ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2002, p. 3065–3067 Vol. 46, No. 9
0066-4804/02/$04.00+0 DOI: 10.1128/AAC.46.9.3065–3067.2002

Antibiotic Susceptibilities of *Parachlamydia acanthamoeba* in Amoebae

M. Maurin,[1] A. Bryskier,[2] and D. Raoult[1]*

Unité des Rickettsies, CNRS UPRES A 6020, Faculté de Médecine, Université de la Méditerranée, 13385 Marseille Cedex 05[1], and Anti-Infective Research Department, Hoechst-Marion-Roussel, 93235 Romainville, France[2]

Received 6 August 2001/Returned for modification 8 April 2002/Accepted 5 June 2002

***Parachlamydia acanthamoeba* are intracellular bacteria of amoebae and are considered potential etiological agents of human pneumonia. We have determined the in vitro antibiotic susceptibilities of two strains (strain Bn_9 and Hall's coccus) in *Acanthamoeba polyphaga*. The two strains were susceptible to tetracyclines, macrolides, and rifampin, but resistant to fluoroquinolones.**

Based on 16S ribosomal DNA sequence comparison, the taxonomic classification of species belonging to the order *Chlamydiales* has been recently reassessed, and a new family, *Parachlamydiaceae*, has been proposed (13). This family now comprises two genera: i.e., the genus *Parachlamydia* with *Parachlamydia acanthamoeba* as a type species (2), and the genus *Neochlamydia* with *Neochlamydia hartmannellae* as the type species (16). Strains belonging to the species *P. acanthamoeba* include the type strain Bn_9 (ATCC VR 1476), isolate $Berg_{17}$, Hall's coccus (21), and unnamed isolates (2, R. J. Birtles, T. J. Rowbotham, C. Storey, T. J. Marrie, and D. Raoult, Letter, Lancet **349:**925-926, 1997), which are all strictly intracellular bacteria with variable Gram staining properties and which display more than 99% 16S rRNA gene similarity (Birtles et al., Letter). *Parachlamydia* spp. naturally infect *Acanthamoeba*. Trophozoites of *Acanthamoeba* hosting chlamydia-like bacteria have been isolated in patients with fever associated with use of humidifiers in Vermont (i.e., Hall's coccus) (21) and from human nasal mucosa (i.e., the Bn_9 and $Berg_{17}$ strains) (2). Moreover, fourfold rising titers of antibodies directed against Hall's coccus have been detected by an immunofluorescence technique in sera from patients suffering from pneumonia of undefined cause in Ohio and Nova Scotia, Canada (Birtles et al., Letter). These sera did not react with *Chlamydia trachomatis*, *Chlamydophila pneumoniae*, or *Chlamydophila psittaci* antigens (Birtles et al., Letter). More recently, Marrie et al. (22) reported detection of anti-*P. acanthamoeba* antibodies (antibody titer of 179 [there]ϕ [there]σ 121:50) in 8 of 376 patients (~2%) with community-acquired pneumonia compared with 0 of 511 healthy controls. Thus, a recent medical interest in *Parachlamydia* strains has arisen because of the potential etiological role in community-acquired pneumonia as well as nosocomial pneumonia, especially in patients with a humidifier (14). In this respect, the knowledge of their antibiotic susceptibilities could be of primary clinical importance. Especially, it is of particular interest to verify that current first-line recommendations for antibiotic therapy of pneumonia (3, 6) apply to this group of pathogens.

* Corresponding author. Mailing address: Unité des Rickettsies, CNRS UMR 6020, IFR 48, Faculté de Médecine, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France. Phone: (33) 4 91 38 55 17. Fax: (33) 4 91 83 03 90. E-mail: Didier.Raoult@medecine.univ-mrs.fr.

