Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother 2010; **65**: 2514–2517
doi:10.1093/jac/dkq383 Advance Access publication 14 October 2010

In vitro susceptibility of *Actinobaculum schaalii* to 12 antimicrobial agents and molecular analysis of fluoroquinolone resistance

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Received 20 August 2010; returned 10 September 2010; revised 10 September 2010; accepted 14 September 2010

Objectives: To assess the *in vitro* susceptibility of *Actinobaculum schaalii* to 12 antimicrobial agents as well as to dissect the genetic basis of fluoroquinolone resistance.

Methods: Forty-eight human clinical isolates of *A. schaalii* collected in Switzerland and France were studied. Each isolate was identified by 16S rRNA sequencing. MICs of amoxicillin, ceftriaxone, gentamicin, vancomycin, clindamycin, linezolid, ciprofloxacin, levofloxacin, moxifloxacin, co-trimoxazole, nitrofurantoin and metronidazole were determined using the Etest method. Interpretation of results was made according to EUCAST clinical breakpoints. The quinolone-resistance-determining regions (QRDRs) of *gyrA* and *parC* genes were also identified and sequence analysis was performed for all 48 strains.

Results: All isolates were susceptible to amoxicillin, ceftriaxone, gentamicin, clindamycin (except three), vancomycin, linezolid and nitrofurantoin, whereas 100% and 85% were resistant to ciprofloxacin/metronidazole and co-trimoxazole, respectively. Greater than or equal to 90% of isolates were susceptible to the other tested fluoroquinolones, and only one strain was highly resistant to levofloxacin (MIC ≥32 mg/L) and moxifloxacin (MIC 8 mg/L). All isolates that were susceptible or low-level resistant to levofloxacin/moxifloxacin ($n=47$) showed identical GyrA and ParC amino acid QRDR sequences. In contrast, the isolate exhibiting high-level resistance to levofloxacin and moxifloxacin possessed a unique mutation in GyrA, Ala83Val (*Escherichia coli* numbering), whereas no mutation was present in ParC.

Conclusions: When an infection caused by *A. schaalii* is suspected, there is a risk of clinical failure by treating with ciprofloxacin or co-trimoxazole, and β-lactams should be preferred. In addition, acquired resistance to fluoroquinolones more active against Gram-positive bacteria is possible.

Keywords: UTIs, *A. schaalii*, quinolone resistance, QRDRs, *gyrA*, *parC*

Introduction

The genus *Actinobaculum* is closely related to the genera *Actinomyces*, *Arcanobacterium* and *Mobiluncus*.[1] These bacteria are curved, non-motile, non-spore-forming Gram-positive bacilli and they are catalase, oxidase and urease negative.[1,2] They grow preferentially at 37°C under strictly anaerobic or microaerophilic conditions.[1] To date, four species have been described (http://www.bacterio.cict.fr/): *Actinobaculum schaalii* (1997); *Actinobaculum suis* (1997); *Actinobaculum massiliae* (2002); and *Actinobaculum urinale* (2003). They are probably part of the commensal flora of the human genital or urinary tract.[1] Because of its slow growth and its similarity to normal bacterial flora on skin and mucosa, *A. schaalii* is difficult to identify by culture and is probably frequently considered as a contaminant. However, *A. schaalii* has been reported to be responsible for numerous urinary tract infections (UTIs), mainly in elderly patients with underlying urological predispositions.[2–4] Interestingly, a recent study showed that 22% of 252 urine samples from patients >60 years were positive for *A. schaalii* using real-time PCR.[3] This microorganism may also cause septic complications such as urosepsis, abscess, osteomyelitis and endocarditis.[2,4–6] Although *A. schaalii* is an emerging uropathogen, *in vitro* susceptibility to antimicrobial agents has been poorly evaluated, particularly in terms of MICs.

Resistance to quinolones is mainly related to the acquisition of point mutations in DNA gyrase (GyrA) and topoisomerase IV (ParC).[7] Alterations predominantly occur in the so-called

quinolone-resistance-determining region (QRDR). Note that mutations appear most frequently at positions 83 and 87 in GyrA and 80 and 84 in ParC (*Escherichia coli* numbering). Since *gyrA* and *parC* sequences of *A. schaalii* are not available, the presence of specific variations in the QRDRs, which might explain fluoroquinolone resistance, has not yet been documented.

