (currently Table 3) and then other aerobic actinomycetes (currently Table 4).
2. Introduction, line 56 - briefly elaborate on the findings of Biehle et al., so the reader doesn't have to stop and look them up to understand the point made by this sentence.
3. Methods, pg 5 line 90 - provide source for the sterile beads.
4. Methods, pg 7 lines 123-126 - one should not use the term they are defining in a definition (for example, don't use VME to define "VME"). Reword to signify that a very major error occurs when the new method indicates a susceptible response and the current AST indicates a resistant response, etc.
5. Methods, pg 7 lines 133-135 - indicated the % similiarity required to identify the organism to the species level. The results of findings are discussed in the results section but no indication is ever given as to what an acceptable lower limit would be.
6. References 18 and 20 have minor formatting errors in the authors list.
7. Methods and Tables - define the criteria for "Essential Agreement". Are the authors using +/- 1 dilution?
8. Table 2 footnote b - why are there 43 MICs on 42 isolates?

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Response to reviewers' comments

Reviewer 1

1. Concerns regards identification of Nocardia species

   The reviewer's concerns are valid primarily from the perspective that definitive identification of Nocardia species has not been conclusively dealt with. The CLSI have published standards for interpretive criteria for identification of bacteria (MM18-A), which states that ≥99.6% identity is sufficient to designate a species. Identities of 99 – 99.5% are considered sufficient for genus and possibly should be reported as "closest to species". Furthermore this document specifies other target sequences that may be more discriminatory. A publication by Woo et al (J Med Microbiol 2009; 58:1030) suggests that 16S rRNA sequencing is insufficient for speciation of Nocardia species. Despite these limitations the literature most often cites the use of 16S rRNA sequences as the means of identifying to species level and we thought it appropriate to maintain the status quo until such time as a definitive recommendation is made. We are currently addressing this issue ourselves by looking at RT-PCR of *gyrB* gene sequences.

   We nevertheless attempted to identify our organisms as accurately as possible by including characteristic phenotypic traits, where the sequencing result was not discriminatory enough, to make a final decision on organism identification. This was stated in the manuscript, but has been added to and expanded on in the methods section. Furthermore we decided to highlight the level of sequence identity in a table as this is often not clear in other studies that focus primarily on AST. We feel this is an important omission given the aforementioned issues and have thus attempted to draw attention to this "grey" area, although it was not addressed in our discussion and we have done so now (see amended manuscript, line 220-228). We have addressed this as a limitation but do not feel that it detracts from the overall objective of the study which is to compare the 2 AST methods for this group of organisms. We do make certain conclusions based on the organism identity but with the exception of N. farcinica our identifications were based on significant homology (>99.5%) with a type strain. Isolates with more than 500 bases sequenced were assessed (added to manuscript, line 138). It should also be noted that only 3 species had isolates with less than 99.5% sequence identity. N. nova is a complex of organisms and it is accepted that 16S rRNA sequencing does not adequately discriminate between species of the complex. N. exalbida is a relatively new species, first described in 2006. N. farcinica is not usually a problem although we had a total of 4 isolates for which sequencing was not sufficiently discriminatory. These isolates did not however give other species options when blasted. This may reflect 16S rRNA diversity in isolates from SA, such as increased microheterogeneity. We believe it requires further investigation.

Our identification of N. transvalensis was 100% (for 764 bases) by 16S rRNA sequencing. It was the only isolate in our entire study that showed elevated MICs to amikacin with a BMD MIC of 2µg/ml and an Etest MIC of 6µg/ml, which is still susceptible according to CLSI guidelines (this has been addressed in the discussion, line 259).

2. Determination of endpoints

   All endpoints were read by 2 people- the authors. Both are experienced in reading endpoints as BMD is an important component of our routine laboratory work (principally done by author 2). Added to methods section, line 127-129.

3. p2, line 20 has been rephrased.
4. p2, line 21 has been rephrased.
5. p2, line 27 omission has been added.
6. p2, line 28 has been rephrased.
7. p2, lines 31-34 has been addressed.
8. p2, line 36 has been rephrased.
9. p3, line 48 has been rephrased.
10. p3, line 51 has been added to.
11. p4, line 74 has been expanded.
12. p5, lines 82-84 were used, as stated above, to assist with identification and resolve discrepancies where possible. Based on our statements above that genotypic characterization can be complemented by phenotypic characteristics we think it is important to retain this information.
13. p5, line 94 is addressed in line 95, which states that colony counts were done on random samples. We have addressed this issue further in the discussion as well.
14. p5, line 96. We used procedure for aerobic actinomycetes, but reference to aerobic bacteria (ref.5) is for preparation of antimicrobials and panels which is not specifically addressed in ref.17
15. p5, line 100, trimethoprim/sulfamethoxazole has been added.
16. p6, line 119, have added information regards AST with supplemented media to the results section. As stated these isolates consistently failed to grow on MHA which is why we used supplemented media.
17. p7, lines 123-126, definitions have been included.
18. p7, line 139, sequences have already been submitted, only for those isolates that generated sequence identities of ≥99.5%. Accession numbers have been included.
19. We included the reference strains but have removed them and the text reflects the table now. We do reflect on the sequencing results of the reference strains further in the text and thus agree that it is better to correlate with the table.
20. p8, line 160 has been reworded.
21. p12, line 235, comment about colony counts has been added to in discussion and has been addressed above.

Reviewer 2

Major concerns

1. Abstract has been corrected to reflect the 51 isolates tested, and materials and methods section has been updated to reflect how many Nocardia spp. vs non-Nocardia spp.
   The total no. of clinical Nocardia spp. isolates tested, as reflected in table 1, is 39. The total of 42 isolates reflected in table 2 is because of the 3 additional reference strains that were incorporated for the AST. We have added footnotes to explain the numbers.

   Table 4 has been updated to reflect the number of isolates and the species included.

   The identities of organisms tested have been reflected in the results section and we believe it is sufficient to discriminate the numbers of Nocardia spp. vs non-Nocardia spp. in the methods section, as requested.

   We have carefully reconciled all the numbers of isolates and believe that the manuscript now accurately reflects the numbers tested.

2. Accession numbers have been included.

Minor concerns

1. Table 4 has been modified to include species tested.
2. Line 56, Biehle et al's findings have been elaborated on.
3. p5, line 90, Sterile beads source added.
4. p7, lines 123-126 has been addressed.
5. p7, lines 133-135. This has been extensively addressed above (reviewer 1, major concern).
6. References 18 and 20 have been corrected.
7. Definitions for categorical and essential agreement have been added to methods section (interpretation of susceptibility results).
8. 43 MICs as the ATCC 19247 reference strain was tested twice on separate occasions. We chose to include this as it made no difference to the $MIC_{50,}$ MIC_{90} or range but reflects on our primary objective in the assessment of categorical and essential agreement. We have added to the footnote to clarify this.

Appendix 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Lowman

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M071050**
PROJECT	Phenotypic Identification and Antibiotic Susceptibility Testing of Nocardia Specieis
Evaluation of Phenotypic	Identification Methods and the Etest for Direct
Susceptibility...	
INVESTIGATORS	Dr W Lowman
DEPARTMENT	Clinical Microbilogy
DATE CONSIDERED	07.10.26
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.10.30 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof AG Duse

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**