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

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Running title: Antibiotic susceptibility of *Parachlamydiaceae*

ABSTRACT

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 amoebophila* [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 inocolum 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'- AGCACGTGTGTAGCCCCA), 100 nM of the fluorescent labeled probe (6-FAM-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* amoeabal 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] cells 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 levofloxacin 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.

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DISCLOSURE STATEMENT

The authors did not report any potential conflict of interest.

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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.

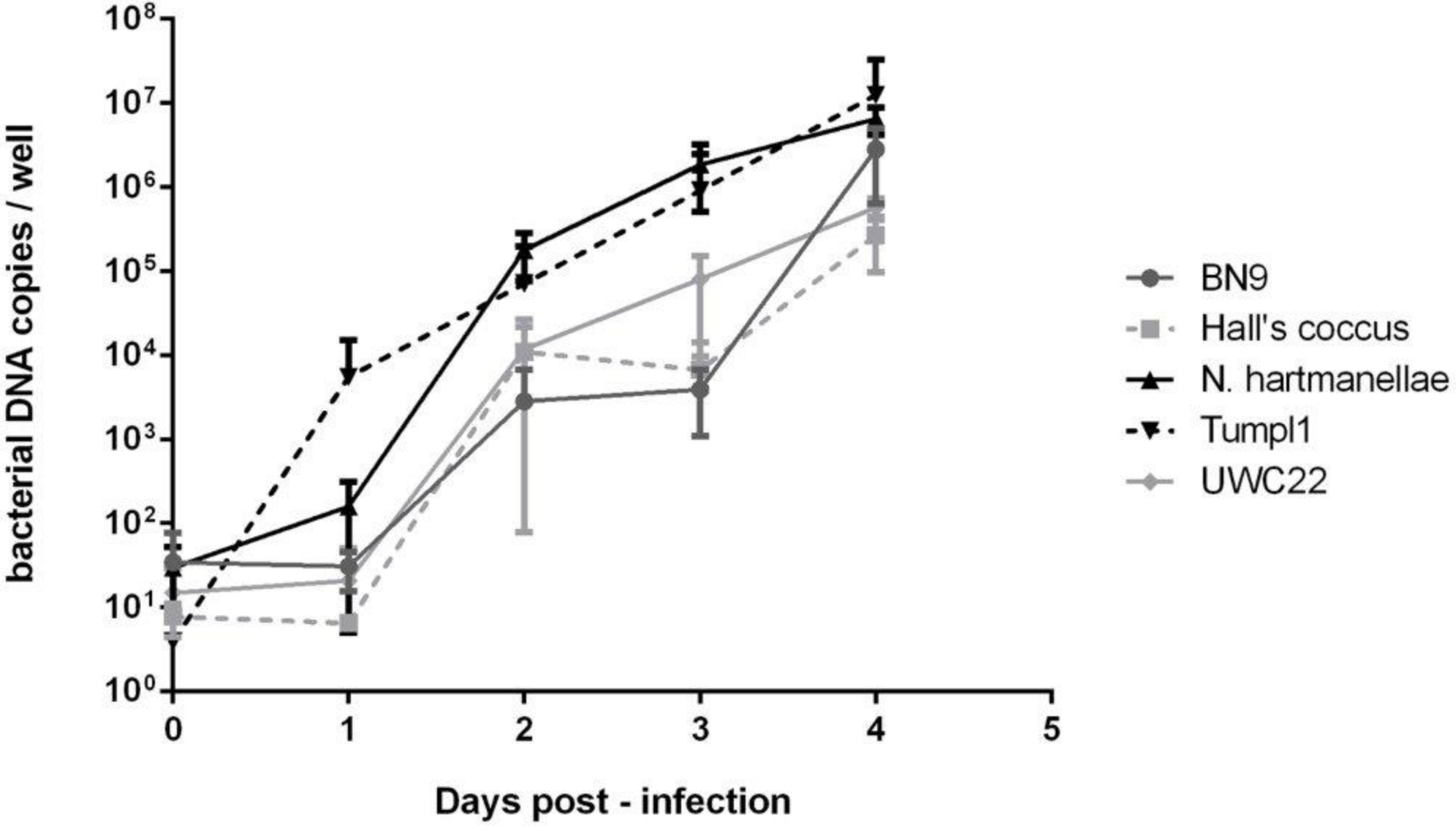
Figure 2: Antibiotic susceptibility of each parachlamydial strains in amoebae

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.