The aim of this study was to determine the *in vitro* susceptibility of 48 *A. schaalii* clinical isolates to 12 antimicrobial agents including those commonly used for the treatment of UTIs. The molecular analysis of fluoroquinolone resistance was also attempted by determining the QRDR sequences of the *gyrA* and *parC* genes.

Materials and methods

Bacterial isolates

We examined 48 clinical isolates of *A. schaalii* recovered from urine ($n=28$), blood ($n=11$), abscess ($n=7$), biopsy ($n=1$) and urethral swab ($n=1$) samples including 10 collected between 2005 and 2008 from Henri Mondor Hospital (Créteil, France), 28 collected between 2004 and 2010 from ADMED (La Chaux-de-Fonds, Switzerland),[8] 9 collected between 2000 and 2010 from the University Hospital of Lausanne (Lausanne, Switzerland) and 1 collected in 2010 from Dianalab (Geneva, Switzerland). All 48 strains were accurately identified as *A. schaalii* by sequencing of the 16S rRNA gene, as previously described.[8]

Antimicrobial susceptibility testing

MICs of the following 12 antibiotics were determined for all strains using the Etest method (bioMérieux, Marcy l'Étoile, France): amoxicillin; ceftriaxone; gentamicin; vancomycin; clindamycin; linezolid; ciprofloxacin; levofloxacin; moxifloxacin; co-trimoxazole; nitrofurantoin; and metronidazole. All tests were performed on freshly poured Schaedler agar supplemented with 5 μg/mL haemin, 1 μg/mL vitamin K1 and 5% sheep blood (lysed sheep blood for co-trimoxazole testing). The plates were inoculated with a bacterial suspension adjusted to a turbidity equivalent to that of a 1 McFarland standard in 0.9% NaCl and incubated anaerobically for 48 h. *Bacteroides fragilis* ATCC 25285 was used as a quality control strain, and interpretation of results was made according to non-related species EUCAST clinical breakpoints (www.eucast.org/), except for co-trimoxazole (Enterobacteriaceae and *Staphylococcus* spp.), nitrofurantoin (*E. coli* and *Staphylococcus saprophyticus* responsible for uncomplicated UTIs) and clindamycin and metronidazole (Gram-positive anaerobes).

PCR amplification and sequencing

Bacterial genomic DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Courtaboeuf, France). The DNA fragments corresponding to the QRDRs of *gyrA* and *parC* genes were first amplified using degenerate primers as previously described.[9] Based on these sequences, a novel pair of specific primers for each gene was specifically designed for *A. schaalii*: gyrA-As-F2 (5′-CGGAGACCGGCCAGATTC-3′) and gyrA-As-R2 (5′-GGTGAG GATGGTCGGTTCC-3′) to give a 316 bp product for *gyrA*; and parC-As-F2 (5′-AGCGCCGTATTCTCTTCCAG-3′) and parC-As-R2 (5′-GTCCAGCGAAGAAAT CATCG-3′) to give a 281 bp product for *parC*.

The PCR mixture (50 μL) contained 1× reaction buffer containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 U of *Taq* polymerase (Q-Biogene, Illkirch, France) and ∼150 ng of DNA template. PCR amplifications were performed using an iCycler thermal cycler (Bio-Rad, Marnes-la-Coquette, France) as follows: (i) an initial denaturation step of 5 min at 95°C; (ii) 35 cycles of PCR, with one cycle consisting of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C; and (iii) a final extension step of 10 min at 72°C. Purified PCR products were then directly sequenced with the same sets of primers in both directions (GATC Biotech, Konstanz, Germany). The nucleotide sequences were analysed using SeqScape™ v2.0 software (Applied Biosystems, Courtaboeuf, France). Multiple alignment and calculation of amino acid identity was carried out using ClustalX software (version 1.83).

Nucleotide sequence accession numbers

The partial nucleotide sequences of *gyrA* and *parC* genes obtained from the *A. schaalii* HM 883 strain were deposited in the GenBank database under accession numbers HQ009514 and HQ009515, respectively.

Results

Antimicrobial susceptibility

According to EUCAST breakpoints, all isolates were susceptible to amoxicillin, ceftriaxone, gentamicin, vancomycin, linezolid and nitrofurantoin (Table 1). All isolates except three (MICs ≥256 mg/L) were highly susceptible to clindamycin (Table 1). All isolates were resistant to ciprofloxacin, whereas 90% and 96% remained susceptible to levofloxacin and moxifloxacin, respectively (Table 1). A single strain was highly resistant to levofloxacin (MIC ≥32 mg/L) and moxifloxacin (MIC 8 mg/L). Finally, all isolates were resistant to metronidazole (MIC ≥256 mg/L), whereas only 15% were susceptible to co-trimoxazole (Table 1).

