Simkania negevensis, an example of the diversity of the antimicrobial susceptibility pattern among Chlamydiales

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Running title: Antibiotic susceptibility of Simkania negevensis
Abstract

Over the last years, several *Chlamydia*-related bacteria have been discovered, including *Simkania negevensis*, the founding member of the *Simkaniaceae* family. We evaluated the antimicrobial susceptibility patterns of this emerging intracellular bacterium and highlighted significant differences with other related *Chlamydiales*. *S. negevensis* was susceptible to macrolides, clindamycin, cyclines, rifampicin and quinolones. Importantly, unlike other *Chlamydiales*, treatment with β-lactams and vancomycin did not induce the formation of aberrant bodies leading to a complete resistant phenotype.
Rapid progresses in diagnostic techniques have enabled the discovery of several novel Chlamydia-related bacteria, including *Simkania negevensis*. Mostly known for the pathogenic *Chlamydia* spp., the *Chlamydiales* order is now composed of at least 9 family-level lineages (1), each harboring specific biological characteristics. *S. negevensis* represents the founding member of the *Simkaniaceae* family and represents an emerging pathogen previously associated with respiratory diseases, at least in the Middle East (2, 3). Infections were empirically treated with a macrolide-based regimen (4). Several differences regarding antimicrobial susceptibility have been highlighted among the different *Chlamydiales* family-level lineage (5, 6). We therefore investigated the antibiotic susceptibility of the *Simkaniaceae* family, which remains poorly studied, using *S. negevensis* as a model. We provide subsequent information on the evolution of antimicrobial resistance in this order as well as potential therapeutic options.

*Simkania negevensis* strain Z was grown within Vero cells in 25cm² cell culture flasks (Corning, Corning, USA) in Dulbecco’s modified essential medium (DMEM; PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS) at 37°C with 5% CO₂. A 6 or 7-days-old co-culture, diluted at 1/1000, was used to inoculate fresh A549 cells or Vero cells, previously seeded at 1.5x10⁵ cells/ml on a 24 wells plate (Corning) as previously described (7). At 2 hours post-infection (p.i.), media was changed for a media containing twofold serial dilutions of various antibiotics. Antibiotic-free wells served as growth controls while uninfected cells served as negative controls. Twelve antibiotics from 8 different classes were used in this study. Minimum Inhibitory Concentrations (MICs) were defined as the minimal concentration that prevented bacterial growth at day 6, when compared to a control infection performed in the absence of antibiotics. Growth at day 2 was also assessed for β-
lactams, fosfomycin and vancomycin to ensure the absence of effect due to instability of the compounds after 48 h at 37°C. An in house specific quantitative PCR targeting the 16S rRNA gene was used to quantify *S. negevensis* DNA as previously described (7). The absence of antibiotics toxicity towards cells was determined by examining the microplates using an inverted microscope (Zeiss Axiovert 25, Carl Zeiss). When other solvents than distilled water were used (Dimethyl sulfoxide (DMSO), 0.1M HCl and 1M NaOH) to suspend antibiotic solutions, the absence of effect of these solvents on *S. negevensis* growth was assessed.

As others *Chlamydiales*, *S. negevensis* was susceptible to macrolides, clindamycin, cyclines and rifampicin (Table 1). Interestingly, *S. negevensis* was susceptible to quinolones. While *Chlamydiaceae* are sensitive, other *Chlamydia*-related bacteria, such as *Waddlia chondrophila*, *Parachlamydia* spp. and *Estrella lausannensis* are resistant (5, 6, 8). A previous work had suggested that *S. negevensis* was resistant to ciprofloxacin (9). In this study, MICs were determined in amoebae as the minimal concentration that prevented amoebal lysis. The observed results might have been due to the presence of an efflux pump present in amoebae and decreasing quinolones bioavailability. Despite identification of several mutations in the *gyrA* and *parC* quinolone resistance-determining regions (QRDRs), these differ from the ones observed in resistant *Chlamydia*-related bacteria and may explain the absence of resistance observed (6, 9).

