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

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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, ciprofloxacin, levofloxacin, moxifloxacin, co-trimoxazole, nitrofurantoin and metronidazole, 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 Actinomycetes, 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...
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quinoIone-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), 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.

Antimicrobial susceptibility testing

MICs of the following 12 antibiotics were determined for all strains using the Etest method (bioMérieux, Marcy l’Etoile, France): amoxicillin; ceftriaxone; gentamicin; vancomycin; 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. Based on these sequences, a novel pair of specific primers for each gene was specifically designed for A. schaalii: gyrA-As-F2 (5′-CGGAACGGCAGATGAT-3′) and gyrA-As-R2 (5′-GTGGAG GATGTCGTTGC-3′) to give a 316 bp product for GyrA; and parC-As-F2 (5′-AGCAGGGTTTCCTCTGCACG-3′) and parC-As-R2 (5′-GTCCAGCGAAGAT CATC-3′) to give a 281 bp product for ParC.

The PCR mixture (50 μL) contained 1× reaction buffer containing 1.5 mM MgCl2, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 U of Taq polymerase (Qi-Biogene, Ilkirch, 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. Although A. schaalii is an emerging uropathogen, particularly in elderly patients, very little information is available about its in vitro
susceptibility to antimicrobial agents, especially those commonly used in the treatment of UTIs. To date, only one study determining MIC50 and MIC90 values for nine strains has been conducted. In the study by Reinhard \textit{et al.}, all isolates were susceptible to penicillin, cefuroxime, amoxicillin, nitrofurantoin, tetracycline and clindamycin with MIC50s and MIC90s of 0.008 and 0.023 mg/L, respectively, whereas reduced activities were seen with ciprofloxacin, with MIC50 and MIC90 values of 1.5 and 2 mg/L. In a Danish study describing 76 strains of \textit{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. In several clinical cases, antimicrobial susceptibility testing has been performed using Etest or disc diffusion methods only for a few antibiotics. An \textit{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). In another clinical case, using the disc diffusion method, the

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>range</th>
<th>MIC50 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>Susceptibility breakpoint (mg/L)</th>
<th>Percentage susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0.03-0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>≤2</td>
<td>100</td>
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<tr>
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<td>0.06</td>
<td>0.12</td>
<td>≤1</td>
<td>100</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.12-2</td>
<td>1</td>
<td>2</td>
<td>≤2</td>
<td>100</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.12-0.25</td>
<td>0.12</td>
<td>0.25</td>
<td>≤2</td>
<td>100</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>≤0.01 to ≥256</td>
<td>0.03</td>
<td>0.06</td>
<td>≤4</td>
<td>94</td>
</tr>
<tr>
<td>Linezolid</td>
<td>0.12-1</td>
<td>0.5</td>
<td>1</td>
<td>≤2</td>
<td>100</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
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<td>≥32</td>
<td>≥32</td>
<td>≤0.5</td>
<td>0</td>
</tr>
<tr>
<td>Levofloxacin</td>
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<td>1</td>
<td>2</td>
<td>≤1</td>
<td>90</td>
</tr>
<tr>
<td>Moxifloxacin</td>
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<td>0.5</td>
<td>0.5</td>
<td>≤0.5</td>
<td>96</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
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<td>2</td>
<td>16</td>
<td>≥32</td>
<td>15</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0.12-32</td>
<td>2</td>
<td>16</td>
<td>≤64</td>
<td>100</td>
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<tr>
<td>Metronidazole</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≤4</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Non-related species EUCAST breakpoints were used for all antibiotics, except for co-trimoxazole (EUCAST breakpoints for Enterobacteriaceae and \textit{S. saprophyticus} responsible for uncomplicated UTIs) and clindamycin and metronidazole (EUCAST breakpoints for Gram-positive anaerobes).

\textbf{GyrA}  

\begin{itemize}
  \item \textit{A. schaalii}: AKIVGDVMGYHHPGDIAIYETMVRVLAPWSRMYPVLGQ
  \item \textit{A. haemolyticum}: VRKIVGDVMGNYHPDGAAIYDTMVRLAVQPWSNMLYPLVAGQ
  \item \textit{A. odontolyticus}: SRVIVGDVMGYNPDIYDIALVRPLMRSYPLVAGQ
  \item \textit{M. tuberculosis}: ARIVGDVMGYAHPDIAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{P. acnes}: SRTVIVGDVMGYHPDGAAIYDTMVRLVQPWSNMLYPLVAGQ
  \item \textit{S. pneumoniae}: ARIVGDVMGYAHPDIAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{S. aureus}: ARIVGDVMGYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{P. aeruginosa}: ARIVGDVMGYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
\end{itemize}

\textbf{ParC}  

\begin{itemize}
  \item \textit{A. schaalii}: VRTVIVGDVLGKYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{A. haemolyticum}: VRTVIVGDVLGKYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{A. odontolyticus}: VRTVIVGDVLGKYHNPDAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{P. acnes}: VRTVIVGDVLGKYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{S. pneumoniae}: AGTVIVGDVLGKYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{S. aureus}: AKTVIVGDVLGKYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
  \item \textit{P. aeruginosa}: ARTVIVGDVLGKYHPDGAAIYDTMVRLAQPWSMRYPLVAGQ
\end{itemize}
isolate was susceptible to penicillin, amoxicillin, ceftriaxone, tetracycline, clarithromycin, clindamycin, nitrofurantoin and co-trimoxazole, whereas it was resistant to ciprofloxacin. Like related species of Actinomyces, *A. schaalii* seems to be intrinsically resistant to metronidazole, as expected for a facultative aerobic species. 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*. 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. 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. Nevertheless, treatment with β-lactams has been successfully used in several cases. 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.

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Transparency declarations
None to declare.

References