QRDR sequences of gyrA and parC genes

The amino acid sequence of fragments corresponding to the QRDRs of GyrA and ParC was compared with those from other bacterial species (Figure 1). All isolates that were susceptible or low-level resistant to levofloxacin/moxifloxacin ($n=47$) showed sequences identical to that of the *A. schaalii* HM 883 isolate (Figure 1). The GyrA QRDR sequence of *A. schaalii* presented an amino acid identity with other sequences ranging from 68% to 85%, with the highest identity with *Arcanobacterium haemolyticum* (85%). The ParC QRDR sequence of *A. schaalii* presented an amino acid identity with the other sequences ranging from 50% to 88%, with again the highest identity with *A. haemolyticum* (88%). More specifically, they possessed an alanine (GyrA) or a threonine (ParC) at position 83/80 and an aspartate at position 87/84 (GyrA and ParC, according to *E. coli* numbering) (Figure 1). In contrast, the single isolate exhibiting high-level resistance to levofloxacin and moxifloxacin possessed a unique mutation in GyrA (Ala83Val), whereas no mutation was present in ParC.

Discussion

UTIs are predominantly caused by members of the family Enterobacteriaceae. However, Gram-positive bacteria and polymicrobial infections are not uncommon in patients with underlying urological conditions. *A. schaalii* is an underestimated opportunistic uropathogen and its search is relevant in cases of unexplained chronic pyuria, especially when there is discrepancy between smear microscopy findings and growth results under aerobic conditions.[2,4]

Although *A. schaalii* is an emerging uropathogen, particularly in elderly patients, very little information is available about its *in vitro*

Table 1. *In vitro* activity of 12 antimicrobial agents against 48 clinical isolates of *A. schaalii*

Antimicrobial agent	MIC (mg/L)			Susceptibility breakpoint (mg/L)[a]	Percentage susceptible
	range	MIC_{50}	MIC_{90}		
Amoxicillin	0.03–0.5	0.12	0.25	≤2	100
Ceftriaxone	≤0.01–0.25	0.06	0.12	≤1	100
Gentamicin	0.12–2	1	2	≤2	100
Vancomycin	0.12–0.25	0.12	0.25	≤2	100
Clindamycin	≤0.01 to ≥256	0.03	0.06	≤4	94
Linezolid	0.12–1	0.5	1	≤2	100
Ciprofloxacin	2 to ≥32	≥32	≥32	≤0.5	0
Levofloxacin	0.5 to ≥32	1	2	≤1	90
Moxifloxacin	0.25–8	0.5	0.5	≤0.5	96
Co-trimoxazole	2 to ≥32	16	≥32	≤2	15
Nitrofurantoin	0.12–32	2	16	≤64	100
Metronidazole	≥256	≥256	≥256	≤4	0

[a]Non-related species EUCAST breakpoints were used for all antibiotics, except for co-trimoxazole (EUCAST breakpoints for Enterobacteriaceae and *Staphylococcus* spp.), nitrofurantoin (EUCAST breakpoints for *E. coli* and *S. saprophyticus* responsible for uncomplicated UTIs) and clindamycin and metronidazole (EUCAST breakpoints for Gram-positive anaerobes).