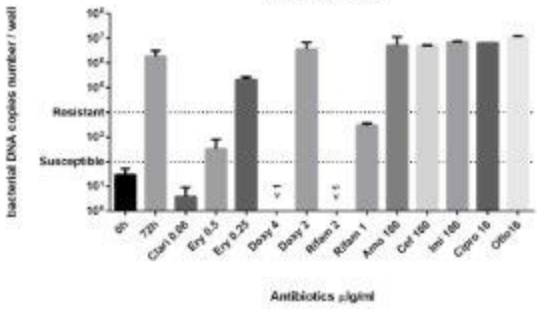
(A) Neochlamydia hartmanellae (B) *Parachlamydia acanthamoebae* strain Hall's coccus (C) *Parachlamydia acanthamoebae* strain BN9 (D) *Parachlamydia* sp. Tumpl1 (E) *Neochlamydia* sp. UWC22.

Table 1: Antibiotic susceptibility of *Parachlamydiaceae* and others *Chlamydiales*

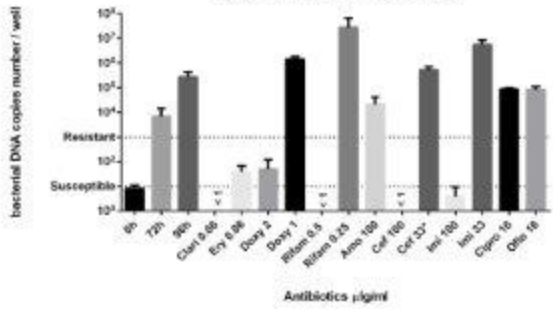
This table represents the MICs in µg/ml of various antibiotics against members of the *Chlamydiales* orders


bacterial DNA copies / well
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
1
2
3
4
5
Days post - infection
BN9
Hall's coccus
N. hartmanellae
Tumpl1
UWC22

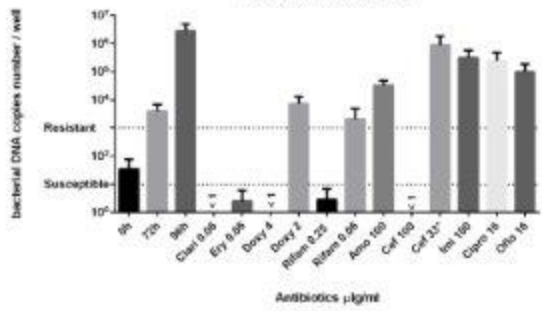
A.


N. hartmanellae
bacterial DNA copies number / well
Resistant
Susceptible
0h
72h
Clari 0.06
Ery 0.5
Ery 0.25
Doxy 4
Doxy 2
Rifam 2
Rifam 1
Amo 160
Cef 160
Imi 160
Cipro 16
Oflo16
Antibiotics µg/ml

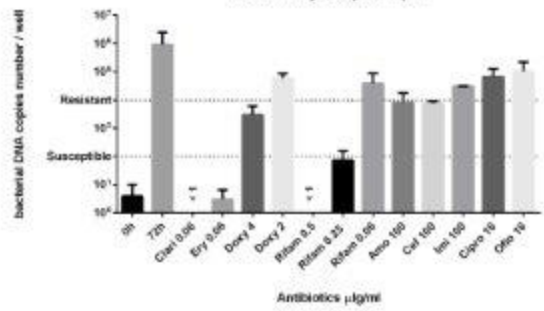
B.