*S. negevensis* was resistant to three kinds of cell wall inhibitors: β-lactams, fosfomycin and vancomycin (MICs > 32 μg/ml). *Chlamydiales* members lack the traditional peptidoglycan (PG) layer. Nevertheless, a partial susceptibility to β-lactams is observed among *Chlamydia* spp., which are known to form aberrant bodies when treated with penicillin-derivatives (10), while *W. chondrophila* is susceptible to high dose of fosfomycin (11). Aberrant bodies
represent enlarged forms of the bacterium, due to abnormal division, despite persisting DNA replication (11). We therefore evaluated the morphology of *S. negevensis* particles treated with β-lactams, fosfomycin and vancomycin by immunofluorescence using an in house rabbit polyclonal anti-*S. negevensis* antibody, as previously described (7). As shown in Figure 1A, no abnormal morphological aspects of *S. negevensis* could be observed under β-lactams treatment even with concentrations as high as 1000 μg/ml. This strikingly contrasted to the abnormal morphology of *Chlamydia trachomatis* observed with 2 μg/ml of β-lactams, making *S. negevensis* unique among *Chlamydiales* members. Indeed, *W. chondrophila* (*Waddliaceae* family) and *E. lausannensis* (*Criblamydiaceae* family) form aberrant bodies under β-lactam treatment (500 μg/ml) (6, 12). Furthermore, unlike *W. chondrophila* (11), *S. negevensis* replication was not inhibited by high dose of β-lactams (1000 μg/ml) (Table 1). This difference could not be explained by the slower replicative cycle, as similar observations were made at day 6 p.i. (Figure 1B). Several β-lactamase motifs are encoded in *S. negevensis* genome (13) and may contribute to the phenotype. Nevertheless, *W. chondrophila* exhibits a partial sensitivity to high dose of β-lactams despite having a class C β-lactamase encoded in its genome (14).

Similarly to *Chlamydia* spp. (11), *S. negevensis* replication was not inhibited by high dose of fosfomycin, which targets the enzyme MurA, implicated in the early steps of PG biosynthesis. Nevertheless, a small fraction of *S. negevensis* particles that increased by day 6, showed abnormal morphological aspects, compatible with aberrant bodies (Figure 1A and B), though remaining significantly less important than what can be observed for *W. chondrophila* (11). *Chlamydia* spp. resistance to fosfomycin is suspected to be related to a single substitution (Cys115 – Asp) in the active site of MurA (11, 15). Conversely, this mutation was not found in *S. negevensis*, supporting the partial observed sensitive phenotype.
Finally, we did not observe aberrant bodies under vancomycin treatment, a drug that inhibits transpeptidation through high affinity binding to the D-alanine precursor (Figure 1A).

Recently, several works have demonstrated the presence of a modified version of PG in Chlamydiales members, which is required for cell division (12, 16, 17), thus explaining their partial sensitivity to cell-wall inhibitors. Interestingly, a recent study has failed to isolate PG-like structures in S. negevensis (18), while such structures were identified in Protochlamydia amoebophila (18) and C. trachomatis (17). In the same work, incorporation fluorescent-labeled D-alanine incorporation could not be highlighted in S. negevensis (18) and correlates with the absence of vancomycin effects observed here. On the other hand, a previous work has shown that, similarly to C. trachomatis, S. negevensis was susceptible to D-cycloserine, a molecule that inhibits the alanine racemase Alr and the alanine ligase Ddl required for D-alanine formation (19). While a predicted Ddl enzyme is encoded in S. negevensis genome, no Alr encoding sequence is present, similarly to Chlamydiaceae (12). It is unknown whether the serine hydroxymethyltransferase glyA encoded in S. negevensis genome could compensate for the absence of Alr as described for Chlamydiaceae (20).

Nevertheless, despite the absence of PG-like structures, the activity of two PG-remodeling enzymes, NlpD and AmiA, was documented in S. negevensis (16) and enzymes implicated in the PG biosynthesis are highly conserved among Chlamydiales, including S. negevensis, supporting their crucial role (12). However, the varying responses to different cell-wall inhibitors, targeting each a specific step of the PG biosynthesis, further supports that despite the likely requirement of modified form of PG for cellular division, some significant differences exist in the PG biosynthesis pathway of S. negevensis and might bring further insights into the mechanisms of Chlamydiales cell division.
In conclusion, we highlighted in this work several differences in the antimicrobial response of *S. negevensis* compared to other *Chlamydiales* members. Though the pathogenic role of *Simkania* spp. remains to be better defined, the precise knowledge of their antimicrobial susceptibility patterns provides significant information in the biology and evolution of the *Chlamydiales* order.