Bacterial and amoebal strains. *P. acanthamoeba* strain Bn_9 was kindly provided by R. Amann (Lehrstuhl für Mikrobiologie, Technische Universität München, Munich, Germany), whereas Hall's coccus was a gift from T. J. Robotham (Public Health Laboratory, Leeds, United Kingdom). *Parachlamydia* organisms were cultured in *Acanthamoeba polyphaga*, grown in 25-cm^2 culture flasks (Becton Dickinson, Le Pont de Claix, France) containing PYG medium (35) until almost complete lysis of amoebae (i.e., 4 days later). Cell supernatants were then recovered and centrifuged at 1,500 rpm (700 × *g*) for 10 min to remove cell debris. A *Parachlamydia* inoculum was prepared for each strain tested by diluting supernatants 1:100 in Page's amoebal saline (28), which corresponded to approximately 10^8 bacteria/ml. Titration of *Parachlamydia* was obtained by inoculating 10-fold serial dilutions of the primary inoculum to uninfected amoebal cultures and determining the highest dilution allowing lysis of amoebal monolayers after 4 days of incubation of cultures at 30°C.

Determination of MICs. Uninfected amoebae, cultured in PYG medium, were harvested by gentle shaking of monolayers and dispensed (160 μl per well of a 5.10^5-organism/ml inoculum) in 96-well microtiter plates (D. Dutcher, Brumath, France). Each well received 20 μl of *Parachlamydia* inoculum (i.e., final inoculum of about 10^6 bacteria/ml). After a 2-h incubation of infected amoebal cultures at 30°C, antibiotics were added (i.e., 20 μl of 10-fold the desired final concentrations). Controls were drug-free uninfected amoebae (as amoebal viability controls), uninfected amoebae with the various antibiotic concentrations tested (as amoebal antibiotic toxicity controls), and drug-free infected amoebae (as *Parachlamydia* growth controls). We also verified that the *P. acanthamoeba* strains tested did not grow in Page's amoebal saline in the absence of amoebae. Drug-free controls received 20 μl of saline instead of the antibiotic solution. Cultures were incubated at 30°C and observed each day under an inverted microscope at a magnification of ×400 until complete lysis of amoebal monolayers in *Parachlamydia*-infected drug-free controls occurred. By this simple technique, MICs corresponded to the lowest antibiotic concentration that prevented *Parachlamydia* growth (i.e., destruction of amoebal cultures after 4 days of incubation). *Escherichia coli* C.I.P. 53.126 and *Staphylococcus aureus* C.I.P. 103811 were obtained from the Pasteur Institute (Institut Pasteur, Marnes La Coquette, France) and were used to control the antibiotic concentrations tested, with Mueller-

TABLE 1. MICs for *Parachlamydia* sp., including the Bn_9 strain and Hall's coccus, as determined in an *A. polyphaga* culture model[a]

Antimicrobial agent	MIC (μg/ml) for:					Reference(s)
	Parachlamydia		*C. trachomatis*	*C. pneumoniae*	*C. psittaci*	
	Bn_9	Hall's coccus				
Penicillin G	>32	>32		>100		18
Amoxicillin	>32	>32	0.25–4	>100		18, 31
Ceftriaxone	>32	>32	>32			15
Imipenem	>32	>32				
Gentamicin	0.5	1	>100			17, 32, 41
Amikacin	1	0.5				
Thiamphenicol	>32	>32				
Doxycycline	0.5	1	0.015–0.063	0.031–0.063	0.05–0.2	1, 7, 24
Erythromycin	0.5	0.5	0.25–1	0.063–0.25	0.125–0.25	24, 37
Clarithromycin	0.5	0.5	0.016–0.031	0.016–0.031	0.016	24
Telithromycin	0.25	0.5		0.015–2		33
TMP/SMX[b]	0.5/2.5	2/10	<0.004–0.125			38
Rifampin	0.25	0.25				
Ofloxacin	>16	>16	0.5–1	0.5–1	0.5	24, 31
Ciprofloxacin	>16	>16	0.78–3.13	1–2	1–2	24, 26
Vancomycin	>16	>16				

[a] MICs for *C. trachomatis, C. pneumoniae,* and *C. psittaci* reported in the literature with corresponding references have been incorporated for comparison.
[b] TMP/SMX, trimethoprim-sulfamethoxazole.

