Antibiotic Susceptibility of *Waddlia chondrophila* in *Acanthamoeba castellanii* Amoebae[▽]

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***Waddlia chondrophila* is an emerging cause of miscarriage in bovines and humans. Given the strict intracellular growth of this *Chlamydia*-like organism, its antibiotic susceptibility was tested by amoebal coculture, cell culture, and real-time PCR. *W. chondrophila* was susceptible to doxycycline and azithromycin but resistant to β -lactams and fluoroquinolones.**

Waddlia chondrophila is a *Chlamydia*-related bacterium belonging to the *Waddliaceae* family (24), together with another species, *W. malaysiensis* (5). The two existing strains of *W. chondrophila* were isolated from aborted bovine fetuses (7, 15). More recently, a role of *W. chondrophila* in human miscarriage was identified (1). The pathogenic role of *W. chondrophila* in humans was further supported by its rapid growth within human macrophages (9). Moreover, like other agents of miscarriage such as *Coxiella burnetii* (23), *W. chondrophila* may also cause pneumonia (11). Consequently, we evaluated the antibiotic susceptibility of *W. chondrophila*. Given the strict intracellular growth of this *Chlamydia*-like organism, its antibiotic susceptibility was tested in amoebae and in Vero cells.

W. chondrophila strain ATCC VR-1470 and strain 2032/99 were cultured in *Acanthamoeba castellanii* grown in 75-cm² cell culture flasks (Corning, Corning, NY) at 32°C in peptone-yeast extract-glucose broth (10) until lysis of amoebae. Cell cultures were then harvested and filtered through a 5- μ m filter (Millipore, Carrigtwohill, Ireland) to eliminate the remaining amoebae. One hundred microliters of this *W. chondrophila* inoculum diluted 1:100 was then inoculated onto *A. castellanii* in microplates containing about 10⁵ uninfected amoebae/well. Inoculated plates were centrifuged at 1,790 $\times$ g for 10 min and incubated at 32°C. Given the strict intracellular lifestyle of *Waddlia*, we did not use aminoglycosides that are often used to remove bacteria not internalized by eukaryotic cells (when testing facultative intracellular bacteria).

Two hours later, 100 μ l of twofold serial dilutions of antibiotics were added. The antibiotics tested in this study were azithromycin (Sigma-Aldrich, Buchs, Switzerland), doxycycline (Clontech, Mountain View, CA), ampicillin (Roche, Zug, Switzerland), ceftriaxone (Sigma-Aldrich), ciprofloxacin (Sigma-Aldrich), and ofloxacin (Sigma-Aldrich). Growth was assessed at 0, 2, 4, and 7 days postinfection. To check antibiotic dilutions and efficacy, MICs for *Staphylococcus aureus* strain ATCC

29213 and *Escherichia coli* strain ATCC 25922 were determined according to the procedure recommended by the Clinical and Laboratory Standards Institute (6). The control strains exhibited the expected MICs.

To assess the growth of *W. chondrophila* in amoebal cells, we used a specific quantitative real-time TaqMan PCR. DNA was extracted with the QIAamp DNA kit (Qiagen, Hilden, Germany). PCRs were performed in a final volume of 20 μ l including 10 μ l of iTaq supermix (Bio-Rad, Rheinach, Switzerland), 200 nM forward primer (WadF3, 5'-CAGTCGAGAATCTTTCGCAAT-3'), 200 nM reverse primer (WadR3, 5'-CAAGGTACGCTCAAATCCAG-3'), 100 nM probe (WadS, 5'-JOE-TGAATGAAGAAGGCCCTTGGGTCGT-BHQ-3'), 4.5 μ l of water, and 5 μ l of DNA. Cycling conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification and detection of PCR products were performed with the ABI Prism 7000 (Applied Biosystems, Rotkreuz, Switzerland). The variability of this real-time PCR is about 0.5 log. Susceptibility was thus defined as a reduction in the number of DNA copies of more than 0.5 log in the presence of a given antibiotic concentration compared to the control growth curve without antibiotic.

W. chondrophila strains ATCC VR-1470 and 2032/99 exhibited doxycycline MICs of 4 μ g/ml (Fig. 1A) and 1 μ g/ml, respectively. Both strains were susceptible to azithromycin. Strain ATCC VR-1470 growth was inhibited by 0.125 μ g/ml in most experiments. In additional experiments, growth was inhibited by 0.06 μ g/ml (Fig. 1B). Thus, the MICs of azithromycin for this strain ranged between 0.06 and 0.125 μ g/ml. *W. chondrophila* strain 2032/99 had an azithromycin MIC of 0.06 μ g/ml. Both strains exhibited resistance to β -lactams (ampicillin, ceftriaxone), with MICs of $\geq$ 32 μ g/ml, and fluoroquinolones (ciprofloxacin, ofloxacin), with MICs of $\geq$ 16 μ g/ml (Fig. 1C, D, E, and F).