P. acanthamoebae Hall's coccus
bacterial DNA copies number / well
Resistant
Susceptible
0h
72h
96h
Clari 0.06
Ery 0.06
Doxy 2
Doxy 1
Rifam 0.5
Rifam 0.25
Amo 160
Cef 160
Cef 32
Imi 160
Imi 32
Cipro 16
Oflo 16
Antibiotics µg/ml

C.


P. acanthamoebae BN9
bacterial DNA copies number / well
Resistant
Susceptible
0h
72h
96h
Clari 0.06
Ery 0.06
Doxy 4
Doxy 2
Rifam 0.25
Rifam 0.06
Amo 160
Cef 160
Cef 32
Imi 160
Cipro 16
Oflo 16
Antibiotics µg/ml

D.


Parachlamydia sp. Tumpl1
bacterial DNA copies number / well
Resistant
Susceptible
0h
72h
Clari 0.06
Ery 0.06
Doxy 4
Doxy 2
Rifam 0.5
Rifam 0.25
Rifam 0.06
Amo 160
Cef 160
Imi 160
Cipro 16
Oflo 16
Antibiotics µg/ml

E.

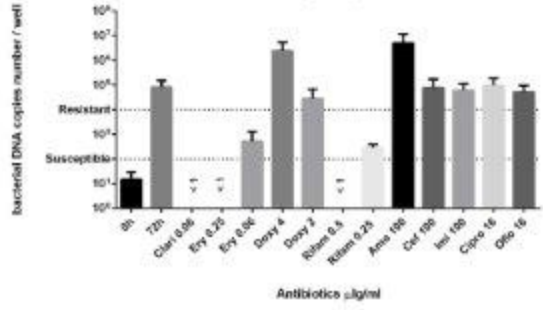

Neochlamydia sp. UWC22
bacterial DNA copies number / well
Resistant
Susceptible
0h
72h
Clari 0.06
Ery 0.25
Ery 0.06
Doxy 4
Doxy 2
Rifam 0.5
Rifam 0.25
Amo 160
Cef 160
Imi 160
Cipro 16
Oflo 16
Antibiotics µg/ml

Table 1: Antibiotic susceptibility of *Parachlamydiaceae* and others *Chlamydiales*

	Parachlamydiaceae						***Waddliaceae***	***Criblamydiaceae***	***Chlamydiaceae***	
		P. acanthamoebae		***N. hartmanellae***	***Parachlamydiaceae* spp.**		***W. chondrophila***	***E. lausannensis***	***C. trachomatis***	***C. pneumoniae***
	BN9 [17]	**BN9**	**Hall's coccus**		**UWC22**	**Tumpl1**	[20]	[19]	[25-29]	[29-31]
Cell lines	Amoebae						Vero	Amoebae	Vero	Mc Coy, Hep2	HeLA
MIC (µl/ml)											
Cyclines											
Tetracycline									0.25	0.25-0.5	0.125-0.5
Doxycycline	0.5	4	2	4	≥ 8	≥ 8	0.25	1-4	0.25	0.06-0.25	0.015-0.5
Macrolides											
Erythromycin	0.5	< 0.06	> 0.06	> 0.5	0.25	< 0.06	ND	ND	ND	<0.125-2	<0.125-0.5
Clarithromycin	0.5	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	ND	ND	ND	<0.125-2	<0.125-1
Azythromycin	ND	ND	ND	ND	ND	ND	0.25	0.006-0.125	2	<0.125-2	<0.125-0.5
β-lactams											
Penicillin derivatives	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	ND	>100
Ceftriaxone	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	<32	ND
Imipenem			>32								
Fluoroquinolones											
Ciprofloxacin	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	32	0.5-2	1-4
Ofloxacine	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	16	0.5-1	0.5-2
Rifamycine											
Rifampicine	0.25	0.25	0.5	1-2	0.25-0.5	0.25-0.5	ND	ND	ND	<0.125-1	<0.125

Abbreviations : MIC, Minimal inhibitory concentration; ND, Not determined