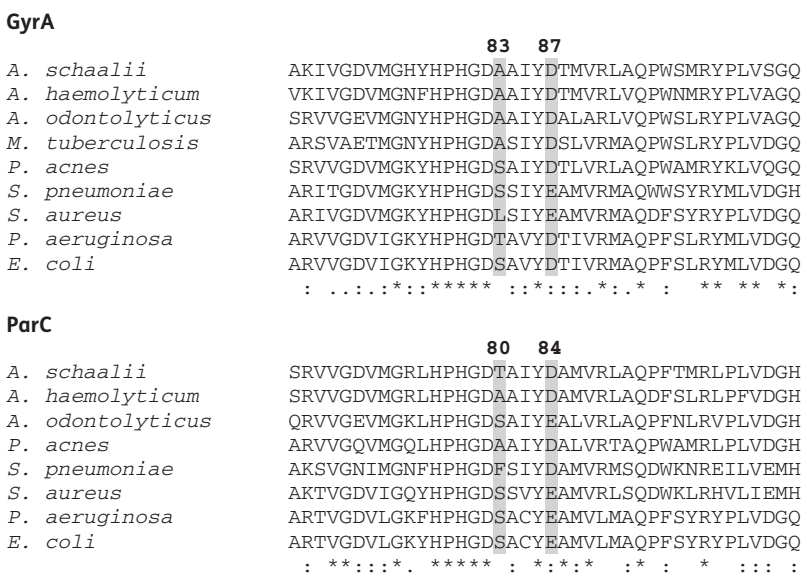

Figure 1. Comparison of the amino acid sequences of GyrA and ParC QRDRs (amino acids 67–106 and 64–103, respectively, in *E. coli* numbering) of *A. schaalii* and other bacterial species. Hot spot positions 83 and 87 in GyrA and 80 and 84 in ParC are highlighted in grey. Similarities in amino acid sequences are marked by asterisks (same amino acid), colons (strong similarity) and dots (family similarity). Multiple sequence alignment was carried out using ClustalX 1.83 software.

susceptibility to antimicrobial agents, especially those commonly used in the treatment of UTIs. To date, only one study determining MIC_{50} and MIC_{90} values for nine strains has been conducted.[2] In the study by Reinhard *et al.*,[2] all isolates were susceptible to penicillin, cefuroxime, amoxicillin, nitrofurantoin, tetracycline and clindamycin with MIC_{50}s and MIC_{90}s of 0.008 and 0.023 mg/L, <0.016 and <0.016 mg/L, 0.5 and 1 mg/L, 0.5 and 1 mg/L, 0.023 and 0.047 mg/L and 0.125 and 0.25 mg/L, respectively, whereas reduced activities were seen with ciprofloxacin, with MIC_{50} and MIC_{90} values of 1.5 and 2 mg/L.[2] In a Danish study describing 76 strains of *A. schaalii* from 55 patients, all isolates were resistant to trimethoprim and ciprofloxacin, whereas they were susceptible to ampicillin and cefuroxime, and showed varying susceptibility to sulphonamides, but MICs were not provided.[4] In several clinical cases, antimicrobial susceptibility testing has been performed using Etest or disc diffusion methods only for a few antibiotics. An *A. schaalii* isolate responsible for a vertebral osteomyelitis was susceptible to amoxicillin/clavulanate (MIC 0.38 mg/L), ceftriaxone (MIC 0.125 mg/L), clindamycin (MIC 0.023 mg/L), rifampin (MIC <0.002 mg/L), doxycycline (MIC 0.125 mg/L) and vancomycin (MIC 0.25 mg/L).[5] In another clinical case, using the disc diffusion method, the

isolate was susceptible to penicillin, amoxicillin, ceftriaxone, tetracycline, clarithromycin, clindamycin, nitrofurantoin and co-trimoxazole, whereas it was resistant to ciprofloxacin.[10] Like related species of *Actinomyces*, *A. schaalii* seems to be intrinsically resistant to metronidazole, as expected for a facultative aerobic species.[11]

The nature of the mutation (Ala83Val) in the QRDR of the *gyrA* gene has already been demonstrated to be involved in the development of quinolone resistance in *Mycobacterium tuberculosis*.[12] Therefore, ParC did not seem to be the primary target of fluoroquinolones in *A. schaalii*, as opposed to other Gram-positive bacterial species, where mutations conferring quinolone resistance occur preferentially in the *parC* gene.[7] Interestingly, the strain exhibiting high-level resistance to levofloxacin and moxifloxacin was recovered from a patient who had received moxifloxacin therapy for UTI before isolation of the resistant strain.

For patients with clinically documented UTIs who do not respond to treatment with ciprofloxacin or co-trimoxazole, infection caused by *A. schaalii* should be suspected, and, if it is the cause of the infection, treatment must be adjusted. Interestingly, one case of chronic pyelonephritis due to *A. schaalii* has been successfully treated after 6 weeks of clindamycin.[13] Nevertheless, treatment with β-lactams has been successfully used in several cases.[2,5,6] However, the optimal duration of antimicrobial drug treatment with β-lactams is not clearly defined even if several weeks of treatment may be required in several cases.[3]

Acknowledgements

We are grateful to Michel Auzou for excellent technical assistance and Nadia Liassine (Dianalab) for sending her strain.

Funding

The study was carried out as part of our routine work.

Transparency declarations

None to declare.

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