Hinton broth as the antibiotic assay medium according to the procedure recommended by the National Committee for Clinical Laboratory Standards (27).

No antibiotic concentration tested displayed a toxic effect against amoebae. MICs for *E. coli* C.I.P. 53.126 and *Staphylococcus aureus* C.I.P. 103811 were compatible with those determined by the Pasteur Institute. *P. acanthamoeba* strains grew well in *A. polyphaga* cells, with complete lysis of amoebal monolayers in drug-free cultures after 4 days of incubation at 30°C. Among the β-lactams tested, penicillin G, amoxicillin, ceftriaxone, and imipenem were ineffective at concentrations up to 32 μg/ml (Table 1). Both strains were susceptible to aminoglycosides, macrolides (including the newer ketolide, telithromycin), doxycycline, cotrimoxazole, and rifampin. In contrast, vancomycin, the activity of which is almost restricted to gram-positive bacteria, was ineffective. Thiamphenicol and, more importantly, the fluoroquinolone compounds ofloxacin and ciprofloxacin were not bacteriostatic at the concentrations tested.

We have evaluated in vitro susceptibilities of two strains belonging to the species *P. acanthamoeba* (i.e., Bn_9 and Hall's coccus), with *A. polyphaga* as an in vitro cell system to support growth of these strictly intracellular bacteria. An amoebal system was used because of the impossibility of growing these bacteria in the other cell systems we currently use in our laboratory, including McCoy cells, Vero cells, P388D1 macrophage-like cells, or human embryonic lung fibroblast cells. Our model was based upon inhibition of amoebal lysis due to bacterial multiplication when antibiotics were added to the culture supernatant compared to that of drug-free controls. Thus, it was critical to verify that amoebal lysis was not related to antibiotic toxicity. Despite these technical limitations, our model allowed us for the first time to define the antibiotic susceptibility pattern of *P. acanthamoeba* and to compare it with those previously reported for *C. trachomatis*, *Chlamydophila pneumoniae*, and *Chlamydophila psittaci*, species that also belong to the order *Chlamydiales*.

P. acanthamoeba strains BN9 and Hall's coccus were found resistant to all β-lactams tested. The in vitro activity of β-lactams against *C. trachomatis* (4, 8, 25), *C. pneumoniae* (18), and *C. psittaci* (23, 40) has been demonstrated. Although these antibiotics are not considered first-line antibiotic therapy for *Chlamydia*-related pneumonia (19), amoxicillin has been used successfully in pregnant women with genital infection due to *C. trachomatis* (9, 20, 39). In contrast, we found aminoglycosides to be bacteriostatic against *P. acanthamoeba* strains, whereas *C. trachomatis* has been reported to be highly resistant to gentamicin (17, 32, 41). Cotrimoxazole could inhibit the growth of *P. acanthamoeba* and is also effective against *C. trachomatis* (38). In contrast, *C. pneumoniae* and *C. psittaci* are resistant to this antibiotic combination. More surprisingly, *P. acanthamoeba* strains were found to be resistant to fluoroquinolones, whereas *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* are highly susceptible to these drugs (24, 26, 31). DNA gyrase is usually the primary target of fluoroquinolones in gram-negative bacteria (11, 29), and resistance to fluoroquinolones due to mutation in *gyrA* (the gene encoding the alpha subunit of DNA gyrase) has been reported in *C. trachomatis* (10). The possibility of *gyrA*-mediated natural resistance to fluoroquinolones in *P. acanthamoeba* should be assessed.

