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Antibiotic susceptibility of *Neochlamydia hartmanellae* and *Parachlamydia acanthamoebae* in amoebae

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Running title: Antibiotic susceptibility of *Parachlamydiaceae*
Parachlamydia acanthamoebae and Neochlamydia hartmanellae are Chlamydia-related bacteria naturally infecting free-living amoebae. These strict intracellular bacteria might represent emerging pathogens. Recent studies report an association with lower respiratory tract infections, especially with pneumonia where they have been identified as a potential causative agent in 1-2% of cases. In this study, we defined the antibiotic susceptibility of Neochlamydia hartmanellae, two strains of Parachlamydia acanthamoebae and two yet unclassified Parachlamydiaceae strains using a quantitative approach. We confirmed the results obtained earlier for P. acanthamoebae strain Bn9 in an observational study. Macrolides (MICs < 0.06 - 0.5 µg/ml), rifampicin (MICs 0.25-0.5, 1-2 µg/ml) and doxycycline were active against P. acanthamoebae strains and Neochlamydia. All strains were resistant to amoxicillin, ceftriaxone and imipenem (MIC ≥32 µg/ml). Similarly to other Chlamydia-related bacteria, all investigated Parachlamydiaceae were resistant to quinolones (MICs ≥ 16 µg/ml). Therefore, we recommend a treatment with macrolides for Parachlamydia-associated pneumonia.

Keywords: Parachlamydiaceae, Antibiotic, Pneumonia, Chlamydia, Intracellular bacteria
INTRODUCTION

In the past few years various Chlamydia-related bacteria, such as Parachlamydia acanthamoebae and Neochlamydia hartmanellae have been discovered, extending our knowledge on the Chlamydiales ecology. Similarly to the pathogenic Chlamydia trachomatis and Chlamydia pneumoniae, these obligate intracellular bacteria are characterized by their biphasic developmental cycle with infectious elementary bodies (EBs) and replicative reticulate bodies (RBs). They have been assigned to the Parachlamydiaceae family level-lineage based on highly taxonomic discriminative genes [1,2] and are known to naturally infect free-living amoebae [3,4].

Various works suggest a role of P. acanthamoebae as a causative agent of pneumonia and other lower respiratory tract infections [5]. A first hint was suggested by its isolation from the water of an humidifier involved in an epidemic of fever in Vermont, USA [6]. This was further confirmed by a positive association with evidence of acute infections to P. acanthamoebae and community acquired pneumonia (CAP) [7], ventilator associated pneumonia (VAP) [8] and nosocomial pneumonia [9]. In addition, P. acanthamoebae DNA was identified in 2 cases of lower respiratory tract infections in children [10] as well as in 13% of children with bronchiolitis [11]. Similar findings were also shown for other members of the Parachlamydiaceae, such as Protochlamydia amoebaphila [12] and Protochlamydia naegleriophila [13]. Despite low prevalence of direct isolation of these organisms (less than 1% in CAP and 8% in VAP), cases of Parachlamydiaceae-associated pneumonia were clearly documented leaving no doubt of the pathogenic role of these species. A low prevalence of Chlamydia pneumoniae-associated pneumonia was also observed in recent studies [14–16]. Its clinical relevance is, nonetheless, not debated.

These findings suggest that Parachlamydiaceae might be responsible for at least some cases of pneumonia of unidentified etiology. Therefore, it is crucial to verify that current
recommended empirical treatments of pneumonia are effective on these emerging pathogens. Partial information is already given by the work performed by Maurin et al. [17]. However, in this study, minimal inhibitory concentrations (MICs) were defined as the lowest concentration that prevented amoebal lysis and therefore provides information based on indirect observations that might be influenced by additional aspects than bacterial growth [18]. In this work, we used a specific real-time PCR to define quantitatively the antibiotic susceptibility of Neochlamydia hartmanellae, two strains of P. acanthamoebae and two yet unclassified Parachlamydiaceae strains. This approach has already been applied to determine antibiotic susceptibility of other Chlamydia-related bacteria [19,20] and is now considered as the standard technique to define antibiotic susceptibility. It should therefore be preferentially used to perform comparisons.