**FUNDING**

This work was supported by Swiss National Science Foundation (SNSF) (MD-PhD grant no. 323530_158123 and grant no. 310030-162603)

**DISCLOSURE STATEMENT**

The authors did not report any potential conflict of interest.

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Figure 1. Effect of cell wall inhibitors on *Simkania negevensis* infection and morphology

Growth of *S. negevensis* observed by immunofluorescence in presence or absence of cell wall inhibitors – A. Effect of β-lactams, fosfomycin and vancomycin treatment in Vero cells at 48 h p.i.. *S. negevensis*, *Chlamydia trachomatis* strain UW-3/Cx and *Waddlia chondrophila* strain WSU 86-1044 (ATCC® VR-1470) were detected using a polyclonal anti-*S. negevensis* rabbit antibody (green) (1:2500) or a mouse anti-MOMP antibody (1:50) (ab20881, Abcam, Cambridge, UK) or anti-*W. chondrophila* rabbit antibody (1:2000) (green), respectively, followed by a secondary antibody (Alexa fluor 488 goat anti-mouse or anti-rabbit antibody (1:500); Molecular probes, Thermo Fisher Scientific, Waltham, USA), mammalian cells (red) are stained with texas red-conjugated Concanavalin A (1:50) and nucleic acids (blue) are stained with DAPI (1:1000). B. Effect of fosfomycin and penicillin treatment in Vero cells at day 6 p.i..
Table 1: Antibiotic susceptibility of *Simkania negevensis* compared to others

**Chlamydiales**

This table represents the MICs in µg/ml of various antibiotics against members of the *Chlamydiales* orders and was adapted from (8).

References: (8) (5, 11) (6)(10, 21–24) (11, 21)

*Criblamydiaceae* present the Cys115 – Asp substitution in the active site of MurA, which is known to confer resistance to fosfomycin in *Chlamydia* spp.

| Simkaniaeae | Parachlamydiaceae | Waddliaceae | Criblamydiaceae | Chlamydiaceae |
|-------------|-------------------|-------------|-----------------|--------------|
| *S. negevensis* | (7) | (4, 10) | (5) | (9, 20-23) |

**Cell lines**

| Cyclines | Tetracycline | 2 | ND | ND | 0.25 | 0.25-0.5 | 0.125-0.5 |
|----------|--------------|---|----|----|------|---------|----------|
|          | Doxycycline  | 0.5 | 2-4 | 0.25 | 0.25 | 0.03-0.25 | 0.02-0.5 |

**Lincosamides**

| Clindamycin | 1 | ND | 2-4 | ND | 0.25-2 | ND |

**Macrolides**

| Erythromycin | ND | 0.06 | ND | ND | 0.02-2 | 0.02-0.25 |
| Clarithromycin | ND | < 0.06 | ND | ND | 0.02-0.125 | 0.004-0.125 |
| Azithromycin | < 0.06 | ND | 0.25 | 2 | 0.6-2 | 0.02-0.5 |

**β-Lactams**

| Penicillin derivatives | > 1000 | > 32 | > 32 | > 32 | 0.25-2 | 5 |
| Ceftriaxone | > 1000 | > 32 | > 32 | > 32 | 16-32 | ND |

**Phosphonic acid derivative**

| Fosfomycin | > 1000 | ND | 500 | ND* | 500-1000 | > 1000 |

**Glycopeptide**

| Vancomycin | > 1000 | ND | ND | ND | 1000 | 1000 |

**Fluoroquinolones**

| Ciprofloxacin | 4 | > 16 | > 16 | 32 | 0.5-2 | 1-4 |
| Ofloxacin | 1 | > 16 | > 16 | 16 | 0.5-1 | 0.5-2 |
| Levofloxacin | 0.5 | ND | ND | ND | 0.12-0.5 | 0.25-1 |

**Rifamycin**

| Rifampicin | < 0.06 | 0.25-0.5 | ND | ND | < 0.125-1 | < 0.125 |

*References: (8) (5, 11) (6)(10, 21–24) (11, 21) |