Our results should be specifically examined considering the potential role of *Parachlamydia* spp. as etiological agents of human pneumonia (2, 21). β-Lactams are considered first-line antibiotic therapy of *Streptococcus pneumoniae*-related pneumonia, but are poorly effective against intracellular pathogens responsible for atypical pneumonia, such as *Chlamydia* spp., *Legionella pneumophila*, *Mycoplasma pneumoniae*, or *Coxiella burnetii* (3, 6). This may also apply for *P. acanthamoeba*, a species resistant in vitro to these agents. In contrast, the susceptibility of *P. acanthamoeba* to macrolides and tetracycline suggests that the current practice of prescribing a macrolide or a tetracycline compound in patients with atypical pneumonia may well apply in case of *Parachlamydia* infection. The new ketolide compound telithromycin, which is active against eryth-

romycin-resistant *S. pneumoniae* (5, 30), as well as against the intracellular pathogens *C. pneumoniae* (33), *L. pneumophila* (12, 36), *M. pneumoniae* (42), and *C. burnetii* (34), was found also active against the *Parachlamydia* strains. Fluoroquinolones, especially ofloxacin and ciprofloxacin, have been advocated as a possible alternative to macrolides in patients suffering atypical pneumonia, although their equivalence to erythromycin in case of legionellosis is still disputed (3, 6). Interestingly, we found *P. acanthamoeba* to be highly resistant to these compounds in vitro.