MATERIAL AND METHODS

Parachlamydia acanthamoebae strain Hall’s coccus, Parachlamydia acanthamoebae strain BN9 (ATCC VR-1476), Parachlamydia sp. TUMPL1 and the Neochlamydia sp. UWC22 were grown within Acanthamoeba polyphaga strain Linc AP-1 as previously described [21]. Neochlamydia hartmanellae (symbiont of Hartmanella vermiformis ATCC 50802) was grown similarly within Hartmanella vermiformis strain BL. After 6 days of incubation, cultures were harvested and the broth was centrifuged at 180 x g for 10 minutes to eliminate most amoebae. The supernatant was then diluted at 1:1000 in Page's amoebal saline (PAS) [21], which corresponds to an approximate final concentration of about 10^3 bacteria/ml. 50 µl of this inoculum was then used to infect Acanthamoeba polyphaga strain Linc AP-1 (Parachlamydia and Parachlamydiaceae-related strain) and Hartmanella vermiformis strain BL (Neochlamydia hartmanellae), respectively, distributed in a 96-wells Costar micro plates (Corning) at a concentration of 5x10^5 amoebae/ml. These amoebae were grown axenically as
previously described [21]. After two hours of incubation, at 32°C, to allow internalization, 50 µl of serial antibiotics dilutions were added. Antibiotics tested in this study were doxycycline [0.06-4 µg/ml] (Pfizer, Neuilly, France), erythromycin [0.06-4 µg/ml] (Abbot, Rungis, France), clarithromycin [0.06-4 µg/ml] (SmithKline Beecham, Nanterre, France), rifampicin [0.06-4 µg/ml] (Cassenne, Puteaux, France). Other antibiotics, that were expected to be ineffective on Parachlamydiaceae based on the work of Maurin *et al* [17], were tested at a single high concentration: ofloxacin [16 µg/ml] (Diamant, Puteaux, France), ciprofloxacin [16 µg/ml] (Bayer Pharma, Sebs, France), amoxicillin [100 and 32 µg/ml] (SmithKline Beecham, Nanter, France), ceftriaxone [100 and 32 µg/ml] (Roche, Paris, France) and imipenem [100 and 32 µg/ml]. Antibiotics were tested in duplicate.

Growth was assessed using a real time TaqMan PCR assay at 2, 24, 48, 72 and 96 hours post infection. Briefly, bacterial co-cultures were incubated at 32°C and wells were harvested at the adequate time. DNA was extracted from 200 µl aliquots of infected amoebal cells using the BioRad Genomic DNA Kit (BioRad Laboratories, Hercules, Ca), as described by the manufacturer. The extracted nucleic acid was resuspended in a final volume of 50 µl and stored at -20°C until used in the quantitative PCR assay.

Antibiotic-free wells served as growth controls while uninfected amoebae wells served as negative controls. The absence of toxicity of antibiotics to amoebal cells was determined by examining the amoebal micro plates once a day under an inverted microscope (Zeiss Axiovert 25, Carl Zeiss). To assess the activity and dilution of the antibiotics used, MICs were determined for *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 49976 (Institut Pasteur, Marnes La Coquette, France) using Mueller-Hinton agar (bioMérieux) incubated at 37°C for 18 hours.

Quantitative PCR was performed using TaqMan technology in a final volume of 25 µl including 12.5 µl of the TaqMan Universal Master Mix (Applied Biosystems, Foster City, Ca)
200 nM of the forward primer (abF 5’-CTCGTGCCGTGAGGTGTT), 200 nM of the reverse primer (abR 5’-AGCACGTGTGTAGCCCCCA), 100 nM of the fluorescent labeled probe (6-121FAM-5’-TCAGGTGGGAACTCTAATGAGACTGCCT 3’-TAMRA, where 6-FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine), 2.5 µl of water and 2.5 µl of DNA. Amplification and detection were performed on the ABI 7900HT sequence detection system (TaqMan system, Applied Biosystems). Cycling conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. DNA extracted from tittered Parachlamydia and water were used as positive and negative controls, respectively. The number of bacteria/ml in each sample was then determined by comparing the threshold cycle (C_T) of the sample to that of the tittered positive control used to establish a calibration curve.

RESULTS

In this study, we evaluated the susceptibility of various members of the Parachlamydiaceae to six classes of antibiotics. MICs were defined using a quantitative PCR as the minimal concentration that prevented bacterial growth. The cut-off used to define a significant prevention of bacterial growth was the presence of less than a 100 bacterial copies at day 3, based on the growth kinetics in the absence of antibiotics (figure 1). Both strains of P. acanthamoebae exhibited a lag in their growth at day 3. Therefore cut-off was adjusted to less than 10 copies to ensure a better discrimination and growth at day 4 was tested to establish the susceptibility to ceftriaxone. No antibiotic toxicity was observed on the amoebae (data not shown). We showed that Parachlamydiaceae were resistant to β-lactams (MIC >32 µg/ml) as well as to quinolones (MIC >16 µg/ml); such concentrations are, indeed, never achieved in the human body. As expected, macrolides were active against all species even at a concentration of 0.06 µg/ml for clarithromycin (MIC <0.06 µg/ml) and 0.06-0.5 µg/ml for
erythromycin. Doxycycline was active against both strains of *P. acanthamoebae* and *Neochlamydia hartmanellae* (MICs 2-4 µg/ml). However, MICs seemed to be higher for the unclassified *Parachlamydiaceae* (≥8 µg/ml). *Parachlamydiaceae* were also susceptible to rifampicin, with a stronger efficacy against *P. acanthamoebae* species (MIC 0.25-0.5 µg/ml) versus *Neochlamydia* (MIC 2 µg/ml) (figure 2).