To confirm the results obtained with amoebae, we tested the antibiotic susceptibility of *W. chondrophila* strain VR-1470 in Vero cells. We used a procedure similar to that used for amoebae, except that infected Vero cells were suspended in RMPI medium-10% fetal calf serum and incubated at 37°C in a 5% CO₂ atmosphere. *W. chondrophila* was susceptible to azithromycin and doxycycline, with a MIC of 0.25 μ g/ml for both antibiotics. Similarly to what was observed in amoebae, *W. chondrophila* was resistant to fluoroquinolones (MICs of $\geq$ 16

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FIG. 1. Susceptibility of *W. chondrophila* (ATCC VR-1470) to the antibiotics doxycycline (A), azithromycin (B), ampicillin (C), ciprofloxacin (D), ofloxacin (E), and ceftriaxone (F) as determined in amoebae. Growth was quantified with a *Waddlia*-specific quantitative real-time TaqMan PCR.

μg/ml) and β-lactams (MICs of ≥32 μg/ml). The MIC of doxycycline was slightly higher in amoebae than in mammalian cells. This may be due to the presence of some efflux machinery in amoebae that protects these protists from chemical com-

pounds present in water. For all other antibiotics, the MICs observed with both types of cells were similar, indicating that amoebae may represent a good alternative to mammalian cells to test the antibiotic susceptibility of strictly intracellular bac-

TABLE 1. MICs for *W. chondrophila* and other species belonging to the order *Chlamydiales* for comparison

Drug	MIC (μg/ml) for:						Reference(s)
	<i>W. chondrophila</i>		<i>Parachlamydia</i>	<i>Chlamydia</i>	<i>Chlamydophila</i>	<i>Chlamydophila</i>	
	Amoebae	Vero cells	<i>acanthamoebae</i> ^a	<i>trachomatis</i> ^b	<i>pneumoniae</i> ^b	<i>psittaci</i> ^b	
Azithromycin	0.06–0.125	0.25	ND ^d	0.125–0.25	0.06–0.25	0.125	4, 8, 14, 28
Doxycycline	1–4	0.25	0.5–1	0.03–0.25	0.03–0.06	0.03–2	2, 25, 27, 28
Ceftriaxone	>32	>32	>32	>32	ND	ND	12
Ofloxacin	>16	>16	>16	0.5–1	0.5–1	0.5	20, 28
Ciprofloxacin	>16	>16	>16	>0.5–2	1–2	1–2	20, 22, 27
Ampicillin	>32	>32	>32 ^c	ND	>100 ^c	ND	16, 18

^a MIC determined in amoebae.
^b MIC determined in mammalian cells.
^c MIC of amoxicillin.
^d ND, not determined.

FIG. 2. QRDR of DNA gyrase A (GyrA) (A) and topoisomerase IV (ParC) (B) of *W. chondrophila* strains ATCC VR-1470 and 2032/99, *P. acanthamoebae* strains BN9 and Hall's coccus, *Protochlamydia amoebophila* UWE25, *Chlamydia caviae*, *C. pneumoniae*, *C. trachomatis*, and *E. coli*. The QRDR are delimited by black bars (*E. coli* numbering). GenBank accession numbers are in parentheses. Amino acid substitutions that may confer resistance to quinolones are in bold.

teria such as *Parachlamydia acanthamoebae* that do not grow in mammalian cells (18).

The susceptibility pattern of *W. chondrophila* is relatively similar to that obtained for other members of the order *Chlamydiales* (Table 1). Indeed, *Chlamydia pneumoniae* and *Chlamydia trachomatis* are both susceptible to macrolides and tetracyclines (4, 8, 14, 17, 25, 27, 28). However, unlike *W. chondrophila*, both of these chlamydiae are susceptible to quinolones (13, 14, 19, 20, 22). Interestingly, *P. acanthamoebae* is, like *W. chondrophila*, resistant to fluoroquinolones (3, 18). The resistance to fluoroquinolones was also observed for other *Chlamydia*-related bacteria, including *Neochlamydia hartmannellae* and *Simkania negevensis* (3). In the latter study, quinolone resistance in these amoeba-resisting bacteria could be explained by mutations in the quinolone resistance-determining regions (QRDR) of the DNA gyrase A (GyrA)- and topoisomerase IV (ParC)-encoding genes. We thus sequenced the QRDR of both genes for the two *W. chondrophila* strains available to date. Both strains exhibited the same QRDR sequences. In GyrA, we observed a Ser-83→Gln substitution, which is known to be associated with the resistance phenotype (Fig. 2A). A Val-70→Ser substitution was also observed. Both substitutions were already observed in the GyrA QRDR of *P. acanthamoebae* (3). In the ParC QRDR, Ser-81→Ala and Glu84→Asp substitutions were observed (Fig. 2B). The latter substitution can also be observed in *Streptococcus pneumoniae*, conferring quinolone resistance on mutant strains (21). The resistance of *W. chondrophila* to quinolones might thus be explained by the mutations present in the GyrA and ParC QRDR.

In conclusion, azithromycin and doxycycline are active in vitro against both strains of *W. chondrophila* whereas quino-

lones and β -lactams are not. Resistance of *W. chondrophila* to penicillin was already documented in BT cells (7).

Consequently, azithromycin should be considered to treat infections due to this intracellular bacterium, given its excellent bioavailability and its documented activity against other members of the order *Chlamydiales* (17, 26). Macrolides might be an especially good first choice to prevent miscarriage, given the contraindication of using doxycycline during pregnancy. Doxycycline might, of course, be an alternative agent for non-pregnant patients. In the future, it will be important to test the activity of these antibiotics against *W. chondrophila* in vivo.

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