REFERENCES

1. **Agacfidan, A., J. Moncada, and J. Schachter.** 1993. In vitro activity of azithromycin (CP-62,993) against *Chlamydia trachomatis* and *Chlamydia pneumoniae*. Antimicrob. Agents Chemother. **37:**1746–1748.
2. **Amann, R., N. Springer, W. Sch[uml]onhuber, W. Ludwig, E. N. Schmid, K.-D. M[uml]uller, and R. Michel.** 1997. Obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydia* spp. Appl. Environ. Microbiol. **63:**115–121.
3. **Anonymous.** 1998. ERS Task Force Report. Guidelines for management of adult community-acquired lower respiratory tract infections. Eur. Respir. J. **11:**986–991.
4. **Barbour, A. G., K.-I. Amano, T. Hackstadt, L. Perry, and H. D. Caldwell.** 1982. *Chlamydia trachomatis* has penicillin-binding proteins but not detectable muramic acid. J. Bacteriol. **151:**420–428.
5. **Barry, A. L., P. C. Fuchs, and S. D. Brown.** 1998. Antipneumococcal activities of a ketolide (HMR 3647), a streptogramin (quinupristin-dalfopristin), a macrolide (erythromycin), and a lincosamide (clindamycin). Antimicrob. Agents Chemother. **42:**945–946.
6. **Bartlett, J. G., R. F. Breiman, L. A. Mandell, and T. M. J. File.** 1998. Community-acquired pneumonia in adults: guidelines for management. The Infectious Diseases Society of America. Clin. Infect. Dis. **26:**811–838.
7. **Butaye, P., R. Ducatelle, P. De Backer, H. Vermeersch, J. P. Remon, and F. Haesebrouck.** 1997. In vitro activities of doxycycline and enrofloxacin against European *Chlamydia psittaci* strains from turkeys. Antimicrob. Agents Chemother. **41:**2800–2801.
8. **Clark, R. B., P. F. Schatzki, and H. P. Dalton.** 1982. Ultrastructural effect of penicillin and cycloheximide on *Chlamydia trachomatis* strain HAR-13. Med. Microbiol. Immunol. **171:**151–159.
9. **Crombleholme, W. R., J. Schachter, M. Grossman et al.** 1990. Amoxicillin therapy for *Chlamydia trachomatis* in pregnancy. Obstet. Gynecol. **75:**752–756.
10. **Dessus-Babus, S., C. M. Bébéar, A. Charron, C. Bébéar, and B. de Barbeyrac.** 1998. Sequencing of gyrase and topoisomerase IV quinolone-resistance-determining regions of *Chlamydia trachomatis* and characterization of quinolone-resistant mutants obtained in vitro. Antimicrob. Agents Chemother. **42:**2474–2481.
11. **Drlica, K., and X. Zhao.** 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol. Mol. Biol. Rev. **61:**377–392.
12. **Edelstein, P. H., and M. A. C. Edelstein.** 1999. In vitro activity of the ketolide HMR 3647 (RU 6647) for *Legionella* spp., its pharmacokinetics in guinea pigs, and use of the drug to treat guinea pigs with *Legionella pneumophila* pneumonia. Antimicrob. Agents Chemother. **43:**90–95.
13. **Everett, K. D., R. M. Bush, and A. A. Andersen.** 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. Int. J. Syst. Bacteriol. **49:**415–440.
14. **Greub, G., and D. Raoult.** 2002. *Parachlamydiaceae*, potential emerging pathogens. Emerg. Infect. Dis. **8:**625–630.
15. **Hammerschlag, M. R., and A. Gleyzer.** 1983. In vitro activity of a group of broad-spectrum cephalosporins and other β-lactam antibiotics against *Chlamydia trachomatis*. Antimicrob. Agents Chemother. **23:**493–494.
16. **Horn, M., M. Wagner, K.-D. Müller, E. N. Schmid, T. R. Fritsche, K.-H. Schleifer, and R. Michel.** 2000. *Neochlamydia hartmannellae* gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmanella vermiformis*. Microbiology **146:**1231–1239.
17. **Johannisson, G., A. Sernryd, and E. Lycke.** 1979. Susceptibility of *Chlamydia trachomatis* to antibiotics in vitro and in vivo. Sex. Transm. Dis. **6:**50–57.
18. **Kuo, C.-C., and J. T. Grayston.** 1988. In vitro drug susceptibility of *Chlamydia* sp. strain TWAR. Antimicrob. Agents Chemother. **32:**257–258.
19. **Lieberman, D., M. Ben-Yaakov, Z. Lazarovich, A. Porath, F. Schlaeffer, M. Leinonen, P. Saikku, O. Horovitz, and I. Boldur.** 1996. *Chlamydia pneumoniae* community-acquired pneumonia: a review of 62 hospitalized adult patients. Infection **24:**109–114.
20. **Magat, A. H., L. S. Alger, D. A. Nagey et al.** 1993. Double-blind randomized study comparing amoxicillin and erythromycin for the treatment of *Chlamydia trachomatis* in pregnancy. Obstet. Gynecol. **81:**745–749.
21. **Mamolen, M., D. M. Lewis, M. A. Blanchet, F. J. Satink, and R. L. Vogt.** 1993. Investigation of an outbreak of 'humidifier fever' in a print shop. Am. J. Ind. Med. **23:**483–490.