**DISCUSSION**

In this paper, we confirmed the results obtained for *P. acanthamoebae* strain BN9 by Maurin et al. [17] using a reliable quantitative approach and extended these observations to additional members of the *Parachlamydiaceae*. We demonstrated that the antibiotic susceptibility of *Parachlamydiaceae* in amoebae is quite similar to what is known for other members of the *Chlamydiales* (see table 1). Macrolides are the treatment of choice. Cyclines might be an alternative, at least for *P. acanthamoebae* strains and *Neochlamydia hartmanellae*, but conclusions are difficult to draw due to the *in vitro* amoebal model used in our study. Indeed, it has already been demonstrated that amoebae are a good alternative to mammalian cells lines to test the antibiotic susceptibility for species that strictly grow in amoebae, as similar results are obtained in both cell types [20]. However, caution should be taken regarding doxycycline, which MIC tends to be higher in amoebae due to the likely presence of an efflux pump [20].

In our study, we found a MIC of 4µg/ml that might be overestimated compared to mammalian cells. Nevertheless, even if a concentration of 4µg/ml is required to inhibit bacterial growth in humans, doxycycline is still an acceptable treatment for *Parachlamydia*-related pneumonia, as it was shown that such lung concentrations were achieved in humans after a single dose of 200 mg IV doxycycline [22]. Confirmation of our results in a mammalian cell model seems to be difficult. Indeed, so far, it has not been possible to grow *Neochlamydia hartmanellae* in mammalian cells *in vitro* and, even if *P. acanthamoebae* was shown to replicate in
pneumocytes, fibroblasts [23], as well as macrophages [24] cell lines in vitro, growth is very limited, in these cell lines, preventing accurate antibiotic susceptibility testing.

Rifampicin was shown to be efficient against Parachlamydiaceae, similarly to what is observed for Chlamydia trachomatis. However, resistance are known to rapidly develop under treatment [25]. Therefore, caution should be taken when using this antibiotic in a single antibiotic regimen.

Of utmost interest, our results confirm that unlike Chlamydia spp.[25–31], Neochlamydia and Parachlamydia spp. are resistant to quinolones, as already demonstrated for several other Chlamydia-related bacteria, including Simkania negevensis, Waddlia chondrophila and Estrella lausannensis [19,20,32]. This resistance is probably due to a mutation in the quinolones Resistance-Determining Region (QRDR) of gyrA, as shown by a recent publication [32]. Indeed, two substitutions were identified in quinolones resistant Chlamydiales when compared to susceptible Chlamydiaceae: (1) at position 70, the presence of a serine and (2) at position 83, the substitution of cysteine by another amino acid might induce resistance [32,33]. Quinolones such as levofloxacina represent one of the alternative treatments recommended for CAP, especially in patients that require in-treatment or patients suffering from additional co-morbidities in the objective to cover both S. pneumoniae and P. aeruginosa infection [34]. Since Chlamydia-related bacteria might represent 1-2% of community-acquired pneumonia, caution should be taken when prescribing quinolones.
ACKNOWLEDGMENTS

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DISCLOSURE STATEMENT

The authors did not report any potential conflict of interest.


FIGURES AND TABLE

Figure 1: Growth kinetic of each parachlamydial strains in amoebae without antibiotic

Kinetics were determined by quantitative PCR. Results are shown in a logarithmic scale as the means +/- standard deviation of triplicate experiments.

Figure 2: Antibiotic susceptibility of each parachlamydial strains in amoebae

Bacterial copy numbers were determined by quantitative PCR at day 3 post-infection, except when indicated by a *, where it was determined at day 4. Only results of significant experiments are shown. Results are shown in a logarithmic scale as the means +/- standard deviation in duplicate experiments. Abbreviations: Clari, clarithromycin; Ery, erythromycin; Doxy, doxycycline, Rifam, rifampicin; Amo, amoxicillin; Cef, ceftriaxone; Imi, imipenem; Cipro, ciprofloxacin; Oflo, ofloxacin.

(A) Neochlamydia hartmanellae (B) Parachlamydia acanthamoebae strain Hall’s coccus (C) Parachlamydia acanthamoebae strain BN9 (D) Parachlamydia sp. Tump11 (E) Neochlamydia sp. UWC22.

Table 1: Antibiotic susceptibility of Parachlamydiaceae and others Chlamydiales

This table represents the MICs in µg/ml of various antibiotics against members of the Chlamydiales orders
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Amoebae</th>
<th>Vero</th>
<th>Amoebae</th>
<th>Vero</th>
<th>McCoy, Hep2</th>
<th>HeLa</th>
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<td>4</td>
<td>≥8</td>
<td>≥8</td>
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<tr>
<td>Erythromycin</td>
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<td>&lt; 0.06</td>
<td>&gt; 0.06</td>
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<td>&lt; 0.06</td>
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<tr>
<td>Azithromycin</td>
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<td><strong>β-lactams</strong></td>
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<td>Penicillin derivatives</td>
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<tr>
<td>Ceftriaxone</td>
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<tr>
<td>Imipenem</td>
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Abbreviations: MIC, Minimal inhibitory concentration; ND, Not determined