22. **Marrie, T. J., D. Raoult, B. La Scola, R. J. Birtles, and E. de Carolis.** 2001. *Legionella*-like and other amoebal pathogens as agents of community-acquired pneumonia. Emerg. Infect. Dis. **7:**1026–1029.
23. **Matsumoto, A., and G. P. Manire.** 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. J. Bacteriol. **101:**278–285.
24. **Miyashita, N., Y. Niki, T. Kishimoto, M. Nakajima, and T. Matsushima.** 1997. In vitro and in vivo activities of AM-1155, a new fluoroquinolone, against *Chlamydia* spp. Antimicrob. Agents Chemother. **41:**1331–1334.
25. **Moulder, J. W.** 1993. Why is *Chlamydia* sensitive to penicillin in the absence of peptidoglycan? Infect. Agents Dis. **2:**87–99.
26. **Nakata, K., H. Maeda, A. Fujii, S. Arakawa, K. Umezu, and S. Kamidono.** 1992. In vitro and in vivo activities of sparfloxacin, other quinolones, and tetracyclines against *Chlamydia trachomatis*. Antimicrob. Agents Chemother. **36:**188–190.
27. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
28. **Page, F. C.**1976. An illustrated key to freshwater and soil amoebae with notes on cultivation and ecology. Scientific publication no. 34. Freshwater Biological Association, Ambleside, Cumbria, England.
29. **Pan, X.-S., J. Ambler, S. Mehtar, and L. M. Fisher.** 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **40:**2321–2326.
30. **Pankuch, G. A., M. A. Visalli, M. R. Jacobs, and P. C. Appelbaum.** 1998. Susceptibilities of penicillin- and erythromycin-susceptible and -resistant pneumococci to HMR 3647 (RU 66647), a new ketolide, compared with susceptibilities to 17 other agents. Antimicrob. Agents Chemother. **42:**624–630.
31. **Rice, R. J., V. Bhullar, S. H. Mitchell, J. Bullard, and J. S. Knapp.** 1995. Susceptibilities of *Chlamydia trachomatis* isolates causing uncomplicated female genital tract infections and pelvic inflammatory disease. Antimicrob. Agents Chemother. **39:**760–762.
32. **Ridgway, G. L., J. M. Owen, and J. D. Oriel.** 1978. The antimicrobial susceptibility of *Chlamydia trachomatis* in cell culture. Br. J. Vener. Dis. **54:**103–106.
33. **Roblin, P. M., and M. R. Hammerschlag.** 1998. In vitro activity of a new ketolide antibiotic, HMR 3647, against *Chlamydia pneumoniae*. Antimicrob. Agents Chemother. **42:**1515–1516.
34. **Rolain, J.-M., M. Maurin, A. Bryskier, and D. Raoult.** 2000. In vitro activities of telithromycin (HMR 3647) against *Rickettsia rickettsii, Rickettsia conorii*, *Rickettsia africae*, *Rickettsia typhi*, *Rickettsia prowazekii*, *Coxiella burnetii, Bartonella henselae*, *Bartonella quintana*, *Bartonella bacilliformis*, and *Ehrlichia chaffeensis*. Antimicrob. Agents Chemother. **44:**1391–1393.
35. **Rowbotham, T. J.** 1983. Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. J. Clin. Pathol. **36:**978–986.
36. **Sch[uml]ulin, T., C. B. Wennersten, M. J. Ferraro, R. C. J. Moellering, Jr., and G. M. Eliopoulos.** 1998. Susceptibilities of *Legionella* spp. to newer antimicrobials in vitro. Antimicrob. Agents Chemother. **42:**1520–1523.
37. **Scieux, C., A. Bianchi, B. Chappey, I. Vassias, and Y. Perol.** 1990. In-vitro activity of azithromycin against *Chlamydia trachomatis*. J. Antimicrob. Chemother. **25**(Suppl. A)**:**7–10.
38. **Segreti, J., H. A. Kessler, K. S. Kapell, and G. M. Trenholme.** 1987. In vitro activity of A-56268 (TE-031) and four other antimicrobial agents against *Chlamydia trachomatis*. Antimicrob. Agents Chemother. **31:**100–101.
39. **Silverman, N. S., M. Sullivan, M. Hochman, M. Womack, and D. L. Jungkind.** 1994. A randomized, prospective trial comparing amoxicillin and erythromycin for the treatment of *Chlamydia trachomatis* in pregnancy. Am. J. Obstet. Gynecol. **170:**829–832.
40. **Tamura, A., and G. P. Manire.** 1968. Effect of penicillin on the multiplication of meningopneumonitis organisms (*Chlamydia psittaci*). J. Bacteriol. **96:**875–880.
41. **Wentworth, B. B.** 1973. Use of gentamicin in the isolation of subgroup A *Chlamydia*. Antimicrob. Agents Chemother. **3:**698–702.
42. **Yamaguchi, T., Y. Hirahata, K. Izumikawa, Y. Miyazaki, F. Maesaki, K. Tomono, Y. Yamada, S. Kamihira, and S. Kohno.** 2000. In vitro activity of telithromycin (HMR3647), a new ketolide, against clinical isolates of *Mycoplasma pneumoniae* in Japan. Antimicrob. Agents Chemother. **44:**1381–1382.